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Reduction of Selenate to Selenide by Sulfate-Respiring Bacteria: Experiments with Cell Suspensions and Estuarine Sediments

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Washed cell suspensions of *Desulfovibrio desulfuricans* subsp. *aestuarii* were capable of reducing nanomolar levels of selenate to selenide as well as sulfate to sulfide. Reduction of these species was inhibited by 1 mM selenate or tungstate. The addition of 1 mM sulfate decreased the reduction of selenate and enhanced the reduction of sulfate. Increasing concentrations of sulfate inhibited rates of selenate reduction but enhanced sulfate reduction rates. Cell suspensions kept in 1 mM selenate were incapable of reducing either selenate or sulfate when the selenate/sulfate ratio was ≥0.02, indicating that irreversible inhibition occurs at high selenate concentrations. Anoxic estuarine sediments having an active flora of sulfate-respiring bacteria were capable of a small amount of selenate reduction when ambient sulfate concentrations were low (<4 mM). These results indicate that sulfate is an inhibitor of the reduction of trace quantities of selenate. Therefore, direct reduction of traces of selenate to selenide by sulfate-respiring bacteria in natural environments is constrained by the ambient concentration of sulfate ions. The significance of this observation with regard to the role sediments play in sequestering selenium is discussed.

Sulfur and selenium are both group VIA elements, and their chemical similarities result in some analogous biochemical and geochemical reactions (2, 6, 24). However, in contrast to the case for sulfur, the biogeochemical cycle of selenium in nature has not been well defined. Reported microbial transformations of selenium include oxidation of reduced species (21, 26), assimilation into organic compounds (22, 24), and the formation and destruction of methylated compounds (3, 4, 15, 20). Reduction of selenite to elemental selenium and selenide was reported for whole cells of aerobically grown *Salmonella heidelberg* (11), as well as for H_2-incubated anaerobic cell extracts of *Micrococcus lactilyticus*, *Clostridium pasteurianum*, and *Desulfovibrio desulfuricans* (29). However, although dissimilatory reduction of sulfate to sulfide by sulfate-respiring bacteria (SRB) is an important aspect of the sulfur cycle in nature, it is not clear if the corresponding reduction of selenate to selenide by these organisms occurs in the environment.

Our recent interest in the biogeochemistry of selenium was prompted by the discovery of toxic concentrations of this element in evaporation ponds of the Kesterson National Wildlife Refuge in California (19). This salt marsh environment is characterized by high sulfate concentrations (50 to 100 mM; 19) and extensive anaerobic bottom sediments (9, 15). Preliminary studies indicated that selenate ions in the waters of the marsh were not chemically conservative (19) and accumulated in the sediments as a reduced precipitate (9). Therefore, circumstantial evidence implied that SRB in the Kesterson sediments may have directly reduced selenate to selenide, which then formed insoluble precipitates, such as iron selenides.

Selenate and other group VI oxyanions are competitive inhibitors of sulfate respiration (1, 16, 18). However, whereas molybdate or tungstate analog of adenosine-5′-phosphosulfate ($APSO_4^{2-}$) (formed from ATP-sulfurylase) were putatively unstable, the selenate analog adenosine-5′-phosphoselenate ($APSeO_4^{2-}$) had greater stability (28). These results implied that trace concentrations of selenate could enter the pathway of dissimilatory sulfate reduction and ultimately be converted to selenide. We now report that washed cell suspensions of *D. desulfuricans* as well as anoxic estuarine sediments were capable of reducing ^{75}Se-selenate to ^{75}Se-selenide. However, sulfate inhibited this reduction. These results have important implications for the ability of SRB to immobilize selenium in sulfate-rich sediments.

