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Formation of Methyl Mercury by Bacteria

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Twenty-three Hg^{2+}-resistant cultures were isolated from sediment of the Savannah River in Georgia; of these, 14 were gram-negative short rods belonging to the genera *Escherichia* and *Enterobacter*, six were gram-positive cocci (three *Staphylococcus* sp. and three *Streptococcus* sp.) and three were *Bacillus* sp. All the *Escherichia*, *Enterobacter*, and the *Bacillus* strain were more resistant to Hg^{2+} than the strains of staphylococci and streptococci. Adaptation using serial dilutions and concentration gradient agar plate techniques showed that it was possible to select a Hg^{2+}-resistant strain from a parent culture identified as *Enterobacter aerogenes*. This culture resisted 1,200 μg of Hg^{2+} per ml of medium and produced methyl mercury from $HgCl_2$, but was unable to convert Hg^{2+} to volatile elemental mercury (Hg^0). Under constant aeration (i.e., submerged culture), slightly more methyl mercury was formed than in the absence of aeration. Production of methyl mercury was cyclic in nature and slightly decreased if DL-homocysteine was present in media, but increased with methylcobalamine. It is concluded that the bacterial production of methyl mercury may be a means of resistance and detoxification against mercurials in which inorganic Hg^{2+} is converted to organic form and secreted into the environment.

There is substantial evidence that small concentrations of inorganic divalent mercury compounds and phenyl mercurials are relatively nontoxic. However, transformation of these compounds in aquatic environments to methyl and dimethyl mercury is a major problem (4). Johnels and Westermark (13) reported that the main types of mercury discharged into aquatic environments are: inorganic divalent mercury, Hg^{2+}; metallic mercury, Hg^0; phenyl mercury, $C_6H_5Hg^+$; methyl mercury (MM), CH_3Hg^+; and methyloxyethyl mercury, $CH_3OCH_2CH_2Hg^+$. Biological methylation of inorganic mercury compounds was suggested as a possible source of the MM that was the causative agent of Minamata Disease in Japan (5, 9, 19). Jensen and Jernelöv (10) showed that MM was formed after incubation of sediment, obtained from fresh water aquaria, with up to 100 μg of Hg^{2+}. Jernelöv (11, 12) again reported that MM could be formed in sediments under anaerobic conditions. Vonk and Sijpesteijn (36) reported that small amounts of MM were produced by various bacterial species growing under aerobic conditions. Wood et al. (39) showed that cell-free extracts of an anaerobic methanogenic bacterium effectively converted inorganic Hg^{2+} into MM with methyl-cobalamine, (a B_{12} derivative) as substrate. Plasmid-mediated resistances to heavy metal have been observed in enteric bacilli, especially in R-factor organisms (26), and have been studied by many investigators (14, 15, 28, 29, 33-35). This research was initiated to examine the Savannah River sediment for mercury-resistant bacteria, ascertain their capacity to form MM, and study factors which influence this biotransformation.

MATERIALS AND METHODS

Analyses. Flameless atomic absorption spectrophotometry was used for quantitative measurements of mercuric ion (18, 32). The method is based on reduction of Hg^{2+} to vapor (Hg^0) by $SnCl_2$ in presence of H_2SO_4; the Hg^0 is then flushed by N_2 through a gas flow-through cell and mounted in a Shimadtzu recording spectrophotometer (MPS-50L), and absorbance at 253.7 nm was measured. Both inorganic and organic mercurial compounds were determined, and their concentrations were ascertained with standard curves. The latter was conducted after oxidation with $KMnO_4$-H_2SO_4. The thin-layer chromatography (TLC) methods used for the separation and detection of mercury compounds were those of Tatton and Wagstaffe (30). Dried silica gel chromatogram sheets (Eastman no. 6061) were spotted and developed at 25 C with a low boiling point petroleum, and acetone mixture (9:1, vol/vol)

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was used as solvent. Spots were located with 0.005% dithizone in benzene, and R_f values consistent with standard methyl, ethyl, and/or dimethyl mercury compounds were obtained. When $^{203}Hg^{2+}$-containing metabolites were present, each spot on the chromatogram was cut into 0.2- to 0.4-cm strips, placed in standard scintillation vials containing 10 ml of toluene-based scintillation fluor, and counted in a Beckman LS-100 C liquid scintillation spectrophotometer. An F & M (model 810) gas-liquid chromatograph (GLC) equipped with an electron capture detector (200 mCi of tritium) was also used for both qualitative and quantitative determination of Hg^{2+} containing metabolites following the procedure of Baughman et al. (2). The analysis was conducted on a Pyrex glass column (100 cm by 12 mm, ID) containing Chromosorb W (60/80 mesh) coated with 5% diethylene glycol succinate. The temperature of the detector, the injection port, and column were 210, 190, and 140 C, respectively. The carrier gas flow rate was 40 ml of nitrogen per min. Benzene extract (5 μl) was injected, and the Hg^{2+}-containing metabolites, their retention time, and peak height were compared to those obtained from standard solutions of methyl or ethyl mercury chloride.

