Biological Mercury Reduction in the Environment and Its Policy Implications for Metals Regulations Based on Speciation

by

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ABSTRACT

Most current U.S. mercury regulations are based on total mercury rather than individual mercury species. Regulations which specify individual species would be more efficient and effective since certain species are more toxic, more easily bioaccumulate and biomagnify, and are more (or less) able to be reduced. Even without regulation of individual species, improved data on fluxes of these substances in the global mercury cycle can lead to improved regulations. For example, regulations which are based upon a global mercury cycling model which includes *mer* operon (a gene system which produces mercuric reductase)-based reduction rates over-predicts aquatic evasion fluxes. The consequence is an over-prediction of the tolerable levels of emissions.

Laboratory experiments were performed to examine reduction rates and mechanisms at more environmentally relevant mercury concentrations (low nanomolar levels). The results indicated that the *mer* operon system was not responsible for mercury reduction in the environment since it was only induced at mercury concentrations greater than 1.5 nM. Environmental reduction rates are thus lower than would be predicted using *mer* operon-based rates of reduction. These rates of reduction should be incorporated into mercury cycling models

upon which mercury regulations are based in order to improve the accuracy of the models and the effectiveness of the regulations.

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1. Introduction

1.1 Science Overview

Mercury is a highly toxic metal which is globally cycled via both natural and anthropogenic mechanisms. Although harmful effects of mercury exposure have long been recognized, an episode of mercury poisoning in Minimata, Japan, in the 1950's focused world attention on the environmental mercury problem. This incident was caused by the release of toxic methyl mercury chloride into Minimata Bay by a chemical plant as part of its normal discharge. The methyl mercury bioaccumulated in the fish and shellfish of the bay, resulting in disease and death among the neighboring population that relied upon the fish as a source of food (D'Itri, 1972).

Since that time, there have been numerous research studies on the toxicity, bioaccumulation, chemistry, and global cycling of mercury, but much still remains unknown. For example, the biogeochemical cycling of mercury, through which the metal circulates in the atmosphere, hydrosphere, lithosphere (the earth's crust), and biosphere (living organisms), has been studied extensively, but the exact mechanisms for some of the transformations which play a major role in its cycling are poorly understood. One of the foci of research on these transformations is the biological component. It is generally accepted that in anoxic environments bacteria play the principal role in transforming mercury to methyl mercury (Compeau and Bartha, 1984, 1985), the form in which it is bioaccumulated (although it is unclear whether there is a similar mechanism in aerobic environments), and a possible role for bacteria in the volatilization of mercury from aquatic systems has also been examined (Barkay, et al., 1989; Mason, et al., 1993). More information is needed in both

cases to satisfactorily elucidate the global biogeochemical cycle.

1.2 Policy Overview

Despite this scientific uncertainty, it is clear that mercury is extremely toxic and must be regulated as a hazardous substance. In the past, mercury has been strictly regulated, using a conservative approach to set emissions limits. The current focus on the effects of environmental regulations on the United States' ability to compete in a global economy is bringing about a re-examination of these regulations. There is increasing concern that some regulations could be more "efficient," i.e., that they could maintain or provide better environmental improvements for less or the same investment. Often, a conservative regulatory approach has been adopted due to the large scientific uncertainty surrounding the environmental transport and impacts of a particular substance. If all of the effects were known there still must be a political decision on the level of deleterious effect that is acceptable to society in exchange for the benefits derived from its use. Once that decision had been made, if there was scientific certainty on the fate and effects of the substance, the regulations could be written more efficiently since the regulators would know exactly what amount of emissions would result in that "acceptable" effect. Additional scientific knowledge can also help in making regulations more geographically specific and appropriate. That is, since the environmental conditions very greatly from location to location, regulations which are designed to meet the needs of the most sensitive area may be overly stringent for other locations, in which case the ability for regulators to design location-specific requirements could also improve the efficiency of the regulatory system.

In the area of metals regulation, this concern for optimizing regulatory efficiency has prompted an interest in examining the actual bioavailability of particular metals species. Although metals can exist in various redox states and/or complex with organic or inorganic compounds, for the most part (organic lead and chromium species excepted) metals are regulated on a total basis. Since certain metals species are more hazardous than others, one current suggestion is to regulate based on the risk posed by individual species or complexes rather than on a total basis. This concept will be examined in greater detail below. Unfortunately, in many cases there are insufficient scientific data available on which to base such regulations. For mercury regulation, additional knowledge concerning its cycling and bioavailability may give regulators the ability to establish better and more efficient regulations.

1.3 Thesis Organization

Some general background information on mercury in the environment is presented first. Then, following a discussion of the global mercury cycle, toxicity, and policy issues, the mechanisms of mercury volatilization via chemical reduction are examined in more depth. Next, there is a description of laboratory experiments which were performed to analyze the hypothesis that one particular biological reduction mechanism is the mechanism which plays the major role in global mercury evasion from surface waters. Following a discussion of the analytical results, there is a section on the policy implications of those results, concluding with policy recommendations.

2. Background

2.1 Mercury in the Environment

Mercury has been used by humans since ancient times in a wide variety of applications. Mercury compounds were used as

drugs in Greece by Hippocrates in about 400 B.C. (Bidstrup, 1964). It has long been used in extracting gold and silver from their ores by an amalgamation process (Bidstrup, 1964). In addition to medical uses, including its use in biocides, mercury has been used in batteries and alkaline energy cells, a variety of lamps (including fluorescent), industrial control instruments (mercury switches, relays, gauges, pump seals, and valves), general laboratory use (diffusion pumps, barometers, manometers, McLeod gauges, thermometers and vibration dampers), mercury cathode cells in the chlor-alkali industry, organomercurials in the paint industry, dental preparations, amalgamation (except for iron and platinum, most metals can be amalgamated with mercury), preserving wood, etching metals, tanning leather, iron and steel works, and as general catalysts, slimicides in the paper and pulp industry, and preservatives in various consumer and industrial products. Although these applications have been useful, an unfortunate end-result is that mercury has been released in association with these activities via discharge and/or accidental release. Mercury is also emitted during fossil fuel combustion since coal, peat, crude oil, and wood all contain mercury (D'Itri, 1972). Especially dangerous organomercurial compounds have been discharged directly in effluents from manufacturing processes (as in Minamata, Japan), in runoff following the use of agricultural mercury-containing chemicals, and from improper use of disinfectants and fungicides (Takeuchi, 1972).

The total mass of mercury in the atmosphere is about 6000 metric tons (Douglas, 1994). Annual global emissions are also about 6000 metric tons; more than half of these emissions arise from human activities. Fossil fuel combustion for non-utility industry applications contributes 1200 metric tons annually. Diffuse sources, such as paint volatilization, manufacturing processes, and disposal of batteries and fluorescent lamps, contribute 1000 metric tons

annually. Waste incineration adds 600 metric tons, followed by electricity generation with 300 metric tons. There are also agricultural inputs via fertilizers, lime, and manure (Anderson, 1987), and land development has remobilized mercury deposited in the past. In all, modern mercury deposition rates are 2-5 times greater than preindustrial rates (Douglas, 1994; Nater and Grigal, 1992).

