APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1987, p. 57–64
0099-2240/87/010057-08$02.00/0

Vol. 53, No. 1

Ecophysiological Adaptations of Anaerobic Bacteria to Low pH: Analysis of Anaerobic Digestion in Acidic Bog Sediments

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Received 3 July 1986/Accepted 16 October 1986

The dynamics of anaerobic digestion were examined in the low-pH sediments of Crystal Bog in Wisconsin. The sediments (pH 4.9) contained 71% organic matter and the following concentrations of dissolved gases (micromoles per liter): CO_2, 1,140; CH_4, 490; and H_2, 0.01. The rate of methane production was 6.2 μmol/liter of sediment per h, which is slower than eutrophic, neutral sediments. Microbial metabolic processes displayed the following pH optima: hydrolysis reactions, between 4.2 and 5.6; aceticlastic methanogenesis, 5.2; and hydrogen-consuming reactions, 5.6. The turnover rate constants for key intermediary metabolites were (h^{-1}): glucose, 1.10; lactate, 0.277; acetate, 0.118; and ethanol, 0.089. The populations of anaerobes were low, with hydrolytic groups (10^6/ml) several orders of magnitude higher than methanogens (10^2/ml). The addition of carbon electron donors to the sediment resulted in the accumulation of hydrogen, whereas the addition of hydrogen resulted in the accumulation of fatty acids and the inhibition of hydrogen-producing acetogenic reactions. Strains of *Lactobacillus*, *Clostridium*, and *Sarcina ventriculi* were isolated from the bog, and their physiological attributes were characterized in relation to hydrolytic process functions in the sediments. The present studies provide evidence that the pH present in the bog sediments alter anaerobic digestion processes so that total biocatalytic activity is lower but the general carbon and electron flow pathways are similar to those of neutral anoxic sediments.

The ecology and microbial physiology of anoxic freshwater sediments near neutral pH have been well characterized over the past several years (6, 9, 14–16, 19, 21, 23, 24). Much less attention has been given to anoxic sediments with pHs below neutrality. This is in part due to the tendency of acidic environments to be oxidizing (e.g., acidic hot springs and acid mine drainage). One set of commonly occurring anoxic, acidic systems are acid peatlands. Peatlands are widespread, having an estimated extent of 420 million to 450 million ha (7).

Studies of near neutral, anoxic sediments have focused on the rates of methanogenesis and sulfate reduction and the turnover of intermediary products of carbon metabolism. Several factors have been shown to influence the rate of anaerobic digestion processes in near neutral sediments, including temperature (19, 24) and the availability of sulfate (1, 10, 12, 24). The rate of methane formation is limited by the availability of electron donors (16, 19), and addition of sulfate to anoxic sediments leads to a pronounced decrease in the rate of methanogenesis (1, 10, 12, 19).

Acetate is the dominant intermediate product of carbon metabolism in anaerobic sewage digestors (3, 5, 8) and in the rumen (4, 20). Lovley and Klug (9) demonstrated that acetate was the dominant intermediary product in Wintergreen Lake. Addition of hydrogen to this ecosystem resulted in the accumulation of volatile fatty acids. However, hydrogen addition stimulated methanogenesis and did not decrease the rate of glucose turnover in the sediments (6). Approximately 65% of the methane in neutral freshwater sediments is derived from acetate, while 35% is derived from hydrogen and carbon dioxide (10, 19). The concept of interspecies hydrogen transfer (20, 21) has led to the understanding that the unfavorable energetics of some organic dehydrogenation reactions are alleviated when the hydrogen concentration is kept low in anaerobic ecosystems. The close association of hydrogen-producing and hydrogen-consuming organisms maintains hydrogen at levels which allow both sets of reactions to proceed. It has become clear that balanced hydrogen metabolism is crucial to the efficient anaerobic digestion of organic matter (22, 23).

In mildly acidic Knaack Lake (pH 6.2), the pH optimum for methanogenesis was reported to be 7.2 to 7.6 (14). The pH optimum for methane production in acidic peat bogs in Minnesota was 6.0 and was well above the in situ pH value of 3.8 (17, 18). Addition of acetate to peat sediments inhibited methanogenesis, and the authors concluded that carbon dioxide and hydrogen were the dominant precursors of methane in this environment.

The present paper examines the dynamics of anaerobic digestion in the low-pH sediments of Crystal Bog in Wisconsin. The purpose of this paper is twofold: to test the hypothesis that the bacterially mediated anaerobic digestion processes in naturally acid ecosystems are "optimally" adapted to function at low pH and to determine the limits that acid conditions place on anaerobic digestion processes.

MATERIALS AND METHODS

Experimental site and sampling procedures. Crystal Bog is a poor fen (receives less than 15% of input from runoff and ground water) located in the Northern Highlands District of north-central Wisconsin. The work focused on the anoxic sediments underlying the open water in the center of the fen. The depth of the overlying water is 2.5 m, and the water column remains anoxic throughout the summer months. The sediment temperature reaches a maximum of 20°C during the summer.

