Association of Hydrogen Metabolism with Methanogenesis in Lake Mendota Sediments

M. R. WINFREY, D. R. NELSON, S. C. KLEVICKIS, AND J. G. ZEIKUS*  

Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706

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Lake Mendota sediments were studied to determine the role of H₂ in sediment methanogenesis. H₂ was generally not detectable in sediment. The addition of H₂ to sediment significantly increased methanogenesis. The amount of methane produced was proportional to the concentration of hydrogen added. H₂ addition stimulated the reduction of CO₂ 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 CO₂ reduction to methane. The addition of formate to sediment resulted in H₂ evolution. H₂ 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 CO₂ to CH₄ 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, H₂ 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 H₂ content. Recently, Oremland (16) showed that methane production in marine sediments incubated with 70% H₂ was stimulated.

The conversion of organic compounds to methane is little understood. Capenberg (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 O₂ 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 O₂ did not

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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 N2 (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 N2-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 N2-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 N2 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. H2 was added to sealed tubes with glass syringes. Tubes were then vortexed to allow mixing of the H2 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 O2-free N2. The gas chromatography–gas proportional counting system described by Nelson and Zeikus (15) was used to detect H2, CH4, 14CH4, CO2, and 14CO2. The efficiency of the proportion counter was 80%. CO2 values were corrected for bicarbonate equilibrium and dissolved CO2. Routine methane analysis was performed on a Varian aerograph model 600-D gas chromatograph as described by Zeikus and Winfrey (23). Trace amounts of H2 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 N2 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. H2 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. N2 carrier gas (60 cc/min) and a thermal conductivity detector were used for maximum H2 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: NaH14CO3 (specific activity, 60 mCi/mmol), 1-14C)sodium acetate (58 mCi/mmol, 2-14C)sodium acetate (58 mCi/mmol, 14C)methanol (60 mCi/mmol), and 14C)sodium formate (51 mCi/mmol).

RESULTS

Effect of hydrogen on methanogenesis. To test the effect of H2 on sediment methanogenesis, varying concentrations of H2 were added to tubes of sediment. Table 1 shows that methanogenesis was greatly stimulated by H2 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 H2 to a 12-fold increase for 474 μmol of H2. Depletion of H2 was monitored in tubes containing 29 μmol of H2 (Fig. 1). Stimulation of methanogenesis corresponded to a decrease of hydrogen in the head space overlying the sediment. After the H2 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, x = 7.8 ± 29% s). This ratio is significantly higher than the theoretical ratio of 4:1 for the reduction of CO2 to CH4 by H2 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, H2 was detected at a concentration of 3 × 10^-6 M (± 30% s) in only one sample.

Effect of organic compounds on methanogenesis. The following compounds were added to the 10-cc portion of freshly collected sediment: 36.7 mmol of 14CO2 (58 mCi/mmol), 36.7 mmol of 14CH4 (58 mCi/mmol), 151.4 mmol of [1-14C]methanol (60 mCi/mmol), 151.4 mmol of 14C)ethanol (60 mCi/mmol), and 151.4 mmol of 14C)ethanol (60 mCi/mmol). The results are shown in Table 2.

Table 1. Effect of varying H2 concentrations on methanogenesis in Lake Mendota sediments

<table>
<thead>
<tr>
<th>H2 in gas phase (μmol)</th>
<th>Rate of methanogenesis (nmol/h)</th>
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<tbody>
<tr>
<td>0.0</td>
<td>4.4</td>
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<tr>
<td>29.0</td>
<td>16.7</td>
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<tr>
<td>59.0</td>
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<td>474.0</td>
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methanogenesis. 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 H2 added. The results are shown in Fig. 2. Initially, formate gave the most rapid rate of methanogenesis, even greater than that from H2 additions. The stimulation by H2, however, may be limited by the rate of diffusion of H2 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 H2 on the conversion of methanogenic precursors to methane. Isotopic experiments were conducted to test the utilization of CO2, acetate, formate, and methanol as methanogenic substrates and the effect of H2 on their conversion to methane. 14C-labeled compounds (1.25 × 10⁶ dpm) were added to sediment in the presence and absence of 118 μmol of H2, and evolved 14CH4 and 14CO2 were monitored with time.