2.2 Metals Speciation

Although certain metals in trace amounts are required for sustaining life, metals as anthropogenic inputs to the environment may be harmful due to their direct or indirect interaction with and disruption of natural biochemical processes. Most metals can exist in a variety of redox states (or species) under environmental conditions, and may also be found in a variety of organic or inorganic complexes. The redox state and/or the nature of its complexation will exert a large effect on the interactions of the metal with other components of the environment (including organisms). As an illustration, iron cycles between the ferrous (Fe^{2+}) and ferric (Fe^{3+}) forms in aqueous environments. Iron oxides in the sediments are used as terminal electron acceptors by anaerobic microorganisms, which reduce the iron to Fe^{2+}. This is a soluble form of the metal which is thereby released into the aqueous phase. After mixing into the aerobic zone, the iron is oxidized to Fe^{3+}, which is insoluble and will settle back to the sediments where it can then be re-reduced by bacteria to continue the cycle. As is the case with iron, particular species of other metals will also be found in different phases and zones within the environment, and, in addition, certain species of metals will be more bioavailable than others, meaning that they are more readily available to interact with biological components in the system.

There is currently much interest among researchers and the regulated community to encourage the regulators to issue

metals regulations on a species basis. An important meeting (the American Chemical Society, Environmental Protection Agency, and Delaware Workshop) on metal speciation was held in June, 1993, as a followup to earlier meetings, in order to examine the current knowledge on metal speciation, local variations, and regulatory approaches (Merian, 1994). Several presentations demonstrated the presence of multiple species and complexes of various metals and their cycling among various phases, as described for iron above. Bioavailability of metals, and the role of organisms in metal cycling were important topics; a consensus was reached that speciation and bioavailability should be of concern to regulators, but more data is necessary.

2.3 Global Cycling

In addition to human inputs detailed above, natural sources and transport also play an important role in the global cycle of mercury. Mercury is transported to oceans by atmospheric deposition and natural soil erosion, and since mercury has a high vapor pressure (1mm at 126°C), metallic mercury readily evaporates from the soil into the atmosphere (see **Figure 1**). In fact, the global cycling of mercury is dominated by atmospheric processes. Mercury volatilization from water bodies to the atmosphere, and deposition from the atmosphere, are far greater than aquatic or sedimentary fluxes. For example, the ocean receives about 90% of its mercury through dry and wet atmospheric deposition (Mason, et al., 1994). The most common species in surface waters is Hg^{2+} (Gavis, 1972), but methyl mercury is the predominant form found in fish (Kamps, 1972; Westoo, 1973). Methylation of Hg^{2+} occurs in the anoxic sediments by sulfate-reducing bacteria, but the factors controlling this process are not well understood. Mercuric ions can also be removed from the environment prior to methylation by chemical reduction to Hg^{0}, which is volatile and escapes from the aqueous environment. Since this process removes mercury from the aquatic environment prior to

methylation and uptake by organisms, in effect playing an important detoxifying role and being a significant component in the global mercury cycle, it is very important to understand and characterize the rates and mechanisms of this reaction. In addition to reduction, some bacteria can also demethylate the organomercurial species. The mercury cycle may thus depend on environmental conditions that determine which bacterial process will dominate.

The total mass of global mercury is obviously fixed, but the mercury exists as a varying population of species. Human intervention has changed the magnitude of the fluxes within the natural cycle. As noted above, human emissions have added a significant burden to the natural global cycle by translocating mercury from the soil to the atmosphere from where it can then be deposited into water bodies, and more importantly have an opportunity to bioaccumulate. Manmade mercury compounds have also perturbed the natural cycle.

2.4 Toxicity

Monomethyl mercury (CH_3Hg^+), which accounts for 95% or more of the mercury found in fish (Douglas, 1994), is a potent neurotoxin. Young children and fetuses are particularly vulnerable, since methyl mercury poisoning can damage growing nerve tissue (Douglas, 1994). The effects of mercury poisoning can include liver and kidney damage (Sprague, 1985), nervous system damage (for example, speech, gait, and visual impairment) and even death (Takeuchi, 1972).

Mercury is of particular concern because of its tendency to bioaccumulate and to biomagnify in the food chain. Bioaccumulation occurs when methylated mercury is taken up by organisms and stored in their tissues, eventually resulting in much higher tissue mercury levels than those present in the aquatic environment. Biomagnification occurs when algae and other organisms low in the food chain take up and store

mercury, and in turn are consumed by organisms higher in the food chain, and so on. The higher organisms continually take up mercury when assimilating the lower organisms but do not expel mercury at the same rate, with the result that methylated mercury is stored in bodily tissues and the mercury concentration in these organisms becomes extremely high. For example, one study found that large fish in a relatively contaminated lake had mercury concentrations of 5.8 ppm, and fish found in their stomachs had mercury concentrations of 3.1 ppm, while the benthic organisms upon which the latter fish fed had concentrations of only 0.3 ppm. A similar comparison from a relatively uncontaminated lake found large fish with 1.2 ppm mercury, smaller fish with 0.6 ppm, and bottom fauna with 0.05 ppm (Jernelov, 1972). This demonstrates why the dangers of bioaccumulation cause serious concern over even very small discharges of mercury.

2.5 Regulatory Policy

Of the 189 substances designated "hazardous air pollutants," or HAPs, under Title III of the 1990 Clean Air Act Amendments, mercury was singled out for special study because of these significant effects on health. One of the main concerns is that humans, high on the food chain, will be harmed by the consumption of methyl mercury that has bioaccumulated in the food chain (Douglas, 1994). In 1963, the United Nations Food and Agricultural Organization and World Health Organization Codex Alimentarius Commission recommended an upper level of 0.05 ppm of mercury in all foods except fish and shellfish. The U.S. Food and Drug Administration (FDA) originally set an advisory limit of 0.5 ppm for mercury in fish flesh, considering the basic data and extrapolations to be "scanty and unreliable" (Goldwater and Stopford, 1977). The U.S. Food and Drug Administration has subsequently adjusted the advisory limit to 1 ppm in fish flesh, but several states have set lower limits (0.5 ppm; Douglas, 1994). In Japan, the limits were established at 0.4

ppm total mercury and 0.3 ppm as methyl mercury (Tsubaki and Irukayama, 1977).

Unlike this example from Japan, most of mercury regulation in the United States does not distinguish different mercury species or compounds. One exception is the Clean Water Act's list of hazardous substances, which includes mercuric cyanide, mercuric nitrate, mercuric sulfate, mercuric thiocyanate, and mercurous nitrate; the regulated mercury compounds are inorganic and represent two redox states of the metal.

Under Section 112 of the Clean Air Act, Congress specifically lists "mercury compounds" as hazardous air pollutants. The criteria given for determining the appropriateness of adding a compound to the list is:

> "... pollutants which present, or may present, through inhalation or other routes of exposure, a threat of adverse human health effects (including, but not limited to, substances which are known to be, or may reasonably be anticipated to be, carcinogenic, mutagenic, teratogenic, neurotoxic, which cause reproductive dysfunction, or which are acutely or chronically toxic) or adverse environmental effects whether through ambient concentrations, bioaccumulation, deposition, or otherwise..."

Thus mercury emissions to the atmosphere can be regulated under the Clean Air not only on the basis of the danger of mercury inhalation (which would be Hg^0 or mercury oxides, which are less hazardous than other forms), but also on the basis of the dangers of mercury bioaccumulation in aqueous environments because a major source of the bioaccumulated mercury is atmospheric deposition.