Dimethyl mercury, methyl, and ethyl mercury salts were obtained from Alfa Ventron. Phenyl mercuric chloride was purchased from Eastman Kodak Co., and mercuric chloride was secured from Baker Chemicals. Methyl-cobalamine was prepared by L. G. Ljungdahl (Biochemistry Department, University of Georgia, Athens) from cyanocobalamin and methyl iodide (7, 20).

Media. Unless otherwise stated, $HgCl_2$ or other constituents sterilized by filtration (0.22-μm filter) were added to sterile culture medium in designated concentrations. Glucose basal salt broth (GBSB) and minimal chloride (M-Cl) broth or their agar were used. The GBSB (pH 7.0) consisted of: KH_2PO_4, 6.8 g; $CaCl_2 \cdot 4H_2O$, 0.09 g; $MgSO_4$, 0.06 g; $MnSO_4$, 0.001 g; $ZnSO_4$, 0.007 g; $FeSO_4 \cdot 7H_2O$, 0.025 g; NH_4Cl, 0.25 g; $(NH_4)_2SO_4$, 0.25 g; $NaMoO_4 \cdot 2H_2O$, 0.001 g; 10 g of glucose per liter of deionized distilled water. The M-Cl medium (pH 7.0) was that reported by Ray and Speck (21), but modified so that most of the inorganic salts were of the chloride form and contained: KH_2PO_4, 5 g; NaCl, 0.1 g; NH_4Cl, 2.04 g; $MgCl_2$, 0.08 g; 10 g of glucose per liter of deionized distilled water. Aqueous standard stock solution of $HgCl_2$ was made daily and further dilutions were performed from the stock to the desired concentration of Hg^{2+}. Standard methyl, dimethyl, and ethyl mercuric chloride solutions were prepared in either benzene or saline.

Microbial analyses. A sediment sample obtained from the Savannah River below the outfall of the Olin Mathison Manufacturing Plant, Augusta, Ga., was used. The sample was subjected to microbial analyses using standard methods for aerobes (1) and the shake culture procedure (6) for anaerobes. The isolates were screened for resistance to Hg^{2+} by inoculating GBSB, containing desired levels of $HgCl_2$, with a 1% 18-h culture previously grown at 37 C in the absence of Hg^{2+}. Cultures exhibiting resistance were further adapted to growth in GBSB containing higher levels of Hg^{2+} with serial dilution and concentration gradient agar plates. Each gradient plate was divided into six zones where zones 1 and 6 represent, respectively, the lowest and the highest levels of Hg^{2+} per unit area of agar medium.

Survival. Mercury-sensitive (S) and -resistant (R) cultures of one isolate (*Enterobacter aerogenes*) were used. The culture was allowed to grow in GBSB for 18 to 24 h at 37 C. Cells were harvested by centrifugation, washed twice with sterile saline, and then resuspended in 15 ml of sterile phosphate buffer (0.05 M, pH 7.0). Five-milliliter cell suspensions were then added to 95 ml of phosphate buffer containing the desired concentration of Hg^{2+}. Samples (1 ml) were withdrawn initially and at selected intervals, and the viable cell populations were determined by plating appropriate dilutions on glucose basal salt agar. Colonies were counted after 48-h incubation at 37 C, and the survival fraction, N/N_0, was determined, where N_0 represents the number of cells per ml of suspension at zero time and N denotes the number of cells surviving after exposure to Hg^{2+}.

Uptake of $^{203}Hg(NO_3)_2$. Two experiments were carried out with the *E. aerogenes*-R culture. The first was concerned with growing cells for 16 h in the presence of 5 μg of Hg^{2+} per ml of GBSB supplemented with $^{203}Hg(NO_3)_2$ to yield a total count of 2×10^6 counts/min per ml of medium. Ryan flasks containing the media were inoculated with a 1% 18-h culture previously grown at 37 C in the absence of Hg^{2+}. Control flasks containing the same basal medium and no added Hg^{2+} were also inoculated, and the growth was measured at 610 nm with a Bausch and Lomb spectronic 20. During incubation, aliquots of culture media were removed and filtered with a preweighed membrane filter (0.22 μm, Millipore Corp.). The cells were washed with concentrated HCl to remove any $^{203}Hg^{2+}$ absorbed to the filter, and both media and HCl wash were collected. The membrane filter was then dried to constant weight and cell weight was determined. The filtrate and cells were examined for radioactivity with a solid scintillation counter. The second experiment was designed to follow the distribution of the $^{203}Hg^{2+}$, derived from $^{203}Hg(NO_3)_2$, among cellular fractions. The technique used was similar to that performed in the first experiment except that the entire culture biomass was used after the desired incubation time. Cells were harvested by centrifugation, washed twice with saline, and subjected to the chemical fractionation procedure reported by Roberts et al. (23).

