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Association of Hydrogen Metabolism with Methanogenesis in Lake Mendota Sediments

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Lake Mendota sediments were studied to determine the role of H_2 in sediment methanogenesis. H_2 was generally not detectable in sediment. The addition of H_2 to sediment significantly increased methanogenesis. The amount of methane produced was proportional to the concentration of hydrogen added. H_2 addition stimulated the reduction of CO_2 to methane, but did not significantly stimulate the conversion of methanol or the methyl position of acetate to methane. Various organic compounds also stimulated sediment methanogenesis. Formate, ethanol, and glucose were shown to serve as electron donors for CO_2 reduction to methane. The addition of formate to sediment resulted in H_2 evolution. H_2 was not detectable after the addition of ethanol or glucose, which is consistent with the phenomenon of interspecies hydrogen transfer. The results indicate that hydrogen is an important intermediate and a rate-limiting factor in sediment methanogenesis.

Methane production in aquatic sediments is a common event (1, 7, 13, 14, 18), yet little is known about the means by which precursors are converted to methane in these environments. Hydrogen gas is utilized in the reduction of CO_2 to CH_4 by all pure cultures of methanogenic bacteria (20). The importance of this substrate in nature, however, is little understood. In anaerobic environments where methanogenesis occurs, H_2 is rarely detected (1, 7, 8, 11, 13), although large numbers of hydrogen-producing organisms are present (18, 24, 25).

It has been proposed that methane bacteria play a key role as terminal organisms in anaerobic food chains (20, 21). Methanogens keep the partial pressure of hydrogen extremely low and thus allow otherwise thermodynamically unfavorable reactions to occur. Metabolic studies coupling methanogens and hydrogen-producing bacteria (3, 17, 21) allow growth of methanogenic bacteria in the absence of detectable hydrogen. From these studies, the concept of interspecies hydrogen transfer was proposed, whereby molecular hydrogen is thought to pass from hydrogen-producing organisms to methanogens. Bell et al. (2) gave further evidence for this phenomenon by showing that hydrogenase is located in the periplasmic space of *Desulfovibrio gigas*. They suggested that it may function as a hydrogen-binding protein required for the transfer of low levels of hydrogen between microorganisms.

Some evidence for the importance of hydrogen during methanogenesis in nature has been given. Hungate (11) showed that methanogenesis in the rumen is proportional to the dissolved H_2 content. Recently, Oremland (16) showed that methane production in marine sediments incubated with 70% H_2 was stimulated.

The conversion of organic compounds to methane is little understood. Cappenberg (4) showed that formate, acetate, and lactate stimulate methanogenesis in lake sediments. Acetate has been calculated to be the major methane precursor in sediments (6, 13) and sewage sludge (12, 19), accounting for about 70% of the methane produced. We report the effect of hydrogen on methanogenesis in Lake Mendota sediments and the importance of hydrogen in the conversion of precursors to methane.

MATERIALS AND METHODS

Sediment sampling procedures. All sediment samples used in this investigation were collected with an Eckman dredge from a site located under 18 m of water. The 18-m site was chosen because previous studies (23) showed that, with respect to temperature, rates of methanogenesis, and numbers of methanogens throughout the year, it had the greatest methanogenic activity and less variation than shallower sites. Sediment was collected throughout the year (March 1975–May 1976) and stored in glass bottles under strict anaerobic conditions as previously described (23).

Although the sediment was briefly exposed (less than 2 min) to O_2 during sampling, control experiments in which sediment was collected using a Jenkins core sampler or a sealed Martex bottom sampler indicated that this brief exposure to O_2 did not

effect methanogenic activity. Furthermore, sediment exposed for up to 1 h to air in the laboratory had no effect on methanogenesis. This was attributed to the small surface area of sediment exposed compared to the volume and the high reducing capacity of the sediment. Although dredge samples varied in size, in situ temperature, and season collected, the same patterns were observed in experiments from different samples.

