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Kinetic Parameters of the Conversion of Methane Precursors to Methane in a Hypereutrophic Lake Sediment†

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The kinetic parameters K_m, V_{max}, T_t (turnover time), and v (natural velocity) were determined for H_2 and acetate conversion to methane by Wintergreen Lake sediment, using short-term (a few hours) methods and incubation temperatures of 10 to 14°C. Estimates of the Michaelis-Menten constant, K_m, for both the consumption of hydrogen and the conversion of hydrogen to methane by sediment microflora averaged about 0.024 μmol g^{-1} of dry sediment. The maximal velocity, V_{max}, averaged 4.8 μmol of H_2 g^{-1} h^{-1} for hydrogen consumption and 0.64 μmol of CH_4 g^{-1} h^{-1} for the conversion of hydrogen to methane during the winter. Estimated natural rates of hydrogen consumption and hydrogen conversion to methane could be calculated from the Michaelis-Menten equation and estimates of K_m, V_{max}, and the in situ dissolved-hydrogen concentration. These results indicate that methane may not be the only fate of hydrogen in the sediment. Among several potential hydrogen donors tested, only formate stimulated the rate of sediment methanogenesis. Formate conversion to methane was so rapid that an accurate estimate of kinetic parameters was not possible. Kinetic experiments using [2-^{14}C]acetate and sediments collected in the summer indicated that acetate was being converted to methane at or near the maximal rate. A minimum natural rate of acetate conversion to methane was estimated to be about 110 nmol of CH_4 g^{-1} h^{-1}, which was 66% of the V_{max} (163 nmol of CH_4 g^{-1} h^{-1}). A 15-min preincubation of sediment with 5.0×10^{-3} atm of hydrogen had a pronounced effect on the kinetic parameters for the conversion of acetate to methane. The acetate pool size, expressed as the term $K_m + S_n$ (S_n is in situ substrate concentration), decreased by 37% and T_t decreased by 43%. The V_{max} remained relatively constant. A preincubation with hydrogen also caused a 37% decrease in the amount of labeled carbon dioxide produced from the metabolism of [U-^{14}C]valine by sediment heterotrophs.

Anaerobic decomposition is an important but little studied route of carbon flow in aquatic ecosystems. Methane production becomes the terminal step when alternative electron acceptors are absent, which is probably the case in most eutrophic lakes. For Wintergreen Lake, the subject of this study, we have estimated that as much as one-third of the primary production could be recovered as methane over a summer season (Strayer and Tiedje, Limnol. Oceanogr., in press), and Molongoski and Klug (unpublished data), in a 2-year study, have evidence that at least 39% of the sedimenting carbon could be accounted for as methane. Thermodynamic considerations and reasoning from interspecies hydrogen transfer theory suggest that the terminal methanogenic step could be the key rate-controlling process for anaerobic decomposition—thus nutrient regeneration—and therefore should be a focal point for study.

Previous work with lake sediments has shown that the addition of hydrogen, which is an immediate precursor of methane and a substrate for all known pure cultures of methanogenic bacteria (30), caused an immediate stimulation of sediment methane formation (28). Thus, hydrogen may be a factor which limits methanogenesis, but not much else is known about its role in the sediment metabolic processes.

Some insight into the importance of hydrogen in anaerobic decomposition has been supplied by experiments with pure and mixed cultures (8, 16, 21, 22, 26, 31). These experiments have shown that when hydrogen-producing heterotrophs are grown in the presence of hydrogen-consuming

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bacteria such as methanogens, a metabolic coupling exists between the two physiological groups (31). This coupling, termed interspecies hydrogen transfer, results in an altered metabolism characterized by an increase in substrate utilization, a decrease in reduced fermentation end products, an increase in hydrogen produced (as methane), and an increase in oxidized fermentation end products such as acetate (31).

Central to the concept of interspecies hydrogen transfer is the maintenance of low partial pressures of H_2, because the fermentative bacteria are assumed to produce molecular hydrogen from reduced pyridine nucleotide (NADH). This reaction is thermodynamically unfavorable at hydrogen partial pressures above 10^{-3} to 10^{-4} atm (31). Since dissolved-hydrogen concentrations of anaerobic sediments are extremely low and often undetectable (28), hydrogen production from NADH and thus interspecies hydrogen transfer could occur in this habitat. Our hypothesis was that the methanogenic bacteria were capable of maintaining these low hydrogen concentrations. We predicted that the K_m for the conversion of hydrogen to methane must be relatively low, thus implying that the affinity of the methanogens for hydrogen was high. Hungate et al. (15) have determined that the K_m for the conversion of H_2 to methane by the bovine rumen ecosystem and for *Methanobacterium ruminantium* in culture was very near the natural dissolved-hydrogen concentration.

Acetate is thought by some to be another important precursor of the methane produced in lake sediments (5–7, 28, 29). Cappenberg and Prins (7) have calculated that about 70% of the methane produced by Lake Vechten sediments comes from acetate, and Winfrey et al. (28) have shown that radioactively labeled [2-^{14}C]acetate is rapidly converted to methane by the microflora in Lake Mendota sediments. It is to be noted, however, that the production and utilization of the oxidized fermentation product, acetate, would also occur in lake sediments if interspecies hydrogen transfer were operative. Kinetic studies of the conversion of acetate to methane (7, 24) have resulted in the estimation of the turnover rate of the natural acetate pool by following the temporal disappearance of ^{14}C-labeled acetate added to this pool. In these studies it was assumed that all of the acetate was being converted to methane, but this assumption was not verified.

In this paper we report on the kinetic parameters, K_m, V_{max}, and turnover time (T_t), for the conversion of hydrogen and acetate to methane by the microflora of an anaerobic pelagic lake sediment. Also reported are the effects of formate on sediment methanogenesis and the effects of hydrogen on the kinetics of acetate conversion to methane.