The conversion of 14CO2 to 14CH4 occurs at a low but constant rate in sediments (Fig. 3A).

This slow rate is influenced by a large dilution of the H14CO2− because Lake Mendota sediments are saturated with carbonate. The addition of 118 μmol of H2 caused a 12-fold stimulation of 14CO2 reduction to 14CH4. 14CO2 did not decrease significantly in the N2 gas phase but showed marked decrease when H2 was present.

It is important to note that CO2 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 14CO2 when H14CO2− was added to sediment (Fig. 3A). Thus, the evolution of 14CO2 in the following experiments is not significantly affected by CO2 turnover.

The addition of [14C]formate (Fig. 3B) to sediments with an N2 gas phase resulted in an immediate release of 14CO2. This result indicates a rapid conversion of formate to CO2. 14CH4 was produced slowly but constantly with time. The addition of hydrogen greatly stimulated 14CH4 production and enhanced depletion of 14CO2.

Similar results were observed when [1-14C]acetate was added to sediments (Fig. 3C). With an N2 atmosphere, 14CO2 is evolved rapidly, indicating that the carboxyl of acetate is released as CO2. Small amounts of 14CH4 are

Fig. 1. H2 depletion and methane production in Lake Mendota sediment. Symbols: □, H2; ◊, CH4 produced with an N2 atmosphere; *, CH4 produced with 29 μmol of H2.

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; O, 5 mM ethanol; □, 5 mM formate; Δ, 5 mM glucose; ★, 50 μmol of H2.
produced under N₂. Under an H₂ atmosphere, however, ¹⁴CO₂ is depleted and ¹⁴CH₄ evolution is stimulated.

Figure 3D shows the conversion of [2-¹⁴C]acetate to ¹⁴CH₄ and ¹⁴CO₂ in Lake Mendota sediments. ¹⁴CH₄ and ¹⁴CO₂ were released very rapidly, mostly of the labeling evolved in the first hour. The addition of H₂ resulted in a slight increase in ¹⁴CH₄ production and a decrease in ¹⁴CO₂. The addition of [¹⁴C]methanol (Fig. 3E) also resulted in a rapid release of ¹⁴CH₄ and ¹⁴CO₂ in the first hour of incubation. The addition of 118 μmol of H₂ resulted in less ¹⁴CO₂ evolved and more ¹⁴CH₄ evolved than from sediment with an N₂ atmosphere. It is important to note that the relative amounts of ¹⁴CO₂ and ¹⁴CH₄ evolved from methanol and the methyl of acetate varied with different sediment samples. The ¹⁴CO₂ produced was generally less than ¹⁴CH₄ 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 x 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 [1$^4$C]methanol or [2-1$^4$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 thermophilum 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; O, $^{14}$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.](http://aem.asm.org/Downloaded from http://aem.asm.org/ on October 27, 2017 by guest)
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 CO₂.

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 production rapidly decreases due to rapid production and depletion of hydrogen. Formate was also shown to effect significant stimulation of CO₂ reduction to methane. This was due to the cleavage of formate to CO₂ and H₂ 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 [¹⁴C]methanol was rapidly converted to methane and CO₂. 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 (H₂) 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 H₂ 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 ¹⁴CO₂ reduction to ¹⁴CH₄ in sediments. Hydrogen is required for this reduction of CO₂ to CH₄ by pure cultures of methanogens. Thus, it appears that these substrates are involved in H₂-producing reactions. H₂, 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 H₂ is a limiting factor in the reduction of CO₂ 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.

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LITERATURE CITED


