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Inhibition of Bacterial Growth by Mercury and the Effects of Protective Agents

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Introduction

Although the toxicity of mercury and many of its compounds has been known for centuries, potential hazards from its use were recognized only after the mercury-poisoning incident in Minamata, Japan (FUJIKI, 1963). Since that event, considerable research has been directed toward understanding the natural pathways by which organic and inorganic mercurials are cycled through the biological and geological components of the environment. The wide distribution of mercury in minute quantities is due to its unusually high vapor pressure at ambient temperature (KAZANTZIS, 1971). Mercury is also concentrated in areas near sewer outfalls and is correlated with the more organic-rich (clay-silt), rather than the sand fraction (APPLEQUIST et al., 1972). It has been reported (DUNLAP, 1971; HAMDY and NOYES, 1975; and WOOD et al., 1968) that bacteria are able to transform inorganic mercury to the organic form which is readily assimilated and concentrated in aquatic food chains (KAZANTZIS, 1971). These microbial communities may also affect the detoxification of the organic mercurials by conversion to elemental or methyl mercury which in turn could be removed from the system through volatilization (BILLEN et al., 1974; SPANGLER et al., 1973; and SPANGLER et al., 1973). However, the release of mercury from polluted sediment may continue to contaminate the water even if discharge of mercury containing waste is halted (HOKANSON, 1974).

The microbial flora of the sediments are important because of their role in recycling inorganic nutrients. If the microbial activity is depressed or destroyed by mercurial compounds the productivity of the entire system may be impaired, resulting in significant decreases in aquatic food. On the other hand, the presence in the aquatic environment of organic compounds containing sulfhydryl groups may antagonize the effects of the mercurials on the aquatic microbial flora. The present investigation was conducted to evaluate the effect of Hg^{2+} on two mercury-sensitive anaerobes and ascertain the antagonistic role offered by the sediment (from which these cultures were isolated)

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toward Hg^{2+} toxicity. The effect of other protective agents (tryptone and ground rubber) in reducing the bactericidal efficiency of Hg^{2+} was also evaluated.

Materials and Methods

Microbial analyses. Anaerobic cultures were isolated from sediments collected at two sites, one near the Allied Chemical Plant on Turkey Creek, Ga., and the other from Par Pond, a large lake near Aiken, S.C. These sediment samples contain 18-20% organic material by weight. Approximately 10 g of each semiment sample was added to 500 ml of sterile thioglycollate broth (Difco) and incubated for 24 h at 28 C. The enriched cultures were examined for anaerobic populations using both thioglycollate pour plates incubated anaerobically in Gas Paks (BBL) and the shake culture tube procedure (HAMDY et al., 1954). The predominant organisms were isolated based on colonial morphology, cultural characteristics and biochemical activities (DOWELL and HAWKINS, 1973). The cultures were tested for Hg^{2+} resistance by the disc sensitivity procedure (HAMDY and NOYES, 1975) using glucose basal salt agar medium (GBSA). The GBSA (pH 7.0) consisted of: K_2HPO_4, 7 g; KH_2PO_4, 3 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $(NH_4)_2SO_4$, 1 g; NaCl, 3 g; yeast extract, 4 g; glucose, 5 g; and agar, 16 g per liter of deionized distilled water.

Cell preparations. Bacterial isolates were grown in thioglycollate broth at 28 C for 20 h. Cells were harvested by centrifugation (10,000 x g for 10 min) and washed three times with sterile saline. They were then resuspended in 10 ml sterile sodium phosphate buffer (pH 7.0, 0.05 M) to the desired concentration as determined by plating on thioglycollate agar.

Survival studies. Ten ml of cell-suspension of each organism was added to 90 ml of sterile Na-phosphate buffer containing the desired level of Hg^{2+}. Viable cells were enumerated in 1.0 ml samples taken initially (0 h) and at selected intervals, by plating appropriate dilutions on thioglycollate agar. Colonies were counted after 48 h incubation at 28 C and the survival fraction, N/N_o, was determined, where N_o represents the number of cells as colony forming units (CFU) per ml of suspension at zero time, and N denotes the number of cells (CFU) after exposure to Hg^{2+}. A stock solution of $HgCl_2$ (25 mg Hg^{2+} per ml of 0.01 N HCl) was made daily (NOYES et al., 1975). Further dilutions using distilled water were prepared from the stock to the desired concentration of Hg^{2+}.

