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The Influence of Sulfide on Solid-Phase Mercury Bioavailability for Methylation by Pure Cultures of *Desulfobulbus propionicus* (1pr3)

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To help understand the mechanism and control of Hg uptake in Hg-methylating bacteria, we investigated the effect of sulfide on Hg methylation by pure cultures of the sulfate-reducing bacterium *Desulfobulbus propionicus* (1pr3). Our previous research in natural sediments has suggested that Hg methylation occurs most rapidly when sulfide concentrations favor formation of neutral dissolved Hg−S species. In this study, the chemical speciation of Hg in culture media was manipulated by growing *D. propionicus* across a range of sulfide concentrations, with inorganic Hg (Hg_I) added in the form of ground ores. A solid-phase, rather than a dissolved source of Hg, was used to simulate the controls on Hg partitioning between solid and aqueous phases found in natural sediments. Methylmercury (MeHg) production by cultures was not related to the absolute solid-phase concentration of Hg in the ores, and it was only weakly related to the dissolved Hg_I concentration in the medium. However, MeHg production was linearly related to the calculated concentration of the dominant neutral complex in solution, HgS°. Furthermore, the diffusive membrane permeability of HgS°, as estimated from its octanol−water partitioning coefficient, was found to be sufficient to support MeHg production by cells. The present paper expands on our previous work by providing experimental support of our hypothesis that sulfide influences methylation by affecting the speciation of dissolved Hg_I and its uptake via passive diffusion.

Introduction

Mercury (Hg) is a contaminant of concern that is known to bioaccumulate through the food web. There is evidence that Hg may cause damage to wildlife populations (*1, 2*), and Hg poses a threat to human health through consumption of contaminated fish (*3, 4*). Although both natural and anthropogenic inputs occur largely in the form of inorganic Hg (Hg_I), it is the organic form, methylmercury (MeHg), that accumulates and causes toxic effects at higher trophic levels. In most aquatic ecosystems, the external supply of MeHg is insufficient to account for the MeHg accumulating in sediments and biota (*5, 6*), and in situ MeHg production plays a key role in determining the amount of MeHg reaching higher trophic levels. Certain types of aquatic ecosystems are susceptible to high levels of MeHg production and bioaccumulation, including wetlands (*7*−*14*), new reservoirs (*15, 16*), lakes impacted by acid deposition (*17, 18*), and lakes with anoxic hypolimnia (*19*).

Sulfate-reducing bacteria (SRB) are the principal methylators of inorganic Hg in estuarine (*20*) and freshwater (*18*) sediments. Sulfate stimulates MeHg production by enhancing the activity of SRB in many freshwater sediments (*18, 21, 22*), except at higher sulfate concentrations where sulfide produced through microbial sulfate reduction severely limits MeHg production (*13, 23*−*26*). Although MeHg production is a function of the activity of methylating bacteria, it is also dependent on the availability of Hg for methylation. Our research to date in aquatic ecosystems also suggests that the chemical speciation of Hg affects methylation rates by controlling uptake into bacterial cells via passive diffusion (*27, 28*). Specifically, we have proposed that in sulfidic pore waters, HgS° most readily crosses the bacterial membrane because it is small and uncharged.

Building on our past results, here we present an experimental approach to investigate the effect of sulfide on Hg methylation by pure cultures of *Desulfobulbus propionicus* (1pr3), a strain that has been extensively studied in our laboratory in terms of its ability to methylate Hg.

Implicit to our analysis is the assumption that Hg must enter cells before it is methylated. Observations which support this assumption include the following: (1) that no methylation of Hg occurs in spent cultures of methylating organisms (*29*) and (2) the mechanism for Hg methylation in the one SRB strain studied, *Desulfovibrio desulfuricans*, is via a side reaction of the Acetyl-CoA pathway, which takes place in the cytoplasm (*30*).