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The sediment was sampled between June and September, during which time the sediments and the immediately overlying water remained anoxic. Samples were collected with an Eckman dredge (24). The headspace of the dredge was flushed continuously with nitrogen gas, and the top 3 cm of the sediment was transferred with a modified 60-ml syringe into 1-liter screw-on cap bottles, which were also flushed with nitrogen gas. The sediments were transported on ice to the laboratory, and all analyses were begun within 6 h of collection. The temperature and pH determinations were made in the field.

The porosity of the sediments was determined from the weight loss of wet sediments dried at 105°C for 36 h. The organic content of the sediments was determined from the weight loss of dry sediments ashed at 550°C for 36 h. Dissolved sulfate and sulfide were quantified as described previously (19).

Samples for dissolved gases were collected by transferring 45 ml of sediment into flasks which had been flushed with nitrogen gas. The gas had been scrubbed to remove hydrogen and oxygen. The sediment was placed into the flask through a stopcock in the bottom, and the gas was displaced through a needle placed in a septum in the top of the flask. The volume of each flask (approximately 90 ml) was accurately predetermined. Each flask was shaken for 2 min to equilibrate the dissolved gases with the headspace. A 10-ml subsample of the equilibrated headspace was removed by syringe and transferred to a flask containing a saturated solution of sodium preserved with 100 μg of mercuric chloride per ml. The flasks were transported to the laboratory without allowing the gas bubble to contact either the septum or the stopcock.

Chemicals, gases, and isotopes. All chemicals used were of reagent grade or better and were obtained from Sigma Chemical Co., St. Louis, Mo., or Mallinckrodt, Paris, Ky. All radioactive compounds were obtained from New England Nuclear Corp., Boston, Mass. The specific labeled substrates were [2-^{14}C]acetate (54 mCi/mmol), [U-^{14}C]lactate (40 mCi/mmol), [U-^{14}C]glucose (384 mCi/mmol), [2-^{14}C]ethanol (50 mCi/mmol), and [U-^{14}C]butyrate (1.8 mCi/mmol). Radioisotopes which were supplied in ethanol were evaporated by gassing with nitrogen gas and redissolved in anaerobic distilled water. N_2, N_2-CO_2 (95:5), H_2, and H_2-CO_2 (80:20) were greater than 99.9% pure (Matheson Gas Co., Joliet, Ill.) and were passed over heated, copper-filled furnaces (Sargent-Welch Scientific Co., Skokie, Ill.) to remove oxygen.

Measurement of in situ concentrations of dissolved gases and intermediary metabolites. Hydrogen was detected with a trace reducing gas analyzer (Trace Analytica, Menlo Park, Calif.) after separation on a gas chromatograph (model 750; GowMac Instruments Co., Bridgewater, N.J.) equipped with a Spherocarb column (GowMac) with nitrogen as the carrier gas at 25 cm^3/min and operated at 150°C. Hydrogen detection is based on the stoichiometric conversion of mercury oxide to mercury vapor by hydrogen and measurement of the mercury spectrum.

Methane was measured with a model 660D Aerograph gas chromatograph (Varian Associates, Palo Alto, Calif.) equipped with a Poropack R column (80/100 mesh; Anspec Co., Warrenville, Ill.) and a flame ionization detector. The column was operated at room temperature with nitrogen as the carrier gas (30 cm^3/min). The amounts of gas were corrected for dissolved gas remaining in the sediments by Henry's Law. Carbon dioxide was determined with a Packard model 417 gas chromatograph (Packard Instrument Co., Downers Grove, Ill.) equipped with a Carbosieve-B column (120/140 mesh; Supelco Inc., Bellafonte, Pa.) and a thermal conductivity detector. The column was operated at 95°C with helium as the carrier gas at a flow rate of 60 cm^3/min. Total dissolved gas was calculated by Henry's Law and the in situ pH.

Pore water for analysis of alcohols and volatile fatty acids was obtained by anaerobically centrifuging sediment at 10,000 × g for 15 min at 5°C. For the analysis of volatile fatty acids, the supernatant was adjusted to pH 10 with 10 N NaOH. One liter of supernatant was concentrated to 100 ml by vacuum distillation. Samples (250 μl) were placed into chilled 0.5-ml centrifuge tubes, acidified with 25 μl of 2 N H_3PO_4, and centrifuged at 3,000 × g for 5 min. Acetate and butyrate were quantified by injecting 2 μl of the acidified supernatant into a Packard model 419 gas chromatograph equipped with a Chromosorb 101 (80/100 mesh) column (Supelco Inc.) and a flame ionization detector. The column was operated at 200°C, and the detector and injector temperatures were 240 and 220°C, respectively.

Ethanol was measured by injecting 2 μl of the pore water supernatant into a Packard model 419 gas chromatograph equipped with a Super Q (80/100 mesh) column (Alltech Associates Inc., Deerfield, Ill.) and a flame ionization detector. The column was operated at 180°C, and the detector and injector temperatures were both 200°C. The carrier gas was helium at a flow rate of 100 cm^3/min.