2.6 Mathematical Cycling Models in Policy Formation

Policy makers must base environmental regulations upon the available scientific data. For regulations concerning emissions levels, regulators seek to understand the fate of the emitted chemical in the environment and the risk associated with that emission. Models which describe the fate and transport of mercury in the environment have been used by regulators in establishing appropriate emissions levels. For example, there has been much concern over the discovery of mercury-contaminated fish in remote lakes with no obvious source of mercury contamination. In these instances, it was determined that the mercury was deposited in the lakes as non-organic, oxidized mercury, via atmospheric deposition originating at distant sources, and then methylated. Without knowledge of the mercury cycle, regulators would not even know who to regulate, much less what emissions levels are appropriate. The accuracy of the predictions from these models are particularly dependent on and sensitive to the values assigned to flux rates. In a model of the global mercury cycle, rates of methylation and of volatilization due to reduction need to accurately reflect conditions in the environment so that appropriate regulatory decisions can be made concerning this toxic metal.

The U.S. EPA uses the Water Analysis Simulation Program (or WASP4) to assess water quality problems in surface waters. A mathematical model to simulate mercury dynamics in a specific lake and to calculate methyl mercury contamination in large fish within the lake has been developed and incorporated into WASP4 [the portion of the model concerning global scale dynamics of atmospheric, terrestrial, and oceanic mercury cycling is presented in a paper by Hudson, et al., (1994; see also Hudson, et al., 1995)]. The mercury model was designed to help decision makers evaluate options for assessing and managing risks associated with mercury contamination

(Douglas, 1994). Hudson et al. suggest that the flux rate chosen for mercury evasion from oceans (representing all aquatic environments) in Mason, et al. (1994), based upon current knowledge of mercury reduction, was apparently too high. This indicates a need for improved quantitation of mercury reduction and evasion.

Historically, in addition to the mathematical models, environmental standards have been based on attainability, and toxicological and epidemiological data. Levels for chronic low level exposure must often be set using an extrapolation of the available data from acute, relatively high level exposure. With some compounds, the detection limit of current analytical technology may constrain the establishment of a low limit. The technologies for measuring mercury levels in environmental samples have evolved with the increasing concern on mercury contamination in the environment. For example, until relatively recently, a colorimetric method using diphenylthiocarbozone was performed to quantify mercury in a sample. As is common with these types of methods (i.e., colorimetric), it was fairly insensitive. Flame atomic absorption methods have also been used, but the method of choice today is cold vapor atomic absorption (CVAA; for details see below under "Methods"), which detects Hg^0 and is extremely sensitive (Fitzgerald, et al., 1974; Fitzgerald and Gill, 1979; Stuart, 1978). Total mercury can be analyzed by first treating the sample with a reducing agent. Organomercurials are detected by gas chromatography in conjunction with CVAA. These are difficult techniques which require a fairly substantial investment in equipment and technical personnel or training.

Sample collection techniques have also advanced. In fact, until recently, when researchers analyzed field samples, fairly high mercury levels were reported (for example, at one time levels of many tens of nanograms per liter in seawater

were reported, versus 20 picograms per liter reported more recently; Bloom and Crecelius, 1983; Bothner, 1973; Fitzgerald and Lyons, 1973; Windom, et al., 1975). The consensus is that these earlier high values were artifacts from contamination introduced by the researchers during sample collection (Bothner, 1973; Bothner and Robertson, 1975; Lo and Wai, 1975; Porcella, 1990; Yamazaki, et al., 1978). These improvements in both sampling and analysis have led to great improvements in the state of knowledge of the global mercury cycle, and also to much better models of the cycle. Policy makers now have useful mathematical tools on which to base regulatory levels, rather than having to base them solely on available control technology and then hoping that those levels would turn out to be safe.

2.7 Mercury Reduction

Since reduction plays such an important role in the global mercury cycle, a close examination of its characteristics and mechanisms is required for reasoned policy decisions as well as for thorough scientific elucidation of the mercury cycle.

2.7a Reduction Mechanisms

The two types of mechanisms for mercury reduction are via abiotic or biotic reactions. The abiotic reactions involve either thermal reduction by humic substances (complex organic matter suspended in aqueous environments, including altered amino acids, sugars, and triglycerides from terrestrial and planktonic sources that have become linked together; Schwarzenbach, et al., 1993; Alberts, et al., 1975; Skogerboe and Wilson, 1981) or photochemical reactions in the presence of H_2O_2 (Schroeder, et al., 1991; Amyot, 1994; Horvath, 1993). While abiotic reduction of mercury has been reported, many investigators have emphasized the role of microorganisms in mercury reduction (see Summers 1986; Foster, 1987; Robinson, 1984). This research has evolved from studies of mercury resistant bacteria which have focused on plasmid-encoded

resistance mechanisms (Summers, et al. 1978; Blaghen, 1983; Booth, 1984; Clark, 1977; see also Summers, 1986). When exposed to high levels of mercury (traditionally 50 μM), the resistant bacteria express proteins which ultimately reduce the mercury to the Hg^0 species. Plasmid-encoded mercury resistance (generally encoded by the *mer* operon) has been found in clinical, industrial, and environmental samples exposed to high mercury levels (see Summers, 1986; Foster, 1987). There has been much effort expended in characterizing this operon (Brown, et al., 1986; Foster, et al., 1979; Foster and Brown, 1985; Griffin, et al., 1987; Hamlett, et al., 1992; Jackson and Summers, 1982a,b; Misra, et al., 1984; Misra, et al., 1985; Nakahara, et al., 1979; Ni' Bhriain, et al., 1983; O'Halloran and Walsh, 1987; Philippidis, et al., 1991; Sahlman and Jonsson, 1992; Sahlman and Skarfstad, 1993; Summers, et al., 1982; Summers and Kight-Olliff, 1980; Summers and Silver, 1972).

2.7b Bacterial Resistance

Mercuric ions are toxic to bacteria because of their strong affinity for sulfhydryl groups in proteins (Albert, 1973). Bacterial resistance to mercury compounds is a common property, especially among bacteria of clinical origin (Foster, 1987). Studies indicate that plasmid-encoded mercury resistance is as common as the antibiotic resistances (Summers, 1986), and in fact many studies have interestingly found the mercury resistant phenotype linked to antibiotic resistant genes in clinical isolates (Porter, et al., 1982).

Mercury resistance is defined as the ability to exist with mercury concentrations at or above 50 μM. This level is much higher than that found in all but the most contaminated of environments (such as mine drainage streams or hospitals). The most common resistance mechanism involves the intracellular conversion of Hg^{2+} to Hg^0, the volatile species (as mentioned above) which diffuses out of the cell.

Resistance which does not involve volatilization has been described in only two species (Pan-Hou and Imura, 1981; Pan-Hou, et al., 1981).

In many cases, the mercury resistant bacteria of clinical origin have been found to carry a specific mercury resistance gene system linked to antibiotic resistance genes on a plasmid; this system has been termed the *mer* operon. The use of mercurials in hospitals as disinfectants may have provided the selection for mercury resistance. Conversely, it has been demonstrated that the incidence of mercury resistance among hospital staphylococci has declined recently, possibly due to the discontinuation of the use of organomercurials as disinfectants (Porter, et al., 1982).

2.7c Emphasis on the *mer* Operon System

With the *mer* operon two types of mercury resistance have been described. In one, termed narrow-spectrum resistance, the cells have the ability to reduce Hg^{2+} to Hg^{0} by expressing mercuric reductase. In the other, referred to as broad-spectrum resistance, the cells have both the ability to demethylate organomercurial compounds, as well as to reduce mercury using mercuric reductase (Foster, 1987). In most cases, the demethylation occurs via organomercurial lyase which cleaves C-Hg bonds by protonolysis (Foster, 1987). Bacteria with this ability could play an important role in the mercury cycle in anoxic sediments, where most methylation occurs, and where the methyl mercury concentration could be high enough (in contaminated areas) to induce a response.