Hg^{2+} antagonists. The following agents were examined for their protective effect on the test cultures against

the bactericidal action on Hg^{2+}:sediment (Sed), ashed sediment (Ash-Sed), Difco tryptone (Try), and ground rubber (GR). The sediment sample was freeze-dried and stored in a dessicator at room temperature until use. Some samples of freeze-dried Sed were ashed at 625 C in a muffle furnace and stored at room temperature. Ground rubber was prepared by drilling holes in rubber stoppers with a 1/4-in. (6.35 mm diameter) using a drill press and collecting the resulting fine powder (NOYES et al., 1975). Rubber particles ranging in diameter from 10 to 200 u were used. The desired concentration of each antagonist was prepared daily in phosphate buffer (pH 7.0, 0.05 M) and sterilized by autoclaving. The effects of the antagonists on survival of the test culture in the presence of Hg^{2+} was determined as previously outlined except for the addition of the necessary concentration of the antagonist just prior to the addition of Hg^{2+}.

Results

Microbial analysis. Twenty-two cultures were isolated from the sediment samples, characterized morphologically and biochemically, and tested for resistance to Hg^{2+} (HAMDY and NOYES, 1975). Of all the cultures examined only two obligate anaerobes identified as a Bacteroides and a Clostridium sp., were found to be sensitive to very low levels of Hg^{2+}. Therefore, these sensitive cultures were used in this investigation. The Bacteroides culture did not produce either indol, H_2S, or acetoin and strongly fermented carbohydrates. The reactions were similar to those of B. fragilis subsp. vulgatus, as described in the 8th edition of Bergey's Manual of Determinative Bacteriology, except for the production of butyric and acetic acids from glucose. The Clostridium also did not produce H_2S but had many characteristics of C. bifermentans. The glucose fermentation products included acetic, isobutyric, isovaleric, and isocaproic acids, but not alcohols.

Effect of Hg^{2+} on growth. Both the Bacteroides sp. and the Clostridium sp. were very sensitive to Hg^{2+} as evidenced by their inhibition by 1.1 ug Hg^{2+} or more per disc (Table 1). However, plates seeded with the Clostridium sp. at a level of 10^6 CFU per ml medium were more sensitive to this level of Hg^{2+} than plates seeded with the Bacteroides sp. This latter culture, at 10^8 CFU per ml medium, was inhibited by 18.5 ug of Hg^{2+} per disc. However, the sensitivity of these two cultures decreased in direct proportion to cell number and the response of either culture to a specific level of Hg^{2+} was variable. In the presence of the same level of Hg^{2+} and at a cell concentration of 10^7 CFU per ml medium, the inhibition zones were greater for the Bacteroides sp. than for the Clostridium sp.

TABLE 1. Effect of cell number on inhibition of the Bacteroides sp. and the Clostridium sp. by H^{2+} as measured by the disc sensitivity procedure.

Concentration of Hg^{2+} (ug/disc)	Zone of Inhibition (mm)[a]					
	Bacteroides sp. - (log CFU/ml)			Clostridium - (log CFU/ml)		
	6	7	8	5	6	7
0.37	0.0	0.0	0.0	0.3 ±0.0[b]	0.0	0.0
0.74	0.0	0.0	0.0	1.5 ±0.6	0.3 ±0.0	0.0
1.11	0.1 ±0.1[b]	0.0	0.0	3.0 ±0.6	0.8 ±0.5	0.0
1.85	0.3 ±0.0	0.3 ±0.0	0.0	4.3 ±1.0	1.6 ±0.6	0.0
3.70	2.3 ±0.5	1.3 ±0.0	0.0	8.0 ±1.5	4.8 ±0.9	0.1 ±0.1
9.25	5.8 ±0.5	4.5 ±0.6	0.0	9.8 ±0.9	6.6 ±0.6	0.5 ±0.3
18.50	8.8 ±0.5	6.3 ±0.5	0.9 ±0.7	13.0 ± 0.6	10.3 ±0.0	5.0 ±0.6

[a] Diameter of the zone of inhibition (mm) minus that of the disc.
[b] Standard deviation.