Hg speciation in culture was manipulated by growing this SRB across a range of sulfide concentrations with Hg_I added in the form of ground ores. We compared the estimated HgS° concentration in solution with MeHg production (presumably) inside cells. This relationship supported the idea that HgS° is the form of dissolved Hg_I taken up by SRB prior to methylation. We were further able to compare the rate of Hg_I uptake required to support MeHg production to the cell membrane permeability of HgS° and found that passive diffusion of this complex was sufficient to support Hg methylation.

Materials and Methods

Previous work in our laboratory showed that in order to simulate the Hg speciation found in natural sediments in pure-culture methylation assays, a solid-phase source of Hg is required (*29*). Therefore, for these experiments, we used ground Hg-containing ores. These ground rocks were originally collected as part of a regional survey of mercury in rocks of the Ouachita Mountains (Arkansas), details of which are given in Stone et al. (*31*). As part of this survey, the Hg content was measured by aqua regia (concentrated HCl + HNO_3) digestion in the presence of potassium permanganate and potassium persulfate with cold-vapor atomic absorption spectroscopy (CVAAS) detection (*31*). The ores for the experiments described here were chosen to span a range in Hg concentration. The mass of ore used and the lithology of each is indicated in Table 1. Aliquots of the various ground ores were weighed out into acid-cleaned glass serum bottles,

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TABLE 1. Experimental Design for Hg Methylation Experiments

ID	rock type	sulfide spike concn	ore Hg concn (μg g^{-1})	mass solid (g)	total Hg solid (μg)	solid Hg (μg mL^{-1})
			Experiment #1			
421A&B	shale, sandstone	none	30	2.01	60	1.2
571A&B	limestone, siltstone	none	490	0.54	265	5.2
432A&B	chert	none	1360	0.53	721	14.4
432C&D	chert	1 mM	1360	0.54	734	14.7
			Experiment #2			
421A&B	shale, sandstone	none	30	2.09	63	1.2
421C&D	shale, sandstone	1 mM	30	2.09	63	1.2
571A&B	limestone, siltstone	none	490	0.5	245	4.9
571C&D	limestone, siltstone	none	490	2.01	985	19.8
432A&B	chert	none	1360	0.51	692	13.9
617A&B	shale, black	none	1150	1.03	1185	23.7

50 mL of fermentative medium was dispensed anaerobically, and the serum bottles were then sealed and autoclaved. This medium was identical to that described in Benoit et al. (*29*) except that 30 mM lactate was used instead of pyruvate, and yeast extract was added at 0.5 g L^{-1}. Titanium-NTA solution was added as a reductant at 0.1 mM (*32*, *33*), and the ore-containing media were allowed to equilibrate for several days. Anaerobic sulfide stock was freshly prepared from saturated Na_2S using the Hungate method to minimize oxidation. This stock was added to selected slurries (see Table 1) to a nominal concentration of 1 mM.

Desulfobulbus propionicus (strain 1pr3), a SRB that methylates Hg under both sulfate-reducing and fermentative conditions, was used in these experiments. Previous cell counts under conditions similar to those of these experiments (*29*) showed that absorbance at 660 nm (A_{660}) can be related to cell density by the factor $1.7 \cdot 10^8$ cells mL^{-1} A_{660}^{-1} and that a typical cell diameter is 0.5 μm. The A_{660} of ore-containing cultures was measured on subsamples of suspended cells overlying the settled ores. Aspects of Hg methylation by this strain have previously been studied in our laboratory (*29*). In long-term incubations of cells grown under the culture conditions of the present experiment, but in the absence of ores, MeHg production followed the increase in A_{660} (hence, cell density) through 13 days of growth (*29*). Therefore, the bulk of the MeHg was produced during log-phase growth—between 3 and 10 days—when cell density is increasing most rapidly.