The induction of the *mer* operon of both narrow- and broad-spectrum resistant bacterial strains causes production of mercuric reductase which is an intracellular, cytoplasmic flavoprotein (Summers and Silver, 1978; Schottel 1978). This enzyme uses NADPH as an electron donor and requires the presence of thiols which inhibit the formation of NADPH-Hg^{2+}

complexes for in vitro activity (Foster, 1987). Mercuric reductase is related to glutathione reductase (Foster, 1987). Glutathione reductase is an abundant disulfide reductase in mammalian tissues, where it functions to maintain a large pool of reduced glutathione (which in turn functions as an antioxidant and has a role in the detoxification of xenobiotics; Alscher, 1989; Halliwell and Gutteridge, 1989; Smith, et al., 1989).

2.7d Description of the *mer* Operon

The genes of the *mer* operon are arranged sequentially as shown in **Figure 2**. The gene products are a group of proteins which function together to capture and reduce mercuric ions (or first to demethylate organomercurials, producing mercuric ions, in the case of broad-spectrum resistance). These proteins and their functions, if known, are listed below:

merR: The regulatory protein of the operon. A 16,000 Dalton protein, it acts as both a repressor and inducer of the *merTPCA* genes as well as negatively regulating its own expression (Foster, 1987; Foster and Brown, 1985; Lund, et al., 1986).

merT: A hydrophobic protein with estimated molecular weight of 12,400 Daltons to 16,000 Daltons (Foster 1987; Ni'Bhriain and Foster, 1986; Jackson and Summers, 1982). It is most likely a membrane protein involved in the transport of Hg^{2+}.

merP: Also involved in Hg^{2+} transport, this protein is probably located in the periplasm. Its molecular weight has been reported as 7,500 to 14,000 Daltons (Jackson and Summers, 1982; Ni'Bhriain and Foster, 1986; Sahlman and Jonsson, 1992). The *merP* protein apparently binds the mercuric ion and passes it to the *merT* protein which

transports the mercury into the cell for reduction by the *merA* protein.

merC: A 14,000 Dalton hydrophobic protein which is not present in all *mer* systems (Foster, 1987; Jackson and Summers, 1982). It has been suggested that since there is some homology between *merT* and *merC*, the *merC* protein may also be involved in Hg^{2+} transport (Summers, 1986).

merA: Codes for the mercuric reductase protein, with a molecular weight of 58,700 - 67,000 Daltons (Summers and Silver, 1978; Brown, et al., 1983: Misra, et al., 1985; Furukawa and Tonomura, 1971). The molecular weight has also been reported as 175,000 Daltons, suggesting a trimer (Schottel, 1978).

merD: A protein with a predicted molecular weight of 13,000 Daltons (Brown et al., 1986) which may play a marginal role in resistance.

2.7e Mercury Reduction Experiments

In general, there has been an assumption that this *mer* mechanism must also be responsible for mercury reduction in the global mercury cycle in the environment, even though environmental mercury levels are generally far below the 50 µM levels (in fact at picomolar levels; Mason, et al., 1993) used experimentally. Barkay, et al. (1989) concluded that the *mer* operon was the important system of mercury reduction in a freshwater isolate, but the experiments were performed at micromolar mercury concentrations. However, this study also reported mercury reduction by bacteria from marine environments at the higher mercury levels in the absence of the *mer* operon which were attributed to other unspecified gene systems. Since induction of the *mer* operon has been reported to occur at 10 nM or higher [Summers, 1986; it may be as low as 2 nM (Summers, personal communication)], levels

which are much higher than are found in the environment, it seems unlikely that this mechanism is responsible for the mercury reduction that occurs at the low mercury levels found in the environment. This possibility was examined by performing experiments at low mercury concentrations in which bacterial mercury reduction rates were measured. These experiments were performed to investigate the role of the *mer* operon at mercury levels which are closer to those levels found in the environment. The reduction rates so obtained could be used to achieve more accurate mercury cycle models, and the information on mechanism may have implications for regulation based on speciation.

3. Materials and Methods

3.1 Materials

Cells: For the *mer* experiments, two separate cultures of *Escherichia coli* strain SK1592 was used, one of which contained the plasmid pDu202 containing the *mer* operon. The cell lines were generously provided by Dr. Anne Summers at the University of Georgia. The cell line without the *mer* operon is designated "SK" in the text and figures, while the one containing the *mer* operon is designated "pD".

Glassware: Pyrex 250 ml bottles, tubing and diffusers.

Mercuric chloride: Purchased from Spex Chemical.

LB media: 10 g NaCl, 10 g Bacto-tryptone, 5 g Bacto Yeast Extract, brought to 1 L with MilliQ water and autoclaved for 15 minutes.

LB plates: As above plus 15 g agar.

Mercury plates: As above plus mercuric chloride at 50 μm.

Antifoam A: 30% emulsion obtained from Sigma.

TFB: 10 mM MES [2-(*N* -morpholino)ethanesulfonic acid], 45mM manganese chloride [$MnCl_2(4H_2O)$], 10mM calcium chloride [$CaCl_2(2H_2O)$], 100 mM potassium chloride (KCl), 3 mM Hexamminecobalt chloride.

DnD: 1.53 g dithiothreitol, 9 ml DMSO (dimethyl sulfoxide), 100 ul of 1 M potassium acetate pH 7.5, and H_2O to 10 mls.
Laemmli sample buffer: 0.0625 M Tris [Tris(hydroxymethyl)-aminomethane]-HCl pH 6.8, 2% SDS (sodium dodecyl sulfate), 10% glycerol, 5% mercaptoethanol, 0.001% bromophenol blue.
Laemmli upper and lower reservoir buffers: 0.025 M Tris, 0.192 M glycine, 0.1% SDS; pH 8.3.
Laemmli stacking gel buffer: 0.125 M Tris-HCl pH 6.8, 0.1% SDS.
Laemmli separating gel buffer: 0.375 M Tris-HCl pH 8.8, 0.1% SDS.
Jovin lysing solution: 7 M urea, 20% Triton X-100.
Jovin upper reservoir buffer: 0.040 M Bis-Tris [2,2-bis-(hydroxymethyl)-2,2',2"-nitrilotriethanol], 0.025 M Tricine [N-tris(hydroxymethyl)methyl-glycine].
Jovin stacking gel buffer: 0.044 M Tris, 0.028 M Tricine, pH 7.4.
Jovin separating gel buffer: 0.096 M KOH, 0.217 M Tricine, pH 7.0.
Jovin lower reservoir buffer: 0.050 M KOH, 0.062 M Tricine.
Coomassie blue stain: 40% methanol, 10% acetic acid, 0.1% Coomassie brilliant blue R-250.

3.2 Methods

3.2a Glassware

Rigorous cleaning procedures were required for all glassware used in cell culturing, preparations, and reduction experiments in order to ensure that it was free of extraneous reductant, mercury contamination, and uninvited bacteria. Glassware was soaked 48 hours in diluted Micro cleaner, then rinsed thoroughly with distilled water. Next it was soaked 24 hours in 1 N HCl, then rinsed thoroughly and soaked 48 hours in MilliQ water. Next it was rinsed again in MilliQ water, then, with a small amount of MilliQ water inside, autoclaved 20 minutes. After the residual MilliQ water was

removed the glassware was ready for use. Trace metal clean and sterile techniques were used in all culturing experiments. For example, pipette tips were presterilized by autoclaving, rinsed in autoclaved ultrapure HCl, then double rinsed in autoclaved MilliQ water before use.