Survival studies. Data on the effect of different levels of Hg^{2+} on survival of the Bacteroides sp. at two levels of cell population (5 x 10^6 CFU and 4 x 10^7 CFU per ml buffer) are shown in Fig. 1 (A and B), respectively.

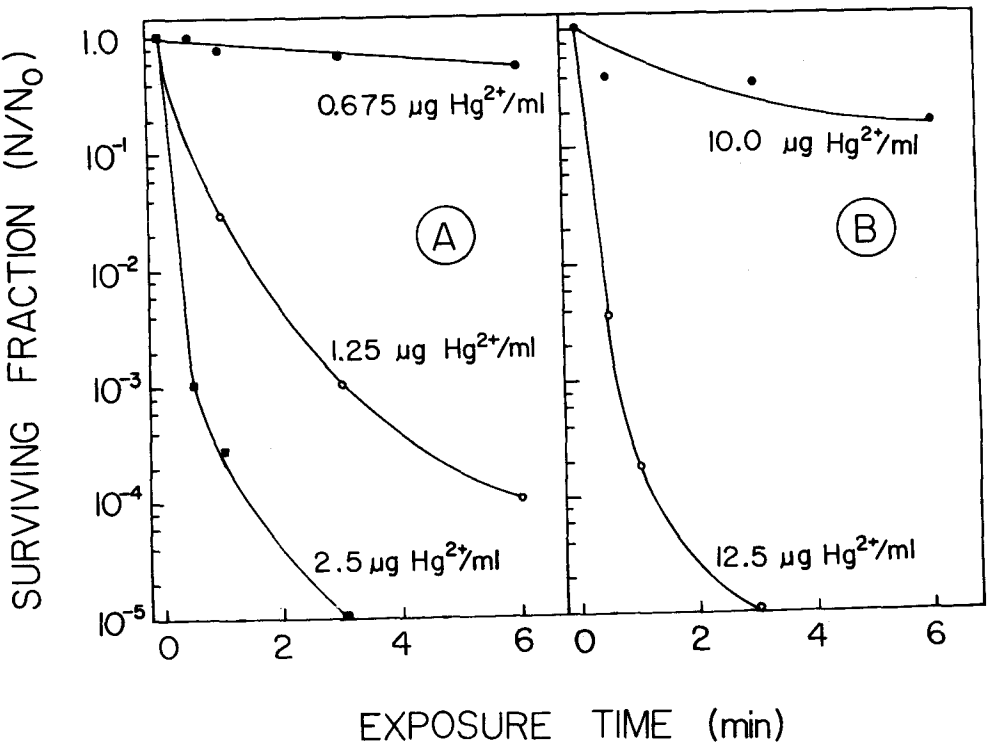

Fig. 1. The effect of cell number on survival of the Bacteroides sp. at various levels of Hg^{2+}. A represents 5 x 10^6 cells, B denotes 4 x 10^7 cells per ml buffer.

Non-exponential inactivation curves for both cell populations were noted at levels higher than 0.7 ug Hg^{2+} per ml. When 10^7 CFU per ml were exposed to 12.5 ug Hg^{2+} per ml, the survival fraction after 3 min exposure was 1.0 x 10^{-5} and similar to that noted when 10^6 CFU were exposed to 2.5 ug Hg^{2+} per ml for the same length of time. Thus, it requires a five-fold increase in the concentration of Hg^{2+} to achieve the same destruction curves with a 10-fold increase in cell number.

