JOURNAL OF BACTERIOLOGY, July 1974, p. 242-249
Copyright © 1974 American Society for Microbiology
Vol. 119, No. 1
Printed in U.S.A.

Cell-Free Mercury(II)-Reducing Activity in a Plasmid-Bearing Strain of *Escherichia coli*

ANNE O. SUMMERS[1] AND LAURENCE I. SUGARMAN

Program in Molecular and Cellular Biology, Washington University, St. Louis, Missouri 63130

Received for publication 21 January 1974

The ability to reduce Hg(II) to Hg(0), which is determined by a plasmid-borne gene in *Escherichia coli*, is conferred by a Hg(II)-inducible activity which is located in the cytoplasm rather than in the periplasmic space of the cell. This Hg(II)-reducing activity can be isolated from the supernatant of a 160,000 × *g* centrifugation after French Press disruption of the cells. The activity is dependent on glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and 2-mercaptoethanol, but is not enhanced by added nicotinamide adenine dinucleotide phosphate. Treatment of the active fraction with *N*-ethylmaleimide causes irreversible loss of the Hg(II)-reducing activity. Unlike the Hg(II)-reducing activity found in intact cells, the cell-free activity is not inhibited by toluene, potassium cyanide, or *m*-chlorocarbonylcyanide-phenylhydrazone; however, it is inhibited by Ag(I) and phenylmercuric acetate to the same extent as the activity in intact cells. Neither phenylmercuric acetate nor methylmercuric chloride is reduced to Hg(0) by the cell-free activity. Au(III), however, is a substrate for the cell-free activity; it is reduced to metallic colloidal Au(0).

Two modes of interaction of bacteria with mercury-containing compounds have been the subject of several recent studies (1-3, 6, 7, 13-17). The ability to methylate Hg(II) is found in several anaerobic species, including *Methanobacter* (15) and *Clostridia* (16, 17). These bacteria effect a non-enzymatic transfer of a methyl group from methylcobalamine to Hg(II). On the other hand, several aerobic or facultative species have been shown to reduce inorganic and organic mercury compounds to metallic mercury via a mechanism which is inducible by mercuric or mercurial compounds. Bacteria using this mechanism to eliminate mercury from their environment include *Escherichia coli* (6, 7, 13, 14), *Staphylococcus aureus* (13), and several species of *Pseudomonas* (1-3, 13). In the *E. coli* and *S. aureus* strains and in some *Pseudomonas* strains, the genes determining the ability to reduce Hg(II) have been shown to map on plasmids or resistance factors (R factors).

This report describes a cell-free Hg(II)-reducing activity from a Hg(II)-resistant R factor-carrying strain of *E. coli*.

MATERIALS AND METHODS

Bacterial strains. *E. coli* AB1932-1(JJ1), a K-12 strain carrying the mercury-resistant plasmid, has been described (5, 14). *E. coli* K10C4 is a K-12 Hfr(Cavalli) prototroph used in this laboratory. It does not carry known plasmids, is *lac*$^+$, will not grow in medium containing 10 μM $HgCl_2$, and is constitutive for the synthesis of alkaline phosphatase.

Growth conditions. Cells were routinely grown in broth containing per liter 16 g of Difco tryptone and 5 g of NaCl. For the induction of Hg(II)-reducing activity in AB1932-1(JJ1), this medium was made 10 μM $HgCl_2$ when the culture was started and again 30 min before harvesting. In all of the experiments described below, the cells were used in the late exponential phase of growth (i.e., after 3 to 3.5 h of growth with aeration at 37 C after a 1/100 dilution of a stationary phase overnight culture).

Preparation of the spheroplasts. One liter each of Hg(II)-induced AB1932-1(JJ1) and K10C4, induced for beta-galactosidase synthesis with 0.1 mM isopropylthiogalactoside, in late exponential phase were mixed and harvested by centrifugation. The cells were suspended in 10 ml of cold (4 C) 50 mM sodium phosphate, pH 7.4. A 5-ml portion of the cell suspension was passed through a French Press at 10,000 to 12,000 lbs/in^2; this material served as a control for maximum activity for all of the enzymes assayed. The remaining 5 ml was diluted to 40 ml with cold 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 8.1, and washed twice with this buffer. These cells were then suspended in 80 ml of 20% sucrose in 0.03 M Tris-hydrochloride, pH 8.1, at 24 C, stirred gnetly, and made 1 mM in ethylenediaminetetraacetate (EDTA), pH 7.0. Lysozyme was then added to a concentration of 20 μg/ml, and gentle stirring was then centrifuged at 5,000 rpm in a Sorvall SS34 rotor

[1] Present address: Department of Microbiology, University of Virginia. School of Medicine, Charlottesville, Va. 22901

at 4 C for 10 min. The supernatant fluid of the spheroplasts was removed, and the intact spheroplasts were suspended in 5.0 ml of 20% sucrose in 0.03 M Tris-hydrochloride, pH 8.1. A 2-ml portion of this spheroplast suspension was added to 8.0 ml of cold deionized water. These ruptured spheroplasts were separated into supernatant and particulate fractions by centrifugation in a Sorvall SS34 rotor at 20,000 rpm for 30 min at 4 C.