Methylation. A model system for production of MM, or ethyl mercury, and/or volatilization of Hg^{2+} to Hg^0 was used. It consisted of a culture chamber (3 liters) connected to a series of traps. The chamber contained 1,500 ml of GBSB media, the desired level of $HgCl_2$ supplemented with $^{203}Hg(NO_3)_2$, other chemicals, and the test culture. The train of traps was designed to scrub volatilized mercury compounds formed by the organism during aeration (submerged culture) or carried by gases given off during incubation in the absence of aeration. The first trap consisted of 200 ml of solution (5% Na_2CO_3 and 5% Na_2HPO_4), to remove organomercury compounds such as ethyl and methyl mercury. The second trap had a 200-ml solution of 1 N H_2SO_4 containing 0.005% dithizone and 10% KBr to remove any organomercury that had escaped the first trap as well as elemental mercury (Hg^0) from the effluent gas. The third trap contained a 200-ml solution of 5% $KMnO_4$ in 2 N H_2SO_4 to remove any Hg^0 that had escaped the second trap. Analyses of culture media (cells and cell free) for the presence of organic and inorganic mercury were performed as shown in the schematic diagram (Fig. 1). The efficiency of the extraction procedure was determined with GBSB containing 50 μg of Hg^{2+} per ml of broth supplemented with either $^{203}Hg(NO_3)_2$ or $CH_3{}^{203}HgCl$ or both. In the presence of $^{203}Hg(NO_3)_2$ alone, 5.4% of the initial $^{203}Hg^{2+}$ activity was found in the first benzene extract (no. 1), whereas, in the presence of $CH_3{}^{203}HgCl$ alone, 92% of its original activity was regained in the benzene extract. If both $^{203}Hg(NO_3)_2$ and $CH_3{}^{203}HgCl$ were present, the total recovery was 87.4%, indicating that 82% of the original $CH_3{}^{203}HgCl$ was retrieved. When benzene extract was subjected to a second extraction (no. 2, Fig. 1), data from radioactive tracer and GLC analysis indicated a 100% recovery of the MM. Aliquots of the various traps were also subjected to benzene extraction following the flow diagram for cell-free media (Fig. 1). Elemental mercury was quantitated in the traps by flushing aliquots of the sample with N_2 to the gas flow-through cell (mounted in the flameless atomic absorption spectrophotometer) directly, without digestion and without the reducing agent. Quantitative trapping of MM and Hg^0 was confirmed experimentally with