3.2b Cells

Cells were cultured in LB media. Purity of cell lines was maintained by subcloning onto LB agar plates and selecting single colonies, which were transferred to 15 ml glass tubes for use in all experiments. SK bacteria were subcloned onto plates without mercury, while pD bacteria were subcloned onto mercury plates.

3.2c Reduction Experiments - General Description

Cells were added to 200 mls total volume LB media plus additives. Mercuric chloride was added if necessary. Sigma Antifoam A 30% emulsion (10 µl) was added to all bottles to prevent foam from entering the soda lime trap and gold column. Blanks contained LB media plus Antifoam A. Spiked blanks contained 10 nM mercuric chloride in addition to LB media and Antifoam A. As a control, LB media and Antifoam A without cells caused no significant reduction in 24 hour experiments.

3.2d Mercury Collection

Any Hg^0 produced via reduction was collected onto gold amalgam traps (see **Figure 3**). Due to its volatility, the mercury could be removed from the sample by sparging with gas. Argon (4.8 grade) was passed through two gold amalgam traps (to remove any mercury contamination in the gas), then into the sample container where it passed through a glass diffuser. The exit gas passed first through a soda lime column (to absorb water which might otherwise interfere with the analysis), then onto the gold amalgam sample collection

column. Fifteen minute sparging was required to remove the volatile mercury from the sample.

Remaining mercuric ion concentration was measured by the addition of tin chloride to the samples after the removal of volatile mercury above. This step was performed in preliminary experiments, but after ascertaining that the mercuric ion concentrations were as expected for these experiments, this step was omitted in most experiments to avoid the possibility of contaminating the glassware with tin chloride. The bottles treated with tin chloride were not reused.

3.2e Mercury Analysis

Collected mercury was analyzed using cold-vapor atomic fluorescence detection (Bloom and Fitzgerald, 1988; Mason 1991). The sample collection gold amalgam column was placed in-line in the mercury analysis apparatus (see **Figure 4**). Helium (grade 5.0) was used as the carrier gas. Two gold amalgam traps were placed before the sample column to capture any mercury contamination in the carrier gas. These traps and the traps from the sparging apparatus were periodically placed in the position of the loading column and heated repeatedly in two minute sessions to remove any mercury which may have collected on them.

The sample collection column was then heated for two minutes. After 30 seconds, the loading column was heated for two minutes. Cooling fans were used to cool the columns after heating was stopped. This sequence was automatically controlled by a ChronTrol box (ChronTrol Corporation, San Diego, CA). The carrier gas from the column fed into an atomic absorption mercury analyzer (Brooks Rand, Ltd., Seattle, WA). This analyzer records maximum peak height values for each run. The output was also fed to an HP integrator (model# 3396 Series II; see sample chromatograph

shown in **Figure 5**). The system was calibrated using Hg^0 standards (50µl and 100 µl saturated Hg^0 vapor; approximately 0.5 ng and 1.0 ng Hg^0), which were injected directly in front of the loading column.

3.3 Specific Experiments

3.3a SK versus pD

Initial experiments were performed to assess any differences in reduction between SK cells (without the *mer* operon) and pD cells (with the *mer* operon). LB media, mercuric chloride, Antifoam A, and cells were added as necessary to each bottle. Cell counts at the start were determined by measuring optical density with a spectrophotometer at 600 nm. Cell counts were previously correlated with O.D. using dilution and plating techniques to ascertain the number of viable cells at each density. Cells were stored overnight at room temperature in the dark in a sealed container. The diffuser with attached columns was introduced and then gas flow applied. Samples for cell density measurements were collected after the sparging was completed. Experiments were conducted over a range of mercuric ion concentrations.

3.3b Live versus Killed Cells

In order to assess any possible abiotic reduction, similar experiments were performed with cells that were killed using microwave heating. The experiments were performed as above, except that each cell line was split into two aliquots immediately prior to adding to the sample bottles. One of the aliquots was microwaved for about two minutes to kill the cells, then cells were added as above.

3.3c Competency Preparation

In order to prove that the difference in reduction between the two cell lines was truly due to the presence of the *mer* operon, and not due to some other difference that had

developed while the cells were grown in the presence of mercury, two types of experiments were performed. First, antisense oligonucleotides (Wagner, et al., 1993) were used to try to block the *mer* activity; second, polyacrylamide gels were used to visualize the *mer* proteins. Prior to performing the antisense experiments, the effects on mercury reduction by the procedure in which the cells would be made amenable to the uptake of the antisense oligo (or "competent") were assessed as a control. Based on the procedure described in Sambrook, et al. (1989) for preparation of competent cells, 30 mls of cell culture were cooled on ice, then centrifuged at 4°C for 10 minutes at 4000 rpm. The pellets were then resuspended in TFB. This step was repeated, followed by addition of DnD solution as recommended. Since large amounts of cells are needed for the reduction experiments, the entire amount of prepared cells was used [in contrast to the Sambrook, et al. (1989) method]. Reduction experiments were conducted at this point to first gauge the effect of this procedure on Hg^{2+} reduction.

3.3d Antisense Oligonucleotide Treatment

A twelve base phosphorothioated single-stranded oligonucleotide (synthesized by The Midland Certified Reagent Company, Midland, TX) to target *merT* was designed based on the gene sequence published by Misra, et al. (1984). After performing the above competency preparation, each cell line was divided into two aliquots, with one incubated with the antisense olionucleotide (at a concentration of 5 μM) while the other received none of the oligo but otherwise underwent the same treatment. Fewer SK samples were chosen than pD samples because each sample required a large amount of antisense oligonucleotide. After incubating for 30 minutes on ice, the cells were heated at 42°C, chilled, then allowed to recover at 37°C as described in Sambrook, et al. (1989). Reduction experiments were then conducted as described above.

3.3e Polyacrylamide Gel Electrophoresis

3.3e.1 Laemmli Buffer System

This commonly used gel system was employed to identify differences in protein expression between SK and pD which indicate the presence of *mer* proteins in pD cells at "higher" mercury levels. Cells were grown for 48 hours in 200 ml aliquots as used for the sparging experiments. Samples were placed on ice, then centrifuged at 4°C for 10 minutes at 6000 x gravity (g). The supernatant was discarded and the cell pellets were transferred to cold 15 ml centrifuge tubes and resuspended in 3 mls ice cold MilliQ water. The samples were sonicated on ice using a Branson Sonifer at output control setting 4 for 30 seconds at 50% duty cycle. The lysates were then centrifuged at 4°C at 16,000 x g for 10 minutes to pellet any insoluble material. The supernatants were removed and a Bradford protein assay performed (using BioRad Protein Assay Reagent; Bradford, 1976). The samples were then added to Laemmli sample buffer, normalized to protein amount (~7 µg protein), and loaded onto a polyacrylamide gradient minigel (4-15% T; 2.6% C; Laemmli, 1970). After electrophoresis for 40 minutes at 200 V, the gel was stained with Coomassie brilliant blue stain solution.

3.3e.2 Jovin Buffer System

Based on the method used by Hamlett, et al.(1992), a native cationic gel system was used to attempt to clearly visualize the mercury transport protein *merP*, and possibly the other transport proteins (*merT* and *merC*), as an indication of *mer* operon activity. This electrophoretic method takes advantage of the high pI (calculated at 9.4, based on amino acid composition) and low molecular weight of the protein. This gel system is expected to provide good separation of *mer* transport proteins. Cells were grown for 48 hours in 200 ml aliquots as used for the sparging experiments. Cell counts were determined using absorbance at 600 nm. The cells were

centrifuged for 10 minutes at 6000 x g. The supernatant was removed, the cells were resuspended in 2 mls MilliQ water, sonicated, and centrifuged as above. Approximately 25 µl of the final supernatant was added to 15 µl of Jovin lysing solution (the amount of sample used was normalized according to cell counts so that extracts corresponding to equal numbers of cells were loaded in each lane), then electrophoresed for one hour at 200 V using the Jovin cationic buffer system 1193 (Jovin 1973a,b,c; Jovin, et al., 1970). The stacking gel was 3.5% T and 2.6% C, while the separating gel was 18% T and 2.6% C. The gels were stained with Coomassie blue protein stain, followed by silver staining using the LabLogix Silver Stain Kit.

