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Electron Donors Utilized by Sulfate-Reducing Bacteria in Eutrophic Lake Sediments†

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Mineralization rates of ^{14}C-labeled substrates were determined in the presence and absence of Na_2MoO_4, an inhibitor of sulfate reduction, in the profundal sediments of a shallow eutrophic lake. Sulfate reduction was inhibited by Na_2MoO_4 at all concentrations tested (0.2 to 200 mM), whereas methane production was inhibited at Na_2MoO_4 concentrations greater than 20 mM. Initial mineralization rates of glucose were unaffected by Na_2MoO_4; however, Na_2MoO_4 decreased the mineralization rates of lactate (58%), propionate (52%), an amino acid mixture (85%), and acetate (14%). These decreases in the rates of mineralization were attributed to inhibition of sulfate reduction. Hydrogen stimulated the reduction of $^{35}SO_4^{2-}$ 2.5- to 2.8-fold, demonstrating potential hydrogen oxidation by sulfate-reducing bacteria. These results indicate that sulfate reducers utilize an array of substrates as electron donors and are of potential significance to the in situ mineralization of lactate, propionate, and free amino acids in these sediments.

In marine sediments, sulfate reduction is the predominant terminal electron-accepting process in carbon metabolism (10). Significant rates of sulfate reduction also occur in freshwater sediments despite low sediment sulfate concentrations (21). Although sulfate reduction is of significance to carbon and electron flow in both of these ecosystems, the key electron donors utilized by natural populations of sulfate-reducing bacteria have not been definitively delineated.

Sulfate-reducing bacteria can potentially compete with methanogenic bacteria for H_2 and acetate in both marine (1, 2, 12) and freshwater sediments (24) and have been demonstrated to oxidize the major portion of added hydrogen in marine sediments (16, 18). Additions of lactate stimulated sulfate reduction in some San Francisco Bay sediments, although additions of acetate, pyruvate, and formate had no effect (17). Lactate has been implicated as the major electron donor for sulfate reduction in freshwater sediments (6), whereas acetate stimulated thermophilic sulfate reduction in the water column of Solar Lake, Sinai (11). Acetate and propionate were mineralized in freshwater sediment enrichments in the presence of 20 mM sulfate but not in the absence of sulfate (13). However, these studies do not represent a systematic determination of electron donors for sulfate-reducing bacteria within a single system at or near in situ concentrations of the electron donors.

This investigation examined the effect of Na_2MoO_4, an inhibitor of sulfate-reducing bacteria, on mineralization rates of tracer additions of ^{14}C-substrates in freshwater sediments to delineate natural electron donors for sulfate-reducing bacteria.

(A preliminary report of this work was presented at the Annual Meeting of the American Society for Microbiology, 1981.)

MATERIALS AND METHODS

Sediment collection. Profundal surface sediments from Wintergreen Lake, a shallow (z_m = 6.5 m) hypereutrophic lake located in Southwestern Michigan (14, 15), were sampled with an Eckman dredge. Jars were completely filled with sediment, sealed, stored at 10°C, and subsampled within 24 h. Sediment was homogenized in the jars with a paint shaker, and 5-ml samples were transferred with a syringe to either 30-ml Wheaton serum bottles or anaerobic pressure tubes (Bellco) which were flushed with O_2-free N_2 throughout the subsampling. Samples for sulfate reduction were sealed with Teflon-lined rubber septa (Supelco). All other vessels were stoppered with butyl rubber anaerobe stoppers (Bellco). For H_2 experiments the headspace gas was flushed with O_2-free H_2 through syringe needles.