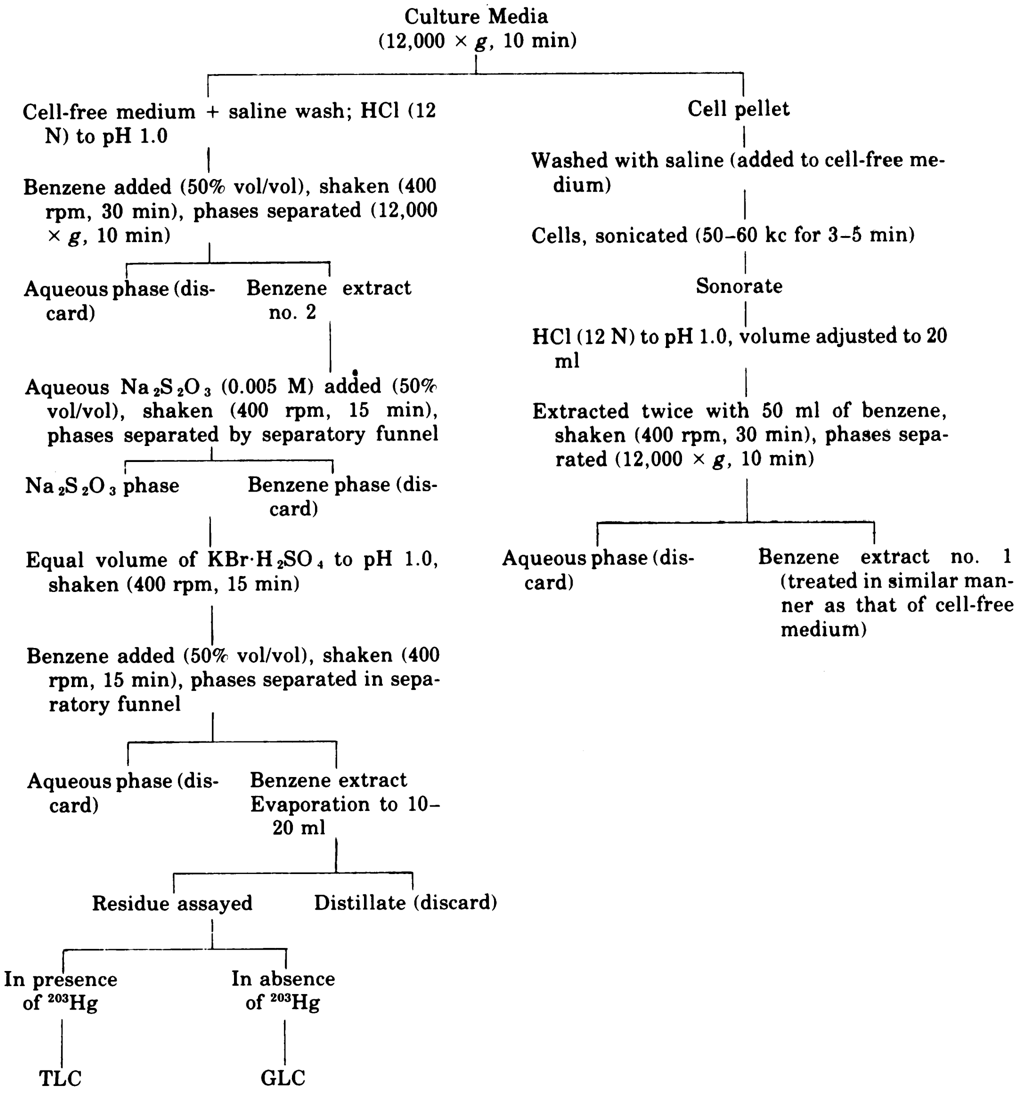

FIG. 1. *Flow diagram for analyses of culture medium (cells and cell free) for organic and inorganic mercury with TLC and GLC.*

$CH_3{}^{203}HgCl$ before and after reduction with $SnCl_2$ in the presence of H_2SO_4.

RESULTS

Microbial analyses. Twenty-three cultures were isolated from the sediment, characterized morphologically and biochemically, and classified into four groups. The first group (14 cultures) were gram-negative short rods belonging to genera *Escherichia* and *Enterobacter*; the second (three cultures) were *Staphylococcus* sp.; the third (three cultures) were *Streptococcus* sp.; and the fourth (three cultures) were *Bacillus* sp. All isolates were tested for resistance to Hg^{2+}. The three staphylococcal cultures were able to grow in the presence of 50 and 100 μg of Hg^{2+} per ml of GBSB and were completely inhibited in the presence of 150 μg of Hg^{2+} per ml. The three *Streptococcus* cultures showed no evidence of growth in GBSB containing 60 μg of Hg^{2+} per ml, whereas all 14 *Escherichia* and *Enterobacter* cultures, as well as the three *Bacillus* sp., exhibited good growth in all media containing up to 400 μg of Hg^{2+} per ml of GBSB. All the Hg^{2+}-R cultures were then subjected to a series of selection and adaptation experiments in the presence of increasing levels of Hg^{2+}. The results revealed that after this manipulation six isolates, two *Escherichia*, three *Enterobacter*, and one *Bacillus*, grew well in GBSB containing 800 μg of Hg^{2+} per ml, and of these one *Enterobacter* and the *Bacillus* were able to grow in GBSB containing 1,200 μg of Hg^{2+} per ml.

All isolates were also screened on freshly prepared concentration gradient agar plates containing various levels of Hg^{2+}. The results also showed that of all the cultures tested, only three *Enterobacter* cultures and one *Bacillus* sp. exhibited very good growth, after 24 h of incubation, on the entire six zones of the gradient plate containing 100 μg of Hg^{2+} per ml of GBSA. The same cultures showed good growth on half the plate (zones 1, 2, and 3) containing 150 μg of Hg^{2+} per ml of agar, some growth on zones 1 and 2 of the plate that had 200 μg of Hg^{2+} per ml of medium, very little growth on zone 1 of the plate at 250 μg of Hg^{2+} per ml of medium, and no visible growth at 300 μg of Hg^{2+} per ml of medium. Again, after manipulation by repeated transfers of these cultures on the gradient plates, two of the six isolates (an *Enterobacter* and a *Bacillus*) grew well in all six zones and resisted the presence of 300 μg of Hg^{2+} per ml of agar, whereas all the other cultures were unable to grow beyond the third zone of these gradient plates. The *Enterobacter*, identified as *E. aerogenes*, was then chosen for further investigations.

Growth of the Hg^{2+}-S-strain of this culture in GBSB containing 20 μg of Hg^{2+} per ml occurred after an 11-h lag (Fig. 2), whereas in the presence of 40 μg of Hg^{2+} per ml of broth growth was delayed for 22 h but then continued to reach control level within 36 h. When GBSB was supplemented with 2% yeast extract (Fig. 3), growth of the S-strain in presence of 25 (line A) or 50 (line B) μg of Hg^{2+} per ml of broth was similar to the control (line C). In the presence of 100 μg of Hg^{2+} per ml of broth, the growth pattern (line D) exhibited a 2.5-h lag followed by a slow but steady growth to reach control (line C) within 8 h. The increase in absorbance (line D) after the addition of 100 μg of Hg^{2+} per ml was due to formation of slight opacity from the reaction of Hg^{2+} with bacterial cells. The effect of 20 and 40 μg of Hg^{2+} per ml of GBSB on the growth pattern of the Hg^{2+}-R-strain was no

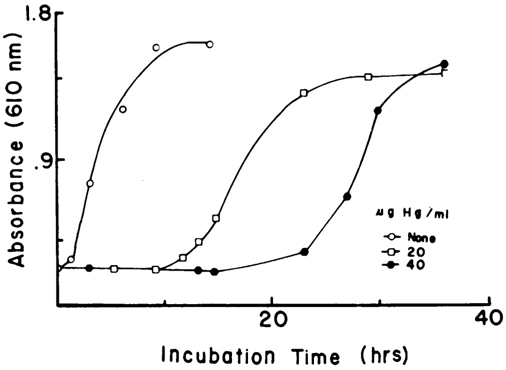

FIG. 2. *Effect of* Hg^{2+} *on growth at 37 C of* Hg^{2+}*-S-strain of E. aerogenes in GBSB.*

