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Mercury Methylation by *Desulfovibrio desulfuricans* ND132 in the Presence of Polysulfides

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The extracellular speciation of mercury may control bacterial uptake and methylation. Mercury-polysulfide complexes have recently been shown to be prevalent in sulfidic waters containing zero-valent sulfur. Despite substantial increases in total dissolved mercury concentration, methylation rates in cultures of *Desulfovibrio desulfuricans* ND132 equilibrated with cinnabar did not increase in the presence of polysulfides, as expected due to the large size and charged nature of most of the complexes. In natural waters not at saturation with cinnabar, mercury-polysulfide complexes would be expected to shift the speciation of mercury from $HgS^0_{(aq)}$ toward charged complexes, thereby decreasing methylation rates.

Sulfate-reducing bacteria are important methylators of mercury in aquatic systems (13, 19). These bacteria are found throughout reduced zones but are commonly concentrated at oxic-anoxic boundaries, where methylation rates in natural sediments and soils are often highest. The speciation of mercury has been shown to be an important determinant of its biological uptake; neutral species entering by passive diffusive transport are believed to be the major cell permeants, as charge hinders partitioning into (and hence diffusion through) a lipid bilayer (8). Passive diffusion of a neutral mercury-chloride complex, $HgCl_2$, has been shown to occur in aerobic environments (2, 22, 27, 28), and estimates of the formation constant for neutral $HgS^0_{(aq)}$ (14), together with field and experimental evidence (3, 5–8), indicate this to be the dominant mercury species taken up by cells at low sulfide concentrations.

Recent studies (25, 30) have demonstrated that polysulfides have a significant effect on the speciation of mercury in sulfidic waters. Several processes can lead to their formation; these include partial oxidation of hydrogen sulfide species, partial reduction of oxidized sulfur species, and the nucleophilic attack of HS^- on elemental sulfur (12, 17, 34) as follows: $HS^- + (x - 1)/8\ S_{8(s)} = S_x^{2-} + H^+$, where x is 3 to 6. The dominant dissolved mercury-polysulfide species in sulfidic water in equilibrium with cinnabar $[HgS_{(s)}]$ and zero-valent sulfur are inferred to be the charged complexes $Hg(S_x)_2^{2-}$ and HgS_xOH^- (15, 25, 31). Recent octanol-water partitioning experiments (25) have confirmed the hydrophilic nature of the dominant complexes, although an uncharged mercury-polysulfide complex (possibly HgS_5^0, referred to here as HgS_x^0) also appears to form at an approximately 10 pM concentration in solutions in equilibrium with cinnabar.

The maximum uptake rate of a cell membrane permeant by passive diffusion, V (amol cell^{-1} day^{-1}), can be estimated by the equation $V = P \cdot C_{(aq)} \cdot A$, where P is the ability of the cell permeant to cross the membrane (cm s^{-1}), $C_{(aq)}$ is its extracellular aqueous concentration (amol cm^{-3}) (the intracellular concentration is neglected), and A is the area of a cell (cm^2 cell^{-1}) (28, 32). P depends on a permeant's tendency to partition into and its diffusivity within a lipid bilayer membrane; a molecule's K_{ow}, or octanol-water partition coefficient [approximately 25 for both HgS^0 and $Hg(SH)_2^0$ (9) to 900 to 11,000 for HgS_x^0 (25)], is thus an important determinant of permeativity. An empirical relationship exists between K_{ow} and $P*$ for membrane permeants (28, 32), with $P*$ defined as the theoretical limit for the ability of a molecule to permeate the membrane. P in turn can be estimated from the empirical relationship $\log P = \log P* - m_v \cdot v$, where m_v is the size selectivity of membranes (0.0546 mol cm^{-3}) and v is the estimated molar volume of the permeant (cm^3 mol^{-1}) (32). This approach has been used in the case of uptake of other uncharged mercury species by phytoplankton (28) and sulfate-reducing bacteria (6). Using molar volumes of 39 (considering that the actual structure is likely HgSHOH), 60, and 105 cm^3 mol^{-1} (6, 10), the resulting P's are 0.07, 0.006, and 0.02 cm s^{-1} for $HgS^0_{(aq)}$, $Hg(SH)_2^0$, and HgS_x^0, respectively.

