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Gas Metabolism Evidence in Support of the Juxtaposition of Hydrogen-Producing and Methanogenic Bacteria in Sewage Sludge and Lake Sediments

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We developed new techniques to measure dissolved H_2 and H_2 consumption kinetics in anoxic ecosystems that were not dependent on headspace measurements or gas transfer-limited experimentation. These H_2 metabolism parameters were then compared with measured methane production rates, and estimates of H_2 production and interspecies H_2 transfer were made. The H_2 pool sizes were 205 and 31 nM in sewage sludge from an anaerobic digestor and in sediments (24 m) from Lake Mendota, respectively. The H_2 turnover rate constants, as determined by using in situ pool sizes and temperatures, were 103 and 31 h^{-1} for sludge and sediment, respectively. The observed H_2 turnover rate accounted for only 5 to 6% of the expected H_2-CO_2-dependent methanogenesis in these ecosystems. Our results are in general agreement with the results reported previously and are used to support the conclusion that most of the H_2-dependent methanogenesis in these ecosystems occurs as a consequence of direct interspecies H_2 transfer between juxtapositioned microbial associations within flocs or consortia.

Hydrogen is an important precursor of methanogenesis in sewage and freshwater sediments (1, 5, 10, 13, 25, 30, 32; Phelps and Zeikus, Appl. Environ. Microbiol., submitted for publication). H_2-consuming microorganisms are of great importance in these anoxic environments, since they maintain a low H_2 partial pressure and thus allow the functioning of biological reactions which otherwise would not occur because of thermodynamic reasons (34). Syntrophic partnerships between H_2 consumers (e.g., methanogens) and H_2 producers (e.g., H_2-producing acetogens) shift the overall fermentation balance of a primary substrate toward better usage of its energy content (28). The term interspecies H_2 transfer has usually been used for systems in which the presence of one or more H_2-consuming species significantly alters the metabolism of a fermentative organism toward production of more oxidized end products (1–4, 15, 16). This alteration can be demonstrated in defined bacterial mixed cultures and in natural environments when the activity of H_2 consumers is inhibited (10, 12, 26).

Previous research on interspecies H_2 transfer has not demonstrated the need for physical contact or juxtapositioning of syntrophic partners, and this process is generally understood as syntrophism via a common pool of H_2. Thus, if syntrophic bacterial associations are randomly distributed within an environment and the bacteria are not in physical contact or close to each other, then the H_2 produced from one partner should be able to equilibrate with the common H_2 pool before it is utilized by the other partner. On the other hand, if the syntrophic bacterial associations are juxtapositioned then the produced H_2 should be partially consumed before it can equilibrate with the common H_2 pool. The purpose of this study was to develop suitable methods to study this problem and to provide evidence in support of the hypothesis that the mechanism of H_2 transfer in anoxic sediments or sewage sludge involves juxtapositioning.

MATERIALS AND METHODS

Chemicals. Purified gases (pressurized air, N_2, H_2, N_2-CO_2) were obtained from Matheson Gas Co., Joliet, Ill. Traces of H_2 were removed from the gases by using a Hopkalit metal oxide converter (Drägerwerke, Lübeck, Federal Republic of Germany). The other chemicals were of analytical quality and were obtained from Mallinckrodt, Paris, Ky., or Sigma Chemical Co., St. Louis, Mo.

Sampling procedures. Anoxic sediment samples were taken with an Eckman dredge from Lake Mendota, Wis. (depth, 24 m) during summer stratification. The anaerobic processes of methanogenesis and sulfidogenesis have been well characterized in this lake (9, 30; Phelps and Zeikus, submitted). The upper 5 cm of the sediment was transferred into glass bottles under an N_2 gas stream. The bottles were completely filled, anaerobically sealed with rubber stoppers, and transported to the laboratory in an ice chest. For measuring H_2 concentrations and turnover rates, sediment samples were transferred into special glass flasks (see below) by using a 60-ml plastic syringe to which latex tubing (length, 7 cm; inside diameter, 5 mm) was connected instead of a needle.

Anoxic samples of sewage sludge were taken from a tap on the Nine Springs Minicipal Sewage Plant anaerobic digestor in Madison, Wis. The end of the tap was inserted into a 1-liter beaker, and the beaker was flushed with sewage for 30 s. Samples were removed from this filled beaker by using a 60-ml syringe and were placed into the special glass flasks.