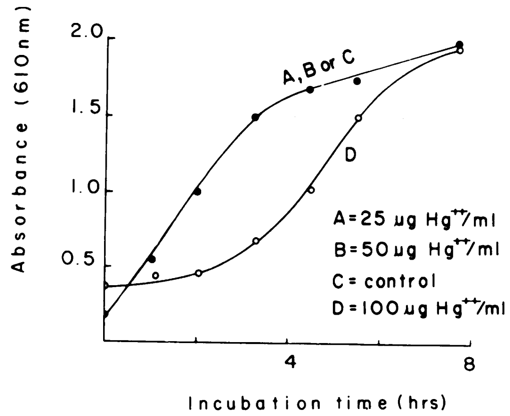

FIG. 3. *Effect of* Hg^{2+} *on growth of* Hg^{2+}*-S-strain of E. aerogenes in GBSB containing 2% yeast extract.*

different from that of control during the entire period of incubation.

Data using the disk sensitivity procedure showed that the S-strain was more susceptible to Hg^{2+} than the R-strain of *E. aerogenes* (Fig. 4) and that the inhibition zone was much less when 2×10^8 cells/ml of medium of either strain were used compared to 2×10^4 cells/ml. The R strain was inhibited to a greater extent on M-Cl agar than on tryptone-glucose-yeast extract (TGE) agar regardless of cell numbers. No significant differences were noted in the diameter of the inhibition zones as a function of Hg^{2+} concentration/disk on M-Cl and TGE media when 2×10^8 cells/ml of media of the S-strain was used.

The comparative survival data of S- and R-strains of *E. aerogenes,* exposed to 20 μg of Hg^{2+} per ml of phosphate buffer, revealed that S-cells were completely inhibited after 0.5-min of exposure to Hg^{2+}, whereas 19.5% of the population of the R-cells survived 0.5-min exposure and reached 1.43, 0.06, and <0.005% after 1.5, 3.0, and 5.0 min, respectively.

Uptake of $^{203}Hg^{2+}$. In absence of yeast extract, 94% of total radioactivity was found in cells of R-strain culture after 4 h (Fig. 5, line B), whereas the activity in cell-free medium (line D) was 6 and 7.8% after 4 and 12 h, respectively. Activities in benzene-soluble, $^{203}Hg^{2+}$-containing metabolites (line E) increased from 0.2 to 1.4% after 4 and 12 h, respectively. When yeast extract was added, the uptake of $^{203}Hg^{2+}$ by the cells (line B) was 73% after 9 h with no changes thereafter. Activity in cell-free medium (line D) gradually decreased to 27% within 9 h and changed very little thereafter, whereas $^{203}Hg^{2+}$-containing metabolites in benzene-extract of cell-free medium (line E) was 0.6 and 0.2% after 4 and 12 h, respectively. Growth of the test culture in GBSB media supplemented with yeast extract (line A in presence of Hg^{2+} and line C in absence of Hg^{2+}) was different compared to growth in media containing no yeast extract. For example, in the presence of yeast extract, the log phase was detected within 2 h and reached an absorbance value of 1.2 after 7 h, whereas in the absence of yeast extract the absorbance was 0.8 and 0.95 after 7 and 12 h, respectively.

Distribution of $^{203}Hg^{2+}$ among various fractions of Hg^{2+}-R-cells grown in the presence or absence of 2% yeast extract (Table 1) showed that after 3-h incubation in GBSB containing no yeast extract, 91% of the total activity of the ^{203}Hg was in the insoluble protein fraction,

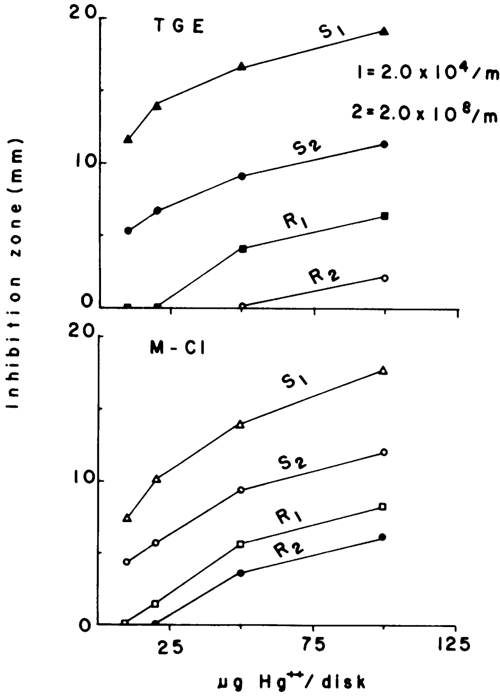

FIG. 4. *Effect of cell number and type of media on inhibition of S- and R-strains of E. aerogenes as measured by the disk sensitivity procedure.*