Preparation of the cell-free activity. Hg(II)-induced AB1932-1(JJ1) cells in late exponential phase were harvested by centrifugation, suspended in 1/200 volume 50 mM sodium phosphate buffer, pH 7.4, containing 1 mM 2-mercaptoethanol at 4 C, and disrupted in a French Press at 10,000 to 12,000 lbs/in^2, 4 C. The disrupted cells were centrifuged at 45,000 rpm for 90 min in a Beckman SW50 rotor at 8 C. The supernatant of this centrifugation (S160) was made 0.1 mM EDTA and 0.2 mM $MgCl_2$ and was incubated with stirring at 37 C with ribonuclease and deoxyribonuclease, each at 10 μg/ml. The nuclease-treated S160 was then concentrated to 1/8 volume by vacuum dialysis against 500 volumes of 50 mM sodium phosphate, pH 7.4, containing 2 mM 2-mercaptoethanol.

Enzyme assays. Mercury reduction was assayed by following the loss of ^{203}Hg from a medium containing 50 mM sodium phosphate buffer, pH 7.4, 5 mM nicotinamide adenine dinucleotide phosphate ($NADP^+$), 25 mM glucose-6-phosphate, 1 unit of glucose-6-phosphate dehydrogenase (EC 1.1.1.49), 1 mM 2-mercaptoethanol, 0.5 mM EDTA, 0.2 mM $MgCl_2$, 10 μM $^{203}HgCl_2$, and 0.005 ml of cell-free extract (unless otherwise noted) in a total volume of 0.5 ml. All determinations were done in duplicate; duplicates differed on the average by not more than 10%. The assay tubes were not sealed but loosely covered, and were shaken at 300 rpm in a New Brunswick gyrotory water bath at 37 C during the assay. One unit of activity represents the reduction of 0.5 nmol of Hg(II) per min at 37 C.

Alkaline phosphatase (EC 3.1.3.1) was assayed by following nitrophenyl phosphate hydrolysis as measured by the increase in absorbance at 410 nm upon the addition of 0.01 to 0.2 ml of the cell fraction to 3.0 ml of 1.0 M Tris-hydrochloride, pH 8.1, containing 0.2 mg of nitrophenyl phosphate per ml at 24 C. One unit of activity represents a change of 1.0 unit of absorbance at 410 nm per min at 24 C.

Beta-galactosidase (EC 3.2.1.23) was assayed by following the hydrolysis of *o*-nitrophenylgalactoside as measured by the increase in absorbance at 410 nm upon the addition of 0.1 to 0.5 ml of cell fraction to 3.0 ml of medium containing 1.4 mg of *o*-nitrophenylgalactoside per ml, 1 mM $MgCl_2$, 0.1 mM 2-mercaptoethanol, and 67 mM sodium phosphate buffer, pH 7.4, at 24 C. One unit of activity represents a change of 1.0 unit of absorbance at 410 nm per min at 24 C.

Both spectrophotometric assays were done with a Beckman DB spectrophotometer equipped with a recorder.

Protein determinations. Protein was determined by the method of Lowry et al. (8).

Reagents. $^{203}Hg(NO_3)_2$, ^{203}Hg-methyl mercuric chloride, and ^{203}Hg-phenyl mercuric acetate were purchased from New England Nuclear Corp., Waltham, Mass. All other chemicals and biochemicals were purchased from Sigma Chemical Co. or Fisher Scientific Co., St. Louis, Mo., and were of the highest purity available.

RESULTS

Cellular location of the Hg(II)-reducing activity. It has been reported that in enteric bacteria, the R factor-determined penicillinases (10) and the streptomycin-adenylating enzyme are located in the periplasmic space and that the R factor-determined chloramphenicol acetyl transferase is a cytoplasmic enzyme (12). To determine whether the Hg(II)-reducing activity is present in the cytoplasm, the periplasm, or both, we examined the distribution of Hg(II)-reducing activity in induced resistant cells that were converted to spheroplasts and then lysed. For these experiments Hg(II)-induced AB1932-1(JJ1) cells were mixed 1:1 with K10C4 cells that are constitutive for the synthesis of alkaline phosphatase, an enzyme known to be located in the periplasmic space (11), and that had been induced with isopropylthiogalactoside for the synthesis of beta-galactosidase, a cytoplasmic enzyme (11). To correlate the mercury-reducing activity with either the cytoplasmic or periplasmic space (as defined by the distribution of the K10C4 enzymes) we assumed that these two strains of *E. coli* K-12 were similarly sensitive to spheroplasting.