Preparation of samples. All experiments were carried out in duplicate in anaerobic tubes (18 by 142 mm, Bellco Glass, Inc.) containing 10 ml of sediment and various gas phases. All manipulations were performed anaerobically using a modification of the Hungate technique (23). Sediment was added to each tube with a glass syringe (10 cc) while being gassed with ultrapure N_2 (approximately 600 cc/min). All additions were made anaerobically, and the volume of each addition was less than 1% of the sediment volume to prevent significant dilution of the sediment. One molar solutions of various organic compounds were prepared and stored in N_2-gassed serum vials, and appropriate volumes were added to the sediment to give the desired final concentration. Radioactive compounds were dissolved in water to give concentrations of approximately 100 μCi/ml and stored in N_2-gassed serum vials. The highest specific activities available were used to minimize the molar quantities of substrate added to the sediment. All tubes were vortexed for 1 min under a flow of N_2 gas to ensure complete mixing of additions and sealed with butyl rubber stoppers. Tubes were gassed for a total of 3 min. This, however, did not affect the pH of the sediment. H_2 was added to sealed tubes with glass syringes. Tubes were then vortexed to allow mixing of the H_2 throughout the sediment. All tubes were incubated at 10°C, as this temperature approximated the in situ temperature (4 to 12°C) throughout the sampling period. All experiments were repeated a minimum of three times.

Detection of gaseous end products. Experimental tubes were sampled at intervals for evolved gases. Gas samples (0.4 cc) were removed from the head space of tubes with a pressure-lock syringe (1 cc) flushed with O_2-free N_2. The gas chromatography-gas proportional counting system described by Nelson and Zeikus (15) was used to detect H_2, CH_4, $^{14}CH_4$, CO_2, and $^{14}CO_2$. The efficiency of the proportion counter was 80%. CO_2 values were corrected for bicarbonate equilibrium and dissolved CO_2. Routine methane analysis was performed on a Varian aerograph model 600-D gas chromatograph as described by Zeikus and Winfrey (23).

Trace amounts of H_2 present in sediment were detected by the following procedure. A 10-cc portion of freshly collected sediment was immediately transferred to anaerobic tubes in quadruplicate (gassed with N_2 for 15 s) and sealed with a butyl rubber stopper. Tubes were vigorously vortexed for 2 min, and 0.4 cc of the gas phase was removed for analysis. H_2 was quantified on a Packard 419 gas chromatograph containing a stainless-steel column (4 ft by one-eighth in [inside diameter]) packed with Carbosieve B. N_2 carrier gas (60 cc/min) and a thermal conductivity detector were used for maximum H_2 sensitivity. The limit of detection was 0.5×10^{-6} M.

Chemicals and radioactive compounds. All chemicals were reagent grade. The following radioactive compounds (Amersham/Searle Corp.) were used in this investigation: $NaH^{14}CO_3$ (specific activity, 60 mCi/mmol), [1-^{14}C]sodium acetate (58 mCi/mmol, [2-^{14}C]sodium acetate (58 mCi/mmol, [^{14}C]methanol (60 mCi/mmol), and [^{14}C]sodium formate (51 mCi/mmol).

RESULTS

Effect of hydrogen on methanogenesis. To test the effect of H_2 on sediment methanogenesis, varying concentrations of H_2 were added to tubes of sediment. Table 1 shows that methanogenesis was greatly stimulated by H_2 additions and that the rate of methanogenesis was proportional to the amount of hydrogen in the tubes. These results and the following data presented are from one experiment that is representative of replicate experiments. Methanogenic rates varied slightly in different experiments, yet the same pattern was exhibited in all replicate experiments. The amount of stimulation ranged from a 4-fold increase in methanogenesis for 29 μmol of H_2 to a 12-fold increase for 474 μmol of H_2. Depletion of H_2 was monitored in tubes containing 29 μmol of H_2 (Fig. 1). Stimulation of methanogenesis corresponded to a decrease of hydrogen in the head space overlying the sediment. After the H_2 was depleted, the rate of methanogenesis returned to the endogenous level. The molar ratio of hydrogen consumed to methane produced ranged from 5:1 to 10:1 ($n = 11$, $\bar{x} = 7.8 \pm 29\%$ s). This ratio is significantly higher than the theoretical ratio of 4:1 for the reduction of CO_2 to CH_4 by H_2 in pure cultures of methanogenic bacteria (20).

As hydrogen was not detected in gases evolved from Lake Mendota sediment (7), more sensitive gas stripping techniques were used to quantify dissolved hydrogen present in the sediment. In seven separate sediment samples, H_2 was detected at a concentration of 3×10^{-6} M ($\pm$ 30% s) in only one sample.