Figure 1 depicts the maximum uptake rate, V, predicted from our speciation model (25) for HgS_x^0, $HgS^0_{(aq)}$, and $Hg(SH)_2$ as a function of total sulfide concentration for solutions in equilibrium with elemental sulfur and cinnabar. The higher calculated K_{ow} of the mercury-polysulfide complex is offset by its large size and relatively lower concentration, such that the contribution of HgS_x^0 to diffusive mercury uptake is expected to be small relative to that of $HgS^0_{(aq)}$. If the hypothesis that the concentration of membrane-permeative species

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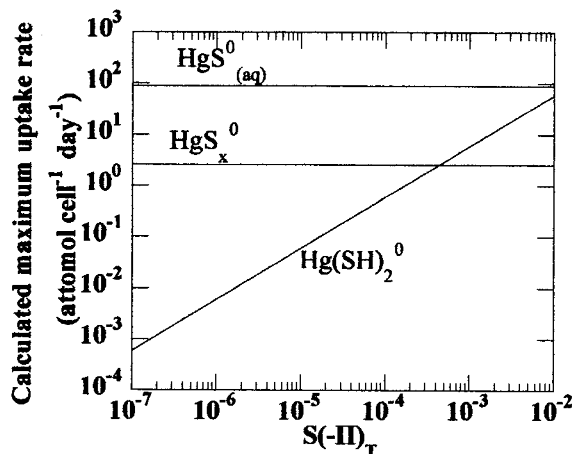

FIG. 1. The calculated maximum uptake rate of a cell membrane permeant at pH 7.3 as a function of $(SII)_T$.

controls bacterial uptake is correct, mercury-polysulfide complexes would be predicted not to contribute appreciably to the biological availability of mercury species via passive uptake processes despite marked increases in the solubility of cinnabar. Rather, $HgS^0_{(aq)}$ would play a dominant role.

For solutions at equilibrium with cinnabar, the concentration of $HgS^0_{(aq)}$ is independent of the concentrations of sulfide species and polysulfides (the intrinsic solubility of cinnabar is estimated to be approximately $10^{-9.3}$ M). Under such conditions, the presence of polysulfides would not be anticipated to affect the availability of mercury by passive diffusion.

By measuring rates of mercury methylation in active cultures of the sulfate-reducing bacterium *Desulfovibrio desulfuricans* ND132 in equilibrium with cinnabar, $HgS_{(s)}$, in both the absence and the presence of polysulfides, we tested the above predictions. We also compared the observed methylation rates with absolute estimates of the ability to permeate the membrane.

Cultures, growth medium, and cell density determination. *D. desulfuricans* strain ND132, isolated from mesohaline Chesapeake Bay (20), was cultured (at 22°C) under sulfate-reducing conditions in a bicarbonate-buffered basal salt medium (29) with 16 mM lactate and 28 mM Na_2SO_4. This strain will be made available upon request. Cell density was determined by direct counts using 4′,6-diamidino-2-phenylindole (Sigma) staining and epifluorescence microscopy and in some cultures by measuring optical density at 600 nm.

Synthesis and measurement of polysulfides. Polysulfides were generated via the reaction of HS^- (prepared using washed crystals of $Na_2S \cdot 9H_2O$; Mallinckrodt) with elemental sulfur (S_8, sublimed; Mallinckrodt). It is convenient to envision each polysulfide dianion as comprising one S(II) atom and $(x - 1)$ S(0) atoms. The total dissolved zero-valent sulfur concentration can be expressed as $S(0)_T = 8 \cdot [S_{8(aq)}] + \Sigma(x - 1) \cdot [(S_x^{2-}) + (HS_x^-)]$; note that $S_{8(aq)}$ is sufficiently low that its contribution to $S(0)_T$ can be neglected under these conditions. The total polysulfide concentration $S(0)_T$ was measured by the triphenylphosphine sulfide (TPPS) method (1, 11, 33). TPPS was then analyzed by high-performance liquid chromatography (33). The total concentration of hydrogen sulfide species, $(H_2S)_T = (H_2S) + (HS^-) + (S^{2-})$, referred to as the sulfide concentration, was determined using the methylene blue method of Cline (21) (detection limit of approximately 1 μM).