All of the Hg(II)-reducing activity was present in the intact spheroplasts rather than in their supernatant fluid, whereas all of the alkaline phosphatase was released into the supernatant fluid of the spheroplasts (Table 1). Because alkaline phosphatase is one of the

TABLE 1. *Distribution of enzymatic activities in various subcellular fractions*

Fraction	Hg(II)-reducing activity (total units)	Alkaline phosphatase (total units)	Beta-galactosidase (total units)
Untreated intact cells	1,130	50	90
Press disrupted cells	1,140	101	2,350
Supernatant of press-disrupted cells	1,590	95	2,150
Tris-washed cells	1,100	104	70
Intact spheroplasts	1,980	23	1,300
Supernatant of intact spheroplasts	60	108	37
Lysed spheroplasts	1,780	10	1,800
Pellet of lysed spheroplasts	180	12	350
Supernatant of lysed spheroplasts	1,640	15	1,650

periplasmic enzymes, this strongly suggests that the Hg(II)-reducing activity is not located in the periplasmic space. When the intact spheroplasts were lysed by osmotic shock, all of the Hg(II)-reducing activity and most of the beta-galactosidase activity were found in the supernatant fluid rather than in the particulate fraction. This suggests that the Hg(II)-reducing activity is, like beta-galactosidase, a cytoplasmic enzyme, although it does not rule out the possibility that this activity is loosely associated with the inner or cytoplasmic membrane. Similar results for the distribution of these enzymatic activities are obtained when the cells are subjected to osmotic shock according to the procedure of Nossal and Heppel (11, data not shown).

Purification of the cell-free activity. The ability of the cell-free preparations to reduce Hg(II) to Hg(0) is, like the activity found in whole cells, unique to the resistant cells which have been grown in the presence of $HgCl_2$ (i.e., induced resistant cells). The supernatants of French Press-disrupted cells show the following activity when assayed in complete medium as described in Materials and Methods: sensitive, 1.9 nmol of Hg(II) reduced per min per ml; uninduced resistant, 7.3 nmol of Hg(II) reduced per min per ml; and induced resistant, 218.0 nmol of Hg(II) reduced per min per ml.

The purification procedure described in the Materials and Methods section results in recovery of 100% of the activity found in intact cells and an approximately threefold purification (Table 2). This isolation method differs from that described for a Hg(II)-reducing activity from *E. coli* that was used by Komura et al. (7) and from that for a Hg(II)- and organomercurial-reducing activity from a *Pseudomonas* species which was used by Furukawa and Tonomura (1-3). We found that we could only recover 20 to 30% of the activity after ammonium sulfate fractionation, as described by both of the above groups. This was true even though the assay medium contained all of the cofactors which these other systems have been shown to require, including reduced nicotinamide adenine dinucleotide phosphate (NADPH), flavine adenine dinucleotide (FAD), and cytochrome *c*. For this reason we chose to use the more gentle method of vacuum dialysis to concentrate the nuclease-treated high-speed supernatant fluid of disrupted cells. (High-speed centrifugation and nuclease treatment remove particulate debris and very high molecular weight material that would gradually block the pores of the dialysis bag and make the procedure unreasonably lengthy.) Because proteins of lower molecular weight are free to pass through the distended pores of the dialysis bag during vacuum dialysis, this step results in a purification as well as concentration of the activity. Further purification-of the activity by gel filtration and ion-exchange chromatography is in progress.