Effect of organic compounds on methano-

TABLE 1. *Effect of varying H_2 concentrations on methanogenesis in Lake Mendota sediments*

H_2 in gas phase (μmol)	Rate of methanogenesis (nmol/h)
0.0	4.4
29.0	16.7
59.0	23.5
148.0	28.0
296.0	36.7
474.0	52.5

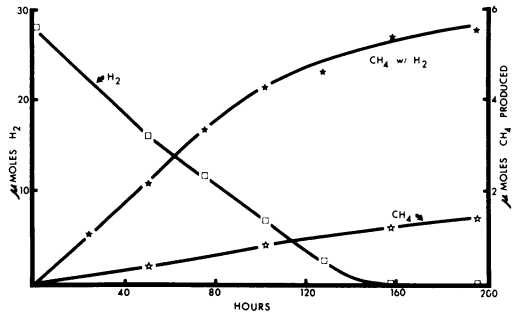

FIG. 1. *H_2 depletion and methane production in Lake Mendota sediment. Symbols: □, H_2; ☆, CH_4 produced with an N_2 atmosphere; ★, CH_4 produced with 29 μmol of H_2.*

genesis. To test the effect of organic compounds on methanogenesis, 50-μmol portions of various organic compounds were added to sediment to give a final concentration of 5 mM. The amount of methane produced was monitored with time. This was compared to sediment having no additions and sediment with 50 μmol of H_2 added. The results are shown in Fig. 2. Initially, formate gave the most rapid rate of methanogenesis, even greater than that from H_2 additions. The stimulation by H_2, however, may be limited by the rate of diffusion of H_2 into the sediment from the gas phase. Ethanol and acetate stimulated methanogenesis six- and threefold, respectively. The greatest stimulation was seen from the addition of 5 mM glucose, although there was a lag of 48 h before a significant stimulation in methanogenesis occurred. This may be indicative of the time required for glucose to be degraded to substrates usable by methanogenic bacteria or an enrichment lag for glucose oxidizers. The addition of 5 mM carbonate showed no stimulation of methanogenesis over the control, and 5 mM methanol did not significantly stimulate methanogenesis over the course of the experiment. However, methanol addition significantly stimulated methanogenesis at incubation times longer than those reported here.

Effect of H_2 on the conversion of methanogenic precursors to methane. Isotopic experiments were conducted to test the utilization of CO_2, acetate, formate, and methanol as methanogenic substrates and the effect of H_2 on their conversion to methane. ^{14}C-labeled compounds (1.25×10^6 dpm) were added to sediment in the presence and absence of 118 μmol of H_2, and evolved $^{14}CH_4$ and $^{14}CO_2$ were monitored with time.

The conversion of $^{14}CO_2$ to $^{14}CH_4$ occurs at a low but constant rate in sediments (Fig. 3A). This slow rate is influenced by a large dilution of the $H^{14}CO_3^-$ because Lake Mendota sediments are saturated with carbonate. The addition of 118 μmol of H_2 caused a 12-fold stimulation of $^{14}CO_2$ reduction to $^{14}CH_4$. $^{14}CO_2$ did not decrease significantly in the N_2 gas phase but showed marked decrease when H_2 was present.

It is important to note that CO_2 is turning over very slowly in Lake Mendota sediments because of the large size of the carbonate pool. A slow turnover is further evidenced by the insignificant decrease in $^{14}CO_2$ when $H^{14}CO_3^-$ was added to sediment (Fig. 3A). Thus, the evolution of $^{14}CO_2$ in the following experiments is not significantly affected by CO_2 turnover.

The addition of [^{14}C]formate (Fig. 3B) to sediments with an N_2 gas phase resulted in an immediate release of $^{14}CO_2$. This result indicates a rapid conversion of formate to CO_2. $^{14}CH_4$ was produced slowly but constantly with time. The addition of hydrogen greatly stimulated $^{14}CH_4$ production and enhanced depletion of $^{14}CO_2$.