Measurement of dissolved H_2 concentration. The procedure used for measuring dissolved H_2 in sediment or sludge samples is shown in Fig. 1. Samples (approximately 30 to 50 ml) were transferred into special 100-ml glass flasks which had been flushed with H_2-free N_2 (steps a and b). The special glass flasks were the same as those used for measuring H_2 or

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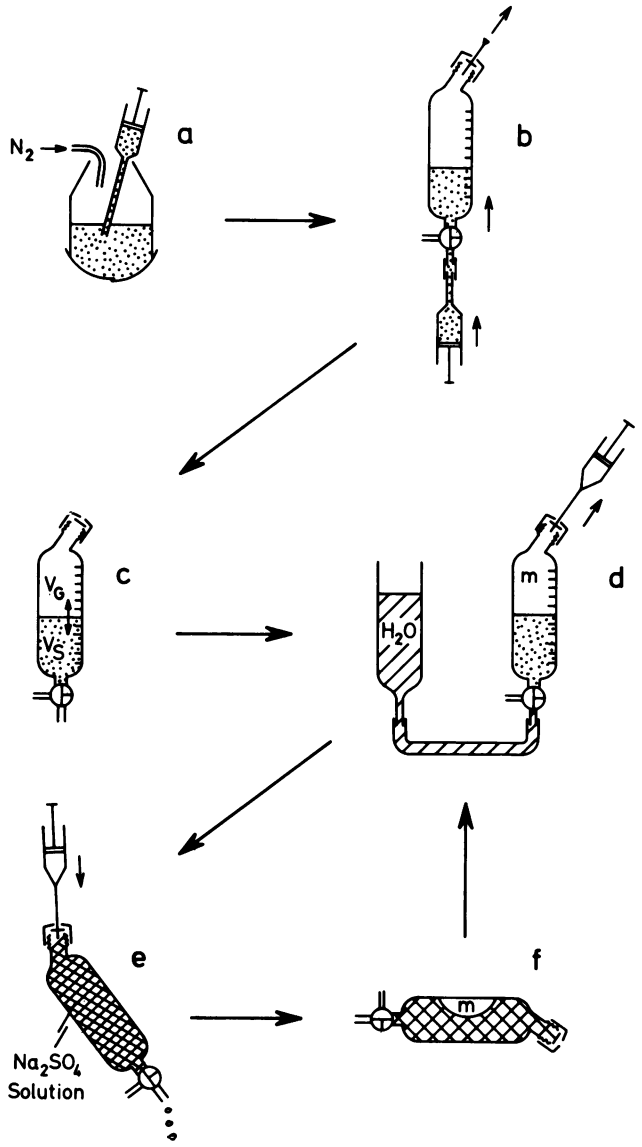

FIG. 1. Schematic diagram of the procedures used to measure dissolved hydrogen in anoxic sewage and sludge samples. See text for details.

CO in water samples (7), except that the openings of the three-way stopcock made of Teflon were enlarged to about 2.5 mm (inside diameter). The H_2 and CH_4 dissolved in the sediment were extracted immediately into the N_2 gas phase by vigorous shaking. The volumes of the sediment or sludge (V_S) and of the gas phase (V_G) were recorded (step c). A cylinder filled with water was connected to the extraction flask to put a slight overpressure on the gas phase, and gas samples were taken with air-tight syringes and transferred into a flask completely filled with a saturated Na_2SO_4 solution containing 100 μg of $HgCl_2$ per ml (steps d and e). The extracted gas was transported into the laboratory as a gas bubble. (step f). There, the gas bubble was analyzed for its mixing ratio (m) of H_2 (step d). The concentration of H_2 dissolved in the sediment (C_{H_2}) was calculated from the mixing ratio by using the following formula: $C_{H_2} = m(V_G/V_S)$ (equation 1).