Radiotracer experiments. The production of methane and carbon dioxide from glucose and intermediary metabolites was determined by transferring 20 ml of sediment by a glass syringe equipped with an 18-gauge needle into a 60-ml serum bottle sealed with a butyl rubber bung that contained a nitrogen atmosphere. A specific ^{14}C-labeled substrate was injected by syringe (Unimetrics, Alltech Associates), and the vial was incubated at 15°C (or the indicated temperature for temperature optima experiments). [^{14}C]methane and [^{14}C]carbon dioxide were analyzed in gas samples (0.4 ml) by a gas chromatographic gas proportional counting technique described previously (13). Liquid samples (0.5 ml) were removed by syringe, immediately introduced into 1.5-ml plastic centrifuge tubes containing 50 μl of 2 N H_3PO_4, and frozen in a dry ice-acetone bath. Prior to analysis, the samples were thawed and centrifuged for 5 min at 3,000 × g. The supernatant (100 μl) was injected onto a Perkin-Elmer series 3 high-pressure liquid chromatograph equipped with a Sigma 10 data station (Perkin-Elmer Corp., Norwalk, Conn.) and a refractive index detector (Laboratory Data Control, Riviera Beach, Fla.). The metabolites were separated on a Bio-Rad Aminex ion-exclusion HPX 87H column fitted with a Microguard precolumn (Bio-Rad Laboratories, Richmond, Calif.) operating at room temperature. The solvent system was 0.01 N H_2SO_4 with a flow rate of 0.6 ml/min. Samples of 5 drops were eluted into scintillation vials on a Gilson FC 100 microfractionator. The vials were filled with 4.5 ml of Instagel scintillation cocktail (Packard) and counted in a Packard Tricarb scintillation counter. The fractions containing specific intermediary metabolites were determined by the addition of internal standards.

The turnover rate constants were determined by the loss of radioactivity in fractions representing specific ^{14}C-labeled substrates. The turnover rate constants (K) were calculated from the relationship $K = 2.303 \times \log_{10}(C_0/C_t)/t$, where C_0 is the initial count and C_t is the count after time interval t.

Hydrogen consumption. The influence of pH on the maximal rates of hydrogen consumption was determined by monitoring hydrogen consumption progress kinetics in the

presence of excess hydrogen. Sediment samples (10 ml) in 26-ml pressure tubes containing a 2% hydrogen headspace were incubated at 15°C with vigorous shaking. Comparison of the hydrogen concentration in the headspace and the dissolved hydrogen by the extraction methods detailed above demonstrated that hydrogen remained equilibrated between the dissolved and gaseous phases throughout the experiment.

Amendment of the sediment pH. The influence of pH on the conversion of glucose, the turnover of intermediate products, and the production of methane, carbon dioxide, and hydrogen was determined by amending sediments with either 4 N HCl or 4 N NaOH. In all cases, NaCl was used as a control to eliminate anion effects. Sediment (10 ml) was added to 26-ml pressure tubes, and the sediments were amended by a predetermined titration curve. The pH was monitored during the course of the experiments and never varied by more than 0.1 pH unit.

Exogenous additions to the sediments. Sodium acetate, sodium lactate, and glucose were tested for their influence on net gas production rates. Serum vials contained 10 ml of sediment and received 1 ml of sterile, anaerobic donors to initial concentrations of 10 mM. The controls used anaerobic sterile water and sodium chloride. The production rates for CH_4, CO_2, and H_2 were determined as described above.

The influence of hydrogen on the conversion of glucose to intermediary products was determined by syringe addition of hydrogen gas to the nitrogen headspace of serum vials prepared as above. Hydrogen was added to a concentration of 400 μmol/liter.

Enumeration techniques. Three-tube most-probable-number (MPN) analyses were performed with tubes (26 ml) containing 9 ml of a phosphate-buffered medium (11) which contained 0.05% yeast extract, 20 mM of an organic carbon energy source, an N_2-CO_2 or H_2-CO_2 (80:20) headspace, and 0.5 μCi of the indicated ^{14}C-labeled carbon source. Titanium was used as the reducing agent because preliminary experiments demonstrated that hydrogen sulfide inhibited the growth of several organisms at low pH. The tubes were incubated at 20°C for 1 month. Positive results were recorded if greater than 1% of the labeled substrate was converted to either $^{14}CO_2$ or $^{14}CH_4$ or, in the case of pectin, if a greater than 0.2-unit increase in optical density at 660 nm over the uninoculated controls occurred.

Isolation of prevalent hydrolytic species. Isolations were made by dilution from positive end dilution tubes with glucose as the substrate. Dilutions (0.1 ml) were plated on appropriate enrichment media solidified with 1.5% purified agar (Difco Laboratories, Detroit, Mich.). Plates were incubated in an anaerobic glove bag (Coy Laboratory Products, Ann Arbor, Mich.). Colonies were picked with sterile glass capillary tubes and inoculated into 2.5 ml of growth medium. The plating procedure was repeated a second time, and strain purity was checked after growth in liquid medium by microscopy and by streaking on plates and observing colony homogeneity.