4. Results

4.1 SK versus pD

Results indicated that there is a difference in mercury reduction between SK (without the *mer* operon) and pD (with the *mer* operon) bacteria, as expected. The two bacterial cultures reduced mercury at the same rate at Hg^{2+} levels below 1.5 nM. At concentrations greater than this, the cells containing the *mer* operon (pD) were able to reduce mercury at a greater rate (see **Figure 6**). Thus, the *mer* operon was induced at ~1.5 nM, a concentration much higher than that found in most environments (Mason, et al, 1993). This suggests that the *mer* operon system is not the mechanism by which mercury reduction and evasion occurs on a large scale in the global environment.

4.2 Live versus Killed Cells

The killed cells demonstrated a very low rate of reduction that did not increase with increasing Hg^{2+} concentration (see **Figure 7**). Both types of cells displayed the same minimal reduction rate. Thus the reduction observed above was not abiotic, but was a result of an active cell process.

4.3 Competency Preparation

In the first experiment to assess the effects of the competency preparation procedure on the reduction rates of the cells, it was observed that the competency protocol inhibited reduction slightly in the cells which did not contain the *mer* operon (SK), even after correction for cell number was performed on the data [see **Figure 8**; the symbol following the cell line designation signifies whether or not the cells underwent the competency protocol (+ = yes; - = no)]. Surprisingly, the competency protocol enhanced the mercury reduction by the cell line with the *mer* operon at higher mercury concentrations. This was most likely due to increased sharing of the plasmid to cells which no longer carried the plasmid. The plasmid likely was lost in the process of serially diluting and growing the cells in the absence of additional mercury to ensure that the cells actually encountered the expected, low mercury concentrations during the reduction experiments.

For subsequent experiments, the pD cells were taken from the mercury plates shortly before each reduction experiment, so that minimal dilution was required and in order to minimize the time during which the cells could lose the *mer* plasmid. In these subsequent experiments, the competency protocol produced smaller impacts on reduction which did not significantly impact the ability to interpret the data (see **Figure 9**).

4.4 Antisense Oligonucleotide Treatment

The level of reduction by bacterial cells lacking the *mer* operon (SK) was not affected by addition of the antisense oligonucleotide [see SK in **Figure 9**; the first symbol following the cell line designation signifies whether or not the cells underwent the competency protocol (+ = yes; - = no), while the second symbol indicates whether or not the

antisense oligo was added]. The rate of mercury reduction by the cells carrying the *mer* operon (see pD in **Figure 9**) was somewhat inhibited by the addition of the antisense oligo at mercury levels above 1.5 nM. It was not expected that the antisense oligo would completely block the *mer* expression, for several reasons: it is unlikely to penetrate 100% of cells; even if it did enter, it might not hybridize successfully in all cases; and finally, the cells that were not blocked by the antisense oligo would reproduce normally (including the plasmid) during the incubation period, leading to more copies of the plasmid than were originally present. Because the mercury levels being measured were so low, it was not possible to substantially shorten the incubation time. It was hoped that the reduction caused by *mer* proteins would still be decreased sufficiently to cause a significant difference in the mercury generated, and prove that the *mer* proteins were indeed responsible for the difference in reduction seen between the cells with and without the *mer* operon, instead of some other mechanism that had developed because the cells were grown in "high" mercury. The data do demonstrate a significant inhibition of reduction with the antisense oligo added to pD, especially when compared, as is appropriate, to the reduction rate of pD following the competency protocol. Thus, there is strong evidence that the *mer* system was responsible for the previously observed difference in mercury reduction between the two cell lines.

4.5 Polyacrylamide Gel Electrophoresis

The polyacrylamide gels provide further evidence that the difference in mercury reduction between the two cell lines was due to the presence of the *mer* operon, rather than to some other difference that had developed while the cells were grown in the presence of mercury. The Laemmli gel showed the presence of a more prominent protein band at ~14,000 Daltons for pD at 2nM and 10nM mercury (see **Figure 10**). Since *merP*, *merT*, and *merC* have been reported at around that molecular

weight, it is possible that all three comigrated in this gel system. The bands at a similar position in pD at 0.5 nM mercury and SK are probably other unrelated proteins in the same size range as the *mer* proteins since the sample represents a total cell extract. Even though the samples had been normalized to protein level, it appears that the loading was not identical between each lane. If amounts corresponding to equal cell numbers had been loaded instead of equal amounts of total protein, the difference in the amount of protein present in the ~14,000 Dalton range between pD at 10 nM and pD at 2 nM would have been even greater. The amount of protein present in the ~14,000 Dalton band apparently was large enough to skew the results of the protein assay. The Jovin gels were loaded based on equal cell numbers instead of equal amounts of protein. In the Laemmli gel, it should be noted that there also appeared to be an enhanced protein at ~70,000 Daltons, which may be mercuric reductase.

With the native cationic Jovin gel system, only proteins with a pI above 7.5 should migrate into the gel, and smaller proteins with high pI's, like the *merP* protein, should migrate the furthest. **Figure 11** clearly shows the presence of such a protein in pD samples at mercury concentrations at and above 2 nM, but not in SK samples or pD samples exposed to lower levels of mercury. The migration position of this protein appears to be the same as that shown by Hamlett, et al. (1992) for the purified *merP* protein. The enhancement of an additional protein band higher in the gel can also be seen in the same sample; a similar band was attributed to be the reduced form of the merP protein by Hamlett, et al. (1992). The Jovin gel system clearly shows that the *merP* protein was induced at mercury concentrations of 2 nM and higher in the pD cells, and thus the *mer* operon was indeed responsible for the mercury reduction demonstrated by these cells.

5. Experimental Conclusions

The difference in the reduction rates between SK and pD above mercury concentrations of 1.5 nM indicate that the *mer*-containing strain is able to reduce mercury more effectively. Below this level, the presence of the *mer* operon did not result in a higher rate of reduction than was performed by cells lacking the *mer* operon. It is of particular interest that the apparent induction of the *mer* operon occurs at 1.5 nM, a level which is much higher than that typically found in the environment, but is less than the induction level reported elsewhere. The antisense and gel experiments confirm that it is the induction of the *mer* operon which is responsible for the increased rate of reduction. Of significance to the global mercury cycle is the result that there is a basal, "background" reduction of mercury by SK cells and non-induced pD cells. These results indicate that the mercury reduction mechanism normally operating in the environment is something other than the *mer* system. This has been shown by performing experiments at concentrations nearer those found in the environment, demonstrating that the assumption that the mechanisms of importance in *in vitro* experiments performed at high concentrations will also be the important mechanisms in the field may be incorrect.

Future work to identify and characterize the real reductive mechanism could include measuring the effect of the addition of known enzyme inhibitors on mercury reduction to investigate whether blocking certain surface enzymes inhibited "SK-type" reduction. Work by Jones, et al. (1987) demonstrated that phytoplankton cell surface redox enzymes (these are ubiquitous enzymes involved in plant to mammalian cell growth, transport, and defense; Crane, et al., 1985) reduce external copper and iron. It is quite possible that these enzymes can also reduce mercury, and are responsible for the mercury reduction in the environment.