The molar concentrations of H_2 were calculated from the concentrations measured as microliters of H_2 per liter of sample and the molar volume of an ideal gas at the in situ temperature and were corrected for the dry matter content of the sample. The same procedure was used to determine the CH_4 concentration. The efficiency of the extraction procedure was checked after equilibration of the sediment with a standard H_2 gas mixture. Because of the low Bunsen solubility coefficient for H_2 in water (≦0.02), the small amounts of H_2 (<2%) that remained dissolved in the sediment and were not extracted into the gas phase were insignificant compared with the overall accuracy of the extraction and analysis procedure and thus were neglected. Tests showed that gas bubbles could be stored for at least 2 days (step f) without significant change of the H_2 mixing ratio (reproducibility, ±5%). However, dramatic changes occurred if the extracted gas was kept in contact with sediment or sludge during transport to the laboratory (e.g., transport at step c). Addition of NaOH (final concentration, 0.5 N) or glutaraldehyde (final concentration, 2.5%) to the sediment to destroy biological activity during the extraction and storage at step c resulted in an increase in H_2 concentration. Hence, it was absolutely necessary to separate the gas phase from the sediment immediately after extraction.

The H_2 mixing ratio was analyzed in an H_2 analyzer based on the HgO-to-Hg vapor conversion technique (23, 24), which has a lower detection limit of 10 parts of H_2 per billion (10 nl of H_2 per liter), allowing the detection of approximately 10 nl of dissolved H_2 per liter of sediment. The precision of the extraction and H_2 analysis was within ±10%. CH_4 either was analyzed in a gas chromatograph equipped with a flame ionization detector or was detected as a second peak in the chromatogram of the H_2 analyzer. The lower detection limits were 0.002 and 0.02% (vol/vol), respectively, allowing the detection of approximately 0.02 to 0.2 ml of dissolved CH_4 per liter of sediment.

Measurement of H_2 turnover rate constants. The procedure used to determine H_2 turnover rates is shown in Fig. 2. Samples were transferred by syringe into a 160-ml serum bottle closed with a black rubber stopper that contained a glass tube. The glass tube was connected to butyl rubber tubing which was closed by a clamp. The empty bottles were evacuated and flushed several times with N_2 and were kept anaerobic under an overpressure of N_2 (approximately 50 kPa). The bottles were almost filled with sediment or sludge, clamped, and transported on ice to the laboratory (steps a through c). Then approximately 20 ml of N_2 containing a calculated amount of H_2 was injected into the bottles, replacing the sediment by the gas headspace (step d). The bottles were vigorously shaken to equilibrate the H_2 between the gas and the aqueous phase (step 3). Then the sediment or sludge (approximately 60 ml) was transferred into a glass syringe (300 ml) with a ground glass barrel by applying N_2 pressure to the headspace of the bottle (step f). The syringe containing the sediment or sludge was then incubated in a water bath kept at the in situ temperature recorded in the field (step g). Steps d through g typically took less than 4 min. Subsamples (2 ml) of sediment or sludge were taken from the big syringe by using a small 10-ml plastic syringe (step h). After the subsample was removed, 8 ml of H_2-free air was sucked into the syringe (step i), and the H_2 dissolved in the sediment or sludge was extracted into the air by vigorous shaking. The gas phase of the syringe was then transferred into a flask that was completely filled with water (step k) to remove the sediment or sludge. The H_2 mixing ratio of this gas bubble was then immediately analyzed (step l). Steps h through l typically took less than 3 min. By