TABLE 2. *Purification of the Hg(II)-reducing activity*

Stage	Total protein (mg)	Total units	Units/mg
Intact cells	372	7360	19.8
Press-disrupted cells	350	5340	15.3
S160	236	7690	32.5
Vacuum-dialyzed S160	125	7560	60.5

Requirements for the activity of the vacuum-dialyzed S160. Only glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and 2-mercaptoethanol are necessary for full activity in the cell-free system (Table 3 and Fig. 1). Neither $NADP^+$, FAD, nor cytochrome *c* enhance the activity at this stage of purification. As noted above, these reagents were examined because the Hg(II)-reducing activity in another strain of *E. coli* has been shown to require NADPH (7) and a Hg(II)- and mercurial-reducing activity in a *Pseudomonas* has been shown to require NADPH, FAD, and a *c*-type cytochrome (1-3). The vacuum-dialyzed S160, which has been boiled for 3 min, is not active even with complete assay medium, as described

TABLE 3. *Requirements for activity in the vacuum-dialyzed S160*

Assay medium[a]					Activity (units/ml[b])
2-Mercaptoethanol	Glucose-6-phosphate dehydrogenase	NADP	Cytochrome *c*	FAD	
−	−	−	−	−	89
+	−	−	−	−	47
+	+	−	−	−	592
+	+	+	−	−	522
+	+	+	+	−	480
+	+	+	+	+	490

[a] In addition to the listed reagents, all assay media included 50 mM sodium phosphate, pH 7.4, 0.5 mM EDTA, 25 mM glucose-6-phosphate, and 0.2 mM $MgCl_2$. The listed reagents were added in the following concentrations: 2-mercaptoethanol, 1 mM; glucose-6-phosphate dehydrogenase, 1 unit/ml; NADP, 5 mM; cytochrome *c*, 1 μM; and FAD, 1 μM.

[b] One unit equals reduction of 0.5 nmol of Hg(II) per min at 37 C.

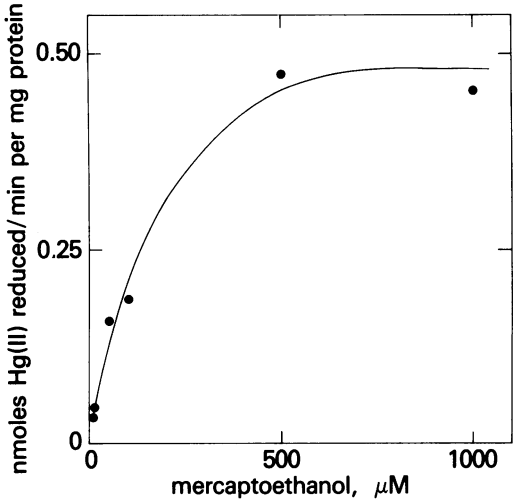

FIG. 1. *Dependence of the activity on 2-mercaptoethanol. The vacuum-dialyzed S160 was diluted 1/1200 into 0.5 ml of assay medium, supplemented with FAD and cytochrome c, each at 1 μM, and containing varying concentrations of 2-mercaptoethanol. Hg(II) reduction was measured as described in Materials and Methods.*

in the Materials and Methods section. The lack of stimulation by $NADP^+$ of the Hg(II)-reducing activity is surprising and may be due either to some low-level contamination of the assay reagents (possibly of the glucose-6-phosphate dehydrogenase preparation) with $NADP^+$ or to a bound pyridine nucleotide or other reducible cofactor in the S160. The identity of the native cofactor in this Hg(II)-reducing activity remains to be established.

There is a clear requirement for a sulfhydryl compound as shown in Fig. 1. Approximately 10% of the activity remains when 2-mercaptoethanol is present at the same molarity as the substrate $HgCl_2$ (10 μM). Increasing the 2-mercaptoethanol to 50 μM restores only about 30% of the maximum activity assayable with 0.5 mM 2-mercaptoethanol.

Incubation of the vacuum dialyzed S160 at pH 8.1 for 15 min at 24 C in the same series of 2-mercaptoethanol concentrations as in Fig. 1 followed by a 100-fold dilution into assay medium, complete except for 2-mercaptoethanol, resulted in a retention of only 5 to 20% of the activity (data not shown). There are two possible explanations of this requirement for a sulfhydryl compound: either there is a very rapidly oxidizable sulfhydryl group on a protein that is involved in the reducing activity; or it may be that the real substrate in the reaction is mercurated 2-mercaptoethanol. This latter possibility must be considered because in the 100-fold molar excess of 2-mercaptoethanol in which the assay is done, essentially no free Hg^{2+} cation will be present; unreduced mercury in this assay medium will be present almost entirely as the mercaptide derivative.

The two possibilities for the role of a sulfhydryl compound are not mutually exclusive. To test the first possibility we asked whether there was a protein-bound sulfhydryl group necessary for the activity in the vacuum-dialyzed S160. The vacuum-dialyzed S160 was thoroughly reduced with an excess of 2-mercaptoethanol at pH 8.1, and then the sulfhydryl groups present were exposed to the alkylating reagent, *N*-ethylmaleimide (NEM). The data in Table 4 show that treatment with sufficient NEM to saturate completely the sulfhydryl groups present destroys the activity. Incubation with 2-mercaptoethanol at pH 8.1 followed by exposure to a less-than-saturating concentration of NEM caused no loss of activity at all. We therefore believe that there is a protein-bound sulfhydryl group that is subject to alkylation by NEM and that is essential for the activity. The possibility that 2-mercaptoethanol is also required to form a Hg-mercaptide which is the recognized substrate remains to be tested.