MATERIALS AND METHODS

Sampling. An Ekman dredge was used to collect samples from the pelagic sediment located within the 6-m-depth contour of Wintergreen Lake. All samples were taken when the sediments were anaerobic and producing methane. Mason jars were filled to overflowing with the collected sediment, and the lid was tightly screwed in place. Sealed jars were transported to the laboratory in well-insulated containers and stored at 4°C. Prior to use, the sediment was transferred from the Mason jars to stoppered 1-liter reagent bottles. Transferral was done within an anaerobic glove chamber (Coy Manufacturing Co., Ann Arbor, Mich.) that had a gas atmosphere of H_2/N_2 (10:90, vol/vol). The rubber stopper that sealed the regent bottle was pierced by glass sampling and gassing tubes to which silicon rubber tubing was attached. The tubing was clamped to prevent the entry of air. After the reagent bottles were removed from the anaerobic chamber, they were flushed with O_2-free argon (passed over hot reduced copper wire) to remove the H_2 in the headspace. Bottles were stored at 4°C. For experiments, the sediment in the bottles was sampled through the outlet tube with a 10-ml, water-lubricated syringe that had been flushed with argon.

Incubation system. Because H_2 is a relatively insoluble gas and because its diffusion from the gas phase into sediment is slow, we devised an incubation system (Fig. 1) that would minimize this limitation. A major feature is that the tubes are rotated at a near-horizontal position so that a thin film (about 2 mm) of sediment accumulates on the sides, thus maximizing the contact between sediment and gas atmosphere. A second important feature is that there is a large headspace volume, which was necessary to ensure that the extremely low concentrations of added hydrogen were not significantly depleted during incubation.

The tube was made from a screw-cap culture tube (20 by 200 mm; Kimble Glass Co., Toledo, Ohio). The top was modified to accept a flange-type butyl rubber Hungate stopper held in place by a Hungate screw cap (Bellco, Vineland, N.J.; items no. 2047-11600 and 2047-16000, respectively). Tubes were flushed with oxygen-free argon, and 5 to 10 ml of sediment was added anaerobically with a glass syringe. The tubes were sealed with the rubber stopper, mixed on a Vortex mixer for 1 min while flushing with argon, and flushed for an additional 2 min. This step was necessary to remove the high concentration of dissolved methane which masked the rate of methane production. Since the sediment pH increased from 6.8 before flushing to 7.4 after flushing, carbon dioxide (9.5% final volume, determined empirically and by calculations based on CO_2 solubilities) was added to return the pH to the original value.

All substrate additions were made anaerobically by syringe. The total volume of solutions, when added, never exceeded 6% of the sediment volume. All tubes were incubated at 10 to 14°C unless otherwise noted. All data are reported on a sediment dry weight basis; the sediments were typically 86% (±2%) water, and 10 ml of wet sediment weighed approximately 10.8 g.

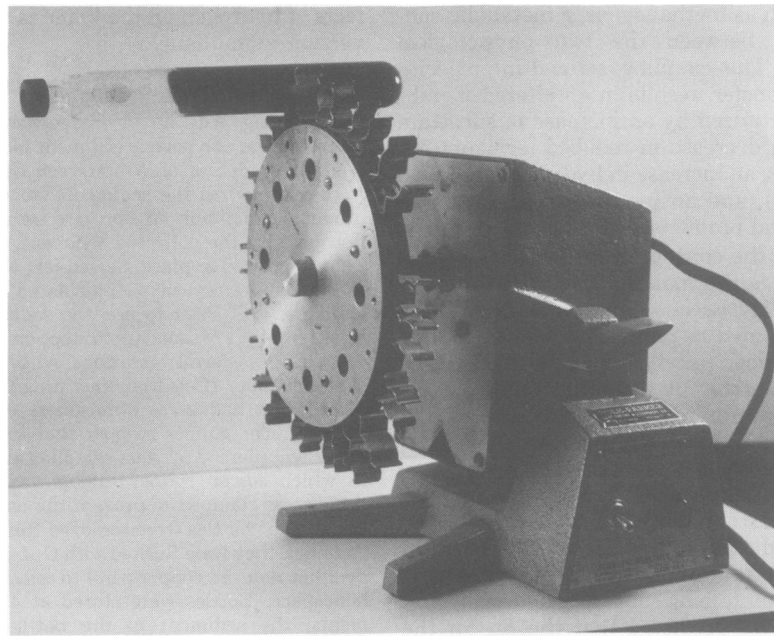

FIG. 1. *Incubation system used to study the kinetics of methane production in lake sediments. The tube was specially made from a 20- by 200-mm culture tube with the top modified to accept a flange-type butyl rubber stopper and Hungate-type screw cap. Tubes with 5 to 10 ml of sediment were incubated in a near-horizontal position on a rotary mixer as shown.*

Gas analysis. Methane was analyzed by a gas chromatograph (GC) equipped with a flame ionization detector and a 2-m Porapak Q column operated at 60°C. Hydrogen was determined with a Carle Basic 8515 GC (Carle Instruments Inc., Fullerton, Calif.) equipped with a microthermistor detector. Gas separation was made with 1-m Porapak Q and 1-m Molecular Sieve 5A columns connected in series. Columns were operated at room temperature, and argon was the carrier gas. For quantitation of low concentrations of H_2, the detector signal was further amplified by a 5/10-× operational amplifier which we had constructed. Hydrogen pool sizes in the sediment were measured by using a method we developed for extraction and concentration of the H_2 prior to GC analysis (R. F. Strayer, J. R. Robinson, and J. M. Tiedje, Abstr. Annu. Meet Am. Soc. Microbiol. 1978, N27, p. 166).