The ore-containing media were inoculated with *D. propionicus* that had been grown on medium containing 28 mM sulfate; 1 mL of inoculum was used in experiment 1 and 2 mL in experiment 2. This inoculation introduced a potential sulfate/sulfide carry-over of $6 \cdot 10^{-4}$ M in experiment 1 cultures and $12 \cdot 10^{-4}$ M in experiment 2 cultures. Cultures were incubated at 27 °C without shaking, and anaerobic conditions were maintained in the cultures at all times. Cell growth was terminated after 10 days by freezing the cultures. Cultures were thawed and shaken, and a small amount of slurry poured out for unfiltered MeHg analysis.

Subsamples for total Hg were taken periodically from the overlying culture via degassed syringe, passed through an 0.2 μm Acrodisc filter unit, dispensed into a Teflon vial, diluted with deionized water, and acidified to 0.5% with HCl as a preservative. To avoid ambiguity, throughout the text we refer to subsamples treated in this way as "filtered" and subsamples analyzed directly (still containing cells) as "unfiltered". The filtered Hg_I concentrations were used as a surrogate for dissolved Hg_I in calculating the concentration of HgS°, although this fraction likely contains some colloids. Unfiltered subsamples were also taken via syringe, preserved in sulfide antioxidant buffer (*34*), and dissolved sulfide concentration was measured using a silver-sulfide ion-specific electrode.

Total mercury (Hg_T) subsamples were digested overnight with BrCl and analyzed by $SnCl_2$ reduction, dual amalgamation, and cold-vapor atomic flourescence spectroscopy (CVAFS) (*35*, *36*). MeHg concentration was determined by distillation, aqueous-phase derivitization, and CVAFS (*37*, *38*). In the second experiment, MeHg was determined in filtered subsamples collected just prior to freezing, and the filtered Hg_I concentration was calculated by difference between and filtered Hg_T and filtered MeHg. In the first experiment, filtered subsamples for MeHg were not taken, so this parameter was estimated based on the following relationship: [filtered Hg_T] = [filtered MeHg] + [filtered Hg_I], which can also be written as $y = mx + b$, where y = [filtered Hg_T], x = [unfiltered MeHg], m = fraction of unfiltered MeHg that is filterable, and b = [filtered Hg_I]. Linear regression of the experimental data yielded $m = 0.42$, $b = 5.1$ pg mL^{-1}, and $r^2 = 0.92$. Unfiltered MeHg was multiplied by 0.42 to estimate the filtered MeHg concentrations in experiment 1.

Results and Discussion

For these experiments, cells were grown on medium made without sulfate. However, some sulfate was carried over in the inoculum, resulting in some sulfate reduction before fermentative growth commenced. The sulfide concentration in the cultures exposed to different Hg-containing ores was initially very low (<10 μM) just after inoculation but increased over time as sulfate reduction proceeded, and it was approximately constant after 3 days. The average sulfide concentration in the cultures between days 3 and 10 are shown in Figure 1. This average concentration is representative of the sulfide concentrations to which cells were exposed during log-phase growth, when the majority of the MeHg was produced.

Different sulfide concentrations were reached in the cultures depending on the ore that was present even though similar cell densities (as indicated by A_{660}) were obtained in all of the cultures within a given experiment. We suggest, therefore, that rather than inhibiting sulfate reduction rate to varying degrees, these ores had differing reactivity toward sulfide. The average sulfide concentration of control cultures, which did not contain any powdered rock, indicated the maximum amount of sulfide produced was a result of reduction of carried-over sulfate. In ore-containing cultures, the final sulfide concentration may have been controlled by oxidation and/or precipitation with metal oxide phases (e.g. Fe and Mn) present in the rocks or other mechanisms that removed sulfide from solution. Sulfide amendments were also made to some cultures to increase the range of the sulfide concentrations to about 1.5 orders-of-magnitude (see Table 1 and Figure 1).