Sulfate reduction. Prereduced (by sparging with O_2-free N_2) carrier-free $Na_2{}^{35}SO_4$ (New England Nu-

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clear Corp., 0.5 ml, containing 1 to 2 μCi), diluted with unlabeled Na_2SO_4 when appropriate, was added through a syringe and needle to each sample. Sediment samples were blended with a Vortex mixer and incubated at 10°C (with shaking when H_2 was present in the headspace) for appropriate periods. Bottles containing an H_2 headspace were preincubated for 30 min before amendment with $Na_2{}^{35}SO_4$, as were controls which had been killed by the addition of 0.5 ml of 10% zinc acetate. Sulfate reduction was terminated by the addition of 0.5 ml of prereduced 10% zinc acetate, and the samples were frozen until analysis. The $H_2{}^{35}S$ produced was trapped with a flushing train as described by Smith and Klug (21). The headspace gas in the serum bottle was flushed with O_2-free N_2 for 2 min to purge the system of O_2 before the addition of 5 ml of O_2-free 3 N HCl to the frozen sediment. Samples were flushed for 30 min into two traps containing 8 ml of 2% $CdCl_2$. Upon completion of flushing, 10 ml of Aqueous Counting Scintillant (Amersham Corp.) was added to each trap (scintillation vials), and the radioactivity was determined with a Beckman LS8000 liquid scintillation counter (Beckman Instruments). Due to the difficulties involved with measuring the sediment sulfate concentrations (21) the sulfate-reducing activity is reported as the rate of $H_2{}^{35}S$ recovered or as the turnover time of the in situ sulfate pool based upon the rate of $H_2{}^{35}S$ recovered and the activity of $^{35}SO_4{}^{2-}$ added to the sediment.

Effect of molybdate on sediment activities. Prereduced Na_2MoO_4 (0.5 ml at appropriate concentrations) was added to sediment subsamples and preincubated for 30 min at 10°C, and sulfate reduction was determined as described above. Due to the formation of insoluble MoS_3 at low pH (5) the $^{35}S^{2-}$ produced was quantified as described by Oremland and Silverman (17). Sediment samples were thawed and filtered through membrane filters (0.45-μm HA filters; Millipore Corp.). The bottles and filters were rinsed several times with 1% Na_2SO_4, the filters were dried, and sediment subsamples were placed in scintillation vials containing 5 ml of H_2O. The vials were shaken overnight to disperse the sediment, 10 ml of Aqueous Counting Scintillant was added, and the samples were dark adapted 24 h before the determination of radioactivity. The counting efficiency of each sample was determined by adding an internal standard of $H_2{}^{35}SO_4$.

The effect of Na_2MoO_4 upon methane production in profundal sediments was measured by dispensing sediment and Na_2MoO_4 into anaerobic pressure tubes (Bellco) as described above. Headspace gas was flushed with 93% N_2–7% CO_2, and the samples were preincubated for 30 min at 10°C. Methane production was determined by analyzing the change in headspace CH_4 concentration at several time points over the course of 12 h. Methane was analyzed on a Varian 600 D gas chromatograph as previously described (15). The effect of Na_2MoO_4 on the mineralization of [2-^{14}C]acetate was measured in a second set of 5-ml subsamples prepared as above. Prereduced [2-^{14}C]-acetic acid, sodium salt (New England Nuclear Corp.; 1.5 μCi, 27.4 nmol; 0.2 ml) was added through a syringe and needle, and the samples were incubated at 10°C for 30 min. Biological activity was stopped by quick-freezing in a dry ice-ethanol bath. $^{14}CH_4$ and $^{14}CO_2$ in the headspace gas were determined with a Varian 3700 gas chromatograph (Varian Instruments) equipped with a thermal conductivity detector connected in series with a gas proportional counter. The gas chromatograph analysis was at 40°C with a stainless steel column (1.8 m by 2-mm inner diameter) packed with Porapak N. The distribution of $^{14}CO_2$ between the aqueous and gaseous phases was determined by adding 0.1 ml of a $NaH^{14}CO_3$ (New England Nuclear Corp.; 1 μCi/ml) solution to the tubes; after equilibration, the radioactivity of the headspace gas was determined a second time. Results are presented as a ratio, termed the respiratory index (RI) by Winfrey and Zeikus (25), where $RI = {}^{14}CO_2/({}^{14}CO_2 + {}^{14}CH_4)$.