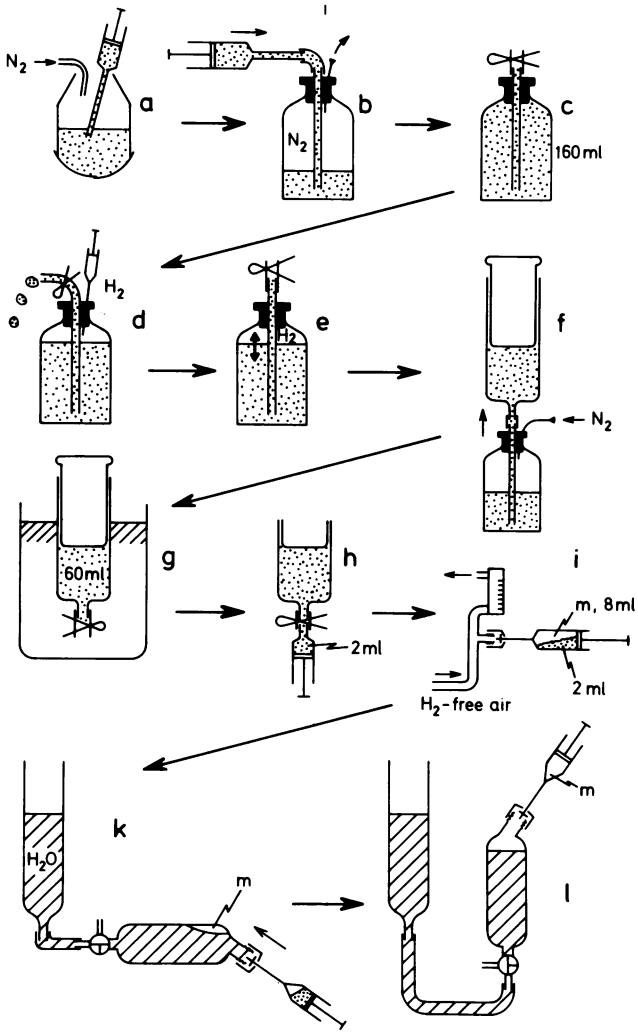

FIG. 2. Schematic diagram of the procedure used to measure the kinetics of dissolved H_2 turnover in anoxic sewage and sediments. Our procedures avoided experimental use of gaseous headspace and gas phase transfer limitations. See text for details.

overlapping samples and extraction and analysis times, it was possible to obtain time point resolutions of 0.5 to 1 min. The H_2 concentration in the sediment was four times the mixing ratio in the gas phase when the small amount of nonextracted H_2 was neglected. The H_2 turnover rate constant was calculated from the time course of the logarithmic decrease in dissolved H_2 level (precision, ≤15%).

Determination of CH_4 production. Sediment samples (20 ml) from Lake Mendota were transferred into 160-ml serum bottles that were flushed with N_2 and sealed with black rubber stoppers. The headspaces of the bottles were pressurized with N_2 to a total pressure of 130 kPa. The bottles were incubated in duplicate at the in situ temperature for 5 days. During this period, 400-μl gas samples were taken with a gas-tight pressure lock syringe twice a day and analyzed for methane in a gas chromatograph equipped with a flame ionization detector. The CH_4 production rate was calculated from the linear increase in the CH_4 concentration in the headspace of the bottles. This increase was linear for at least 2.5 days, and no lag phase was observed. The CH_4 production rate in the sewage sludge was determined by the same procedure, and these results were compared with data on gas production, gas composition, and digestor volume provided by the municipal sewage treatment plant in Madison. These latter values were within 20% of the CH_4 production values determined in our laboratory.

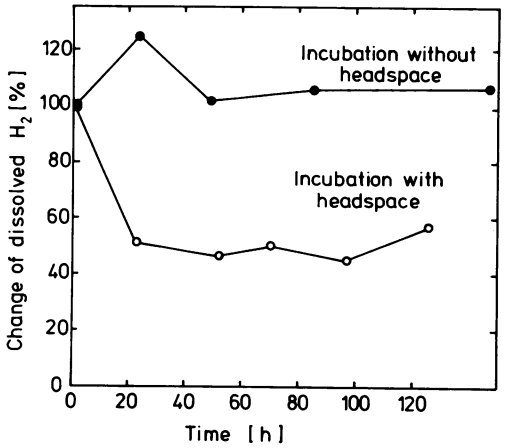

FIG. 3. Comparison of dissolved H_2 changes in experimental sediment samples incubated in the presence and absence of a headspace. Sediment incubation at in situ temperatures took place in 160-ml serum vials that were flushed with N_2 and contained either 20 ml of sediment (with headspace) or 160 ml of sediment (without headspace). The dissolved H_2 concentration (i.e., the in situ concentration) was measured immediately after sampling, and this value was set at 100%.