RESULTS

General analysis of anaerobic digestion. Physicochemical parameters were measured to characterize the Crystal Bog sediment as an environment for anaerobic digestion of organic matter. The sediments had a porosity of 95% and a high organic matter content of 71%. The pH of the sediments was 4.9. The temperature ranged from 9.9 to 20.2°C, and the conductivity, measured on the water immediately overlying the sediments, was 0.2 μS/cm. The dissolved sulfate concentration was extremely low (0.2 μmol/liter), and dissolved sulfide was undetectable (<1 μmol/liter). Dissolved concentrations of carbon dioxide, methane, and hydrogen were 1,140, 490, and 0.01 μmol/liter, respectively. Carbon dioxide had a greater solubility, even at low pH, than either methane or hydrogen. The molar ratio of steady-state methane to hydrogen was 490,000.

TABLE 1. Turnover of intermediary metabolites in the sediments of Crystal Bog[a]

Metabolite	Rate constant (h^{-1})	Concn (μmol/liter)	Turnover rate (μmol/liter per h)
Ethanol	0.089	<5	ND[b]
Lactate	0.277	<10	ND
Acetate	0.118	34	4.0
Glucose	1.10	<1	ND

[a] Pore waters were separated anaerobically from 1 liter of sediment by centrifugation. The turnover rate constants were determined by the disappearance of radiolabeled compounds from the sediments with high-pressure liquid chromatography.

[b] ND, Not determined. Turnover rates could not be determined for metabolites whose concentrations were undetectable.

Experiments were designed to determine the rate of methane production. The rate of methane production was 6.2 μmol/liter of sediment per h at the in situ temperature (15°C), but as expected it was higher at 35°C (24). We demonstrated previously that the steady-state rate of hydrogen production was 0.03 μmol/liter per h (Goodwin and Zeikus, submitted for publication). Therefore, the ratio of methane to hydrogen production rates in these sediments was 207.

Acetate was the only intermediate metabolite detected in significant quantities in the sediment (Table 1). Glucose, lactate, and ethanol were below the limits of detection. Turnover rate constants for these intermediary metabolites were determined from the rate of loss of ^{14}C-labeled glucose, acetate, lactate, and ethanol. Glucose had the fastest turnover rate constant (1.10 h^{-1}), followed by lactate, acetate and ethanol (0.277, 0.118, and 0.089 h^{-1}, respectively). The turnover rate for acetate was determined to be 4.0 μmol/liter per h.

Acetate appeared to be the major methane precursor because its turnover rate was 64% of the methane production rate. Both [2-^{14}C]acetate and $^{14}CO_2$ were transformed to [^{14}C]methane in the bog sediments. It was difficult to assess whether carbon dioxide was transformed directly to methane because high activities of [^{14}C]acetate production from $^{14}CO_2$ were detected in these sediments.

Experiments were initiated to determine the influence of pH on the anaerobic digestion processes in the sediments. Radiolabeled substrates ([^{14}C]glucose and [2-^{14}C]acetate) were added to sediments in trace quantities. The rates of production of $^{14}CO_2$ and [^{14}C]methane were monitored from sediments amended to pHs between 3.8 and 7.0. The pH optima for the conversion of glucose to gaseous end products is presented in Fig. 1. The pH ranges for significant conversion of glucose to carbon dioxide versus methane were 3.5 to 5.0 versus 4.7 to 6.2, respectively. The pH optima for methane and carbon dioxide production from glucose were approximately 5.2 and 4.5, respectively. The pH optimum for acetate conversion to methane was also near 5.2 (Fig. 2). The rate of methane production from acetate was fivefold lower at pH 3.7 than at pH 4.7 to 5.6. The pH optimum for hydrogen-consuming processes was estimated by comparing the influence of pH on hydrogen

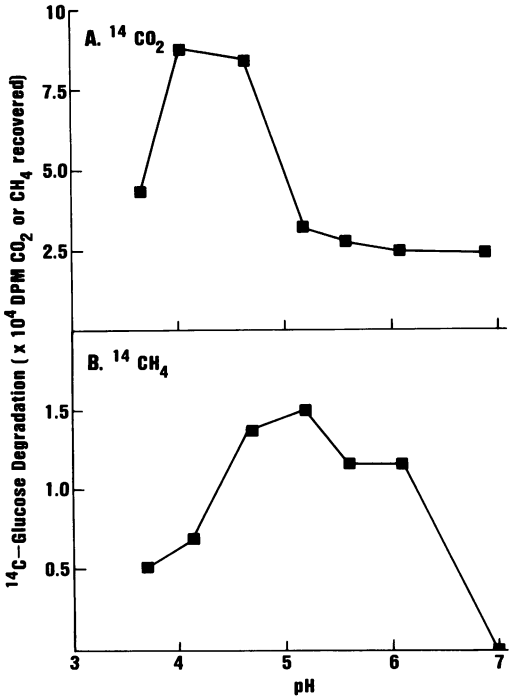

FIG. 1. pH optima for the conversion of [^{14}C]glucose to $^{14}CH_4$ and $^{14}CO_2$. Sediment samples (10 ml) in 26-ml pressure tubes received 1 μCi of [U-^{14}C]glucose and were incubated for 6 h at 15°C.

consumption (Table 2). Hydrogen consumption displayed an optimum at 5.6 and was detectable from pH 3.5 to 7.0.