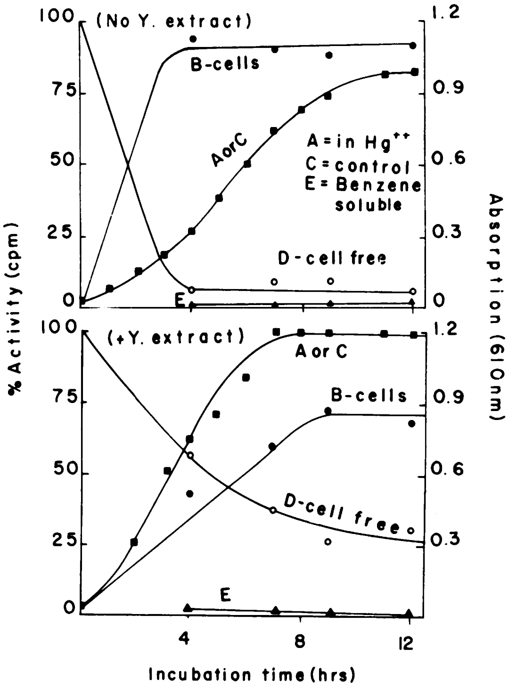

FIG. 5. *Uptake of ^{203}Hg during growth of Hg^{2+}-R-strain of E. aerogenes at 37 C in presence or absence of yeast extract in GBSB containing 5 μg of Hg^{2+} per ml of media supplemented with $^{203}Hg(NO_3)_2$.*

TABLE 1. *Effect of yeast extract on distribution of $^{203}Hg^{2+}$ among cellular fractions of R-E. aerogenes during growth in GBSB containing 5 μg of Hg^{2+} per ml supplemented with $^{203}Hg(NO_3)_2$*[a]

Cellular fraction	Incubation time (h)											
	3		5		7		9		11		21	
	A	B	A	B	A	B	A	B	A	B	A	B
Cold trichloroacetic acid soluble[b]	2.0	11.3	0.5	2.4	0.7	1.1	0.1	0.6	0.1	0.4	0.3	0.2
Alcohol soluble (lipids and proteins)	3.1	1.0	0.7	1.0	1.9	1.0	0.2	0.2	0.2	0.3	0.3	0.1
Alcohol-ether soluble[c]	2.0	0.0	0.2	0.0	2.1	0.0	0.1	0.3	0.1	2.3	0.2	0.5
Hot trichloroacetic acid soluble (nucleic acid)	1.9	16.9	10.7	16.2	2.5	2.8	0.6	8.5	0.3	3.9	0.9	1.2
Residual precipitate (protein)	90.9	70.7	87.7	80.4	92.8	94.9	98.7	90.2	98.9	92.9	98.1	97.9

[a] Results are presented as percentage of activity recovered in the absence (A) or presence (B) of 2% yeast extract.

[b] Transient intermediates and inorganic cations.

[c] Small quantities of lipid and proteins.

reaching 98% after 21 h. The activity in the other soluble fractions (cold trichloroacetic acid, alcohol, alcohol-ether, and hot trichloroacetic acid) ranged from 0.1 to 10.7% during the 21-h incubation. However, in presence of 2% yeast extract 71, 17, and 11% of the total activity were found after 3 h in the protein, hot trichloroacetic acid-, and cold trichloroacetic acid-soluble fractions, respectively. These values changed as follows: the insoluble protein fraction increased to 90 and 98% after 9 and 21 h, respectively, and the hot trichloroacetic acid-soluble fraction remained at 16% after 5 h, decreasing to 8.5 and 1% after 9 and 21 h, respectively. The activity in the cold trichloroacetic acid-soluble fraction, on the other hand, decreased to 2.4 and 0.2% after 5 and 21 h, respectively. The ether-alcohol-soluble fraction did not contain any $^{203}Hg^{2+}$ until after 11 h when 2.3% of the total activity was detected, which then decreased to 0.5% after 21 h. The activity in the alcohol-soluble fraction remained constant (1%) until 9 h of incubation. By 21 h of incubation, the $^{203}Hg^{2+}$ activity had decreased to 0.1%.

Formation of MM. Initial investigation centered on qualitative determination of MM in a model system in which GBSB containing 50 μg of Hg^{2+} per ml was inoculated with the Hg^{2+}-R-*E. aerogenes* and incubated at 37 C. After 3 days, the entire culture medium was analyzed for MM using TLC and GLC and results confirmed the presence of MM. It was also established that GLC quantitation of MM after TLC was impractical due to volatilization and/or evaporation of MM from TLC plate during storage (i.e., within 5 h no MM could be detected by GLC from standard MM reference spot containing 1 μg/0.1 ml of benzene on TLC plate). Quantitation was possible, however, if either TLC or GLC analyses were performed alone but not from spots on TLC plates to GLC. In this investigation, TLC was used when $^{203}Hg^{2+}$ was present and GLC was used in the absence of isotope in samples.

Several factors which might effect the microbial methylation of Hg^{2+} by the R-culture were also examined. These factors included: time course, presence or absence of oxygen, and supplementation of the media with DL-homocysteine and/or methyl-cobalamine (CH_3-B_{12}).