MATERIALS AND METHODS

Preparation of washed cell suspensions. *D. desulfuricans* subsp. *aestuarii* (ATCC 17990) was grown in 50-ml batches in serum bottles (150 ml) sealed under N_2. The lactate-yeast extract medium of Mara and Williams (10) was used as modified by Oremland and Silverman (14). Late-log-phase cultures (100 ml; ~50 μg of cell protein per ml) were combined and centrifuged under N_2. The pellet was suspended in 100 to 140 ml of sulfate-free mineral salts (see below), centrifuged, and suspended again in fresh mineral salts. The final cell suspensions were dispensed in 15-ml portions into serum bottles (30 ml) which were then crimp sealed under N_2 with black butyl rubber stoppers. All manipulations were made in an anaerobic glove box (Coy Manufacturing Co., Ann Arbor, Mich.) to avoid exposure to O_2. The mineral salts solution was composed of the following (millimolar): NaCl (431), $MgSO_4 \cdot 7H_2O$ (8.0), NH_4Cl (18.9), $FeSO_4 \cdot 7H_2O$ (1.8), KH_2PO_4 (1.8), K_2HPO_4 (1.4), $CaCl_2 \cdot 2H_2O$ (1.4). The solution was adjusted to a final pH of 7.5. All bottles were flushed with O_2-free N_2 (~150 ml/min for 5 min) prior to initiation of the experiments to ensure anaerobiosis. Additions of Na_2SO_4, Na_2SeO_4, or Na_2WO_4 (concentrations given in the text) and ^{35}S-sulfate, ^{75}Se-selenate, or ^{75}Se-selenite (see below) were made just prior to the start of the experiments via syringe injection from anaerobic stock solutions. Tungstate was used as an inhibitor of sulfate respirers instead of molybdate to avoid the formation of molybdosulfide complexes, which interfere with acid volatilization of sulfides (1, 14). Sodium lactate

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(final concentration, 63 mM) was added at the start of the experiment (unless indicated otherwise) to provide an electron donor for the SRB. Experimental bottles were incubated at room temperature (~20°C), and subsamples (2 ml) were withdrawn periodically for extraction of radioactive selenide and sulfide (see below). Rates of sulfate or selenate reduction by washed cell suspensions were calculated from the respective linear portions of the ^{35}S-sulfide or ^{75}Se-selenide production curves.

Preparation of sediment slurries. Sediments were taken from a San Francisco Bay salt marsh and homogenized under N_2 with an equal volume of sulfate-free artificial bay water (13). The slurry was dispensed (20 ml) into serum bottles (30 ml) sealed under N_2. Selected experimental bottles were (i) autoclaved (121°C and 250 kPa for 45 min), (ii) supplemented with sodium lactate (1 mM) or sodium selenate (1 mM), or (iii) incubated under an atmosphere of H_2 instead of N_2. Experimental conditions were tested in duplicate (selenate reduction) or triplicate (sulfate reduction) sets of bottles. Bottles received additions of either ^{35}S-sulfate or ^{75}Se-selenate (see below) and were incubated in the dark at 20°C with constant rotary shaking (~150 rpm). Subsamples (5 ml) were withdrawn periodically for extraction of radioactive sulfide or selenide (see below). "Low-sulfate" slurries contained less than 4 mM sulfate, as calculated from the possible carry-over of interstitial sulfate.

Radioisotopes. ^{35}S-sulfate (carrier free; 1.505 Ci/μmol), ^{75}Se-selenate (1.722 Ci of Se per mmol), and ^{75}Se-selenite (15.089 Ci of Se per mmol) were obtained from ICN Radiochemicals, Irvine, Calif. The purity of the Se isotopes was >99% and was checked by high-pressure liquid chromatography separation (Waters model 6000A solvent delivery system with a Nova-pak C-18 column and a model 480 UV-visible detector) of selenite and selenate followed by fraction collection and counting. No contamination was noted, and counts were confined to only the injected species. Stock solutions (10 μCi/ml) were prepared in water under N_2. Syringe injections of radioisotopes (2 μCi) were made into the cell suspensions or into the sediment slurries to yield final activities of 0.13 μCi/ml (cells) or 0.10 μCi/ml (sediments). Concentrations of selenate and sulfate achieved by isotope additions were 58 to 77 nM and 66 to 89 pM, respectively.