Effect of protective agents. Since the results obtained using the two Sed samples were almost the same, only the data obtained from Par Pond are reported. Figure 2A summarizes the results obtained when 5 x 10^6 CFU of the Bacteroides culture per ml buffer containing Sed were exposed to two

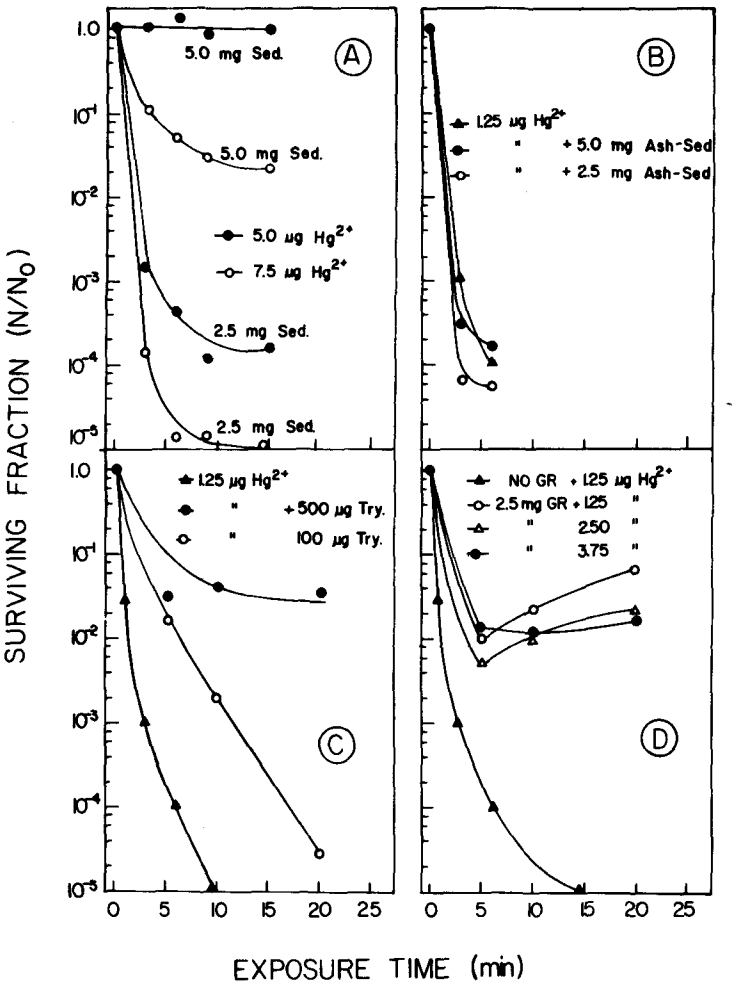

Fig. 2. The effect of protective agents on the survival of the Bacteroides sp. (5 x 10^6 cells) exposed to various levels of Hg^{2+} per ml. A, two levels of sediment (Sed); B, ashed sediment (Ash-Sed); C, tryptone (Try); and D, ground rubber (GR).

different levels of Hg^{2+}. Maximum protection of the entire cell population was noted against 5.0 ug Hg^{2+} per ml when the buffer had 5.0 mg Sed. In the presence of 7.5 ug Hg^{2+} per ml buffer the protective effect of Sed (5.0 mg per ml) decreased slightly. The protective effects of Sed continued to diminish at lower concentrations (2.5 mg Sed:7.5 ug Hg^{2+} per ml). Although not represented on the graph, the surviving fraction in the presence of 2.5 mg Sed and 2.5 ug Hg^{2+} remained fairly constant (0.81) during the entire 15 min

exposure, indicating that this level of Sed completely protected the entire cell population against the bactericidal action of that level of Hg^{2+}. The levels of Hg^{2+} which reduced N/N_o to 1×10^{-4} after 6.0 min exposure were: 1.25 ug Hg^{2+} per ml buffer containing no Sed, 5.0 ug Hg^{2+} per ml buffer containing 2.5 mg Sed per ml, and 10.0 ug Hg^{2+} per ml buffer with 5.0 mg Sed per ml. Thus, a two-fold increase in Hg^{2+} concentration necessitated a doubling of Sed concentration to achieve an equivalent surviving fraction. To determine if the protective effect of Sed on the cells of the Bacteroides sp. was due to the presence of organic fraction, ashed sediment was tested. The Ash-Sed offered no protection (Fig. 2B). Figure 2C depicts the protective effects of tryptone. When the Bacteroides sp. was exposed to Hg^{2+}, in the presence of GR, the N/N_o decreased to 1.3×10^{-2} within 5 min after exposure to 3.75 ug Hg^{2+} per ml (Fig. 2D). This value was similar to results noted following 2 min exposure of the cell population to 1.25 ug Hg^{2+} in absence of GR. The protective effect of GR remained evident, as after 5, 10 and 20 min of cellular exposure to 3.75 ug Hg^{2+} the N/N_o was unchanged, whereas cells plus GR and either 1.25 and 2.5 ug Hg^{2+} per ml buffer showed pronounced recovery (Fig. 2D).