Radioactive isotopes and analysis of radioactive gases. The following radioactive compounds were used: sodium [2-^{14}C]acetate, specific activity, 50 mCi mmol^{-1}; sodium [2-^{14}C]acetate, specific activity, 57.7 mCi mmol^{-1}; [*U*-^{14}C]valine, specific activity, 280 mCi mmol^{-1}. Radioactive methane and carbon dioxide (after acidification) in headspace gases were separated by injection of 1-ml samples into the Carle GC. Effluent gases from the GC were passed through a combustion furnace (Packard Instruments Co., Downers Grove, Ill.) where methane was converted to CO_2 by passage over hot (750°C) oxidized copper wire. Carbon dioxide in the gas exiting the furnace was trapped in a solution that contained 9 ml of methanol, 2 ml of ethanolamine, and 6 ml of scintillation solution [15 g of 2,5-diphenyloxazole, 1 g of *p*-bis(*O*-methylstyryl)benezene, 1 liter of toluene]. The radioactive CO_2 trapped in this solution was counted in a scintillation counter (Packard Tri-Carb) with an efficiency of 91%.

Theory. The assumption was made that the conversion of substrates to methane by the sediment microflora followed Michaelis-Menten kinetics according to the equation

$$v = \frac{V_{max} \cdot S}{K_m + S} \quad (1)$$

The term v is the initial rate of methane production or substrate utilization, S is the initial concentration of substrate, V_{max} is the maximum initial velocity that can be attained, and K_m, the Michaelis constant, is the concentration of substrate that would give a rate equal to one-half the maximum velocity. To estimate the kinetic parameters, K_m and V_{max}, we used the direct linear plot of Eisenthal and Cornish-Bowden (12). This nonparametric statistical method has the advantages that less assumptions are needed than with the methods which use least-squares analysis, that it is simple and direct, and that the K_m and V_{max} estimates are less sensitive to outliers (10). Data pairs of substrate (S_i) and initial velocity (v_i) are plotted as lines in K_m-V_{max} parameter space. Each intersection of these lines is considered an estimate of the K_m and V_{max}, and the median value of these estimates is taken as the best estimate.

We also used progress curves (substrates or product versus t) (23) as a second, independent method for determining the kinetic parameters for the conversion of hydrogen to methane. The following equation is an integrated solution to the Michaelis-Menten equation (23):

$$V_{max} \cdot t = K_m \cdot \ln \frac{S_0}{S_t} + (S_0 - S_t) \quad (2)$$

with S_0 equal to the initial substrate concentration and S_t equal to the substrate concentration at time, t. Equation 2 can be converted to the form of a linear regression by dividing by t and rearranging (23) such that

$$\frac{\ln S_0/S_t}{t} = \frac{1}{K_m} \cdot \frac{S_0 - S_t}{t} + \frac{V_{max}}{K_m} \quad (3)$$

Equation 3 was used to analyze the data of both H_2 and CH_4 progress curves.

For kinetic experiments which involved the addition of labeled substrates and analysis of labeled gaseous products, the equation of Hobbie and Crawford (14) was used:

$$\frac{t}{f} = \frac{(K_m + S_n)}{V_{max}} + \frac{1}{V_{max}} \cdot A \quad (4)$$

with t equal to the incubation time, f equal to the fraction of added label that was converted to product, S_n equal to the in situ (natural) substrate concentration, and A equal to the concentration of added (labeled and unlabeled) substrate. A regression of t/f versus A yields values for the $K_m + S_n$ at the x intercept, for the slope $(K_m + S_n)/V_{max}$, and for the turnover time, T_t, at the y intercept (32). If S_n is known, the natural rate of substrate utilization can be calculated from

$$v = S_n/T_t \quad (5)$$

RESULTS

Concentrations of dissolved hydrogen in the lake sediment were determined for samples collected at various times. The concentrations ranged from 2 to 6 nmol of dissolved H_2 g^{-1} of dry sediment (0.2 to 0.7 μmol of dissolved H_2 $liter^{-1}$ of wet sediment).

A summary of the kinetic experiments that we performed with H_2 as substrate is presented in Table 1. Included are the approximate dates on which sediment was collected, the substrate or product analyzed, the range of substrate concentrations, the estimates of K_m and V_{max}, and the method used to estimate these kinetic parameters.

Stimulation of the rate of sediment methanogenesis by increasing levels of hydrogen in the headspace gas usually resulted in a rectangular hyperbola (Fig. 2). Incubation times were kept short to ensure that initial hydrogen concentrations had not decreased by more than 10% at the end of the experiment. Incubation times were usually 1.25 h. The range of initial hydrogen concentrations was variable but never less than 1.3×10^{-3} atm or greater than 2.0×10^{-2} atm. The kinetic parameters, K_m and V_{max}, were estimated from direct linear plots of the v,S data pairs (10) for each experiment. The data presented in Fig. 2 are shown as a direct linear plot in Fig. 3. Although K_m and V_{max} estimates can be made directly from the graph, we calculated the coordinates of each intersection from equations 5 and 6 of Cornish-Bowden and Eisenthal (10). Values for the K_m ranged from 1.3×10^{-3} atm (0.010 μmol of dissolved H_2 g^{-1}) to 4.3×10^{-3} atm (0.033 μmol of dissolved H_2 g^{-1}). The V_{max} ranged from 0.45 to 0.69 μmol of CH_4 g^{-1} h^{-1} for methane production and 4.3 to 5.9 μmol g^{-1} h^{-1} for H_2 consumption.

The alternative approach of using progress curves to obtain kinetic data was used because this method allows the determination to be done in a single experimental vessel and does not require concern over rapid substrate depletion at low substrate concentrations. An example of a progress curve for H_2 consumption and CH_4 production is presented in Fig. 4. The methane concentrations at each sampling have been corrected for the methane produced by the control, which did not have hydrogen added. Concentrations of hydrogen and methane in the headspace were analyzed every 30 to 45 min depending on the number of replicates in each experiment. For the H_2 progress curve experiments (Table 1) the K_m estimates ranged from 2.2×10^{-3} atm of H_2 (0.014 μmol of dissolved H_2 g^{-1}) to 4.8×10^{-3} atm of H_2 (0.034 μmol of dissolved H_2 g^{-1}), and the V_{max} ranged from 2.44 to 6.38 μmol of H_2 consumed g^{-1} h^{-1}. For the H_2 progress curve shown in Fig. 4 the K_m was 2.9×10^{-3} atm and the V_{max} was 4.73 μmol of H_2 g^{-1} h^{-1}. For the methane progress curve the K_m was 4.0×10^{-3} atm and the V_{max} was 1.13 μmol of CH_4 produced g^{-1} h^{-1}. Although the ratio of H_2 V_{max} to CH_4 V_{max} is approximately 4 (the theoretical ratio for conversion of 4 mol of H_2 to 1 mol of CH_4), the ratio of hydrogen consumed to methane produced increased as the hydrogen concentration in the headspace decreased (Table 2).