Inhibitors of the cell-free activity. Several of the inhibitors which had been used to exam-

TABLE 4. *Effect of N-ethylmaleimide on the activity of the vacuum-dialyzed S160*

Preparation[a]	NEM treatment[b]	Activity (units/ml)
A	None	2,520
	0.5 mM NEM	2,570
	2.0 mM NEM	121
B	None	3,850
	0.5 mM NEM	4,000
	2.0 mM NEM	33

[a] Preparations A and B were both prepared as described in Materials and Methods. Preparation A had been stored at 4 C for 1 week when the above experiment was done; preparation B was freshly prepared.

[b] The vacuum-dialyzed S160 was diluted 1:1 with 2 mM 2-mercaptoethanol in 0.1 M Tris-hydrochloride, pH 8.1, and incubated at 24 C for 10 min. A 20-μliter portion of this reduced vacuum-dialyzed S160 was then added to 0.1 ml of either 0.5 mM or 2.0 mM NEM in 50 mM sodium phosphate buffer, pH 7.0, containing 0.75 mM 2-mercaptoethanol, and incubated at 24 C for 15 min. A 5-μliter portion of this NEM-treated vacuum-dialyzed S160 was then diluted into 0.5 ml of assay medium supplemented with FAD and cytochrome *c*, each at 1 μM, and assayed for Hg(II)-reducing activity as described in Materials and Methods.

ine this activity in whole cells (14) were examined for their effect on the cell-free activity (Table 5). The inhibitor of the terminal oxidation of the electron transport chain, KCN, and the uncoupler of oxidative phosphorylation, *m*-chlorocarbonylcyanide-phenylhydrazone, do not inhibit the activity of the S160 fraction at the same levels which cause inhibition of the activity in whole cells. This suggests that there is some energy-dependent process necessary for Hg(II)-reducing activity in whole cells which is bypassed in the cell-free system.

The data on toluene inhibition of the activity in whole cells has been variable. Initial experiments showed clearly that exposure to 1% toluene (vol/vol) at 37 C did not affect the cells' ability to reduce successive additions of 10 μM $HgCl_2$ (14). Subsequent experiments showed a loss of 90% of the Hg(II)-reducing activity in whole cells after 8 min of exposure to 1% toluene at 37 C. However, it is clear from the data in Table 5 that exposure to toluene for approximately 8 min has no effect on the activity of the S160.

Both $AgNO_3$ and phenylmercuric acetate inhibit the activity of the S160 fraction to the same extent that they inhibit the activity in whole cells. We therefore examined more thoroughly the interaction of the cell-free activity with organomercurials with Ag(I) and with Au(III), which had also been shown to inhibit the activity in whole cells (14).

TABLE 5. *Effect of inhibitors on the S160*[a]

Inhibitor[b]	Units/ml[c]
None	342
KCN, 10 mM	455
m-Chlorocarbonylcyanide-phenylhydrazone, 0.1 mM	377
Toluene, 1%	380
$AgNO_3$, 0.1 mM	34
Phenylmercuric acetate, 0.1 mM	214

[a] The supernatant of a 160,000 $\times$ *g* for 90-min centrifugation of French Press-disrupted induced resistant cells.

[b] The assay medium consisted of: 10 mM sodium phosphate buffer (pH 7.3); 0.5 mM EDTA; 0.2 mM $MgCl_2$; 1 mM 2-mercaptoethanol; 5 mM $NADP^+$; 25 mM glucose-6-phosphate; 1 unit of glucose-6-phosphate dehydrogenase per ml; 1.0 μM FAD; 1.0 μM cytochrome *c*.

[c] A 5.0-μliter portion of the indicated material was added to duplicate 0.5-ml samples of the indicated medium containing the inhibitor at the indicated concentration at 4 C. These tubes were then warmed at 37 C for 5 min, and at time zero 10 μliters of 0.5 mM $^{203}HgCl_2$ was added for a final concentration of 5.0 nmol of Hg(II)/0.5 ml. Twenty-five-microliter samples were taken at 2.5, 7.5, and 16.5 min of incubation at 37 C (300 rpm agitation). The initial rate of mercury reduction in terms of percent of the total mercury lost per min per 5.0 μliters of material assayed is converted to units (1.0 unit equals 0.5 nmol of Hg(II) reduced per min) per milliliter of material assayed by multiplying by 20.