Total dissolved mercury and methylmercury determination. Syringe-filtered (0.02-μm-pore-size Anotop) aliquots were digested with bromine monochloride (BrCl) and analyzed by stannous chloride reduction and cold-vapor atomic fluorescence spectrometry (9, 18). Reagent blanks (0.05 to 0.2 ng of mercury) were consistent throughout a given day using the same batch of reagents. Detection limits were 0.5 to 2.0 ng/liter. Monomethyl mercury in syringe-filtered samples was determined by gas chromatography with cold-vapor atomic fluorescence spectrometry detection (26). Samples were purged after acetic acid addition to remove sulfide and extracted into dichloromethane. The detection limit for this procedure (extracting 2 ml of sample, with no preconcentration) was 40 ng/liter.

Growth of *D. desulfuricans* in the presence of polysulfides. Before studying the effect of polysulfides on mercury methylation, we investigated their effect on the growth of sulfate-reducing cultures. Bottles containing medium in the presence or absence of S_8 (all solids were ethanol washed and subjected to tyndallization [16]) at pH 7.3 with an initial sulfate concentration of 28 mM were equilibrated with an initial $(H_2S)_T$ of 3.5 mM for 1 week prior to inoculation. After inoculation, the polysulfide concentration began to decrease immediately in bottles with live samples, with no lag time (Fig. 2a). For killed controls, the polysulfide concentration increased slightly (probably due to the additional sulfide transferred with the inoculum). The decrease in polysulfide concentration appears to be due to utilization by the organism (23). This has been observed under S(0)-reducing conditions, under which the addition of a small amount of $(H_2S)_T$, which solubilizes S(0) to polysulfides, increases the reduction rate of S(0) (24). In the experiments of Fig. 2, sulfide production from polysulfide reduction was small compared to that generated by reduction of sulfate, and the resulting increase was not significant. Sulfide production leveled off after day 4, apparently due to lactate limitation, and polysulfide concentration rebounded to its original value. Neither $(H_2S)_T$ production nor bacterial growth were significantly affected by the presence of polysulfides (Fig. 2b).

Comparison of mercury methylation rates in the presence and absence of polysulfides. Mercury methylation by *D. desulfuricans* ND132 was measured in cultures equilibrated with cinnabar in the presence and absence of polysulfides. The initial $(H_2S)_T$ in two separate experiments was 4 or 7.5 mM, which reacted with S_8 to generate polysulfides at 0.22 or 0.45 mM $S(0)_T$, respectively. The medium was reduced with 0.1 mM titanium(III)–nitrilotriacetic acid (which does not affect the mercury speciation) so that sulfide would not serve as a reductant. Dissolved methylmercury, total dissolved mercury, $(H_2S)_T$, and cell density were measured over time.

In triplicate bottles, initially at 4 mM $(H_2S)_T$ and pH 7.0, total dissolved mercury at inoculation was eight times greater in the presence of S_8. Total dissolved mercury concentration in bottles in the absence of added S_8 remained fairly constant, while that in bottles with S_8 decreased after inoculation; at the lowest measured concentration, however, the dissolved mer-