Similar results were observed when [1-^{14}C]acetate was added to sediments (Fig. 3C). With an N_2 atmosphere, $^{14}CO_2$ is evolved rapidly, indicating that the carboxyl of acetate is released as CO_2. Small amounts of $^{14}CH_4$ are

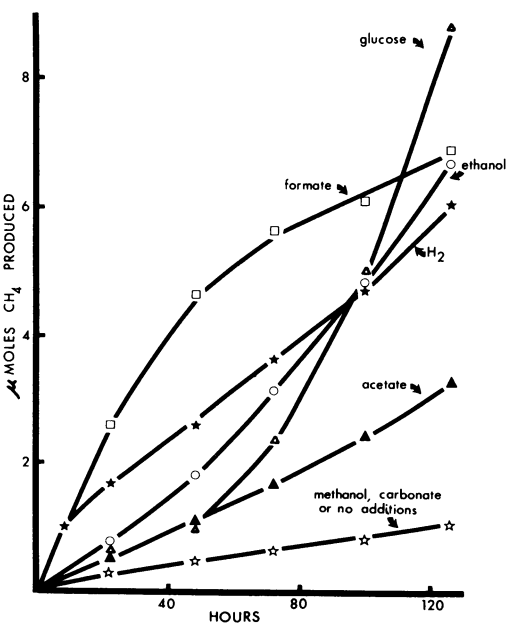

FIG. 2. *Effect of organic additions on methanogenesis in Lake Mendota sediments. Symbols: ☆, no additions of 5 mM methanol or 5 mM carbonate; ▲, 5 mM acetate; ○, 5 mM ethanol; □, 5 mM formate; △, 5 mM glucose; ★, 50 μmol of H_2.*