Filtered Hg_T concentrations in the cultures varied with ore type but were fairly constant over time. The final filtered Hg_I concentrations are plotted against solid-phase Hg

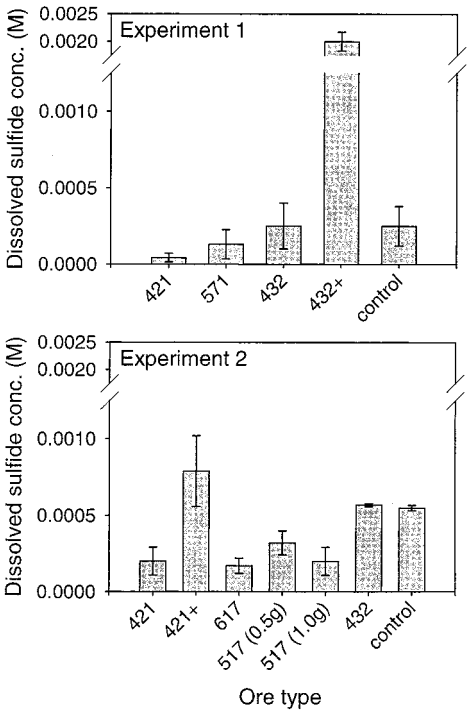

FIGURE 1. The average sulfide concentration between days 3 and 10 in cultures from the Hg methylation experiments; error bars represent 1 standard deviation. The error bars largely represent variability over time, and duplicate cultures agreed with each other within <5%. A plus sign denotes that sulfide was added to the cultures.

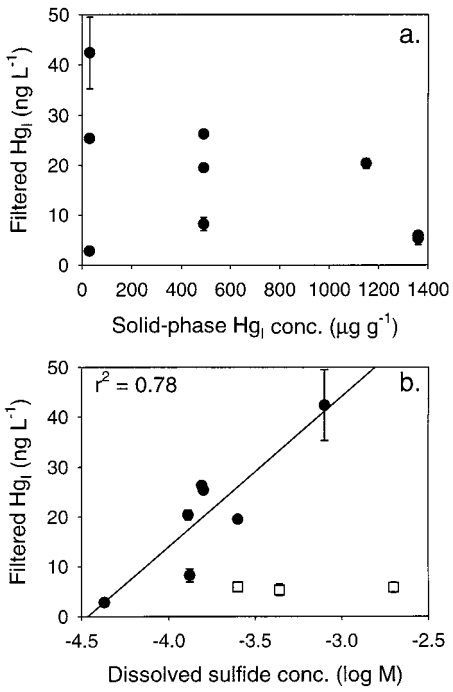

FIGURE 2. Filtered Hg$_I$ concentration from the combined experiments, shown as a function of (a) solid-phase Hg$_I$ and (b) dissolved sulfide concentration. The white squares represent ore 432, and these points were not included in the regression. Averages for duplicate cultures are shown, and error bars represent 1 standard deviation. Where error bars do not appear, they fall within the symbol.

concentration in Figure 2. Notice that the filtered Hg$_I$ was not simply a function of the concentration of Hg$_I$ in the rock powders, and at a given solid Hg$_I$ concentration, a range of filtered concentrations was observed. As shown in Figure 2b,

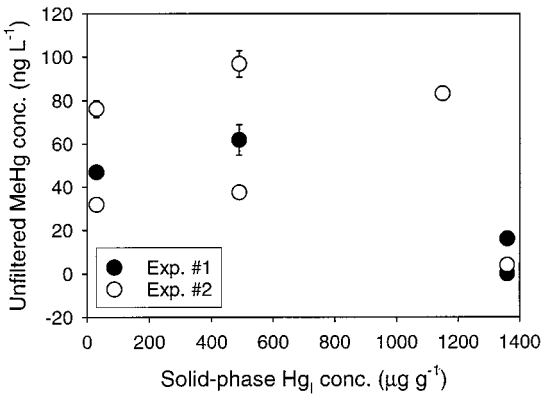

FIGURE 3. Methylmercury produced in the ore-containing cultures, shown versus solid-phase Hg$_I$.

filtered Hg$_I$ increased with sulfide concentration for all but ore 432, which had extremely low filtered Hg$_I$ even at the highest dissolved sulfide concentration of the experiment. The filtered Hg$_I$ concentrations in these experiments were similar to those measured in pore waters from a number of aquatic sediments (*39*−*41*), and these concentrations are well below that expected from the precipitation and dissolution of $HgS_{(s)}$ (*27*).