RESULTS

The average in situ concentrations of H_2 measured in five replicate samples of sewage sludge and lake sediment were 205 and 31 nM, respectively (Table 1). Preliminary experiments were performed to test the influence of incubation

TABLE 1. Comparison of dissolved H_2 concentrations and H_2 turnover rate constants in relation to methanogenesis parameters in sewage sludge and sediment[a]

Anoxic ecosystem	Dissolved H_2 concn (nM)	H_2 turnover rate constant (h^{-1})	H_2 production rate (μM/h)	CH_4 production rate (μM/h)	% of CH_4 originating from H_2	% of CH_4 originating from CO_2	Interspecies H_2 transfer rate for methanogenesis (μM/h)
Sludge	205 ± 62	100 ± 9	21	390	1.4	30[b]	450
Sediment	31 ± 13	31 ± 6	1	15	1.7	21–35[c]	18

[a] Values were measured or calculated as described in the text for samples taken from the Nine Springs Anaerobic Digestor and from Lake Mendota, Madison, Wis. (depth, 24 m).

[b] Data from studies by Boone (1), Mountfort and Asher (17), and Smith and Mah (25).

[c] Data from studies by Phelps and Zeikus (in press) and Winfrey and Zeikus (30).

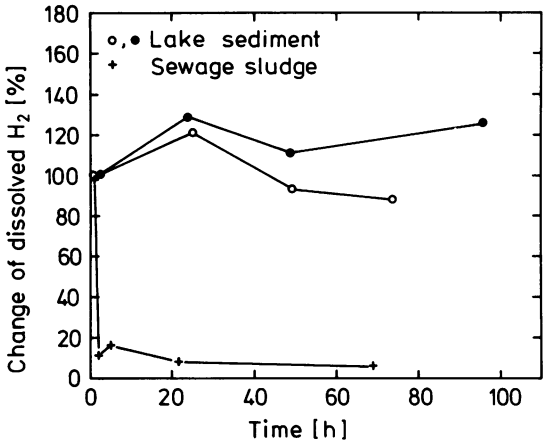

FIG. 4. Comparison of dissolved H_2 concentration changes in representative sludge and lake sediment samples. Incubation of samples at in situ temperatures was performed without a headspace, as described in the legend to Fig. 3.

conditions on the steady-state H_2 pool size in sewage and sludge samples. Figure 3 shows that the H_2 pool size dropped to a lower steady-state concentration when the sediment sample was incubated with an N_2 headspace (sediment to headspace ratio, 1:8), compared with no significant change when incubation was without the use of a headspace. The former result was probably a consequence of the fact that the headspace constituted a much larger reservoir for H_2 than the water phase because of the low Bunsen solubility coefficient (0.02 at 8°C). For these reasons, the remaining experiments were performed without a headspace.

Figure 4 shows the relationship between incubation time and change in the dissolved hydrogen concentration in sediment and sludge samples. The concentration of dissolved hydrogen in sewage sludge dropped to 10% of the in situ value after 1 to 5 h. On the other hand, the dissolved hydrogen concentration in Lake Mendota sediment stayed within 90 to 120% of the initial value for 100 h. The difference in these two anoxic ecosystems is most probably a reflection of the quantity and quality of the organic electron donors and the actual carbon and electron flow patterns in an anaerobic digestor compared with a lake sediment. These results demonstrate the necessity of immediate sample processing at the field site to measure H_2 concentrations that approximate in situ conditions.

Figure 5 shows the consumption kinetics of exogenous hydrogen additions in samples incubated without a headspace at in situ temperatures. At saturating H_2 concentrations, the H_2 concentration decreased linearly with time, indicating zero-order kinetics and giving the V_{max} of H_2 consumption. When H_2 utilization was followed with time to lower H_2 concentrations, the H_2 decrease shifted from a linear rate to a logarithmic rate, indicating first-order kinetics. The logarithmic rate of H_2 decrease was the H_2 turnover rate constant (K_t), which was 103 and 31 h^{-1} for sludge and sediment, respectively. When the H_2 concentration had decreased so much in sewage sludge that it approached the in situ concentration, the rate of H_2 decrease gradually became smaller and finally reached zero change. This observation indicates that the in situ concentration of the H_2 pool (C_{H_2} was a steady-state concentration which resulted from simultaneous production and consumption of H_2. The rate of H_2 production (P) could be calculated from the following equation: $P = C_{H_2} K_t$ (equation 2). For steady-state conditions, the H_2 production rate must be equal to the H_2 consumption rate or equivalent to the turnover rate of the H_2 pool at in situ H_2 concentrations.