Other hydrogen donors and the stimulation of methanogenesis. Table 3 shows a survey of the effect of various potential hydrogen donors on the rate of methanogenesis. Since only formate immediately stimulated methanogenesis, we attempted to determine the kinetic parameters for the conversion of this substrate to methane (Fig. 5). In this experiment, the response approaches a hyperbolic relationship. However, assuming that 4 mol of formate is needed to produce 1 mol of methane, at the lowest concentration of 1.16 μmol of formate g^{-1} 87% of the formate was converted to methane within the 1-h incubation period. This percentage does not include other nonmethane fates of

TABLE 1. *Summary of kinetic experiments on the consumption of hydrogen and the conversion of hydrogen to methane by Wintergreen Lake sediments*

Date of expt	Date sediment was collected	Substrate or product analyzed	Initial H_2 concn (atm × 10^{-3})	Type of kinetic expt	Incubation time (h)	K_m as partial pressure of H_2 (atm × 10^{-3})	K_m (μmol of dissolved H_2 g^{-1})	V_{max} (μmol of H_2 consumed or μmol of CH_4 produced g^{-1} h^{-1})
13 Dec. 1976	Oct. 1976	CH_4	2.6, 19.3	v vs S[a]	1.25	2.4	0.014	0.45
12 Jan. 1977	Jan. 1977	H_2	5.5	Progress curve[b]	0–6	2.2	0.014	2.44
		H_2	5.5	Progress curve	0–6	2.4	0.015	3.00
5 Mar. 1977	Feb. 1977	H_2	2.2, 1.0	Progress curve	0–3	2.1	0.010	1.18
		H_2	2.2, 1.1	Progress curve	0–3	2.2	0.010	1.14
10 Mar. 1977	8 Mar. 1977	H_2	1.3–10.6	v vs S	1.25	3.7	0.027	4.27
		CH_4				4.5	0.033	0.47
11 Mar. 1977	8 Mar. 1977	H_2	1.4–7.0	v vs S	1.25	3.8	0.028	5.51
		CH_4				1.3	0.010	0.49
12 Mar. 1977	8 Mar. 1977	H_2	1.4–7.1	v vs S	1.25	4.7	0.033	5.88
		CH_4				3.8	0.027	0.69
15 Mar. 1977	8 Mar. 1977	H_2	5.1	Progress curve	0–4.5	2.9	0.021	4.73
		CH_4				4.0	0.029	1.12
25 Mar. 1977	8 Mar. 1977	H_2	6.6	Progress curve	0–5.25	4.8	0.034	6.38
		H_2	6.6	Progress curve	0–5.25	4.3	0.031	6.01

[a] Direct linear plot used to estimate K_m and V_{max}.
[b] Integrated rate equation of Segel (23) used to estimate K_m and V_{max}.

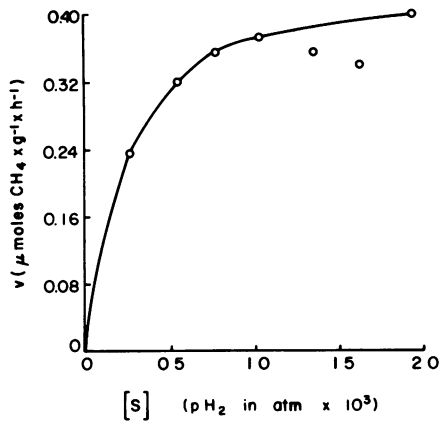

FIG. 2. *Effect of increasing hydrogen concentration, [S], on the rate of sediment methane production, v. The rates have been corrected for the control (no hydrogen added). Incubation time was 1.25 h at 10°C. Sediment was collected in the early winter.*

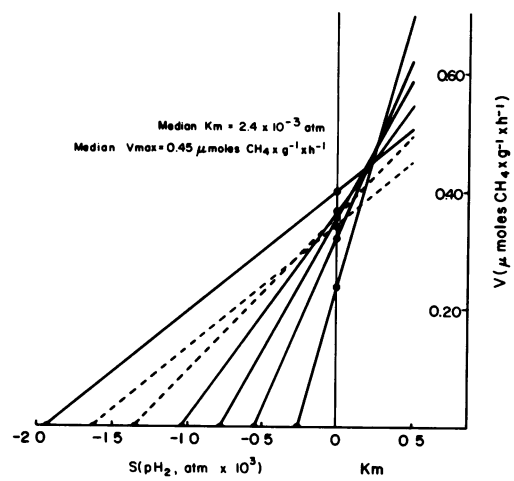

FIG. 3. *Direct linear plot of the substrate-velocity data pairs plotted in Fig. 2.*

formate. In an attempt to achieve a more valid temporal resolution, we used progress curves at several formate concentrations and with 2-min sampling intervals. The experiment was, of necessity, performed at room temperature (23°C) next to the GC. The rates of methane production, calculated for the 10-min sampling time (Table 4), still indicated that at least 47% of the formate had been consumed within this short time. At sampling times under 10 min the rates were variable but suggested that maximum velocities of formate conversion to methane were reached somewhere between formate concentrations of 260 and 400 nmol g^{-1} of sediment. K_m values could not be estimated.