Mineralization of ^{14}C-labeled substrates. A 0.5-ml amount of a prereduced solution of one of four different ^{14}C-labeled substrates was added by syringe and needle to 5-ml subsamples of sediment. The ^{14}C-substrates added were: [*U*-^{14}C]lactic acid, sodium salt (New England Nuclear Corp.; 1 μCi, 7.2 nmol); [1-^{14}C]propionic acid, sodium salt (New England Nuclear Corp.; 0.2 μCi, 20 nmol); [*U*-^{14}C]glucose (New England Nuclear Corp.; 1 μCi, 5.5 nmol); and *U*-^{14}C-amino acid mixture (New England Nuclear Corp.; 0.15 μCi, 1.3 to 2.8 nmol, containing 15 individual L-amino acids). Controls were killed by the addition of 0.5 ml of prereduced 50% glutaraldehyde and preincubated for 30 min before the addition of ^{14}C-labeled substrate. A second set received 0.5 ml of a prereduced solution of Na_2MoO_4 of the appropriate concentration and was also preincubated 30 min. Sediment samples were blended with a Vortex mixer and incubated at 10°C for appropriate lengths of time, and the biological activity was terminated by quick-freezing in a dry ice-acetone bath. The samples were kept frozen at −10°C until analysis. Incubation intervals and/or the position of the ^{14}C label (i.e., [1-^{14}C]propionic acid) was chosen to ensure minimal $^{14}CH_4$ production. Frozen samples from the longest incubation periods were placed in a boiling water bath for 10 min and then allowed to cool. Headspace gas from these samples was analyzed for $^{14}CH_4$ with the gas chromatograph-gas proportional counter system described above.

$^{14}CO_2$ was trapped in the flushing train as previously described, except that a third trap was added, 1 N KOH was substituted for $CdCl_2$, and the volume was increased to 18 ml in each trap. Upon completion of flushing a 1-ml sample of each trap was added to 1 ml of a saturated $BaCl_2$ solution contained in scintillation vials, followed by the addition of 5 ml of 0.4 N tris(hydroxymethyl)aminomethane buffer (pH 1.3) and 8 ml of Aqueous Counting Scintillant. The scintillation vials were dark adapted for several hours, and the radioactivity was determined as described. Counting efficiency of the $BaCO_3$-gel suspension was 84%.

RESULTS

Effect of Na_2MoO_4 on sediment processes. Na_2MoO_4 completely inhibited sulfate reduction during a 30-min incubation in Wintergreen Lake profundal sediments at all concentrations of Na_2MoO_4 tested (Table 1). Total methane pro-

TABLE 1. *Effect of Na_2MoO_4 on methane production and sulfate reduction in Wintergreen Lake profundal sediments*[a]

Addition[b]	Methane production[c]	% Inhibition	Sulfate reduction[d]	% Inhibition
Control	35 (3)	0	420 (12)	0
0.2 mM MoO_4^{2-}	30 (10)	14	0	100
2 mM MoO_4^{2-}	32 (3)	9	0	100
20 mM MoO_4^{2-}	28 (2)	20	0	100
200 mM MoO_4^{2-}	17 (2)	51	ND	

[a] Sediments were collected September 1980.
[b] Final concentration of Na_2MoO_4. Control was amended with O_2-free water.
[c] Micromoles of CH_4 produced per liter of sediment per hour. Numbers within parenthesis indicate the standard error; $n = 5$.
[d] Microcuries of $^{35}S^{2-}$ produced per liter of sediment per hour at in situ sulfate concentration. Numbers within parentheses indicate the standard errors; $n = 3$; ND, not determined.

duction was inhibited 51% by 200 mM Na_2MoO_4 (Table 1); however, at lower concentrations (<20 mM) methane production was only slightly inhibited (10%). Production of $^{14}CH_4$ from [2-^{14}C]acetate was essentially unaffected by Na_2MoO_4 concentrations below 20 mM (Table 2), whereas nearly complete inhibition (98%) was noted after addition of 200 mM Na_2MoO_4. Mineralization rates of [*U*-^{14}C]glucose, as measured by $^{14}CO_2$ production, were unaffected by a final concentration of 20 mM Na_2MoO_4 (Fig. 1). No $^{14}CH_4$ was detected during the time course of the experiment in either the presence or the absence of the inhibitor.