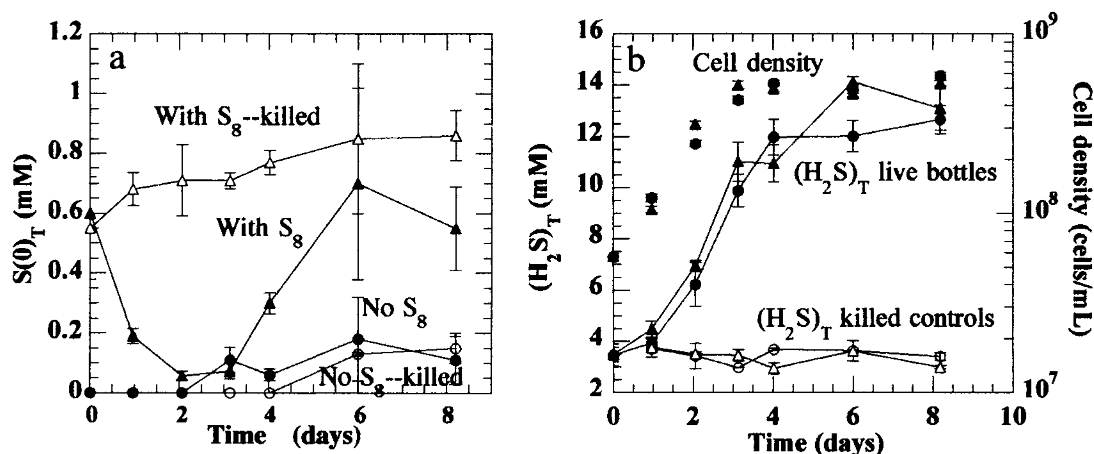

FIG. 2. $S(0)_T$ (a) and cell density and $(H_2S)_T$ (b) over time in triplicate growing cultures of *D. desulfuricans* ND132, in pH 7.3 medium with 28 mM sulfate (ionic strength = 0.2 M), in the absence (●) and presence (▲) of elemental sulfur. Open circles and triangles indicate killed controls for each group. Concentration of $S(0)_T$ was measured by the triphenylphosphine derivatization with high-performance liquid chromatography separation and UV absorbance detection. Error bars represent the standard deviations of measurements.

cury concentration in the presence of polysulfides was still higher, by a factor of 3, than in the absence of added S_8. This temporal pattern of total dissolved mercury is consistent with the transient decrease of polysulfides during cell growth.

At 4 mM initial $(H_2S)_T$, growth rates were similar in the absence and presence of S_8 (95% confidence intervals of 1.3 ± 0.4 day^{-1} and 1.2 ± 0.6 day^{-1}, respectively; data not shown), as were final $(H_2S)_T$ concentrations (11.5 and 12.5 mM). The methylation rate, K_{meth} (amol cell^{-1} day^{-1}), was calculated according to the following equation: $K_{meth} = [CH_3Hg^+]_t\mu/(x_t - x_0)$, where μ is the specific growth rate (day^{-1}) and x_0 and x_t are numbers of cells per milliliter at time zero and t, respectively. K_{meth} was not significantly influenced by polysulfides, being 0.021 ± 0.004 and 0.016 ± 0.004 amol of $HgCH_3^+$ cell^{-1} day^{-1} in their absence and presence, respectively. (The means of replicate methylmercury analyses for each bottle were averaged for each group [absence and presence of S_8] and the relative standard deviation of 15% was used in error propagation.)

Similar results were obtained at an initial $(H_2S)_T$ of 7.5 mM, pH 7.3. Growth rates in the absence and presence of S_8 were 0.34 ± 0.09 and 0.50 ± 0.05 day^{-1}, respectively (Fig. 3). Total dissolved sulfide $(H_2S)_T$ was 20 mM for each treatment at the end of the experiment, although it was somewhat higher at intermediate times in the presence of S_8 (Fig. 3). In these cultures, methylmercury was measured at the beginning and the end of the experiment. The rates of methylation were 0.027 ± 0.008 and 0.020 ± 0.003 amol of $HgCH_3^+$ cell^{-1} day^{-1} in the absence and presence of polysulfides, respectively, again not significantly different at the 95% confidence level.

The addition of elemental sulfur in the presence of cinnabar markedly increased the concentration of dissolved mercury in the culture medium, as expected. This was not accompanied, however, by an increase in methylation rate, consistent with the hypothesis that the concentration of membrane-permeative species controls the methylation rate. Methylation rates observed in these systems (at saturation with cinnabar) agree well with data by Benoit et al. (6) relating the concentration of $HgS^0_{(aq)}$ to the bacterial methylation rate (Fig. 4).