Kinetics of acetate conversion to methane. The addition of 1.23 to 12.1 μmol of acetate g^{-1} of sediment did not stimulate the rate of methanogenesis by sediments beyond the rate of the control with no addition (Fig. 6). This rate, calculated from the slope of the linear portion of the lines plotted in Fig. 6, averaged 0.307 μmol of CH_4 produced g^{-1} h^{-1} (±4% *s*, *n*

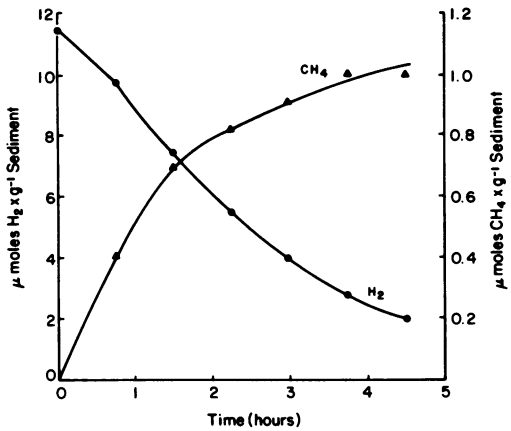

FIG. 4. *Progress curves of the headspace hydrogen and methane concentrations with time. Methane concentrations have been corrected for the control, which had no hydrogen added. Incubation temperature was 12°C. Headspace (vapor) volume was about 65 ml. Sediment was collected in late winter.*

TABLE 2. *Ratio of hydrogen consumed to methane produced with decreasing hydrogen concentration calculated from the progress curves of Fig. 4*

Time (h)	H_2 in headspace (atm × 10^{-3})	H_2 consumed/CH_4 produced
0.00	5.0	
0.75	4.3	4.1
1.50	3.3	5.7
2.25	2.4	7.2
3.00	1.8	8.1
3.75	1.2	8.1
4.50	0.9	8.8

TABLE 3. *Effect of potential hydrogen donors on the rate of sediment methanogenesis*[a]

Substrate	Concn range (μmol liter^{-1})	Incubation time (h)	Stimulation[b]
Formate	25–150	0.25	+++
	100–1,000	1.0	+++
Lactate	40–280	6.0	0
		24.0	0
Propionate	40–280	6.0	0
		24.0	0
Valine	40–280	6.0	0
		24.0	0
Leucine	40–280	6.0	0
		24.0	0

[a] Incubation temperature was 12°C.

[b] Relative stimulation; 0 = no stimulation over control to +++ = threefold stimulation.

= 11). This lack of stimulation prompted us to attempt short-term progress curves at lower concentrations and with methane measurements every 2 min. The results for the 10-min sampling

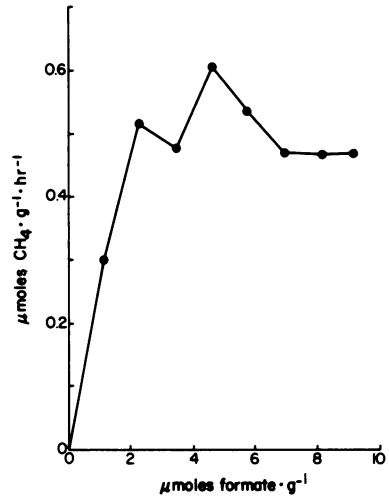

FIG. 5. *Effect of initial concentrations of dissolved formate on the rate of sediment methanogenesis. Methane production rates have been corrected for the rate of the control, which received an identical volume of oxygen-free water but no formate. Incubation time was 1 h at 12°C. Sediment was collected in midwinter.*

TABLE 4. *Effect of dissolved-formate and dissolved-acetate concentrations on the rate of sediment methanogenesis*[a]

Substrate concn (nmol g^{-1})	Rate of methane formation	
	Formate (nmol of CH_4 g^{-1} h^{-1})	Acetate (nmol of CH_4 g^{-1} h^{-1})
0	220	250
130	340	170
270	520	330
400	776	280
530	850	300
660	ND[b]	310
800	950	330

[a] Incubation time was 10 min at 23°C. Sediment was collected in the late winter.

[b] ND, Not determined.

time are presented in Table 4. Even under these conditions we would not detect significant acetate stimulation of methanogenesis.

To determine if the lack of acetate stimulation was due to the absence of acetate-utilizing methanogens, we monitored the production of radioactively labeled methane and CO_2 from [2-^{14}C]acetate with time (Fig. 7). Also plotted is the production of labeled methane by sediment that had been preincubated with 5.0×10^{-3} atm of H_2 for 15 min prior to the addition of labeled acetate. In both the presence and absence of added H_2, acetate was rapidly converted to

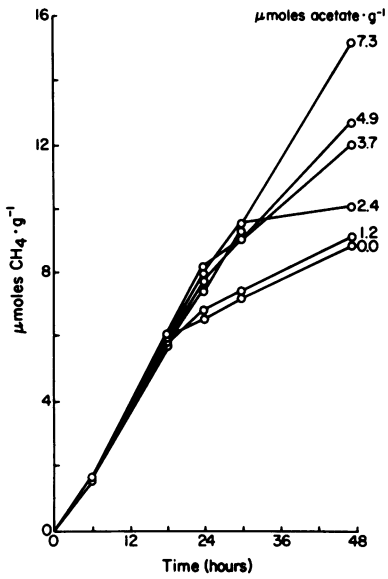

FIG. 6. *Amount of methane produced by sediment with time at different concentrations of added dissolved acetate. Incubation temperature was 12°C. Sediment was collected in midwinter.*

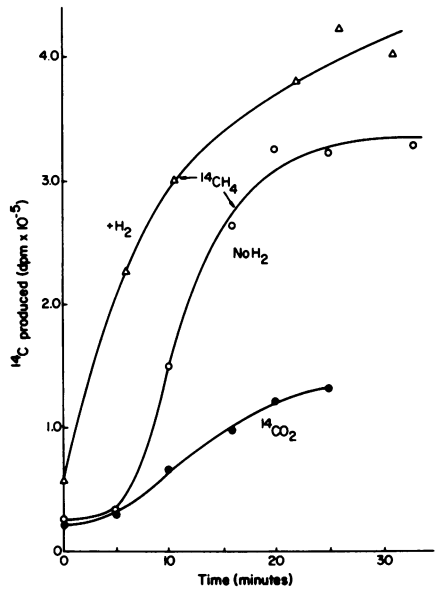

FIG. 7. *Production of labeled methane and carbon dioxide from [2-^{14}C]acetate (1 μCi). Total acetate added amounted to 0.02 μmol 10 ml^{-1} of wet sediment. Symbols: ○, labeled methane produced in the absence of hydrogen; ●, labeled carbon dioxide produced in the absence of hydrogen; △, labeled methane produced in the presence of 5.0 × 10^{-3} atm of hydrogen. Hydrogen was added 15 min prior to the addition of labeled acetate. Incubation temperature was 12°C. Sediment was collected in June.*

methane, although the addition of H_2 caused an immediate conversion.