Past observations of the relationship between Hg$_I$ and sulfide in sediments include no apparent effect of sulfide on filtered Hg$_I$ concentration in pore waters in the Florida Everglades (*13*) and a positive relationship with sulfide in pore waters from the Patuxent River Estuary (*39*) and Lavaca Bay (*42*). We have modeled dissolved Hg$_I$ in sulfidic pore waters as a competition between sorption to solid surfaces and complexation by dissolved sulfide (*27*). This model predicted constant or increasing dissolved Hg$_I$ with increasing sulfide, depending on the nature of the solid matrix. The relationships between filtered Hg$_I$ and sulfide observed in these experiments and the low filtered Hg$_I$ concentrations measured in the presence of ground ores (Figure 2b) are both consistent with this model. Sorption and aqueous complexation, rather than cinnabar precipitation/dissolution, appear to be the major controls on dissolved Hg in both natural sediments and in these experiments.

The final MeHg concentration in these experiments is plotted against solid-phase Hg$_I$ in Figure 3. Since MeHg in freshly inoculated medium is undetectable, the final concentration was equal to the net MeHg produced. If Hg$_I$ were directly available from the solid phase, a positive relationship between solid-phase Hg$_I$ and MeHg would be expected; however, no such relationship was observed (Figure 3). In fact, when a particular rock was used more than once, as was the case for 421 (30 μg Hg g^{-1}) and 571 (490 μg Hg g^{-1}), a range in MeHg concentrations resulted.

There was a very weak positive relationship between filtered Hg$_I$ and final MeHg concentration (Figure 4a). As indicated in Figure 4b, sulfide concentration also affected MeHg production, but, in this case, the effect was inhibitory. Since both filtered Hg$_I$ and sulfide varied across these experiments, it was necessary to reconcile the effect of these two variables on MeHg production by the SRB cultures.

Our pore water model (*27*) predicted a decrease in the fraction of dissolved Hg$_I$ present as the neutral complex HgS° with increasing sulfide concentration. Furthermore, an observed decrease in the octanol−water partitioning coefficient (D_{ow}) of Hg in the presence of increasing dissolved sulfide suggested that passive diffusion of Hg across lipid membranes declines with increasing sulfide (*28*). We have hypothesized that, under sulfidic conditions, HgS° is the form of Hg$_I$ taken up by SRB prior to methylation and that increased sulfide concentration decreases the bioavailability of Hg$_I$ to

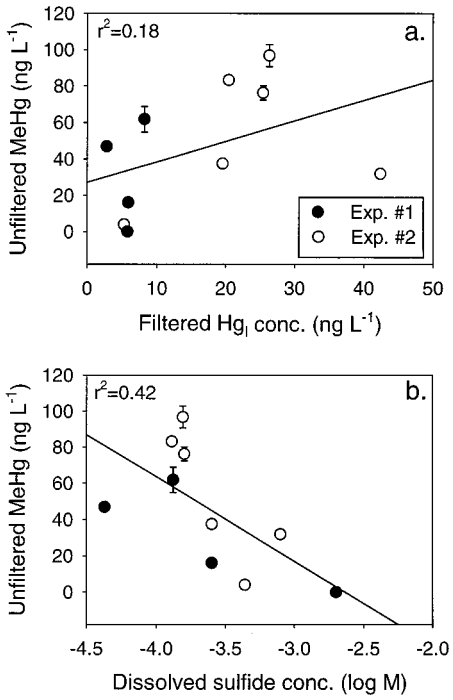

FIGURE 4. Methylmercury produced in the ore-containing cultures, shown versus (a) filtered Hg_I and (b) dissolved sulfide.