Extraction of ^{75}Se-selenide and ^{35}S-sulfide. Subsamples of the cell suspensions (2 ml) or the sediment slurries (5 ml) were injected into sealed reaction bottles (20 ml) connected to an N_2 stripping-trapping train described elsewhere (23). The subsamples were acidified by injection of an equal volume of 6 N HCl. Liberated acid-volatile ^{35}S-sulfide and ^{75}Se-selenide were trapped by bubbling through two sequential 16 mM zinc acetate traps. More than 85% of the extracted radioisotope was recovered in the first sequential trap. Teflon tubing was used throughout the stripping-trapping system. When ^{75}Se-selenide was extracted, it proved necessary to change the tubing after each extraction because ^{75}Se adsorption on the walls of the tubing resulted in high background levels (adsorbed activity was usually less than 5% and not more than 15% of the quantity trapped). For the determination of ^{35}S, 10 ml of scintillation cocktail (Aquasol II; New England Nuclear Corp., Boston, Mass.) was added to an equal volume of trapping fluid and counted in a Beckman scintillation counter. Samples were corrected for quench by using the external standard method. The efficiency of trapping of ^{35}S-sulfide was 85% (23). ^{75}Se-selenide was trapped in 15 ml of the zinc acetate trapping fluid, the subsequent gamma radiation was quantified on a Beckman Gamma 8000 counter, and background counts (~129 cpm) were subtracted from experimental data. The efficiency of trapping of ^{75}Se-selenide was determined by reducing ^{75}Se-selenate to ^{75}Se-selenide with sodium borohydride. A glass scintillation vial containing distilled water (3 ml) and sealed with a Teflon-lined stopper was connected to the zinc acetate trapping system with Teflon tubing. A constant flow of N_2 was maintained through the reaction vial and traps. ^{75}Se-selenite (2 μCi), sodium borohydride solution (1 ml of 0.16 M $NaBH_4$ in 0.025 N NaOH), and 6 N HCl (3 ml) were injected in sequence into the reaction vessel. The reaction proceeded for 2 to 5 min. The radioactivity left in solution, adsorbed to the Teflon stopper and tubing, and present in the traps was then determined. The quantity of radioactivity lost from the solution upon acidification of the borohydride-treated ^{75}Se-selenite was compared with the amount captured in the traps. This resulted in an efficiency of ~97 ± 6% (n = 15).

The efficiency of extraction of ^{75}Se-selenide from the sediment slurries and washed cell suspensions was not investigated. A significant fraction of ^{35}S-sulfide in sediments may not be volatilized upon acidification of the sediments owing to the formation of elemental sulfur or to processing artifacts and isotopic exchange with pyrite (7, 8). A similar situation may exist for ^{75}Se-selenide, and therefore, our measured rates probably underestimate the true rate of ^{75}Se-selenide production. However, these experiments were designed to determine whether this reaction occurs rather than to estimate in situ rates.

RESULTS

Washed cell suspensions. Cell suspensions of *D. desulfuricans* were capable of reducing sulfate, selenate, or selenite. Reduction of these compounds was greatly stimulated by the presence of lactate. For example, in the case of selenate, after 4 h of incubation, ~6,000 dpm/ml was recovered in lactate-amended suspensions, while only ~200 dpm/ml was recovered in suspensions lacking an electron donor. Similarly, sulfate reduction was enhanced by lactate (~40,000 dpm/ml with lactate versus ~4,000 dpm/ml without lactate). Reduction of sulfate and selenate proceeded at a constant rate (Fig. 1). However, selenite reduction was erratic, although it generally increased with time (data not shown). Nonetheless, significant counts were recovered from lactate-amended selenite suspensions (4,000 to 7,000 dpm/ml after 2 to 5 h of incubation), as opposed to suspensions incubated without an electron donor (<500 dpm/ml).