Because labeled methane appeared so rapidly after the addition of labeled acetate, we ran kinetic experiments that were similar in approach to the kinetic experiments described by Wright and Hobbie (32). A different amount of unlabeled acetate was added to each tube of sediment. Each tube then received the same amount (1 μCi) of [2-^{14}C]acetate. After an incubation time of 10 to 15 min, the amount of label in the headspace methane was determined. This radioactivity was used to calculate the term t/f of equation 4, which was plotted against the amount of labeled plus unlabeled acetate added to each tube. This plot is equivalent to a kinetic plot of S/v versus S. The results of this experiment, for the two cases of plus and minus preincubation with 5.0 × 10^{-3} atm of H_2, are presented in Fig. 8. The kinetic parameters estimated from this plot and a similar experiment are presented in Table 5.

Since the addition of hydrogen had a pronounced effect on the conversion of acetate to methane, the effect of H_2 on $^{14}CO_2$ production from L-[*U*-^{14}C]valine, a potential H_2 donor (Table 3), was examined. Four tubes, each containing 10 ml of sediment, were incubated at 12°C for 15 min prior to the addition of 1 μCi of labeled valine. Hydrogen (5.0 × 10^{-3} atm) was added to two of the tubes at the start of the preincubation period. The amount of $^{14}CO_2$ present in the headspace gas was determined 15 min after the addition of the label. In the absence of hydrogen, 6.3 × 10^4 dpm of $^{14}CO_2$ was detected in the headspace. The sediment exposed to 5.0

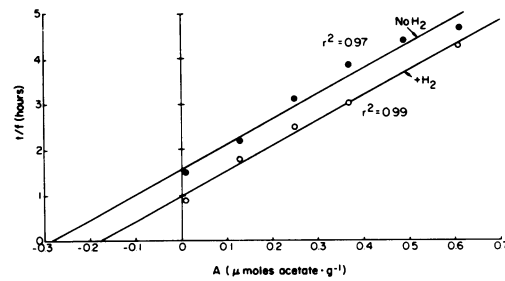

FIG. 8. *Effect of increasing acetate concentration (A) and 5.0 × 10^{-3} atm of hydrogen on the term t/f, where t equals the incubation time and f equals the fraction of added radioactive [2-^{14}C]acetate (1 μCi) converted to $^{14}CH_4$. Symbols: ●, t/f versus A in the absence of added hydrogen, t = 0.25 h; ○, t/f versus A in the presence of 5.0 × 10^{-3} atm of added hydrogen, t = 0.167 h. The hydrogen was added 15 min prior to the labeled plus unlabeled acetate addition. Incubation temperature was 12°C. Sediment was collected in June. The r^2 values on the figure are the coefficients of determination for the linear regression lines.*

TABLE 5. *Kinetic parameters estimated from the regression of t/f versus A (Fig. 8)*[a]

Expt	Preincubation with 5.0 $\times 10^{-3}$ atm of H_2	$K_m + S_n$ (μmol of acetate g^{-1})	V_{max} (μmol of CH_4 g^{-1} h^{-1})	T_t (h)
1	−	0.281	0.181	1.56
	+	0.177	0.181	0.98
2	−	0.258	0.145	1.78
	+	0.165	0.189	0.91

[a] Experiment 1 was run within 4 days of collecting sediment in early July. Experiment 2 was run within 2 weeks of collecting sediment in late June.

$\times 10^{-3}$ atm of hydrogen produced only 4.0×10^4 dpm of $^{14}CO_2$. This represents a 37% decrease in the production of labeled carbon dioxide from uniformly labeled valine.

DISCUSSION

The K_m estimates for the conversion of hydrogen to methane (Table 1) demonstrate that if present in sufficient numbers, the methanogenic bacteria are capable of maintaining low concentrations of hydrogen in the sediment. Evidence that the methanogenic population is sufficient is indicated by the progress curve (Fig. 4), where moderately high initial hydrogen levels were decreased by 83% within 4.5 h. This rapid rate of consumption suggests that hydrogen limits sediment methanogenesis and that hydrogen concentrations that may inhibit heterotrophic hydrogen production are quickly lowered to more favorable concentrations. In spite of the heterogeneity of sediments, we were able to obtain fairly reproducible K_m estimates among experiments using different methods of analysis and with sediments collected on different dates (Table 1). These estimates must be considered the upper limit, since transfer of hydrogen from the vapor phase to the aqueous sediment phase may still be rate limiting despite the design to optimize the transfer. The mean K_m for hydrogen consumption for the winter sediments was 0.025 μmol g^{-1} (or 3.0 μmol liter^{-1} of wet sediment). The mean K_m for methane production was 0.023 μmol g^{-1} (or 2.7 μmol liter^{-1} of wet sediment), which does not vary significantly from the one for H_2 consumption. These average K_m estimates compare favorably to the mean K_m value of 1 μmol liter^{-1} determined by Hungate et al. (15) for bovine rumen fluid and for *M. ruminantium*.