Reaction with organomercurials. The mercury-reducing activity isolated by Furukawa and Tonomura from a *Pseudomonas* species will reduce not only Hg(II) but will also reduce various organomercurials, including methylmercuric chloride and phenylmercuric acetate, and release metallic mercury (1, 2). We had examined the ability of whole cells of AB1932-1(JJ1) to release metallic mercury from methylmercuric chloride and found that they did not have this ability (14). Therefore, by using both phenylmercuric acetate and methylmercuric chloride, labeled with ^{203}Hg, we asked whether the S160 fraction prepared from AB1932-1(JJ1) could release metallic mercury from these compounds. Under conditions where we observe typical Hg(II)-reducing activity in the S160 fraction, there is essentially no release of metallic mercury by this preparation from either of the organomercurials (Table 6). Therefore, neither of these compounds will serve as a substrate for the Hg(II)-reducing activity in AB1932-1(JJ1).

Reaction with Ag(I) and Au(III). Both Ag(I) and Au(III) salts markedly inhibited the Hg(II)-reducing activity in whole cells (14), and as shown above Ag(I) inhibits the cell-free activity (Table 5). We have also found that mercury-resistant cells will bind four to six times more radioactive Ag(I) (added as ^{110m}Ag)

TABLE 6. *Reaction of the S160 fraction with phenylmercuric acetate and methylmercuric chloride*[a]

Mercury compound	Units/ml[b]
Phenylmercuric acetate, 10 μM[c]	0.2
Methylmercuric chloride, 10 μM[d]	0.4
Mercuric chloride, 10 μM[e]	342.0

[a] The reaction mixture consisted of 10 mM sodium phosphate, pH 7.3; 1 mM 2-mercaptoethanol; 0.5 mM EDTA; 0.2 mM $MgCl_2$; 5 mM $NADP^+$; 25 mM glucose-6-phosphate; 1 unit of glucose-6-phosphate dehydrogenase per ml; 1.0 μM FAD; 1.0 μM cytochrome *c*; and 0.01 ml of the S160 in a total volume of 1.0 ml.

[b] One unit equals the reduction of 0.5 nmol of mercury/min at 37 C. Each determination was done in duplicate.

[c] Specific activity, 0.93 Ci/mol.

[d] Specific activity, 3.0 Ci/mol.

[e] Specific activity, 8.5 Ci/mol.

per cell than sensitive cells. In the course of attempting to isolate the ^{110m}Ag-binding component from induced resistant cells, we noted that the dark colloid of silver metal was found in the presence of induced resistant cells much more rapidly than in the presence of sensitive cells or by the action of light alone. We subsequently found that this was also true for the formation of the dark purple colloid of gold metal from Au(III). This suggested that Ag(I) and possibly Au(III) were not only inhibitors of the Hg(II)-reducing activity but substrates for the reduction as well. We have encountered considerable technical difficulty in measuring directly the reduction of Ag(I) or Au(III). The difficulty arises primarily from the fact that the high concentration of protein in the case of whole cells, and the requirement of 1 mM 2-mercaptoethanol for the activity of the cell-free system, prevent efficient and reproducible partition of ionic and reduced forms of the metals by solvent- or resin-extraction techniques.

Currently the best measure of the interaction of the crude extract with these metal cations is the increase in optical density at 600 nm which occurs as the dark purple colloid of metallic gold is formed from Au(III) in the presence of the vacuum-dialyzed S160 from induced AB1932-1(JJ1) (Fig. 2). The background reduction of Au(III) by the assay medium alone which contains high concentrations of biological reducing agents is rather large, but the observation of more rapid and more extensive colloid formation by the vacuum-dialyzed S160 is consistently observable. Similar experiments with $AgNO_3$ show a change from a milky white suspension to a brown precipitate in the same period of time in the presence of the vacuum-dialyzed S160. This suggests that both Au(III) and Ag(I) will serve as substrates for this Hg(II)-reducing activity.

We have not examined the ability of the cell-free extract of sensitive cells to effect the formation of these colloidal metals; therefore, we cannot assert that this is a property of the cell-free extract of the induced resistant cells alone and not simply a result of some nonspecific interaction of cell proteins, the assay reagents, and Ag(I) or Au(III) salts. We think it is very likely, however, because of the inability of sensitive cells to effect the formation of the colloidal metals, that this activity, like the Hg(II)-reducing activity, is unique to the cell-free extract of the induced resistant cells.