In two separate experiments, cell suspensions of *D. desulfuricans* reduced either selenate to selenide (Fig. 1A) or sulfate to sulfide (Fig. 1B). Formation of the reduced radioisotopes was generally linear for over 2 h in both cases. The addition of 1 mM sulfate slightly retarded selenate reduction (Fig. 1A); in contrast, sulfate reduction was enhanced (Fig. 1B). The addition of either 1 mM selenate or 1 mM tungstate strongly inhibited the reduction of either isotope.

The effects of various concentrations of sulfate upon the rate of reduction of either sulfate or selenate are shown in Table 1. Increased sulfate concentrations stimulated the rate of sulfate reduction, while they had an inhibitory effect upon the reduction of traces (~68 nM) of selenate. Sulfate concentrations of 50 mM inhibited the rate of selenate reduction by 99.4%.

The effect of various concentrations of sulfate ions (1, 5, or 50 mM) upon the inhibition by 1 mM selenate of the reduction of either selenate or sulfate was examined. Total

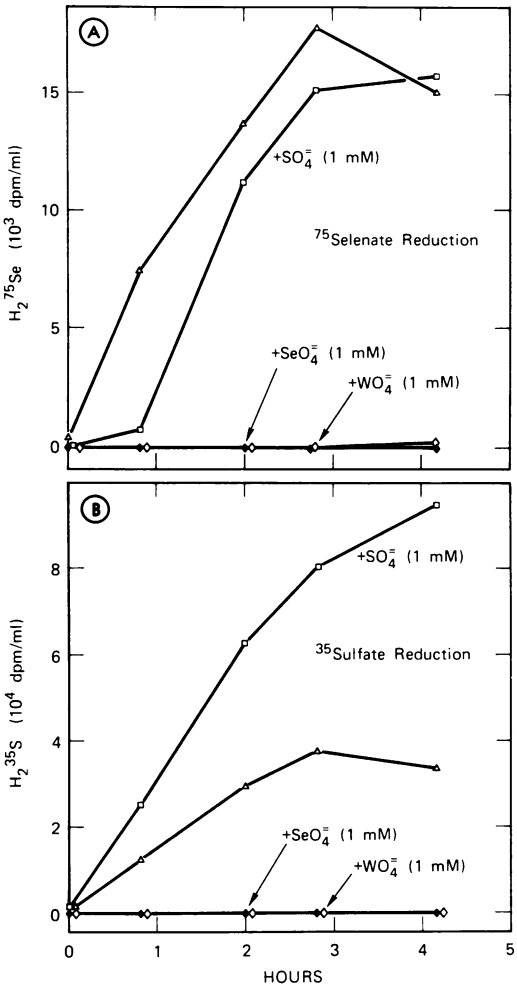

FIG. 1. Formation of ^{75}Se-selenide (A) and ^{35}S-sulfide (B) in parallel experiments with washed cell suspensions of *D. desulfuricans*. Cells were incubated without sulfate (△), with 1 mM sulfate (□), with 1 mM selenate (◆), or with 1 mM tungstate (◇).