Studies were undertaken to determine the influence of exogenous electron donors (i.e., hydrogen and reduced carbon compounds), known to stimulate methanogenesis in neutral sediments, on gas metabolism in Crystal Bog sediments. Table 3 compares the influence of adding acetate, lactate, and glucose on hydrogen concentration and the rate of carbon dioxide and methane production. The rate of methane production was not stimulated by these organic additions. Notably, the addition of glucose and lactate stimulated the rate of carbon dioxide production and led to the accumulation of hydrogen in the headspace to concentrations far in excess of steady-state levels in the bog sediments. In control experiments, sodium chloride addition did not alter gas metabolism.

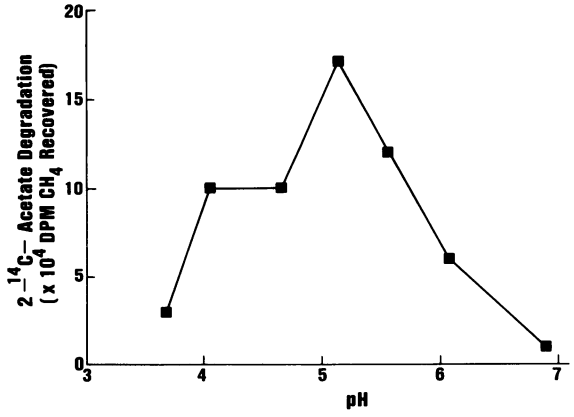

FIG. 2. pH optimum for the conversion of [^{14}C]acetate to $^{14}CH_4$. Sediment samples (10 ml) in 26-ml pressure tubes received 0.5 μCi of [2-^{14}C]acetate and were incubated for 6 h at 15°C.

TABLE 2. Influence of pH on hydrogen consumption in sediments of Crystal Bog[a]

pH	Hydrogen consumption (μmol/liter per h)
3.1	ND[b]
3.5	5.0
4.2	5.3
4.8	6.0
5.6	6.9
6.2	4.4
7.0	4.2

[a] Pressure vials similar to those described in the legend to Fig. 1 were equilibrated with a 2% hydrogen atmosphere. This concentration was shown to saturate the rate of H_2 uptake. H_2 consumption was determined from the loss of H_2 headspace. Dissolved hydrogen was shown to be in equilibrium with the headspace throughout the experiment.
[b] ND, Not detectable.

Analysis of bacterial populations. The different trophic groups present in the bog sediments were enumerated on three separate occasions. Table 4 presents the three-tube MPN enumerations of hydrolytic, acetogenic, and methanogenic populations at pH 5.0 and 7.0. Although the population levels were much lower than reported for neutral sedimentary ecosystems, the higher levels of hydrolytic bacteria than methanogens was expected (14). MPN determinations underestimate population levels in all ecosystems, but perhaps this is augmented in this acid ecosystem by the potential for enhanced species survival in close association in microniches that would be disturbed by these techniques. This was of interest because the first dilution tubes at pH 5.0 served as good methanogenic enrichments. Undoubtedly, special techniques need to be developed to isolate and grow methanogens and acetogens which function in this bog ecosystem.

Hydrolytic, but not acetogenic or methanogenic, organisms were isolated from the end tube dilutions used to enumerate trophic groups. Three interesting hydrolytic bacteria that grew at pH 5.0 were isolated from the bog sediments. The first organism was a gram-positive rod, devoid of catalase, which fermented glucose predominantly to lactate. This organism was identified as *Lactobacillus* sp. strain CBP1 and grew at pHs as low as 4.7. A second prevalent organism was a strictly anaerobic, gram-positive, sporeforming rod that fermented glucose to acetate, ethanol,

TABLE 3. Influence of exogenous carbon electron donors and hydrogen on gas production in Crystal Bog sediments[a]

Addition	Production (μmol/liter per h)		Hydrogen concn[b] (μmol/liter)
	CH_4	CO_2	
Distilled water	6.2	3.1	0.01
Sodium chloride	8.9	3.3	0.01
Sodium acetate	6.8	2.2	0.01
Sodium lactate	6.5	8.5	67
Glucose	8.1	247	400
Hydrogen	6.6	3.2	—[c]

[a] Pressure tubes (26 ml) contained 10 ml of sediment and received additions from sterile anaerobic solutions to final concentrations of 10 mM.
[b] Hydrogen concentrations were determined after 120 h.
[c] —, Not determined.