To test whether either Ag(I) or Au(III) might induce the Hg(II)-reducing activity in whole cells, AB1932-1(JJ1) was subcultured twice in

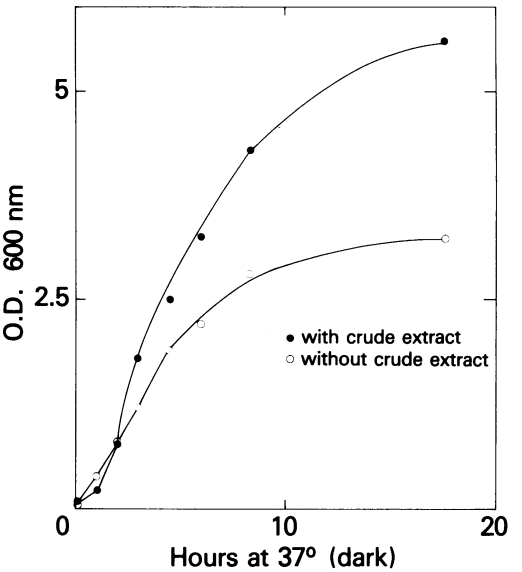

FIG. 2. *Formation of colloidal gold by the vacuum-dialyzed S160. A 30-ml portion of crude extract was added to 3 ml of assay medium as described in Materials and Methods which was supplemented with 1 μM FAD and 1 μM cytochrome c and which contained 50 μM $HAuCl_4$ rather than $HgCl_2$. The reaction mixtures in covered 3-ml cuvets were incubated in a light-proof box at 37 C and their optical density at 600 nm was read periodically against a blank containing complete medium without $HAuCl_4$ in a Beckman DB spectrophotometer. Dilutions of reactions with an optical density (600 nm) greater than 1.0 were made in deionized water. All manipulations were done in a dimly lit room.*

medium without $HgCl_2$ and then, in late exponential phase, exposed to either $HgCl_2$, $HAuCl_4$, or $AgNO_3$, each at 10 μM in fresh tryptone broth at 37 C. At various times up to 90 min, samples of the cells were treated with 1% toluene and assayed immediately for their ability to reduce Hg(II). At no time did the cells incubated with either $AgNO_3$ or $HAuCl_4$ develop any Hg(II)-reducing activity. Control cells incubated with $HgCl_2$ developed normal levels of Hg(II)-reducing activity after 15 min of exposure to $HgCl_2$. Therefore, only Hg(II) appears to be an inducer of the reducing activity.

DISCUSSION

In addition to the Hg(II)-reducing system we are studying, we have referred to two other bacterial mercury-reducing systems which are currently being examined in other laboratories. Komura and co-workers (6, 7) have described a Hg(II)-inducible R factor-determined Hg(II)-reducing activity in a strain of *E. coli*. The

cell-free activity prepared from this strain (28,000 × *g* supernatant of sonicated cells) requires NADPH and a sulfhydryl compound. Furukawa and Tonomura have described a mercury- and mercurial-reducing activity in a strain of *Pseudomonas* (1–3). Nothing is as yet known about the genetics of this latter system. Unlike the activity we have investigated in *E. coli*, this system is inducible by and will reduce both Hg(II) and organomercurials, such as phenylmercuric acetate and methylmercuric chloride. The cell-free activity from *Pseudomonas* requires NADPH, cytochrome *c*, and a sulfhydryl compound and has a bound FAD.

The substrate specificity is perhaps the most outstanding of the differences in these three systems. We are currently examining phenylmercuric acetate-resistant and methylmercuric chloride-resistant strains of *E. coli* for their ability to reduce either of these compounds to metallic mercury. We would like to resolve the question of whether or not there are two distinct mechanisms of detoxification of mercury-containing compounds in the enteric bacteria.

Mercury resistance occurs in 10 to 20% of the clinically isolated R factor-carrying strains of the enterics and in 20 to 30% of the resistance plasmid-carrying strains of the staphylococci (Frank Young, personal communication). The question of whether, and in what manner, these bacteria protect their hosts from heavy metals is an important one which is as yet unresolved. The existence of mercury detoxification systems in normal flora might provide a strategy for prophylaxis and/or treatment of humans and domestic animals accidentally or chronically exposed to high levels of mercury compounds.