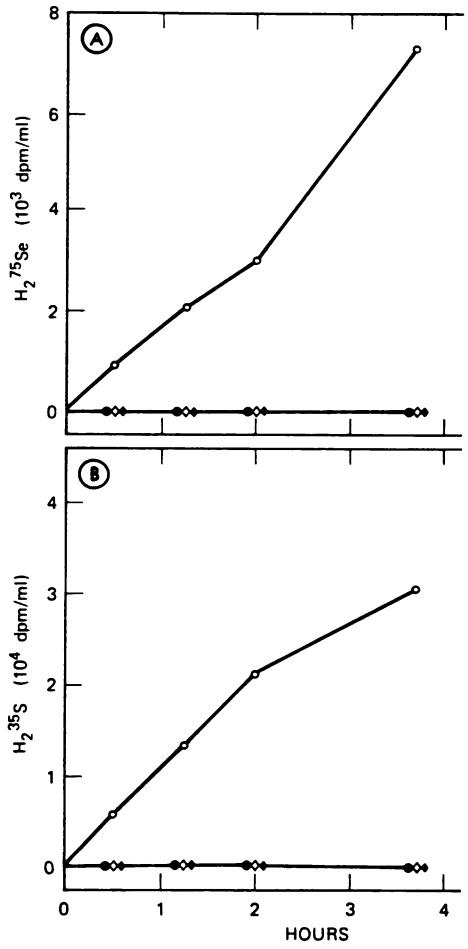

FIG. 2. Formation of ^{75}Se-selenide (A) and ^{35}S-sulfide (B) by washed cells of *D. desulfuricans*. Cells were incubated with 1 mM sulfate but without selenate (○), 1 mM selenate plus 1 mM sulfate (◆), 1 mM selenate plus 5 mM sulfate (◇), and 1 mM selenate plus 50 mM sulfate (●).

inhibition of the reduction of either radioisotope occurred at all the added sulfate levels (Fig. 2A and B). Significant reduction of these radioisotopes proceeded only in the absence of selenate (sulfate concentration, 1 mM). Therefore, inhibition by 1 mM selenate was irreversible by sulfate.

Experiments with anoxic sediment slurries. Reduction of selenate and sulfate occurred in parallel experiments with low-sulfate sediment slurries (Fig. 3). The production of acid-volatile selenide increased with time and was stimulated by H_2 or lactate, while no significant increases were noted in the autoclaved controls (Fig. 3A). Significant production of acid-volatile sulfide occurred over the course of incubation in a parallel set of low-sulfate slurries (Fig. 3B). This activity was inhibited 93% by 1 mM selenate, and total inhibition occurred with autoclaving. Only a very small quantity of ^{75}Se-selenide was formed from the added ^{75}Se-selenate, accounting for at most only ~0.14% of the radioisotope added (Fig. 3A). In contrast, significantly higher conversions (maximum, ~3.6%) of ^{35}S-sulfate to ^{35}S-sulfide occurred (Fig. 3B).

TABLE 1. Effect of sulfate on the rates of sulfate reduction and selenate reduction in two separate experiments with washed cell suspensions of *D. desulfuricans*

Sulfate (mM)	Reduction of:	
	Sulfate (nmol/ml per h)	Selenate[a] (pmol/ml per h)
0.01	0.6	ND[b]
0.20	26.8	5.17
1.0	130.0	4.40
10.0	317.0	1.21
50.0	340.0	0.03

[a] Selenate concentration, 68 pmol/ml.
[b] ND, Not determined.

DISCUSSION

Washed cells of *D. desulfuricans* subsp. *aestuarii* were capable of reducing either selenate or sulfate to selenide or sulfide, respectively (Fig. 1 and 2 and Table 1). These reductions were dependent upon the inclusion of an electron donor (lactate), indicating that they were respiratory in nature. Reduction of either compound was strongly inhibited by 1 mM selenate or tungstate (Fig. 1), both of which stop