Mineralization of ^{14}C-substrates. The principal mineralization product of [2-^{14}C]acetate in Wintergreen Lake sediments was $^{14}CH_4$, as evidenced by an RI value of 0.2 (Table 2). In the presence of 0.2 mM Na_2MoO_4 the RI value for acetate mineralization was 0.05. This fourfold decrease is due to a decrease in $^{14}CO_2$ production relative to $^{14}CH_4$ production. At higher concentrations of Na_2MoO_4, $^{14}CO_2$ production remained relatively constant, whereas $^{14}CH_4$ production was inhibited, resulting in an increasing RI value for acetate mineralization in the sediments as the Na_2MoO_4 concentration increased. The mineralization rate of [1-^{14}C]propionate was linear for 20 min in the presence ($r^2 = 0.83$) or absence ($r^2 = 0.96$) of 20 mM Na_2MoO_4 (Fig. 2). The mineralization rate was 38% lower in the presence of Na_2MoO_4. Table 3 summarizes the results of similar experiments for sediments amended with four different ^{14}C-labeled substrates. Na_2MoO_4 significantly inhibited the mineralization rates of lactate, propionate, and a mixture of amino acids (2.2, 0.6, and 0.3 μCi per min per liter sediment respectively), but not the mineralization of glucose (1.6 μCi per min per liter of sediment). In each case mineralization rates were linear without an apparent time lag. The inhibition of both lactate and propionate mineralization was the same at 1 or 20 mM Na_2MoO_4. No $^{14}CH_4$ was detected in any set of samples within the time period examined.

Effect of H_2 on sulfate reduction. Sulfate reduction in Wintergreen Lake profundal sediments was stimulated by the addition of H_2 to the reacting flask headspace (Table 4). A stimulation was evident in sediments amended with both carrier-free $^{35}SO_4^{2-}$ and 1 mM $^{35}SO_4^{2-}$ (final concentration). The reduction rate was linear for

TABLE 2. *Effect of Na_2MoO_4 on mineralizaton of [2-^{14}C]acetate in Wintergreen Lake profundal sediments*[a]

Addition[b]	Total gas produced (nCi)[c]		RI value
	$^{14}CO_2$	$^{14}CH_4$	
Control	48 (4)	196 (20)	0.20
0.2 mM MoO_4^{2-}	9 (1)	160 (7)	0.05
2 mM MoO_4^{2-}	22 (2)	229 (25)	0.09
20 mM MoO_4^{2-}	15 (2)	105 (7)	0.13
200 mM MoO_4^{2-}	14 (5)	4 (4)	0.78

[a] Sediments were collected September 1980.
[b] Final concentration of Na_2MoO_4. Control was amended with O_2-free water.
[c] Numbers within parentheses indicate the standard errors; $n = 4$.

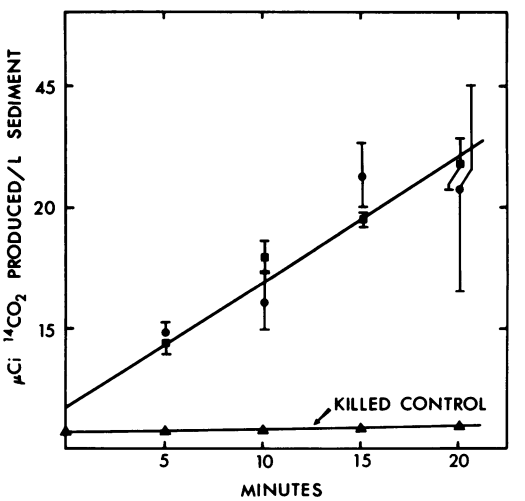

FIG. 1. *Effect of Na_2MoO_4 on the mineralization of [U-^{14}C]glucose at 10°C. Controls were pretreated with glutaraldehyde. Data points are the mean of triplicates. Symbols: ●, with MoO_4^{2-}; ■, without MoO_4^{2-}; error bars represent ±1 standard error.*