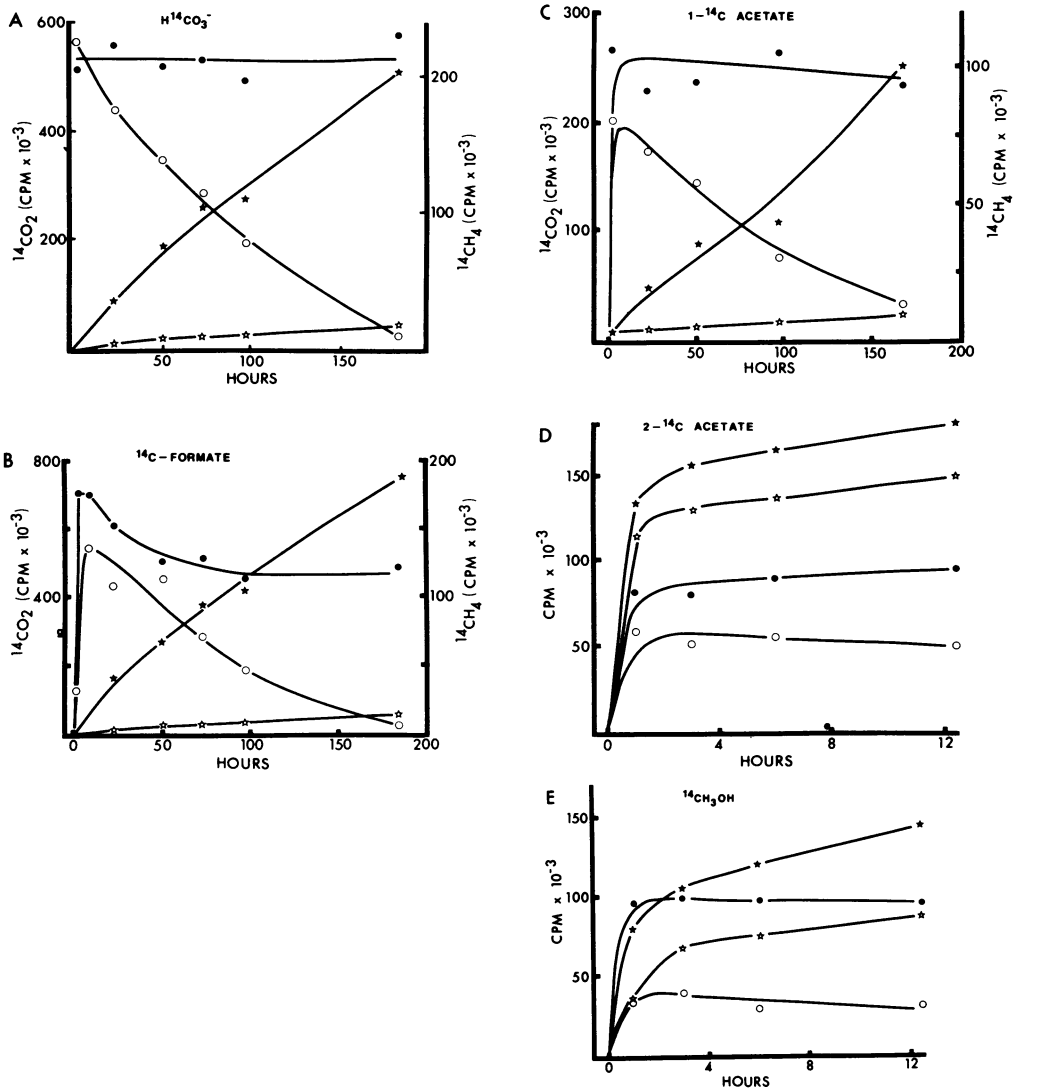

FIG. 3. *Effect of H_2 on the conversion of ^{14}C-labeled compounds to methane in Lake Mendota sediment. (A) Addition of 1.25 × 10^6 cpm of $H^{14}CO_3^-$. (B) Addition of 1.25 × 10^6 dpm of [^{14}C]formate. (C) Addition of 10^6 dpm of [1-^{14}C]acetate. (D) Addition of 1.25 × 10^6 dpm of [2-^{14}C]acetate. (E) Addition of 1.25 × 10^6 dpm of [^{14}C]methanol. Symbols: ☆, $^{14}CH_4$ produced with an N_2 atmosphere; ★, $^{14}CH_4$ produced with 20% H_2 added; ●, $^{14}CO_2$ produced with an N_2 atmosphere; ○, $^{14}CO_2$ produced with 118 μmol of H_2 added. Time zero values were 0 for (B) through (E) as determined by killed controls.*

produced under N_2. Under an H_2 atmosphere, however, $^{14}CO_2$ is depleted and $^{14}CH_4$ evolution is stimulated.

Figure 3D shows the conversion of [2-^{14}C]acetate to $^{14}CH_4$ and $^{14}CO_2$ in Lake Mendota sediments. $^{14}CH_4$ and $^{14}CO_2$ were released very rapidly, most of the label being evolved in the first hour. The addition of H_2 resulted in a slight increase in $^{14}CH_4$ production and a decrease in $^{14}CO_2$. The addition of [^{14}C]methanol (Fig. 3E) also resulted in a rapid release of $^{14}CH_4$ and $^{14}CO_2$ in the first hour of incubation. The addition of 118 μmol of H_2 resulted in less $^{14}CO_2$ evolved and more $^{14}CH_4$ evolved than from sediment with an N_2 atmosphere. It is important to note that the relative amounts of $^{14}CO_2$ and $^{14}CH_4$ evolved from methanol and the methyl of acetate varied with different sediment samples. The $^{14}CO_2$ produced was generally less than $^{14}CH_4$ produced, but ranged from

20 to 175% of the $^{14}CH_4$. The results presented are representative data.

Effect of organic electron donors on CO_2 reduction to methane. To test the ability of various organic compounds to serve as electron donors during methanogenesis, 10 mM formate, 10 mM ethanol, 5 mM glucose, and 0.10 mmol of H_2 were added to tubes containing 1.25 $\times$ 10^6 dpm of $H^{14}CO_3^-$. Sediment containing $H^{14}CO_3^-$ alone was used as a control. Figure 4 shows that glucose, ethanol, and formate stimulated CO_2 reduction to methane, although the greatest stimulation came from H_2. Formate appeared to be the best organic electron donor initially, whereas glucose caused a marked stimulation of CO_2 reduction to CH_4 after a lag.

To detect possible hydrogen evolution from the above electron donors, H_2 in the gas phase of reaction tubes was monitored with time (Fig. 5). The addition of 5 mM formate resulted in a rapid release of hydrogen gas, which was soon depleted. No H_2 was detected in the gas phase of sediment containing no additions or sediment containing 5 mM ethanol or 5 mM glucose.