DISCUSSION

The sensitivities of various microorganisms to mercurials (STEEL, 1960) as well as to other antimicrobial substances (BAUER et al., 1966; FAREWELL and BROWN, 1971; and JAWETZ et al., 1962) have been reported and the data showed that proliferation is usually depressed at fairly low concentrations. However, interpretation of the results are invariably complicated by the fact that the organism and its environment are constantly interacting independently of the presence of the inhibitor. COOK and STEEL (1959) and others (HAMDY and NOYES, 1975) showed that the amount of $HgCl_2$ necessary to produce bacteriostasis to E. coli increased with an increase in number of cells in the inoculum. The presence of sediment also provided an additional protective effect to the Bacteroides cells against the action of Hg^{2+}. The mechanism of this protection is not clear. The increased resistance could be mediated by the adsorption of the bacterial cells onto the colloidal fraction of the sediment or the Hg^{2+} may be inactivated by binding to various sites in the organic matter of the sediment thus decreasing the effective concentration of Hg^{2+}. HAMDY and NOYES (1975) reported that the decrease in uptake of $^{203}Hg^{2+}$ by E. aerogenes was apparently due to complexing of mercury with the constituents of the yeast extract added to the medium, thus retaining the mercury to some degree outside the bacterial cell. RAMAMOORTHY and KUSHNER (1975) reported that fulvic acid, the most important soluble humic substance in natural water, was able to bind Hg^{2+} ions. REIMERS and KRENKEL (1974) showed that the ad-

sorption of $HgCl_2$ was affected by the constituents of the sediment (organic-SH clay sands). This may explain the behavior of the ashed sediment in not protecting the bacterial community against the action of Hg^{2+}.

Previous results (NOYES et al., 1975) have shown that ground rubber could bind organic and inorganic mercury compounds from aqueous solution and suggested that ground rubber be used for the effective removal of Hg^{2+} from rivers and lakes. These results prompted an examination of the behavior of ground rubber in protecting the cells of Bacteroides sp. against Hg^{2+}. The results were different from those obtained on the other materials tested. In the presence of ground rubber, the survival fraction decreased to approximately the same value for all three Hg^{2+} levels used. It appeared that the ground rubber protected the bacterial cells by its chemi-sorptive behavior towards Hg^{2+} (NOYES et al., 1975). A second phenomenon associated with ground rubber was manifested after 20 min exposure to Hg^{2+}. The bacterial population showed evidence of recovery which appeared to be dependent on Hg^{2+} concentration. Mercury is known to affect cells in three stages. The first stage is noted when recovery is facilitated by a simple wash with saline. The second is observed when cells must be washed with a solution containing a mercury binding agent such as H_2S, and the third stage is detected when there is no recovery even if the cells are repeatedly washed with stronger binding agents. The third state represents the time when mercury has reacted irreversibly with the cellular proteins. Rubber appeared to act in a manner similar to the sulfur compounds in the second stage, however, because of its relatively slow binding rate, the effect of rubber was evident only after a 5 min interval. It appeared that rubber was able to reverse the action of Hg^{2+} on the bacterial cells. This was in contrast to the protective effect of sediment where recovery of bacterial cells was not observed and presumably no exchange of mercury occurred once the heavy metal had interacted with the cells. The apparent difference in mechanism of protection between sediment and ground rubber was probably due to the particulate nature of the ground rubber.

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