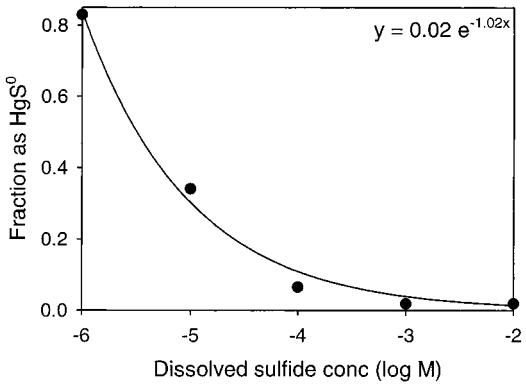

FIGURE 5. The fraction of dissolved Hg_I present as HgS° with increasing sulfide concentration, calculated using the MINEQL$^+$ program. See text for details.

SRB by causing a shift in speciation away from HgS° toward charged complexes, mainly $HgHS_2^-$, near neutral pH (*27*, *28*). This hypothesis predicts that the amount of MeHg produced should be proportional to the concentration of HgS° in solution.

To test the hypothesis, the dissolved Hg_I speciation in the cultures was modeled using the MINEQL$^+$ chemical equilibrium program (*43*). The model included all of the Hg−S complexes considered in our previous model for Hg speciation in sulfidic pore waters (*27*). The filtered Hg_I concentrations in the cultures were below that expected from dissolution of $HgS_{(s)}$, as is consistent with the notion that the dissolved Hg_I concentration was controlled by surface sorption. The predicted change in the fraction of Hg_I present as HgS° across a range of sulfide concentration is shown in Figure 5. From this relationship, and the filtered Hg_I concentrations, it was possible to calculate the concentration of HgS° for each culture type.

The concentration of HgS° is plotted against final MeHg concentration in Figure 6. In both experiments, there is a strong linear relationship between these parameters (r^2 = 0.8). The different slopes reflect differences in cell growth

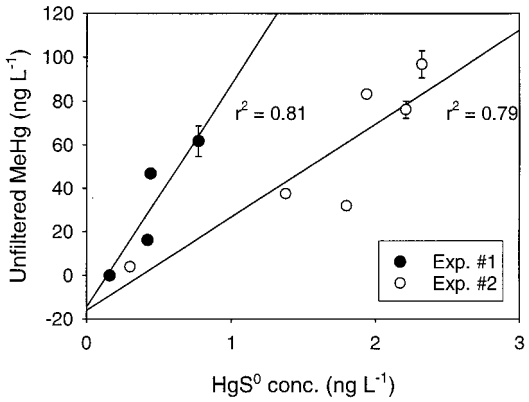

FIGURE 6. The relationship between estimated HgS° concentration and the final MeHg concentration in experimental cultures.

over the course of the two experiments. The average final A_{660} in experiment 1 was 0.402, compared to 0.185 in experiment 2. As discussed below, when MeHg production is expressed on a per cell basis, the relationship between MeHg production and HgS° is quite similar for the two experiments.

A linear relationship between MeHg and HgS° was also seen in the Florida Everglades and Patuxent River estuary (*28*), where about 70% of the variability in the bulk sediment MeHg concentration could be explained by the modeled pore water HgS° concentration. Interestingly, the slope of the line relating HgS° to MeHg concentration was much steeper for the Everglades data, which may be caused by greater microbial activity in these warmer, more organic rich sediments. The field and experimental results taken together suggest that microbial activity and available Hg concentration are both important in controlling MeHg production in aquatic ecosystems. In natural sediments, relationships between MeHg and sulfide concentrations may be obscured by differences in microbial activity and population structure within and across ecosystems. However, sulfide speciation appears to be a primary control on Hg uptake and methylation.