DISCUSSION

These results indicate that hydrogen metabolism is an important factor in methanogenesis from Lake Mendota sediments. Hydrogen was undetectable or present only in extremely small amounts in sediment samples. The addition of hydrogen caused an immediate stimulation of methanogenesis. The amount of stimulation was proportional to the concentration of H_2 added. When added hydrogen was depleted in sediments, the rate of methanogenesis returned to the endogenous level.

Methane bacteria are believed to maintain low hydrogen concentrations in anaerobic environments by rapidly converting it to methane (20, 21). The theoretical ratio of hydrogen uptake to methane produced from CO_2 is 4:1. However, in Lake Mendota 5 to 10 μmol of H_2 was consumed for every μmol of CH_4 produced. This may be indicative of hydrogen metabolism by non-methanogenic bacteria in lake sediments.

The addition of H_2 to sediments stimulated CO_2 reduction to methane and caused a 12-fold increase in $^{14}CH_4$ derived from $H^{14}CO_3^-$. H_2 also stimulated methane formation from the carboxyl of acetate and formate. The carboxyl groups, however, were rapidly released as CO_2 in sediments, indicating that the effect of H_2 was on the CO_2 produced from the carboxyl position rather than on the intact carboxyl of acetate or formate.

Hydrogen caused a slight stimulation of the conversion of methanol and the methyl of acetate to methane. This, however, was accompanied by a concomitant decrease in CO_2 evolved from these methyl groups. As H_2 was found to greatly stimulate the conversion of CO_2 to methane (Fig. 3A), this decrease was probably due to an increase in $^{14}CO_2$ conversion to $^{14}CH_4$. Therefore, the increase in $^{14}CH_4$ evolved from [^{14}C]methanol or [2-^{14}C]acetate was probably a result of stimulation of $^{14}CO_2$ conversion to methane and did not arise from the intact methyl positions. Thus, hydrogen addition does not appear to cause a significant stimulation of methanol or acetate conversion to methane.

Stimulation of methanogenesis by various organic compounds indicate that they may play an important role in sediment methanogenesis. Acetate additions caused a significant stimulation of methane production; thus, acetate may be a factor that limits sediment methanogenesis. Isotopic studies revealed that the methyl position of acetate was rapidly converted to methane and CO_2. The formation of CO_2 from the methyl of acetate may be a result of methanogenic activity or metabolism of acetate by other sediment bacteria. This phenomenon was also observed by Cappenberg and Prins (6) in Lake Vechten sediments and by Zeikus et al. (22) in cultures of *Methanobacterium thermoautotrophicum* grown in the presence of H_2. Recently, Pfennig and Biebl (Arch. Mikrobiol.,

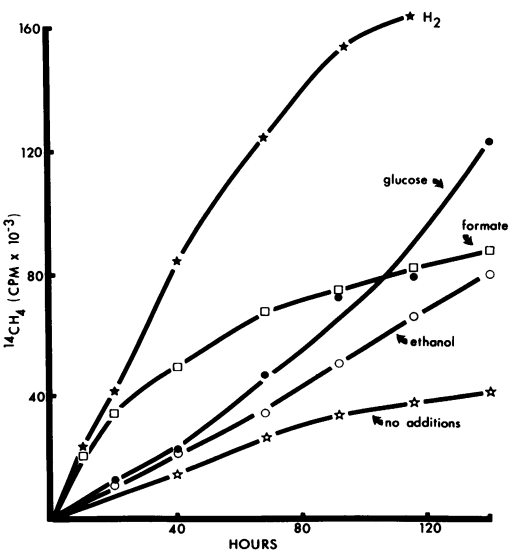

FIG. 4. *Effect of organic additions on the conversion of $H^{14}CO_3$ to methane in Lake Mendota sediment. Symbols: ☆, $^{14}CH_4$ produced with no additions; ○, $^{14}CH_4$ produced with 10 mM ethanol; □, $^{14}CH_4$ produced with 10 mM formate; ●, $^{14}CH_4$ produced with 10 mM glucose; ★, $^{14}CH_4$ produced with 100 μmol of H_2 added.*