The Hg(II)-reducing systems are interesting biochemically as well as ecologically. They constitute a new class of metabolic interaction with heavy metals. Such metals as cobalt, iron, nickel, molybdenum, and zinc have long been known to serve as co-catalysts in the activity of the metalloenzymes and the metal-ion activated enzymes. As catalysts they may be oxidized or reduced in a given reaction, but return to their original state when the reaction is completed. In addition to this widely found catalytic role of metals in metabolic processes, there is the narrower function of two metal cations, Fe(II) and Mn(II), which serve as oxidizable substrates in the metabolism of the chemoautotrophic bacteria, such as the *Thiobacilli* and *Metallogenium*. These bacteria use the energy derived from the oxidation of these metals from the 2+ to the 3+ state to drive CO_2 fixation and other metabolic processes. The mercury-reducing enzymes thus constitute a third class in which the metal ion substrate is reduced, and thereby detoxified, at the expense of metabolic energy.

The mercury-reducing enzymes in the enterics and staphylococci are plasmid determined, and, therefore, non-essential. They are, however, equipped with a Hg(II)-specific induction mechanism and are therefore more "sophisticated" in their control than the constitutively synthesized antibiotic modifying enzymes. Each of these enzymes (and its respective control system) may have been derived by selection from an enzyme which occurs naturally in the cell complement to perform a different but closely related (enzymatically) function. The Hg(II)-reducing activity described in this report will be further purified in order to examine questions of both ecological and biochemical importance.

ACKNOWLEDGMENTS

This work was submitted in partial fulfillment of the requirements for the Ph.D. degree by A.O.S., who gratefully acknowledges the guidance of Simon Silver and valuable discussions with Oscar Chilson and Roy Vagelos. The work was supported in part by U.S. Public Health Service grant AI08062 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. Furukawa, K., and K. Tonomura. 1971. Enzyme system involved in the decomposition of phenylmercuric acetate by mercury-resistant *Pseudomonas*. Agr. Biol. Chem. **35**:604–610.
2. Furukawa, K., and K. Tonomura. 1972. Induction of a metallic mercury releasing enzyme in mercury-resistant *Pseudomonas*. Agr. Biol. Chem. **36**:2441–2448.
3. Furukawa, K., and K. Tonomura. 1972. Metallic mercury releasing enzyme in mercury-resistant *Pseudomonas*. Agr. Biol. Chem. **36**:217–226.
4. Harwood, J. H., and D. H. Smith. 1969. Resistance factor-mediated streptomycin resistance. J. Bacteriol. **97**:1262–1271.
5. Harwood, J. H., and D. H. Smith. 1971. Catabolic repression of chloramphenicol acetyl transferase synthesis in *E. coli* K12. Biochem. Biophys. Res. Commun. **42**:57–62.
6. Komura, I., and K. Izaki. 1971. Mechanism of mercuric chloride resistance in microorganisms. I. Vaporization of a mercury compound from mercuric chloride by multiple drug resistant strains of *Escherichia coli*. J. Biochem. (Tokyo) **70**:885–894.
7. Komura, I., T. Funaba, and K. Izaki. 1971. Mechanism of mercuric chloride resistance in microorganisms. II. NADPH-dependent reduction of mercuric chloride by a multiple drug resistant strain of *Escherichia coli*. J. Biochem. (Tokyo) **70**:895–901.
8. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
9. Magos, L., A. A. Tuffery, and T. W. Clarkson. 1964. Volitalization of mercury by bacteria. Brit. J. Ind. Med. **21**:294–298.
10. Neu, H. C., and E. B. Winshell. 1970. Purification and

characterization of penicillinases from *Salmonella typhimurium* and *Escherichia coli*. Arch. Biochem. Biophys. **139**:279-290.
11. Nossal, N. G., and L. A. Heppel. 1966. The release of enzymes by osmotic shock from *Escherichia coli* in exponential phase. J. Biol. Chem. **241**:3055-3062.
12. Shaw, W. 1971. Comparative enzymology of chloramphenicol resistance. Ann. N.Y. Acad. Sci. **182**:234-242.
13. Summers, A. O., and E. Lewis. 1973. Volitalization of mercuric chloride by mercury-resistant plasmid-bearing strains of *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. J. Bacteriol. **113**:1070-1072.
14. Summers, A. O., and S. Silver. 1972. Mercury resistance in a plasmid-bearing strain of *Escherichia coli*. J. Bacteriol. **112**:1228-1236.
15. Wood, J. M., F. S. Kennedy, and C. G. Rosen. 1968. Synthesis of methyl-mercury compounds by extracts of a methanogenic bacterium. Nature (London) **220**:173-174.
16. Yamada, M., and K. Tonomura. 1972. Formation of methylmercury compounds from inorganic mercury by *Clostridium cochlearium*. J. Ferment. Technol. **50**:159-166.
17. Yamada, M., and K. Tonomura. 1972. Further studies of formation of methyl-mercury from inorganic mercury by *Clostridium cochlearium* T-2. J. Ferment. Technol. **50**:893-900.