Using the information gleaned in the culture experiments, we calculated the minimal uptake rate of Hg_I required to support methylation inside the cell. The MeHg production rate was expressed on a cellular surface area basis (pg cm^{-2} s^{-1}) to normalize for differences in cell growth in the two experiments. In this calculation, final MeHg concentration (pg cm^{-3}) was divided by the average cell density (cell cm^{-3}) during log-phase growth and cell surface area (cm^2 cell^{-1}). Cell density was estimated based on the experimentally determined relationship with A_{660}, and surface area was calculated by assuming a spherical cell using the average measured cell diameter of 0.5 μm. The concentration of HgS° (pg cm^{-3}) is plotted against this MeHg production flux in Figure 7. The slope of the line has units of cm s^{-1}, and it represents the rate of HgS° passage into the cell needed to provide sufficient Hg substrate for MeHg production. The strong linearity of the relationship shown in Figure 7 supports the idea that HgS° is the form of Hg taken up prior to methylation. The results presented here do not directly address the relationship between HgS° and MeHg at the low sulfide concentrations frequently encountered in anaerobic sediments. However, our previous field studies (*27*) and experimental D_{ow} determinations (*28*) suggest that this relationship holds to at least low μM sulfide concentrations.

For the sake of comparison, we calculated the membrane permeability (P, cm s^{-1}) of HgS°, which is the rate at which this complex will cross cell membranes by passive diffusion. The size-corrected membrane permeability (P^*) of HgS° was estimated from its octanol−water partitioning coefficient

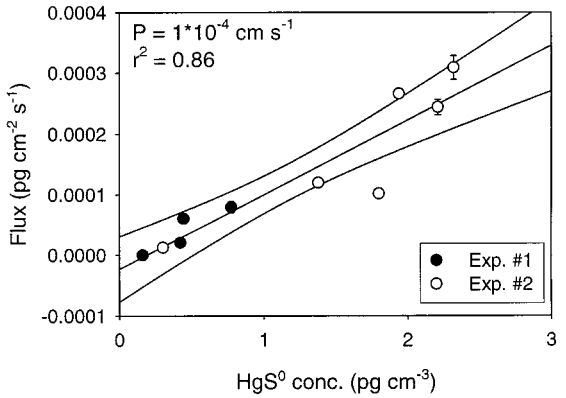

FIGURE 7. The relationship between estimated HgS° concentration and MeHg production expressed on a cellular surface area basis.

(K_{ow}) using a previously determined empirical relationship between these two parameters for organic molecules (*44*, *45*). The K_{ow} for HgS° is 25 (*28*) compared to 3.3 for $HgCl_2$ (*45*) and 28 for CH_3HgSH (*46*). Accepting the limitations of such a calculation, we estimated *P* for HgS° using the equation: $\log P^* = \log P + mv$, where *m* is a constant (0.0546 mol cm^{-3}), and *v* is the van der Waals volume in cm^{-3} mol of an uncharged Hg complex (*44*, *45*). We estimated a van der Waals volume of 39 cm^3 mol^{-1} for HgS° (*47*) compared to 51 and 43 cm^3 mol^{-1} for $HgCl_2$ and for $Hg(OH)_2$, respectively (*45*). Our calculated P for HgS° is $7.4 \cdot 10^{-2}$ cm s^{-1}, which is greater than the value of $7.4 \cdot 10^{-4}$ cm s^{-1} reported for $HgCl_2$ by Mason et al. (*45*) and the value of $4.7 \cdot 10^{-4}$ cm s^{-1} reported for CH_3HgSH by Lawson and Mason (*46*) based on short-term uptake experiments with the algae *Thalassiosira weissflogii*.