TABLE 4. Estimated populations of anaerobic bacteria in Crystal Bog sediments[a]

Population	pH	Substrate	Relative population level (MPN/ml of sediment)
Hydrolytic	7.0	Glucose	1.5×10^6
	5.0	Glucose	4.3×10^4
	7.0	Pectin	4.3×10^3
	5.0	Pectin	4.3×10^2
Acidogenic	7.0	Lactate	1.0×10^1
	5.0	Lactate	$<10^1$
	7.0	Ethanol	$<10^1$
	5.0	Ethanol	$<10^1$
Methanogenic	7.0	CO_2-H_2	2.0×10^2
	5.0	CO_2-H_2	$<10^1$
	7.0	Acetate	4.0×10^2
	5.0	Acetate	$<10^1$

[a] All enumerations were three-tube MPN determinations. Positive results were determined from the conversion of radiolabeled substrate to the appropriate products.

butyrate, hydrogen, and carbon dioxide. The organism was identified as *Clostridium* sp. strain CB5; it displayed a pH optimum of 6.0 and grew at pHs as low as 3.9.

The fermentation end products of *Clostridium* sp. strain CB5 varied as a function of the culture medium pH (Table 5). At pHs below 5.0, the production of hydrogen and butyrate significantly decreased while the production of ethanol increased. The addition of 1 atm (101.29 kPa) of hydrogen to the headspace of cultures of this organism resulted in the stimulation of butyrate production relative to acetate production (data not shown).

The third hydrolytic organism isolated from the bog sediments by standard procedures (2) was identified as *Sarcina ventriculi* JK. This strict anaerobe grew as packets in three planes and produced acetate, ethanol, hydrogen, and carbon dioxide but not butyrate from glucose. *S. ventriculi* JK grew at pHs from 2.0 to 9.0. Detailed studies on the relation of pH and the physiology of this organism are presented elsewhere (Goodwin and Zeikus, submitted for publication).

Influence of pH and hydrogen concentration on glucose degradation. Experiments were performed to determine the influence of pH on the glucose turnover rate constant and the production of intermediary metabolites (Table 6). Radiolabeled [^{14}C]glucose was added to sediments of amended pH. The turnover rate constant for glucose was determined from the rate of loss of labeled glucose. Although the turnover rate constant for glucose displayed an apparent pH optimum of near 5.6, glucose turnover at pH 3.7 was still greater than one-half maximal velocity.

The influence of pH perturbation on conversion of radiolabeled glucose to intermediary metabolites is presented in Fig. 3. At the in situ pH of 4.9, acetate was the predominant intermediary metabolite, the amounts of ethanol and lactate were equivalent at 8 h, and the ethanol pool was constant from 12 to 15 h. When the sediment pH was increased to 6.7, the acetate and lactate pool sizes were equivalent at 8 h, the ethanol pool was lower, and net ethanol consumption occurred between 11 and 15 h. When the sediment pH was decreased to pH 4.1, the acetate and the ethanol pool sizes became equivalent at 8 h and the ethanol pool increased between 11 and 15 h.

TABLE 6. Influence of pH on the glucose turnover rate constant in Crystal Bog sediments[a]

pH	Glucose turnover rate constant (h^{-1})
6.9	1.39
6.1	1.35
5.6	1.45
5.2	1.19
4.9	1.10
4.1	0.89
3.7	0.85

[a] Glucose turnover rate constants were determined from the rate of loss of [^{14}C]glucose. The experimental treatments were the same as described in the legend to Fig. 1.

Because a prevalent glucose-hydrolytic organism isolated from this bog displayed a pH-dependent metabolic shift from high hydrogen and butyrate production at neutral pHs to high ethanol production at more acidic pHs, it was decided to test the effect of hydrogen addition on the intermediary metabolism of glucose degradation to determine whether butyrate production was enhanced in the sediments. Figure 4 compares the intermediary path of [^{14}C]glucose degradation in the absence and presence of 400 μmol of exogenous hydrogen per liter. Hydrogen addition notably altered the carbon and electron flow from glucose in that butyrate became a major metabolite in the presence of exogenous hydrogen. Apparently hydrogen-producing and -consuming processes are normally active in the sediments and prevent butyrate accumulation, but the hydrogen-consuming processes are not active enough to prevent butyrate accumulation in the presence of exogenous hydrogen. The overall rate of glucose conversion into intermediary metabolites was markedly decreased in the presence of hydrogen. The turnover rate constant for glucose in the absence and presence of exogenous hydrogen was 1.11 and 0.28 h^{-1}, respectively.

To understand the influence of perturbations caused by exogenous glucose (Table 3) and hydrogen (Fig. 4) on the intermediary metabolism of glucose in bog sediments, the

TABLE 5. Influence of medium pH on fermentation end products of *Clostridium* sp. strain CB5, a prevalent hydrolytic anaerobe isolated from Crystal Bog[a]

Medium pH	Fermentation balance[b] (mmol/100 mmol of glucose)				
	Acetate	Ethanol	Butyrate	CO_2	H_2
7.0	42.5	ND[c]	88.4	222	277
6.0	42.1	2.9	85.1	216	266
5.0	40.5	12.1	73.6	200	224
4.0	39.8	17.3	59.0	178	160

[a] Cells were grown in pressure tubes (26 ml) containing 10 ml of phosphate-buffered medium described previously (11). The medium contained 20 g of glucose per liter.