The in situ rate of hydrogen consumption can be calculated from the in situ hydrogen concentration, which was 2 to 6 nmol g^{-1}, and the K_m and V_{max} (Table 1) according to equation 1. The resulting range in estimated natural velocities of H_2 consumption was 250 to 1,000 nmol of H_2 g^{-1} h^{-1}, and for H_2 conversion to methane it was 30 to 200 nmol of CH_4 g^{-1} h^{-1} (equivalent to 120 to 800 nmol of H_2 g^{-1} h^{-1}). In their kinetic study of bovine rumen fluid, Hungate et al. (15) determined that hydrogen was converted to methane at half the maximal rate, since the in situ hydrogen concentration was equal to the K_m. We have estimated the V_{max} of this reaction from the $1/v$ versus $1/S$ plots of these investigators (15) and obtained an average value of 2.9 μmol of CH_4 g^{-1} of wet rumen fluid h^{-1}. The natural rate would then be half of this rate, or 1.45 μmol of CH_4 g^{-1} h^{-1}. This rate is 60 times higher than our estimate of the upper limit of the natural rate of hydrogen conversion to methane by sediment microflora, when both are compared on a per-gram (wet weight) basis.

The differences in estimated natural rates of H_2 consumption and H_2 conversion to methane by the sediment microflora were due to the differences in V_{max} estimates (Table 1). The average V_{max} for hydrogen consumption (4.8 μmol of H_2 g^{-1} h^{-1}) was 7.4 times higher than the V_{max} for hydrogen conversion to methane (0.64 μmol of CH_4 g^{-1} h^{-1}). This difference in average maximal rates, and thus estimated natural rates, indicates that methane may not be the only fate of hydrogen in the sediment, since the theoretical ratio for hydrogen conversion to methane is 4. Further evidence for the nonmethanogenic utilization of hydrogen by the sediment microflora is that the ratio of hydrogen consumed to methane produced increased from 4 to 9 with time and decreasing hydrogen concentration during a progress curve experiment (Table 2). Winfrey et al. (28) have also suggested that sediment microflora may use hydrogen for purposes other than methane production. A possible explanation is that the H_2 (and CO_2) is also being converted to acetate by organisms similar to *Clostridium* isolated by Ohwaki and Hungate (18) from sewage sludge or *Acetobacterium* isolated by Balch et al. (1) from sediments. A second possibility is that with short-term incubations, hydrogen consumption may be due to heterotrophic bacteria and the reaction $H_2 + NAD^+ \rightarrow NADH + H^+$. However, this reaction will be limited by the availability of organic electron acceptors, which are needed to recycle the catalytic amounts of NAD^+.

The potential hydrogen donors listed in Table 3 were selected for the following reasons. Formate was chosen because some methanogens can use it directly (30) and because it can be converted to hydrogen and carbon dioxide by a variety of heterotrophs (11). Lactate was selected because many heterotrophs can ferment it (11) and because Bryant et al. (4) have shown

that with lactate, and in the absence of sulfate, interspecies hydrogen transfer occurs between *Desulfovibrio* and H_2-using methanogens. Propionate was selected as a representative volatile fatty acid because Bryant (3) has hypothesized that there is an acetogenic population in anaerobic habitats that can convert these compounds and alcohols to acetate and hydrogen. The amino acids valine and leucine were selected because Molongoski and Klug (17) have shown that proteolytic clostridia are the predominant isolatable heterotrophs in Wintergreen Lake sediments. The lack of significant stimulation of methane production by all but formate (Table 3) may indicate either that these substrates are unimportant hydrogen donors or they are already being turned over at maximum rates.

Although kinetic constants could not be estimated for the conversion of formate to methane, the almost immediate stimulation by low levels of formate (Table 4) suggests that this substrate could also be important in methane production. The rapid stimulation of sediment methanogenesis by formate has also been observed by Winfrey et al. (28). Although strict comparisons between the sediment and the bovine rumen ecosystem are not possible, Hungate et al. (15) have estimated that the K_m for formate conversion to methane by rumen contents averaged 30 nmol g^{-1} of wet weight. This value was 2.5 times the intercellular concentration of formate. These same authors also determined that the average turnover of formate in rumen contents was 11 μmol g^{-1} h^{-1} and that 18% of the rumen methane comes from formate via H_2. Thus, the general importance of formate as a precursor of methane has been established, but the kinetic parameters for this substrate in the sediment remain unknown.

Formate concentrations in Wintergreen Lake sediments have been measured by Molongoski and Klug (unpublished data) and ranged from 13 to 63 nmol g^{-1} during May to October 1976. As with H_2, the methanogens may be responsible, in part, for maintaining these low concentrations. During short incubations and at added formate concentrations in excess of 130 nmol g^{-1}, we have detected only very low concentrations of hydrogen in the headspace gas. Since these hydrogen levels were not high enough to account for the observed stimulation of methanogenesis, we believe that formate may be directly used by the methanogenic bacteria present. This finding is consistent with our fluorescent-antibody studies, which showed a formate- and H_2-utilizing methanogen to predominate in these sediments (25). Another possible explanation for our failure to observe the stoichiometric production of H_2 from formate is that a portion of this hydrogen was consumed by the sediment microflora before it had a chance to enter into the gas phase.

The rate of conversion of acetate to methane appears to be quite close to the V_{max} for this reaction (Table 4), which would explain the apparent lack of stimulation by additions of unlabeled acetate (Fig. 6). Natural concentrations of acetate in these sediments have been determined by Molongoski and Klug (unpublished data) to range from 180 to 550 nmol g^{-1} over the summer of 1976. Using the minimum measured acetate pool size and an average turnover time of 1.67 h (Table 5), an estimate of the minimum natural velocity (equation 5) would be 108 nmol of CH_4 produced g^{-1} h^{-1}. This minimum rate estimate is 66% of the average V_{max} (Table 5) of 163 nmol of CH_4 produced g^{-1} h^{-1}, which further indicates that acetate is being converted to methane at rates very near the maximum. This discovery implies that the only way for an increase in the rate of acetate conversion to methane to occur would be by an increase in the population of acetate-utilizing methanogens. But these bacteria have very slow growth rates (20; A. Zehnder, personal communication), so an increase in the rate of heterotrophic acetate production may lead to an increase in the acetate pool size.