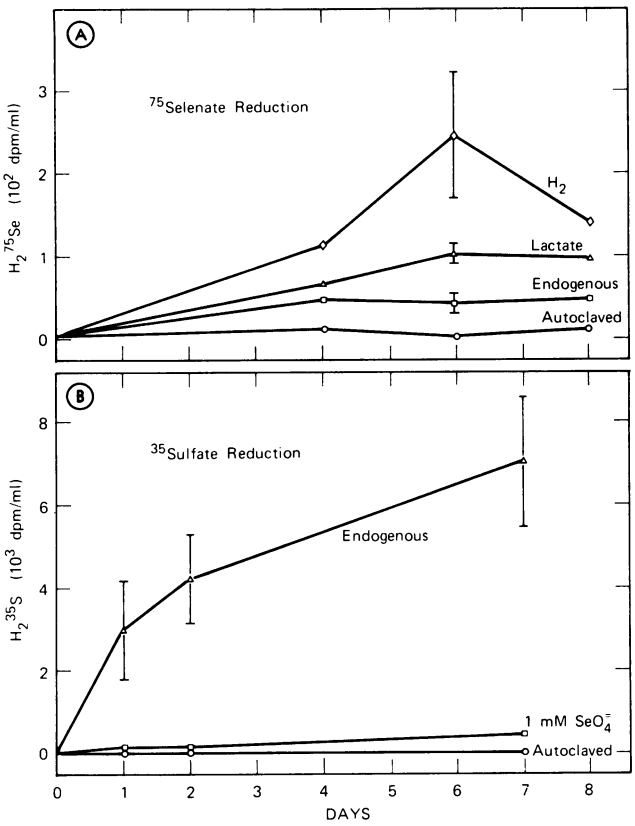

FIG. 3. Formation of ^{75}Se-selenide (A) and ^{35}S-sulfide (B) in parallel experiments with low-sulfate (<4 mM) estuarine sediments. Results represent the average of duplicates (selenate reduction) or triplicates (sulfate reduction), and bars indicate the range in values or standard deviations, respectively. The absence of bars indicates that errors were smaller than the symbols.

sulfate respiration (16, 17). In addition, 1 mM sulfate did not block the reduction of either sulfate or selenate. Therefore, the inhibition of ^{75}Se-selenate reduction by 1 mM selenate was due to a true enzymatic disruption rather than merely a lower specific activity with added selenate.

High levels of selenate (1 mM) inhibited both selenate reduction and sulfate reduction (Fig. 1). Apparently, the low concentrations of selenate (~58 to 77 nM) obtained upon addition of the label to the cell suspensions were below inhibitory levels, and reduction proceeded via the pathway of sulfate respiration. Since the $APSeO_4^{2-}$ product of ATP-sulfurylase is relatively stable (28), further sequential reduction of the activated selenate can occur, provided that the level of selenate is not too high. High levels of selenate as well as of other group VI oxyanions deplete ATP pools of SRB (25), apparently via the action of the pyrophosphatase associated with the ATP-sulfurylase enzyme system (27). Growth of SRB is inhibited by selenate, but since this compound is reported to be a competitive inhibitor (17), there is an ameliorating influence of sulfate (18). Transport processes (5) may be associated with competitive inhibition at low selenate concentrations, while high concentrations probably inhibit ATP-sulfurylase (16).

It is not clear from our results at what concentration the break between inhibition and reduction occurs. We noted a strong inhibition of both selenate and sulfate reductions at selenate concentrations of 1 mM and at selenate/sulfate ratios as low as 0.02 (Fig. 2). Postgate (17) reported that selenate was not reduced by cell suspensions of *D. desulfuricans*. The absence of selenate reduction in that study was probably due to his use of micromolar to millimolar quantities of the compound rather than the nanomolar levels we were able to use with radiotracers. In addition, Postgate (17) reported only a partial inhibition of sulfate reduction at selenate/sulfate ratios between 0.02 and 0.1 (the selenate concentrations ranged between 0.04 and 4.0 mM), although these levels also inhibited growth (18). In contrast, our results with radiotracers indicated a complete inhibition at ratios between 0.02 and 1.0 (at selenate concentrations of 1 mM) (Fig. 2). Thus, inhibition by selenate appears to be related to its absolute concentration and not just the selenate/sulfate ratio. The fact that we found inhibition by 1 mM selenate to be irreversible even in the presence of 50 mM sulfate suggests that noncompetitive inhibition may occur at high selenate concentrations. A similar situation was reported for molybdate inhibition of sulfate reduction in marine sediments (1).