[b] Carbon and electron recoveries were greater than 95%, assuming balanced fermentation.

[c] ND, Not detectable.

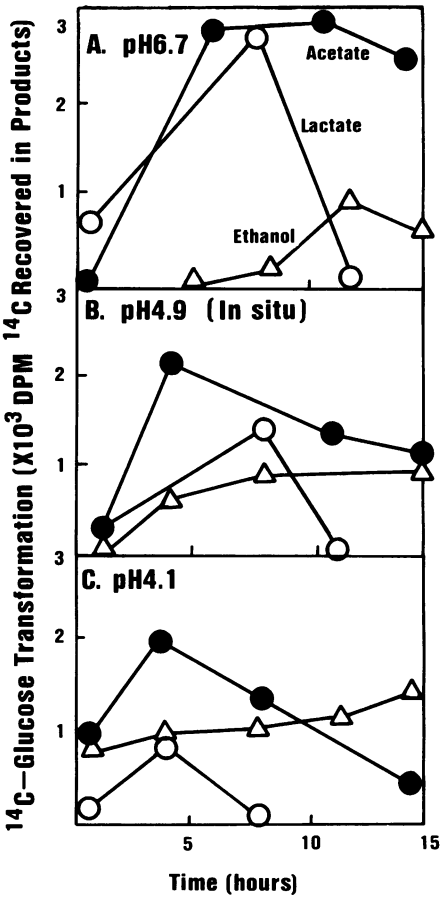

FIG. 3. Influence of pH on the conversion of glucose to intermediary metabolites. Sediment (10 ml) in 26-ml pressure tubes received 0.05 μCi of [U-^{14}C]glucose and were incubated at 15°C.

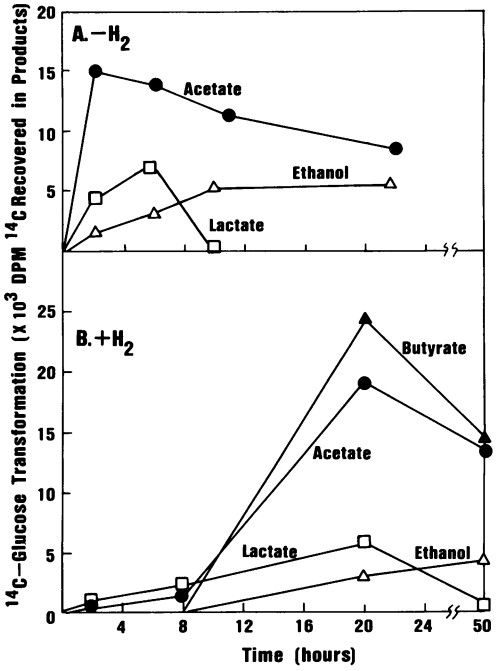

FIG. 4. Influence of hydrogen on the conversion of glucose to intermediary metabolites. Sediments (10 ml) in 26-ml pressure tubes received 0.25 μCi of [U-^{14}C]glucose and were incubated at 15°C. The headspace of the tubes represented in panel B contained 400 μmol of hydrogen per liter.

available free energy for the reactions leading to the consumption of lactate, butyrate, and ethanol were determined for both the in situ conditions and those with exogenous hydrogen perturbation (Table 7). The available free energy for all of the reactions leading to the consumption of intermediary metabolites was negative at in situ hydrogen concentrations. In the presence of excess hydrogen caused by the degradation of 10 mmol of glucose per liter, however, the degradation of either butyrate or ethanol, but not lactate, to acetate and hydrogen was no longer energetically favorable.

TABLE 7. Crystal Bog sediment reaction energetics for the utilization of intermediary metabolites derived from glucose at in situ and hydrogen-perturbed concentrations[a]

Reaction	Available free energy (ΔGr)	
	In situ	H_2 perturbed
0.54 butyrate$^-$ + 0.46 butyric acid + H_2O → 1.16 acetate$^-$ + 0.84 acetic acid + $2H_2$ + 0.62H^+	−50.2	+17.5
1 ethanol + 1H_2O → 0.58 acetate$^-$ + 0.42 acetic acid + $2H_2$ + 0.58H^+	−50.1	+17.4
0.92 lactate$^-$ + 0.08 lactic acid + $2H_2O$ + 0.30H^+ → 0.58 acetate$^-$ + 0.42 acetic acid + 0.04HCO_3^- + 0.96H_2CO_3 + $2H_2$	−88.5	−54.5

[a] Calculations were made with the Nernst equation and the following concentrations of products and reactants (micromolar): acetate, 34; lactate, 10; ethanol, 5; butyrate, 10; CO_2 1,140 (pH 4.9); and hydrogen, 0.01 (in situ) or 400 (hydrogen perturbed).