The actual rates of mercury methylation in our cultures, however, were approximately 10^{-4} of the calculated maximum possible diffusive rate of uptake of $HgS^0_{(aq)}$ through a lipid bilayer membrane. Possibly the vast majority of mercury passing into the cell becomes bound (such as to sulfhydryl groups in proteins), in which case transport limitation per se could limit the intracellular concentration of the substrate for methylation (4). However, this result suggests an alternative mechanism by which the concentration of neutral species in the culture medium may control mercury availability for methylation. Without a large nonmethylation sink within the cell, $HgS^0_{(aq)}$ may equilibrate across the cell membrane. In this

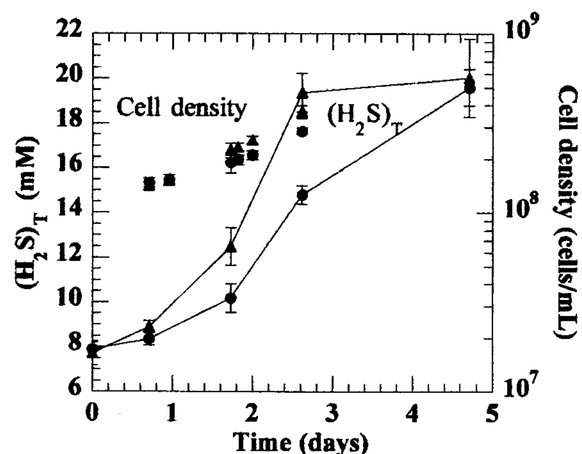

FIG. 3. Cell density and $(H_2S)_T$ throughout a methylation experiment with triplicate cultures of *D. desulfuricans* ND132 (I = 0.2 M) with initial $(H_2S)_T$ of 7.5 mM in the absence (●) and presence (▲) of elemental sulfur. Open circles and triangles indicate killed controls for each group. Cinnabar was present in all bottles. Error bars represent the standard deviations of measurements.

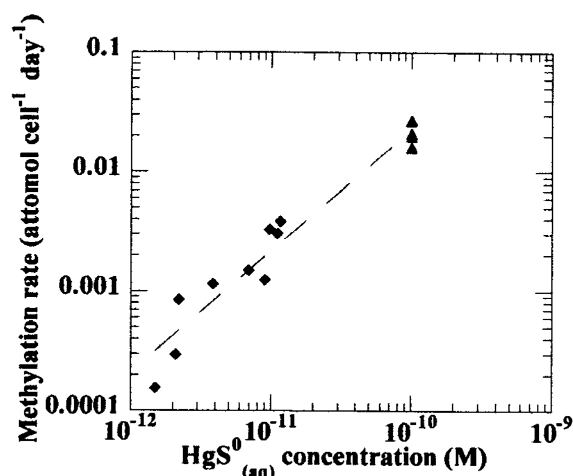

FIG. 4. Observed methylation rates as a function of $HgS^0_{(aq)}$ concentration. Diamonds and triangles represent data from Benoit et al. (7) and this study, respectively. A formation constant of 10^{-10} for $HgS^0_{(aq)}$ has been used in all calculations for consistency.

case, the extracellular concentration of $HgS^0_{(aq)}$ would thus determine the intracellular concentration of $HgS^0_{(aq)}$, and equilibria within the cell would then fix the mercury species (e.g., Hg^{2+}) that serves as a substrate for methylation.

Regardless of the mechanism by which the extracellular concentration of membrane-permeative species controls methylation rate, these results lead directly to quantitative predictions about the effect of polysulfides on environmental mercury methylation in systems not at equilibrium with cinnabar. In the absence of polysulfides, $HgS^0_{(aq)}$ is the dominant species at low sulfide concentrations. At higher sulfide levels, HgS_2H^- becomes the dominant complex, resulting in a marked decrease in $HgS^0_{(aq)}$ and a consequent decrease in the calculated maximum uptake rate for mercury. This model has been employed to explain trends in methylmercury concentrations in the Florida Everglades and the Patuxent River Estuary (3, 7) as well as experimental mercury methylation results (6).

At saturation with elemental sulfur, the dominant species are HgS_xOH^- at low sulfide levels and $Hg(S_x)_2{}^{2-}$ as sulfide levels increase. The $HgS^0_{(aq)}$ concentration is expected to be lower than in the purely sulfidic case due to the dominance of these charged complexes, resulting in a greatly reduced estimated ability to permeate the membrane. Other models for the speciation of mercury in porewater (that consider, for example, sorption to surface thiols [3]) would also show a proportionate decrease in the concentration of $HgS^0_{(aq)}$ with the addition of polysulfides. These considerations imply that the formation of polysulfides in natural waters would be expected to decrease methylation rates, except when cinnabar is present.

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