Table 1 shows the measured rates of methane production, the in situ concentrations of dissolved H_2 and the H_2 turnover rate constants with the calculated rates of H_2 production, the amounts of methane originating from H_2, the amounts of CH_4 originating from CO_2 reduction, and the interspecies H_2 transfer rates in sewage sludge and lake sediment. The higher values for in situ H_2 concentration, H_2 turnover rate, H_2 production rate, and methane production rate in sewage sludge than in lake sediment are indicative of the higher turnover and anaerobic mineralization of organic matter in sewage. The percentage of methane which was derived from hydrogen was calculated by using the following stoichiometric relationship: $4H_2 + CO_2 = CH_4 + 2H_2O$ (equation 3).

As a result, only about 1.5% of the total methane produced in either sewage or sediment could have originated from the H_2 consumed out of the common H_2 pool (i.e., the measured dissolved H_2 concentration). The amount of methane produced from H_2-CO_2 via equation 3 was estimated from the

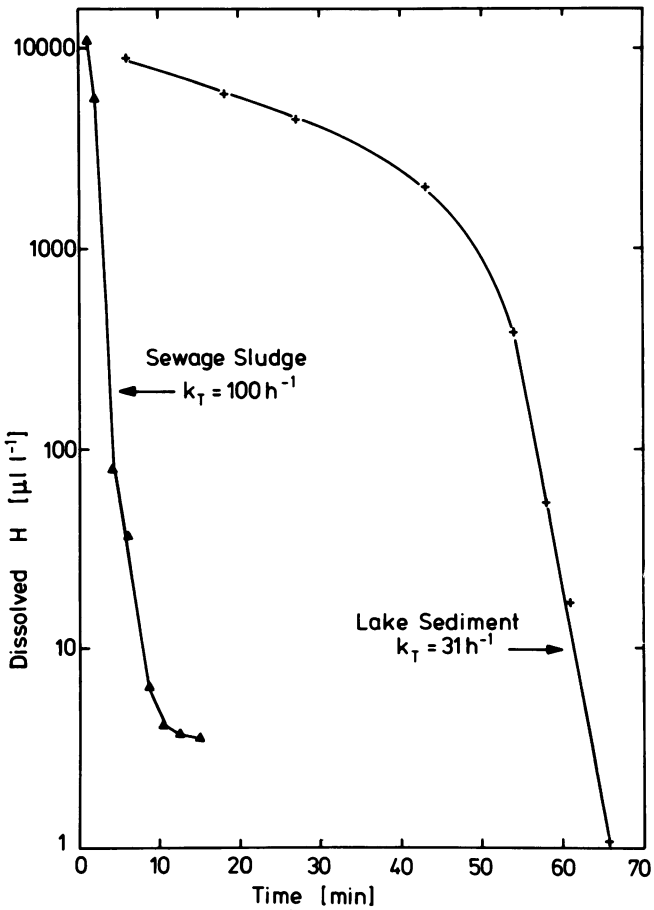

FIG. 5. Comparison of dissolved H_2 consumption kinetics in sewage sludge and lake sediment. Sediments were incubated without a headspace at the in situ temperature by using the procedures described in the legend to Fig. 2. At rate-limiting H_2 concentrations, the H_2 concentration decreased logarithmically, indicating a first-order reaction whose constant was taken as the H_2 turnover rate constant (K_t).