DISCUSSION

In general, the data show that the overall dynamics but not the routes of carbon and electron flow during anaerobic digestion in bog sediments differ from those in neutral eutrophic lake sediments and that they are markedly slowed by low pH. The data also indicate that bacterially mediated anaerobic digestion processes are adapted to low pH, including the processes of methanogenesis and the coupling of hydrogen-producing and -consuming processes that are linked to fatty acid degradation. Under normal steady-state conditions, the flux of carbon through the ecosystem is slow enough that intermediary carbon metabolites and hydrogen do not accumulate. However, the processes that couple fatty acid degradation and hydrogen production to hydrogen consumption and methanogenesis are very sensitive to pH and hydrogen inhibition. The levels of the microbial populations that perform anaerobic digestion are lower than in neutral ecosystems, which is in accord with the measured biocatalytic activities in the bog. Finally, the hydrolytic activities of the prevalent hydrolytic bog organisms in pure culture and in sediments correlate with thermodynamic predictions of fatty acid and hydrogen metabolism.

Figure 5 compares the in situ flux values for carbon and electrons in Crystal Bog sediments with published values for eutrophic Lake Mendota sediments (15). Anaerobic digestion of organic matter (e.g., glucose and intermediary metabolites) involves three distinct stages: a hydrolytic stage during which polymers and sugars are hydrolyzed to fatty

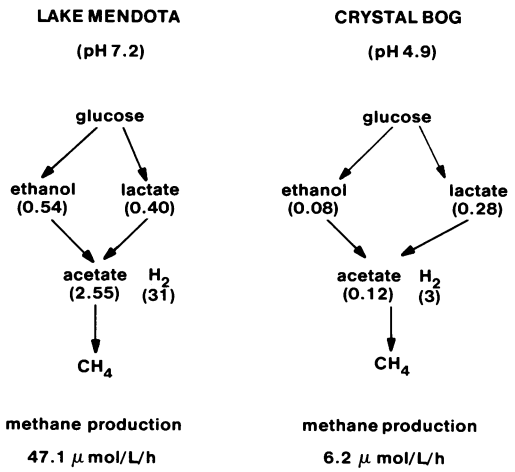

FIG. 5. Comparison of carbon flow in neutral Lake Mendota sediments and acidic Crystal Bog sediments. The values given in parentheses are the turnover rate constants (h^{-1}) for key intermediary metabolites. The values for Lake Mendota are taken from Phelps and Zeikus (15).

acids and alcohols, a hydrogen-producing acetogenic stage during which fatty acids and alcohols are converted to acetate and hydrogen, and a methanogenic stage during which acetate, hydrogen, and carbon dioxide are consumed. The rates of carbon and electron turnover at each of these stages are significantly slower in acid bog sediments than in neutral lake sediments. The ultimate result of these slower turnovers at low pH is a considerably decreased rate of carbon removal and consequentially an enhanced rate of carbon accumulation.

The data support the hypothesis that bacterially mediated anaerobic digestion processes in naturally acidic ecosystems are "optimally" adapted to function at low in situ pH but not at high carbon flux rates. The pH optima for glucose consumption (hydrolytic stage) and both hydrogen and acetate consumption (methanogenic stage) were higher than 4.9, but were within 0.8 pH unit of the in situ pH value. However, because the rate of methane production was not stimulated by the addition of either acetate or hydrogen, the methanogenic population is not substrate limited. The hydrolytic populations do appear to be substrate limited because carbon dioxide production was enhanced by substrate addition. Nonetheless, the consequences of a rapid flux of glucose in the bog sediments would be hydrogen and fatty acid accumulation. Thus, it appears that low acid pH values in the bog limit overall biocatalytic functions of hydrolytic, hydrogen-producing acetogenic, and methanogenic populations.

Carbon flux in the bog ecosystem was inhibited by low pH, as were the overall processes that enable coupling of hydrogen production with hydrogen consumption (i.e., interspecies hydrogen transfer). Addition of excess lactate or glucose to sediments led to the accumulation of hydrogen at pH 4.9 and shifted carbon flux to fatty acid production. Hydrogen accumulation does not accumulate to high enough levels to shift carbon flux when carbon electron donors are added to neutral freshwater sediments (6, 19) because it stimulates carbon dioxide reduction and methanogenesis. In other physically stressed anoxic environments, such as sediments from the Great Salt Lake (F. S. Lupton and J. G. Zeikus, manuscript in preparation) and a thermal acidic pool (pH 2.0) (Goodwin and Zeikus, manuscript in preparation), hydrogen has also been shown to accumulate after the addition of electron donors, because the flux rates of hydrogen-consuming reactions were inhibited. At the low pH in the bog sediments, one might predict that carbon would tend to accumulate as intermediary metabolites (i.e., fatty acids), as is observed during the souring of anaerobic digestors. That fatty acids did not accumulate in this system suggests that polymer hydrolysis is also inhibited in the bog ecosystem.

ACKNOWLEDGMENTS

We thank the staff of Trout Lake Biological Station, University of Wisconsin, for logistical support. The manuscript benefitted from discussions with F. S. Lupton, T. J. Phelps, and M. Chartrain.

This research was supported by Department of Energy grant DE-FG02-85ER13376. Steve Goodwin was supported by a Public Health Service Cellular and Molecular Biology training grant from the National Institutes of Health.

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