Cappenberg and colleagues have also determined kinetic parameters for acetate but not H_2 conversion to methane in Lake Vechten sediments. They determined first-order rate constants (k values) of 0.35 h^{-1} in early studies (7) and of 0.244 and 0.07 h^{-1} for 5- and 1-cm-deep sediments, respectively, in later studies (T. E. Cappenberg and E. Jongejan, *in* W. E. Krumbein, ed., *Proceedings of the Third International Symposium on Environmental Biogeochemistry*, Braunschweig, Germany, 1977, in press). This compares with a k value of 0.6 h^{-1} for our studies, which is consistent with the higher productivity of our lake. Our range of estimated natural rates of acetate conversion to methane is 17 to 25 nmol g^{-1} of wet sediment h^{-1}. Using their k values, the Dutch workers determined apparent rates of acetate conversion to methane of 23 nmol g^{-1} of wet sediment h^{-1} in the earlier studies and approximately 2.5 and 0.4 nmol g^{-1} of wet sediment h^{-1} for 5- and 1-cm-deep sediments, respectively, in their later study. In their earlier study, the total rate of methane production was found to be 34 nmol g^{-1} of wet sediment h^{-1}; thus, acetate was reasoned to account for 70% of the methane production, with $H_2 + CO_2$ presumably contributing the remaining 30%. The variability of these rates with experiment and depth makes it difficult to

generalize about the contribution of each precursor, particularly when the difference method is used.

Our goal is to measure the rates of $H_2 + CO_2$ and acetate conversion to methane independently. Comparisons are difficult because of the broad range of rates we obtained, especially for H_2 conversion to methane, and because the samples were from different seasons. Nonetheless, rough comparisons show similar rates of conversion of the two precursors to methane. A striking difference, however, is in the turnover times of the precursor pools, 25 s for H_2 versus 1.7 h for acetate.

Previous experiments on the rate of acetate conversion to methane with both sediments (7) and sludges (24) have used incubation vessels with a large headspace. This could result in loss of dissolved hydrogen to the headspace, thus reducing the $H_2 + CO_2$ contribution to total methane production and thereby causing an underestimate of the importance of H_2. Using the equations of Flett et al. (13) for phase distribution of gases and the appropriate Bunsen absorption coefficient for hydrogen, we calculate that >98% of the initially dissolved hydrogen would have been transferred to the gas phase at equilibrium in the above-cited experiments. How much transferral of H_2 to headspace occurred, and thus reduction of the H_2 contribution to methane production, cannot be answered. However, because of the experimental design used, the possibility of a significant effect cannot be eliminated.

Methane may not be the only fate of acetate in the sediment. Only 15% of the labeled acetate appeared as methane by the time labeled methane production ceased (Fig. 7). In other experiments (not shown) more of the label was converted to methane, 23 and 32%, but production also terminated at these values regardless of the length of incubation. Additional evidence for another fate of acetate comes from Winfrey et al. (28) in their study of Lake Mendota sediments. We have calculated that only 12% of the methyl-labeled acetate that they added was converted to methane. Other possible acetate-consuming organisms are the recently described sulfur reducer *Desulfuromonas acetoxidans* (19) and the sulfate reducer *Desulfotomaculum acetoxidans* (27). However, only 6% of the labeled acetate we added was converted to carbon dioxide (Fig. 7), which still leaves nearly 80% to an unknown fate. Radioactive label remaining in the acetate pool was not determined.

Hydrogen appears to stimulate the conversion of acetate to methane (Fig. 7), an observation also made by Winfrey et al. (28) for Lake Mendota sediments. However, the kinetic experiments (Fig. 8, Table 5) show that the preincubation period with 5.0×10^{-3} atm of hydrogen caused a decrease in the acetate pool, as $K_m + S_n$, and a concomitant decrease in the acetate turnover time. The apparent stimulation in Fig. 7 is more likely due to a lower acetate pool and thus higher specific activity of the acetate than to an increase in the methanogenic conversion of acetate. This explanation fits with the hypothesis that interspecies hydrogen transfer occurs in the sediment, since, in an uncoupled system, less oxidized substrate such as acetate is produced (31). Thus, a decrease in the acetate pool would occur if the added hydrogen inhibited the heterotrophic production of acetate.

Further evidence that interspecies hydrogen transfer occurs in the lake sediment is that preincubation with hydrogen causes a 37% decrease in the amount of radioactive CO_2 produced from the heterotrophic metabolism of labeled valine. Under anaerobic conditions, valine is normally metabolized via the Stickland reaction to isobutyrate, with NAD^+ being reduced to NADH + H^+ (2). The NADH + H^+ is oxidized back to NAD^+ by a transferral of the electrons to an electron-accepting amino acid such as glycine or proline. The moderately high hydrogen concentrations that we added to the sediment may have caused most of the NAD^+ to be in the reduced form and unable to accept electrons from valine, thus decreasing the production of $^{14}CO_2$. A second possibility is that the hydrogen had no effect on the Stickland reaction but instead inhibited the further degradation of isobutyrate, which is the product of valine degradation via the Stickland reaction.

Cleland (9) has stated "... it is not practical to have the substrate which is an intermediate in a metabolic pathway present in levels much above its apparent Michaelis constant. Maintenance of a stable flow through a metabolic pathway is favored if each enzyme operates in the proportional region of its velocity versus concentration curve, so that a momentary rise in reactant concentration results in an increased reaction rate and tends to prevent further increase in concentration." Our experimental evidence shows that for the anaerobic sediment habitat, short-term increases in hydrogen concentrations lead to increased reaction rates and thus prevention of further increase in dissolved-hydrogen concentrations. However, for acetate, a short-term increase in the pool size cannot cause a significant increase in the reaction rate, since the rate is already near the maximum. Therefore, further increases in acetate concentration are not prevented.

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