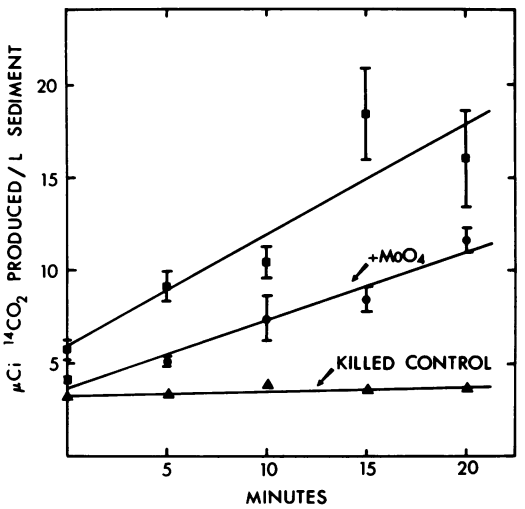

FIG. 2. *Effect of Na_2MoO_4 on the mineralization of [1-^{14}C]propionate at 10°C. Controls were pretreated with glutaraldehyde. Data points are the mean of triplicates. Error bars represent ±1 standard error.*

TABLE 3. *Effect of Na_2MoO_4 on mineralization rates of ^{14}C-labeled substrates in Wintergreen Lake profundal sediments*[a]

Substrate	Na_2MoO_4 concn (mM)	% Inhibition[b]
Lactate	20	47.0
	1	58.3
Propionate	20	52.8
	1	51.7
L-Amino acid mixture	20	84.6
Glucose	20	5.7

[a] Sediments were collected in October 1979 and July, August, and September 1980.

[b] For 20 mM Na_2MoO_4, $n = 2$; for 1 mM Na_2MoO_4, $n = 1$.

10 min at in situ SO_4^{2-} concentrations and the entire time course (20 min) at a 1 mM SO_4^{2-} concentration (data not shown).

DISCUSSION

Molybdate is well established as an inhibitor of sulfate-reducing bacteria (8, 20, 23). It is stereochemically similar to sulfate and has been demonstrated to inhibit adenosine triphosphate sulfurylase, the first enzyme in the sulfate-reducing pathway (19). Since inhibition is specific for biochemical processes involving sulfate, molybdate appears to be well suited for studies of sulfate-reducing bacteria in natural habitats when conducted on a short-term basis (≪1 generation time). In such a situation the effect of molybdate upon the total metabolism of dissimilatory sulfate-reducing bacteria would be far greater than corresponding effects upon assimilatory sulfate-reducing bacteria. Taylor and Oremland (23) have demonstrated that organisms reducing sulfate were much more sensitive to molybdate than were other physiological types of bacteria.

Few studies have employed the selective inhibition by molybdate to investigate the role of sulfate-reducing bacteria in natural habitats. The inhibitor was used to investigate the interaction of sulfate-reducing bacteria and methanogenic bacteria in marine sediments (23). Huisingh and Matrone (7) demonstrated that molybdate inhibited sulfate reduction in sheep fed Na_2SO_4 but stimulated sulfide production from methionine (9). The concentration of molybdate necessary to effect inhibition in natural habitats is dependent upon the sulfate concentration, since inhibition is competitive in nature. It may also be dependent upon the sulfide concentration as complexes of $MoO_2S_2^{2-}$ and MoS_4^{2-} are formed (26). The concentration of sulfate and sulfide in Wintergreen Lake surface sediments was 0.05 and 0.2 mM, respectively, during summer stratification (21). Sulfate reduction in marine sediments is completely inhibited by 20 mM molybdate (the only concentration reportedly tested) (17). However, in freshwater sediments, where the sulfate concentration is much lower, sulfate reduction was completely inhibited by 0.2 mM molybdate (Table 1).

High concentrations (>20 mM) of Na_2MoO_4 inhibited both total methane production and $^{14}CH_4$ production from [2-^{14}C]acetate in profundal sediments. A comparison of total methane

TABLE 4. *Effect of H_2 on sulfate reduction in Wintergreen Lake profundal sediments*[a]

Expt	Sulfate concn[b]	Headspace gas	T_t[c] (h)	% Stimulation
1	In situ	N_2	1.0	
		H_2	0.4	250
	1 mM	N_2	326	
		H_2	204	160
2	In situ	N_2	4.8	
		H_2	1.7	282

[a] Sediments were collected April 1980 for experiment 1 and July 1980 for experiment 2.

[b] Final sediment sulfate concentration.