Effects of growth conditions. The data (Table 2) indicates that the aerobic growth stimulated the production of MM compared to the unaerated system, and that the formation of MM was cyclic during the 20 days of incubation. Benzene extraction of the solution in each trap, followed by GLC or TLC analyses, indicated the absence of MM and Hg^0 in the traps during the entire period of incubation under both aerobic and unaerated systems.

Effect of DL-homocysteine. Homocysteine was added in equimolar amounts to that of Hg^{2+} (0.25 μmol of Hg^{2+} per ml of media) present in GBSB and the medium was then incubated aerobically. The results (Table 2) showed the absence of MM, as detected by TLC or GLC, in the benzene-extract of control medium (noninoculated flask). It was also noted that the presence of homocysteine decreased MM formation in the culture media after 3 and 20 days of incubation. However, more MM was detected in the cell-free media and, again, MM production was cyclic during the 20-day incubation.

TABLE 2. *Effects of aerobic and unaerated systems on MM production by Hg^{2+}-R-E. aerogenes incubated at 37 C in GBSB containing 0.25 μmol of Hg^{2+} per ml of broth*[a]

Incubation (days)	Growth system	MM (ng/ml)		
		Cell free	Cells	Culture
3	Aerobic	246.5	184.9	431.9
10		14.5	11.8	26.2
20		45.5	134.1	179.6
3	Aerobic[b]	321.5	29.8	351.3
10		26.8	45.9	72.7
20		102.0	ND[c]	102.0
3	Unaerated	149.2	243.6	392.8
10		0	0	0
20		28.0	100.2	128.2

[a] Average of three experiments. MM analysis was conducted by TLC.

[b] Broth supplemented with DL-homocysteine (0.25 μmol/ml).

[c] ND, Not determined.

Effect of methyl-cobalamine. Methyl-cobalamine (a methyl donor) was also added to the extent of 0.018 μmol/ml of GBSB containing 0.25 μmol of Hg^{2+} per ml, and both the control (no test culture) and experimental culture were then incubated under the unaerated system. The results (Table 3) showed the presence of MM in both uninoculated (control) and inoculated culture media after 3 days of incubation. The former contained 339 ng of MM per ml of media and the latter had 372 ng of MM per ml of media (33.5 ng in cell-free medium and 338.6 ng in cells/ml of media). When both DL-homocysteine and CH_3-B_{12} were present in GBSB containing Hg^{2+}, no MM was found in absence of the test culture after 3 days of incubation as detected by TLC. On the other hand, in the presence of the test culture, both the cells and cell-free medium contained MM.

DISCUSSION

The decreased effect of Hg^{2+} on S-strain of *E. aerogenes* in the presence of yeast extract may be due to complex formation between the Hg^{2+} and yeast extract. It is also possible that the resistance of this culture to Hg^{2+} may be mediated by some protective mechanism to retain more Hg^{2+} ions outside the cell. Many authors (24, 27, 31) noted a decrease of antibacterial activity of Hg^{2+} in the presence of amino acids, proteins, peptone, glutathione, and H_2S. Webb (38) stated that resistance of bacterial cells to Hg^{2+} was not due to a reduction of cell permeability to the mercurials. Benigno and Santi (3) observed that Hg^{2+}-tolerant *Staphylococci* grew when they had taken up much more Hg^{2+} than the amount required to inhibit the Hg^{2+}-S-strain. Kondo et al. (16) reported that resistance of *S. aureus* to Hg^{2+} may be due to a process of changing the Hg^{2+} incorporated in the cell into a somewhat innocuous form.

TABLE 3. *Effect of methyl-cobalamine on formation of MM by Hg^{2+}-R-E. aerogenes incubated under nonaerated conditions at 37 C in GBSB containing 0.25 μmol Hg^{2+} per ml supplemented with 0.018 μmol per ml*[a] *of methyl-cobalamine*

Incubation time (days)	MM (ng/ml)		
	Cell free	Cells	Culture
3	33.5	338.6	372.1
3 (control)[a]	339.0		339.0
3[b]	80.3	218.0	298.3
3 (control)[a] [b]	0.0		0.0

[a] In absence of culture. Analysis of MM was conducted by TLC.

[b] 0.25-μmol amount of DL-homocysteine per ml was included in the medium.

Data on the uptake of $^{203}Hg^{2+}$ by cells of R-*E. aerogenes* showed that the movement of $^{203}Hg^{2+}$ from GBSB into cells was a function of both time and growth conditions. In absence of yeast extract, $^{203}Hg^{2+}$ tracer moved almost immediately (4 h) into cells (90%) and this value remained constant throughout growth, indicating either adsorption of the Hg^{2+} onto the surface of the cell, uptake of tracer by the cell, or both. On the other hand, the decrease in uptake of $^{203}Hg^{2+}$ by cells in presence of yeast extract was apparently due to complexing of mercury with constituents of yeast extract and thus retaining Hg^{2+} to some degree outside the cell.