6. Policy Discussion

Scientific knowledge of the fate and transport of individual mercury species can impact policy formation in two ways. First, this knowledge when used in global cycling models can help regulators improve current regulation of total mercury levels. Second, regulations could be promulgated which specified emissions limitations of specific compounds or species. Both of these applications will lead to improved protection of health and the environment.

Cycling models are often used by regulators in establishing appropriate emissions levels. Since the *mer* operon system is not responsible for the mercury reduction and evasion that occurs on a large scale in the global environment, models which base an aquatic evasion flux on the rate of reduction by mercuric reductase will be in error. Under the Clean Water Act (National Pollutant Discharge Elimination System subpart), the EPA has promulgated toxics criteria for certain pollutants within Section 131: Water Quality Standards. The Criteria Maximum Concentration (CMC; the highest concentration of a pollutant to which aquatic life can be exposed for a short period of time [one hour average] without deleterious effects) for mercury is 2.4 ppb. The Criterion Continuous Concentration [CCC; the highest concentration of a pollutant to which aquatic life can be exposed for an extended period of time (four days) without deleterious effects] is 0.012 ppb, with the added specification that,

> "If the CCC for total mercury exceeds 0.012 (ppb) more than once in a 3-year period in the ambient water, the edible portion of aquatic species of concern must be analyzed to determine whether the concentration of methyl mercury exceeds the FDA action level [1.0 (ppm)]. If the FDA action level is exceeded, the State must (take appropriate action)."

However, in a lake with mercury concentrations well below this guideline, fish were found to have mercury concentrations of up to 2.4 ppm, well above the 0.5 ppm guideline (Derryberry, 1972). This indicates that the model used to estimate mercury concentrations in fish from concentrations in the water body apparently was not accurate. One possible explanation is that there may be more mercury available to be methylated in the water body than predicted by the model. That is, if high reduction rates were assumed, this would lead to an additional assumption of less mercury available for methylation. Using a lower estimate for reduction rates, as suggested by the above experimental results, would lead to a lower allowable level in the water body. This apparent error in regulatory level should be of great concern. In a recent survey of fish from lakes in the upper peninsula of Michigan, 15% of fish were found to exceed the Michigan state regulatory level of 0.5 ppm, while 60% of lakes contained at least one fish that exceeded this level (Porcella, 1990). This substantiates the assertion that the regulatory level established for water bodies is not stringent enough. It is hoped that the reduction rates obtained here could be used to refine current mercury cycle models.

The experimentally determined reduction rates and information on mechanism may also have implications for regulation based on metal speciation. Reduction and volatilization removes mercuric ions from aquatic environments before they can be methylated and bioaccumulated. The rate at which reduction occurs in the environment should have an impact on the policy-making process based on speciation in the following way: if reduction did occur at the high rates observed when the *mer* operon is induced, more mercuric ions could be allowed in discharges to a water body than if only the basal reductase system is operating. If policy-makers use models with a volatilization flux based on the *mer* operon system,

but in reality only the basal system is actually operating, the result will be an aquatic system in which there is significantly more bioavailable mercury than the regulators intended. Regulators should assume a lower rate of mercury reduction and evasion when establishing emissions levels.

With regard to mercury, regulations would be more efficient and possibly more effective if they were based on speciation rather than total levels. There are significant differences in the toxicity of various mercury species, for example, between Hg^0 and methyl mercury. Different mercuric compounds can even have different bioavailabilities (Mason, et al., 1994). Since organomercurial species are taken up by aquatic organisms, these species should be regulated at very low levels, unless it can be demonstrated that the receiving water body contains high levels of bacteria which are capable of demethylating and reducing these compounds before they are absorbed. Mercuric ions could potentially be released at higher levels in a particular water body if such reducing bacteria were present in sufficient numbers to reduce the mercury before it reaches the anoxic sediments (the particle size to be released is therefore also of concern since it influences the settling velocity and hence the time available for aerobic bacteria to reduce the mercury). Mercury species which are bound tightly to competing ligands and those which bacterial species cannot degrade could be permitted to be released at higher levels since they are not bioavailable.

Regulations specifying individual mercury species may be more cost effective than regulations based on total mercury. For example, the Criterion Continuous Concentration described above specifies that total mercury concentrations be analyzed, and if they exceed a certain level, fish tissue samples must be analyzed. Since performing mercury analyses on fish tissue samples can be quite expensive [$33 for total mercury in aqueous samples versus $69 for tissue samples

(only if it is assumed that all of the mercury found in the tissues is methyl mercury, otherwise, analysis specifically for methyl mercury would be even more expensive); NUS Corporation, 1990], and since so many lakes appear to have contaminated fish (see above), it may be more cost effective to analyze the water samples for reactive mercury species and reduction rates, rather than analyzing the fish.

7. Policy Implementation

In general, the EPA Administrator has the capacity to extend mercury regulations to specific species. For example, under Section 1314 of the Clean Water Act, the Administrator is directed to "publish and revise as appropriate information identifying each water quality standard in effect under this chapter or State law, (and) the specific pollutants with such water quality standard..." [Section 1314 (a)(6)]. Thus the Administrator, in the normal course of revision, could list specific species under the mercury standard. Similarly, for the Clean Water Act effluent standards, "...the Administrator may revise such list and the Administrator is authorized to add or remove from such list any pollutant" [Section 1317 (a)]. Finally, for the hazardous air pollutant list of the Clean Air Act, "(t)he Administrator shall periodically review the list established by this subsection and publish the results thereof, and, where appropriate, revise such list by rule..." [Section 112 (b)(2)].

This more accurate approach based on species bioavailability and cycling could lead to more efficient and more environmentally beneficial regulations. This would come at some cost, however. The EPA would need to dedicate more staff to the issues of mercury cycling and bioavailability. Permit decisions would become even more technical and perhaps take longer (in opposition to the current trend to attempt to consolidate and simplify permit requirements). The dischargers themselves would have to pay higher analytical

monitoring costs. They would have to develop the capacity to perform these analyses in-house, or push their subcontractor laboratories to invest in the appropriate equipment and technical expertise. Despite these issues, mercury regulation based on speciation would be beneficial overall.

8. Overall Conclusions

Most current U.S. mercury regulations are based on total mercury rather than individual mercury species, but models used to establish the regulatory levels do include consideration of individual species. Regulations which specify individual species would be more efficient and effective since certain species are more toxic, more easily bioaccumulate and biomagnify, and are more (or less) able to be reduced. Even without regulation of individual species, improved data on levels and fluxes of these substances and their interactions in the global mercury cycle can lead to improved regulations. For example, regulations which are based upon a global mercury cycling model which includes *mer* operon-based reduction rates will over-predict aquatic evasion fluxes. This leads to over-prediction of the tolerable levels of emissions because the amount of mercury available for methylation and bioaccumulation will be greater than expected with less lost via volatilization.

Laboratory experiments were performed to examine reduction rates and mechanisms at more environmentally relevant mercury concentrations (low nanomolar levels). The results indicated that the *mer* operon system was not responsible for mercury reduction in the environment. The *mer* operon was only induced at mercury concentrations greater than 1.5 nM. Environmental reduction rates are thus lower than would be predicted using *mer* operon-based rates of reduction. These rates of reduction should be incorporated into mercury cycling models upon which mercury regulations are based in

order to improve the accuracy of the models and the effectiveness of the regulations.