[c] Turnover time of the $^{35}SO_4^{2-}$ pool, based on initial rates of sulfate reduction.

production (Table 1) and production from acetate (Table 2) indicates that methane production from acetate was inhibited to a greater extent by Na_2MoO_4 than was methane production from H_2 and CO_2. Both sources of methane production were relatively unaffected at lower concentrations of Na_2MoO_4 (i.e., <20 mM). Heterotrophic metabolic processes not involving immediate precursors for sulfate reduction or methane production appeared to be unaffected by Na_2MoO_4, as rates of glucose mineralization were unaltered in the presence of 20 mM Na_2MoO_4 (Fig. 1).

The RI value for [2-^{14}C]acetate mineralization indicates that 20% of the total mineralization of the methyl group of acetate was oxidation to CO_2. Na_2MoO_4 inhibited 69% of this oxidation, indicating that 14% of the acetate mineralization in the absence of Na_2MoO_4 could be attributed to sulfate reduction, while 6% of the methyl group of acetate was mineralized to CO_2 by some other group of organisms in the sediment.

The mineralization rates of acetate, lactate, propionate, and an amino acid mixture in the presence and absence of Na_2MoO_4 strongly imply that sulfate-reducing bacteria are directly involved in the mineralization of these substrates in Wintergreen Lake profundal sediments (Tables 2 and 3). The actual mineralization rates of glucose, lactate, and free amino acids cannot be extrapolated from these data, since although low concentrations of ^{14}C-labeled substrates were added, these exogenous additions are considered to have altered the natural concentration (<1 μM). Additions of [^{14}C]propionate or [^{14}C]acetate did not significantly alter the natural sediment concentrations of 14 μM and 100 μM for propionate and acetate, respectively (D. R. Lovley and M. J. Klug, submitted for publication). Although 52% of the total propionate mineralization could be attributed to sulfate reduction compared to 14% of the total acetate mineralization, conversely these data do not indicate the relative contribution of acetate and propionate to sulfate reduction since the sediment propionate concentration is approximately 10-fold lower than the sediment acetate concentration. The inhibition of mineralization by Na_2MoO_4 at natural substrate concentrations represents a minimum estimate of mineralization by sulfate-reducing bacteria since inhibition of sulfate reduction can increase the availability of a given substrate to other sediment microorganisms.

Hydrogen can also serve as an electron donor for sulfate-reducing bacteria (3). Cocultures of *Desulfovibrio* can participate in interspecies H_2 transfer as well by oxidizing H_2 and reducing sulfate (1, 4). Oremland and Taylor (18) determined that sulfate-reducing bacteria were primarily responsible for H_2 consumption in marine sediments, whereas a threefold stimulation of sulfate reduction by hydrogen addition was reported in salt marsh sediments (2). H_2 stimulated sulfate reduction 2.5- to 2.8-fold in Wintergreen Lake profundal sediments, indicating potential hydrogen oxidation by sulfate reduction in freshwater sediments. Methane production in these sediments is the primary H_2-consuming process (22), yet occurs concurrently with sulfate reduction within the same sediment interval (M. J. Klug, G. M. King, R. L. Smith, D. R. Lovley, and J. W. H. Dacey, manuscript in preparation). A lack of an increase in total methane production in the presence of Na_2MoO_4 (Table 1) indicates that hydrogen oxidation by sulfate-reducing bacteria at in situ hydrogen concentrations is relatively minor. In these sediments it is likely that the methanogens are maintaining the natural hydrogen concentration significantly below the K_m for hydrogen utilization by sulfate reducers.

Few studies have determined natural electron donors for sulfate reduction in anoxic sediments. Most evidence has been determined indirectly, with lactate, acetate, and hydrogen being commonly implicated as electron donors (18, 24). The data presented here indicate that lactate, acetate, free amino acids, and propionate potentially provide electrons for sulfate reduction in the freshwater sediments examined. Lactate and Casamino Acids can be utilized for growth by several sulfate-reducing isolates from Wintergreen Lake profundal sediments (R. L. Smith and M. J. Klug, manuscript in preparation). Thus, it appears that an array of substrates serve as natural electron donors for sulfate reduction in these sediments and that sulfate reducers are of potential significance to the in situ mineralization of lactate, propionate, and free amino acids, though mineralization of any given substrate may represent only a small fraction of the total electron flow through sulfate reduction.

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