A higher *P* for HgS° is consistent with its smaller size and greater K_{ow}. It has previously been suggested that this complex may be hydrated in solution, possibly as HgSHOH (*48*), so the molar volume may be larger than 39 cm^3 mol^{-1}. The *P* calculation is extremely sensitive to the chosen value of *v*. For example, if the volume were two times larger, the estimated *P* would be two orders of magnitude smaller, i.e., $5.5 \cdot 10^{-4}$ cm s^{-1}. The important point about *P* for HgS°, in the context of this study, is that the uptake of this complex by passive diffusion is more than sufficient to account for the production of MeHg inside the cell, even if the larger molar volume is used. The *P* required to support methylation in the ore experiments ($1 \cdot 10^{-4}$ cm s^{-1}; Figure 7) is less than the estimated uptake of HgS° by passive diffusion, so active transport of Hg_I is not required to support the measured methylation rates.

These experiments indicate that Hg_I in solid phases is not directly available for microbial methylation, but it becomes bioavailable through exchange with the dissolved pool. Although partitioning of Hg_I into the aqueous phase may be enhanced by dissolved sulfide complexation, high concentrations of sulfide tend to inhibit methylation. Therefore, Hg in solid phases is most available for methylation by SRB under very mildly sulfidic conditions, generally below 10 μM. The field conditions that are most conducive to methylation of Hg in ores will occur in aquatic sediments underlying waters of low to moderate sulfate concentration. Based on research in a variety of ecosystems, the optimal conditions for MeHg production in sediments are high availability of organic carbon and a balance between sulfate reduction and sulfide sequestration and reoxidation such that sulfide does not build up to inhibitory concentrations (*13*, *40*). Wetlands may be especially favorable because of rapid microbial sulfur cycling and rapid reoxidation of sulfide in root zones.

While the data presented here indicate that HgS° concentration controls MeHg production, the mechanism linking these two parameters remains somewhat conjectural. Hg methylation has been shown to occur as part of a cytoplasmic biochemical pathway (*30*), and Hg methylation does not occur in spent culture medium (*29*), which contains extracellular enzymes excreted by growing cells. Therefore, it appears that methylation takes place inside the cell and that Hg must be taken up prior to methylation. It is unlikely that an active transport mechanism for Hg has evolved in SRB given that (1) Hg has no known physiological function in bacteria; (2) the ability to methylate Hg does not confer added resistance (*33*); and (3) Hg methylation occurs as an accidental side reaction (*30*). As passive diffusion of neutral $HgCl_2$ has been demonstrated across artificial (*49*) and diatom membranes (*45*), it is probable that passive diffusion also occurs across bacterial membranes. In an *Escherichia coli* strain engineered to report Hg bioavailability to bacterial cells using a *mer-lux* fusion, preferred uptake of neutral Hg species was consistent with diffusive transport across the cell membrane (*50*). While the gram-negative bacterial cell wall and membranes are complex, the outer lipopolysaccharide and peptidoglycan layers are relatively permeable to low molecular weight compounds (*51*).

Although most metals are taken up by cells via facilitated or active transport of the free cation, there are a few examples of diffusive uptake of neutral metal complexes. Silver uptake and toxicity to phytoplankton is inversely proportional to salinity (*52*), suggesting that $AgCl_{(aq)}$ is the principal bioavailable species of inorganic Ag. Recent studies of silver uptake by algae, done at the picomolar concentrations found in natural waters, showed directly that $AgCl_{(aq)}$ is the principal bioavailable species by measuring the octanol−water partitioning of Ag concomitantly with uptake across a salinity gradient (*53*). Small neutral organo-metal complexes (of Cu, Cd, Ni, and Pb) have also been shown to diffuse rapidly across phytoplankton cell membranes (e.g. refs *54* and *55*).

The present study is consistent with the hypothesis that, unlike many other metals, Hg_I is not available to cells as the free cation. Instead, small, neutral, hydrophobic Hg complexes freely diffuse across cell membranes. In the experiments presented here, the Hg methylation rate in SRB cultures was related to the concentration of the HgS°. Therefore, this study supports the hypothesis that sulfide inhibits MeHg production in sediments by shifting speciation away from HgS° toward more polar (negatively charged) species, thereby decreasing the bioavailability of dissolved Hg_I to cells.

Acknowledgments

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