It is of interest to compare the above results to reports of selenate concentrations in the environment to predict whether this compound will be reduced or if it will inhibit the metabolism of sulfate respirers. We recognize that extrapolation of bacterial responses to selenate in washed cell experiments to field conditions is tenuous, but such comparisons are useful in recognizing the dimensions of the environmental contamination problem. Measured concentrations of selenate in unimpacted California waters are low: San Joaquin River (~1.3 to 21.2 nM), Sacramento River (~0.4 to 1.3 nM), and San Francisco Bay (<2.0 nM) (G. Cutter and M. Lourdes, Eos (Am. Geophys. Union) **67**:937, 1986). These values fall well within the range in which we observed selenate reduction by *D. desulfuricans*. By comparison, the selenium-impacted waters of the Kesterson National Wildlife Refuge contain 1.8 to 18 μM selenate and 50 to 100 mM sulfate (19), which would produce selenate/sulfate ratios between 4×10^{-5} and 4×10^{-4}. While these levels of selenate approach the inhibitory range for sulfate reduction (17, 18), it is not clear if this would be compensated for by the very low selenate/sulfate ratios.

Decreased rates of selenate reduction by *D. desulfuricans* occurred with sulfate concentrations above 1.0 mM (Table 1). The decrease in the rate of production of selenide was probably caused by competitive inhibition by sulfate. Nanomolar quantities of selenate were reduced by *D. desulfuricans* in the presence of 1 mM sulfate (Fig. 1A and Table 1). However, higher concentrations (>10 mM) of sulfate inhibited this reduction, with almost complete inhibition occurring at 50 mM (Table 1). Indeed, we did not observe ^{75}Se-selenate reduction by a growing culture of *D. desulfuricans*, apparently because the medium contained high levels of sulfate (~25 mM; unpublished data). We only observed a small amount of such reduction when the slurries were incubated in low-sulfate (<4 mM) water (Fig. 3A). The fact that selenate reduction was stimulated by lactate or hydrogen (Fig. 3A), both of which stimulate sulfate reduction in sediments (12, 14), implies the involvement of SRB. Further evidence consisted of the ability of these sediments to reduce sulfate (Fig. 3B) and the fact that this activity was inhibited by 1 mM selenate, an effective inhibitor of sulfate reduction in marine sediments (1). However, we did not observe selenate reduction in a preliminary experiment with slurries kept in 20 mM sulfate (unpublished data). It is indicated from the above results that the reduction of selenate by SRB in sediments is constrained by the presence of

sulfate ions. Therefore, we predict that in environments having high sulfate concentrations, (e.g., salt marshes), reduction of selenate to selenide by SRB will take place at depths in the sediments where sulfate concentrations are much lower than at the surface. In contrast, freshwater sediments represent a more likely environment for SRB to carry out direct reduction of traces of selenate near the sediment-water interface.

SRB can be involved indirectly in the reduction of selenite by producing hydrogen sulfide, which chemically reduces selenite to elemental selenium (11). Thus, free sulfide formed from sulfate reduction in anaerobic sediments can reduce any selenite entering the sediments. Precipitation of elemental selenium from selenite was observed during incubation under H_2 of extracts of *D. desulfuricans* (29). Postgate (18) reported the precipitation of elemental selenium during growth of *D. desulfuricans* with sulfite (~10 mM) as the electron acceptor in the presence of 1 to 20 mM selenate. Under these conditions, selenate was probably biologically reduced to selenite, and precipitation of elemental selenium was likely the result of chemical reduction by sulfide generated from the respired sulfite ions. We also observed that cell suspensions of *D. desulfuricans* can directly reduce ^{75}Se-selenite to selenide; however, such reduction was far more erratic than that of ^{75}Se-selenate. This was due to the greater chemical reactivity of selenite than of selenate, which interfered with the biological reduction we were attempting to measure.

In summary, we have found that SRB can directly reduce traces of selenate and selenite to selenide. In the case of selenate, this reduction is inhibited by high concentrations of the compound itself, owing to its toxicity for SRB. In addition, sulfate is an inhibitor of the reduction of traces of selenate by SRB. Therefore, the fact that insoluble forms of selenium occur in relative abundance in the sulfate-rich surface (upper 5 cm) sediments of the Kesterson National Wildlife Refuge (O. Were and L. Tsao, Eos (Am. Geophys. Union) **67**:941, 1986) suggests that some reaction other than direct reduction by SRB occurs in this selenium-impacted system.

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