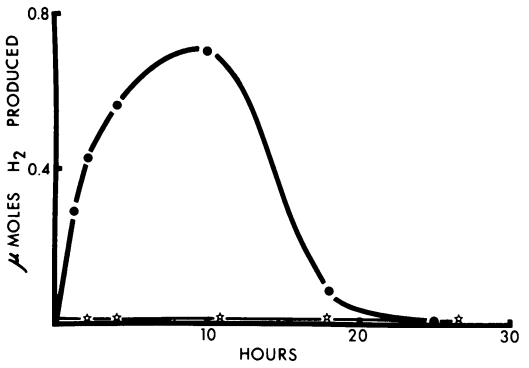

FIG. 5. *Evolution of H_2 from Lake Mendota sediment after organic additions. Symbols: ●, H_2 produced from 5 mM formate; ☆, H_2 produced from 10 mM glucose or 10 mM ethanol or by no additions.*

in press) described an obligate sulfur-reducing anaerobe that oxidizes acetate. The intact carboxyl of acetate does not appear to be a direct carbon precursor in methanogenesis because it was rapidly released as CO_2.

Cappenberg (4) showed stimulation of methanogenesis in Lake Vechten by formate, lactate, and acetate; the greatest stimulation came from acetate and the least from formate. Cappenberg, however, used long incubation times and did not examine early rates of methanogenesis. The results presented here indicate that formate initially stimulated methanogenesis to a greater extent than did other organic additions. However, methane prodution rapidly decreases due to rapid production and depletion of hydrogen. Formate was also shown to effect significant stimulation of CO_2 reduction to methane. This was due to the cleavage of formate to CO_2 and H_2 upon addition of formate to sediment.

The importance of methanol during methanogenesis in anaerobic environments has not yet been established, although it is generally not considered to be an important precursor (12). The addition of 5 mM methanol to sediments did not significantly stimulate methanogenesis over the endogenous level. This may be due to toxic effects, as [^{14}C]methanol was rapidly converted to methane and CO_2. Ethanol and glucose greatly stimulated methanogenesis. As these compounds are not directly metabolized by methanogenic bacteria, further trophic levels are involved in their mineralization. Although the degradation of organic compounds in sediments is ill defined, the amount of stimulation caused by added organics may be related to the amount of reducing equivalents (H_2) and carbon equivalents (acetate) derived from their degradation. Glucose caused the greatest stimulation of methanogenesis at long incubations. Glucose can potentially give rise to more carbon and reducing equivalents than can the other organic substrates tested. Both acetate and H_2 can be formed from ethanol degradation (3), which may explain its stimulatory effect. Acetate addition resulted in the least stimulation, probably because it can only supply a carbon precursor for methanogenesis.

The addition of glucose and ethanol resulted in a stimulation of $^{14}CO_2$ reduction to $^{14}CH_4$ in sediments. Hydrogen is required for this reduction of CO_2 to CH_4 by pure cultures of methanogens. Thus, it appears that these substrates are involved in H_2-producing reactions. H_2, however, was not detected in sediments after the addition of 5 mM glucose or ethanol. Thus, the hydrogen may be used immediately via interspecies hydrogen transfer (21) and not allowed to accumulate. It is important to note that ethanol may not be a significant intermediate in the degradation of sediment organic matter. In the rumen ecosystem, ethanol is not an important intermediate because electrons are preferentially channeled to methanogens rather than to other electron sink products such as lactate or ethanol (10).

The results presented here indicate that H_2 is a limiting factor in the reduction of CO_2 to methane in Lake Mendota sediments. Hydrogen concentrations in sediment were extremely low or undetectable, yet carbonate was abundant (23). Hydrogen produced by non-methanogenic organisms as a result of organic fermentations is probably used by methanogens and perhaps other hydrogen-oxidizing anaerobes before it accumulates in sediments. In addition, these data suggest that interspecies hydrogen transfer reactions described in mixed culture studies (3, 17, 21) may be operative in the sediment ecosystem. By necessity, several laboratory manipulations were required to perform these experiments. Therefore, the results presented here may not represent exact in situ activities. These data do, however, provide valuable information on the role of hydrogen in anaerobic environments.

Organic compounds such as acetate are also very important in sediment methanogenesis, although their relative importance was not investigated here. Studies are in progress to ascertain the importance of acetate and other organic substrates during methanogenesis in Lake Mendota sediments.

ACKNOWLEDGMENTS

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