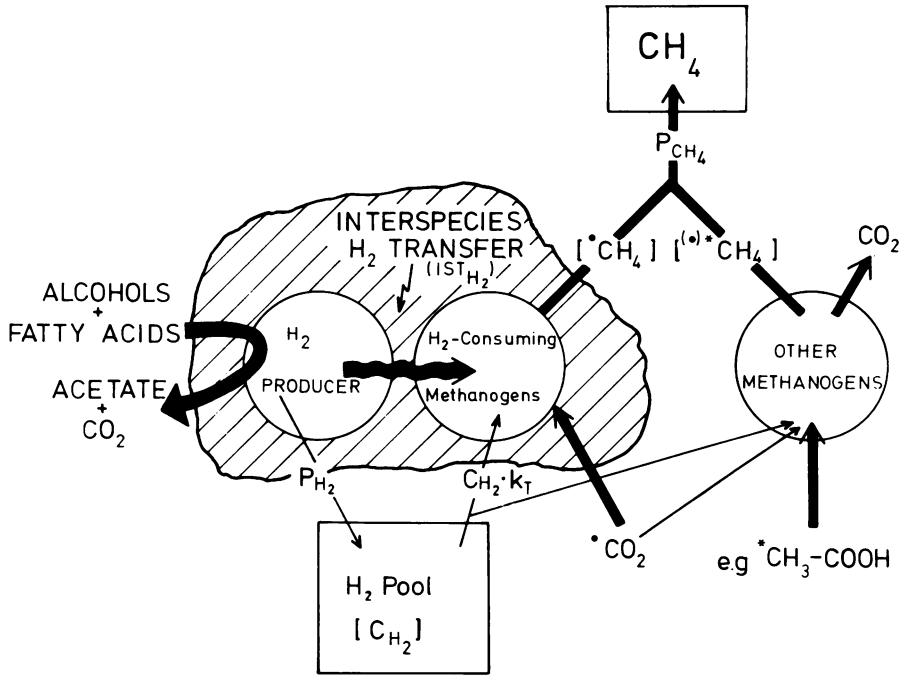

FIG. 6. Carbon and electron flow diagram showing the importance of juxtapositioning of H_2 producers and methanogens in nature. The fact that only 5 to 6% of the total expected methane formed in sewage or sludge samples can be accounted for by the free pool of dissolved H_2 suggests that syntrophic partners are spatially located in a matrix or floc wihich limits diffusion of hydrogen into the aqueous phase as a consequence of close spatial localization of H_2 producers with methanogens which consume the H_2 before it leaves the matrix.

amount $^{14}CH_4$ produced in experiments that contained $^{14}CH_4$. When these procedures were used, approximately 30% of the total CH_4 production observed in sewage (1, 17, 25) or Lake Mendota sediment (30; Phelps and Zeikus, submitted for publication) was from CO_2 reduction by hydrogen. Thus, only 5 to 6% of the methane derived from H_2-CO_2 could have been due to H_2 consumption from the common H_2 pool. Therefore, we suggest that the rest of the H_2-CO_2-derived CH_4 (94 to 95%) originated as a result of interspecies H_2 transfer between juxtapositioned syntrophic partners associated within microbial consortia or flocs. The interspecies H_2 transfer rate was determined by subtracting the calculated hydrogen production rate from the amount of H_2 production needed to account for methanogenesis from H_2-CO_2. The interspecies H_2 transfer rates which we calculated are much higher than the calculated H_2 production rate in either sewage sludge or lake sediment.

DISCUSSION

Figure 6 shows a schematic diagram which summarizes the interpretive conclusions of this study on H_2 metabolism and methanogenesis. In well-studied anaerobic ecosystems, such as sewage sludge digestors, microbial associations or consortia develop as flocs of different species embedded within an exopolymer matrix (8, 31, 34, 35). Juxtapositioning of H_2 producers and methanogens in sludge and sediment was supported by comparing the turnover of the common H_2 pool with the rate of H_2-CO_2-derived CH_4 production. In contrast to the expected contribution of 30%, the H_2 turnover rates accounted for only 5 to 6% of the methane production rates. Therefore, we assume that the rest of the H_2 was directly transferred between syntrophic associations of H_2 producers and methanogens that were juxtapositioned within a floc or consortium. In this manner, most of the H_2 produced did not equilibrate with the common pool of H_2 (i.e., the measured dissolved H_2 concentration); rather, it was directly consumed by the adjacent methanogens before it diffused out of the floc matrix. Therefore, we conclude that the juxtapositioning during interspecies H_2 transfer is of great importance for explaining methanogenesis in nature. Other studies (J. Thiele, M. Chartrain, and J. G. Zeikus, submitted for publication) on the H_2 metabolism in flocs derived from a whey-processing methane digestor have provided direct physical and microscopic evidence for juxtapositioning during interspecies H_2 transfer that supports the gas analysis approach described here.