When cells grown in presence of yeast extract were subjected to fractionation, high levels of $^{203}Hg^{2+}$ were noted in the cold and hot trichloroacetic acid-soluble, but not in the insoluble, protein fractions, especially after 3 to 5 h of incubation. The cold trichloroacetic acid fraction represents transient intermediates and inorganic cations, whereas the hot trichloroacetic acid fraction contains nucleic acids. The insoluble-protein fraction of cells grown in absence of yeast extract contained the greatest level of tracer (90 to 98%) and a low concentration in all other fractions, indicating that most of the $^{203}Hg^{2+}$ incorporated in the cells was bound to protein and became less available for methylation. No tracer was found in the alcohol-ether-soluble fractions (which contained lipid) of cells grown in the presence of yeast extract until 9 to 11 h of incubation, when the level reached

0.3 and 2.3%, respectively. This may be due to the presence of a "pool" of organic $^{203}Hg^{2+}$-containing metabolites stored in the lipid material of the cell. The steady loss of activity in the lipid-soluble fraction of cells grown in absence of yeast extract, coupled with the increase in benzene-soluble $^{203}Hg^{2+}$-metabolites in culture media, suggest the possibility of their movement from the lipid to the culture media.

More MM was produced under submerged aerated culture than in absence of aeration and this was cyclic in nature. Spangler et al. (26) reported that MM production by a mixed culture in Hg^{2+}-containing sediment was cyclic and suggested that the MM was degraded through bacterial action to methane and Hg^0 which was then volatilized. However, no volatilization of $^{203}Hg^{2+}$ was detected in this investigation. Wallace et al. (37) stated that methylation of Hg^{2+} might be carried out more efficiently under aerobic systems, thus confirming the results of Rissanen et al. (22).

Landner (17) showed that addition of DL-homocysteine increased MM production by *Neurospora crassa*. In the present investigation no significant increase in level of MM was noted in presence of DL-homocysteine. Chemical formation of MM was reported by Imura et al. (8), and was also confirmed in our studies, in presence of methyl-cobalamine (CH_3-B_{12}). Upon addition of DL-homocysteine to the CH_3-B_{12}-Hg^{2+} system, no MM was found in absence of test culture. However, in presence of test culture and DL-homocysteine, the concentration of MM decreased. It is postulated that the resistance of the test culture to Hg^{2+} may be due, in part, to detoxification of Hg^{2+} by methylation. Landner (16) suggested that the formation of MM by *N. crassa* may be due to incorrect synthesis of methionine. Wood et al. (39) presented several possible schemes for the microbiological methylation of mercury, one of which involves the use of the cobalamine-dependent methionine synthetase enzyme. Wood et al. (39) further suggested that CH_3-B_{12} binds to the methionine synthetase apoenzyme and DL-homocysteine to give the active enzyme-substrate complex, and that both aerobes and facultative conditions which use the cobalamine-dependent methionine synthetase enzyme are capable of synthesizing MM. It is possible that the MM which is formed in the cell removes the substrate homocysteine from the active site of the enzyme. This could explain why MM produced by the test culture was depressed in presence of Hg^{2+} and DL-homocysteine. If a Hg^{2+}-homocysteine complex existed in the media and was incorporated into the bacterial cell, this complex might not be able to become attached to the active enzyme site as would free homocysteine. It is suggested that mercury which enters the biosynthetic pathway of methionine is methylated by CH_3-B_{12}. Other mercury that does not happen to enter this pathway is probably attached at other enzyme and protein sites leading to a decrease in enzymatic activity of the microorganism at critical sites.

It is of interest to point out that, after 48-h incubation, growth was observed around edges of plates where a high level of Hg^{2+}/unit area was located. Repeated streaking of forward cells detected in an area of low Hg^{2+} to areas of higher Hg^{2+} concentration on the same plate resulted in exceptionally good growth. GBSB plates containing Hg^{2+} stored for 48 h before use also showed exceptionally good growth when used for screening of resistant cultures when compared with freshly prepared Hg^{2+}-agar plates.

The use of the concentration gradient plate technique provides a relatively simple method for selection and/or adaptation of bacteria to Hg^{2+}. However, in the presence of Hg^{2+}, it was very important that the plates be used immediately after their preparation. It became apparent that the reason for good growth of the test culture around the edge of the plastic petri dish, the active growth after restreaking the area of no previous growth, as well as the luxurient growth on 48-h-old plates were due to the decrease in concentration of Hg^{2+} in these areas of the plate through binding of the Hg^{2+} to the walls of the plate (19).

There were large differences noted in concentrations of MM produced under a given set of experimental conditions after analyses by TLC and GLC. The level of MM detected by TLC was higher than noted with GLC. This may be due to the inherent character of the TLC method and the possible decomposition of MM during GLC. Baughman et al. (2) reported that ionic MM compounds undergo decomposition during GLC analysis and that the rate of decomposition increased as the sample size decreased. Another reason for the low concentration of MM detected by GLC could be due to the adsorption of $^{203}Hg^{2+}$-containing compounds to the walls of the column used in the chromatograph (O. R. Noyes, Ph.D. thesis, Univ. of Georgia, Athens, 1974). The response of the GLC to known concentrations of standard MM and ethyl mercury changed frequently (from day to day) perhaps due to poisoning of the tritiated detector by methyl mercury or other compounds found in samples injected for analyses.

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