We also describe new methods for measuring in situ H_2 pool concentrations in anoxic environments and for determining H_2 consumption kinetics. Until now, measurements of in situ H_2 concentrations have almost been lacking. This is mainly because the low concentration of H_2 present are below the detection limit of the analytical equipment usually used (26, 30). Recently, steady-state concentrations of headspace H_2 were determined in sewage and lake sediments by new techniques (12, 18, 19). However, in situ concentrations of dissolved H_2 have so far been similarly reported only in the anaerobic hypolimnium of a eutrophic lake (6) and in the anoxic water of a salt pond (22). In previous studies on H_2 uptake in anoxic exosystems, a headspace analysis was performed (11, 12, 14, 18, 20, 27). However, the technique used is sensitive to limitation of H_2 transfer from the gas phase to the aqueous phase and may result in an underestimate of the H_2 turnover rate constant. The new methods described in this paper overcome these limitations and, in addition, have the advantage of a higher sensitivity compared with headspace analysis because of the low Bunsen solubility coefficient of H_2 in water.

The in situ concentrations of the H_2 pool in sewage and Lake Mendota sediments were 30 and 200 nM (1.5 and 30 Pa), respectively. These concentrations are the result of

simultaneous H_2 consumption and production reactions which are in steady state under in situ conditions. The ratio of production to consumption was higher in sewage than in lake sediments. Our data compare well with other values reported for steady-state H_2 concentrations measured during incubation of a eutrophic lake sediment (12) and sewage sludge (19). To explain the higher H_2 concentrations in sewage than in sediment, we suggest that H_2-producing activities may increase more with increased input of degradable organic matter than H_2-consuming activities, thus resulting in increased steady-state concentrations of H_2.

H_2-producing reactions are either exergonic or endergonic when examined under standard chemical conditions (28, 33, 35). Examples of endergonic H_2-producing reactions are fermentations of ethanol, propionate, and other fatty acids by the so-called obligate proton-reducing bacteria to acetate, CO_2 and, H_2 (2–4, 15, 16, 28). The latter reactions become exergonic and thus thermodynamically possible in anoxic ecosystems as soon as the H_2 partial pressure drops below a particular value (34). High concentrations of substrates such as alcohols and fatty acids, high pH values, and low concentrations of the products acetate and CO_2 allow a higher H_2 partial pressure than vice versa. Therefore, we may hypothesize that the relatively high H_2 partial pressure in sewage is due to the relatively high contents of alcohols and fatty acids compared with the Lake Mendota sediments (8, 21; Phelps and Zeikus, submitted for publication). The H_2 partial pressures (1.4 to 30 Pa) which have been measured in Lake Mendota sediments and sewage are low enough to allow the fermentation of alcohols and fatty acids, but growth of propionate oxidizers would be limited by these relatively high H_2 partial pressures. However, it is possible that obligate proton-reducing propionate oxidizers are protected from the high H_2 concentration of the common H_2 pool by living in microniches provided by juxtapositioning of H_2 consumers which keep a very low H_2 concentration in the microniche. Thus, biopolymer floc formation in consortia may proceed parallel to juxtapositioning of different species from various trophic groups.

The importance of the H_2 transfer between juxtapositioned partners within sewage sludge flocs is also inferred from the observation that the common H_2 pool concentration (i.e., dissolved H_2 concentration) dropped rapidly during the incubation of sewage sludge, whereas the rate of methanogenesis stayed constant. Apparently, overall methanogenesis is not as dependent on the common H_2 pool. Although the excess hydrogen of the common H_2 pool is used up when the sample is removed from the steady-state digestion system, this excess H_2 may be a result of electron donor-driven hydrogen production capacity which is not associated with syntrophic H_2 transfer interactions. In lake sediment, the H_2 pool size stayed constant for several days as long as the sample was incubated without a headspace, indicating a balance between hydrogen-producing and -consuming reactions, perhaps as a consequence of available electron donors or more balance between syntrophic H_2 transfer interactions or both. It will be interesting to understand more completely the microbial basis of syntrophic H_2 transfer interactions in these anoxic ecosystems, including the role of different bacteria in consortia and the exact basis for competition between different types of H_2-consuming bacteria.

The importance of juxtapositioning is also in agreement with recent observations of Van Bruggen et al. (29). These authors observed that there are as many symbiotic methanogens inside sapropelic protozoa cells as there are free-living methanogens in the sediment. As they suggest, it might well be that a large part of the methane of a sediment is produced by methanogens which use the H_2 originating inside the protozoan cells.

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