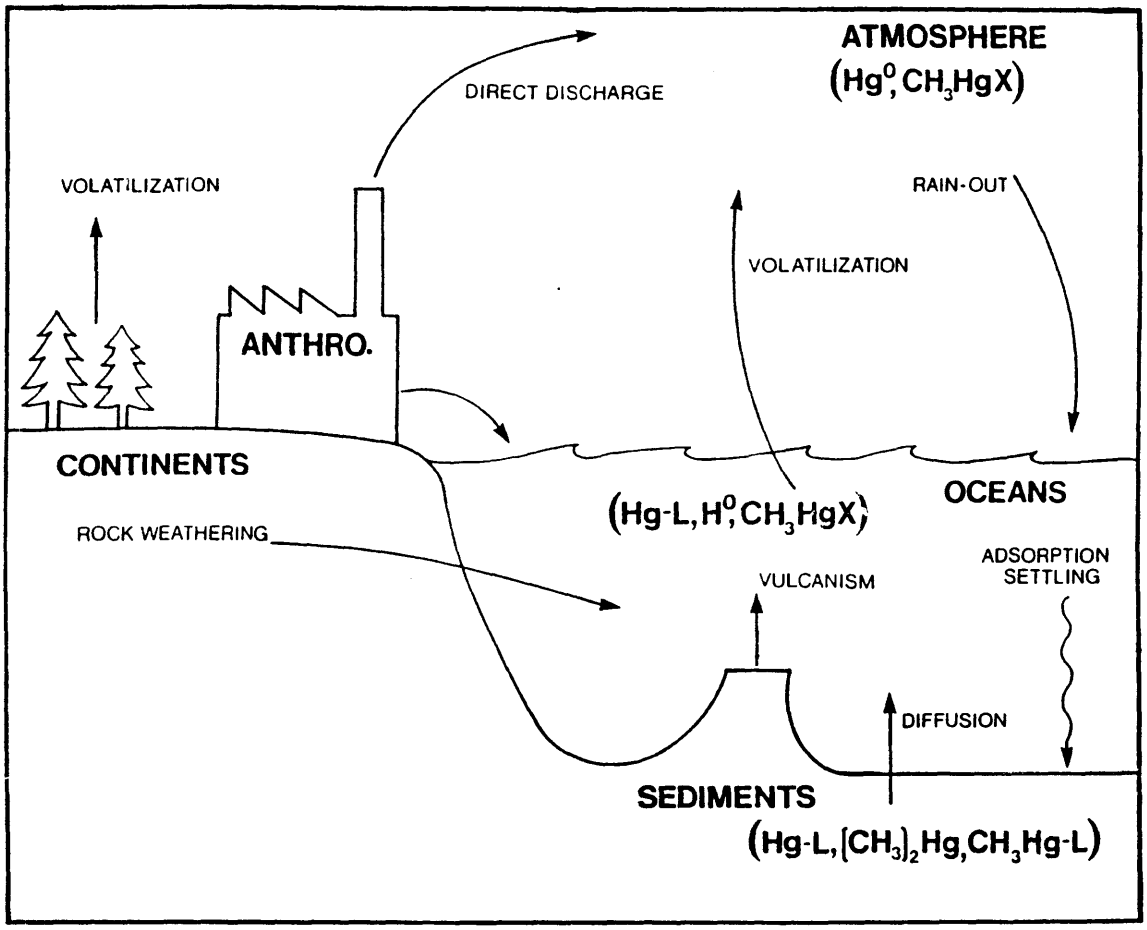

Figure 1 Global Mercury Cycle
(from Brooks Rand sponsored workshop on mercury analysis, Seattle, Nov. 1990)

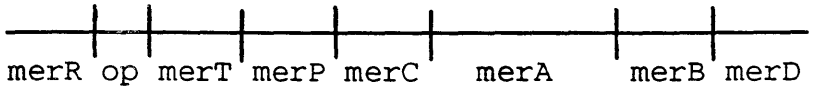

Figure 2 Schematic of *mer* Operon

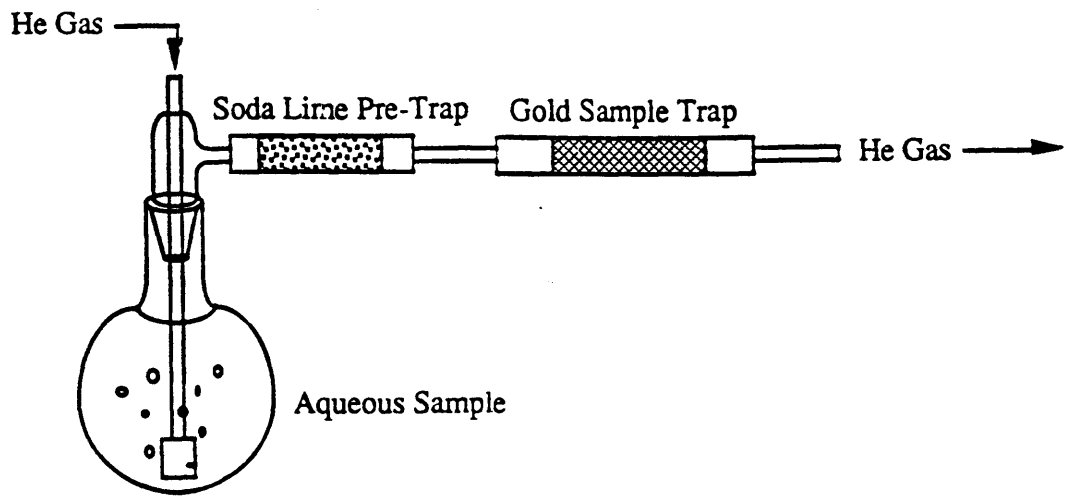

Figure 3 Mercury Collection Apparatus
(from Brooks Rand sponsored workshop on mercury analysis, Seattle, Nov. 1990)

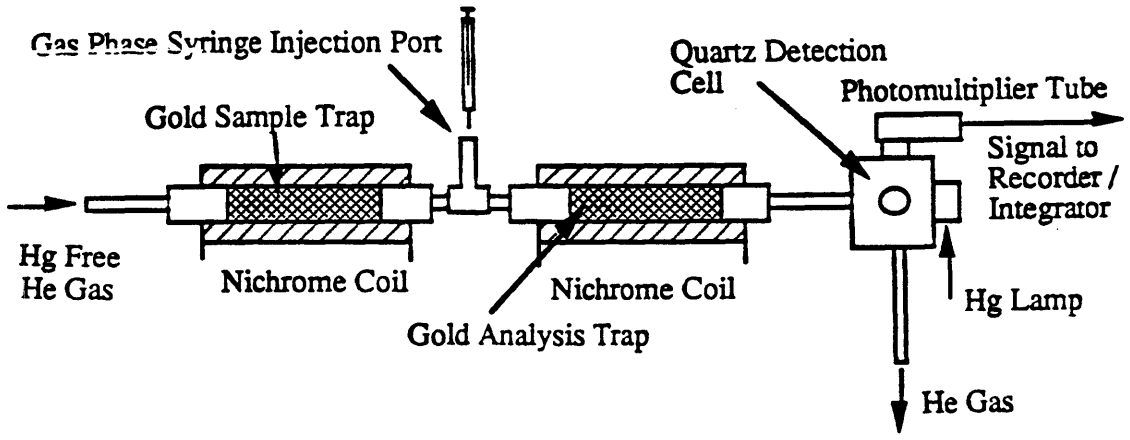

Figure 4 Mercury Analysis Apparatus
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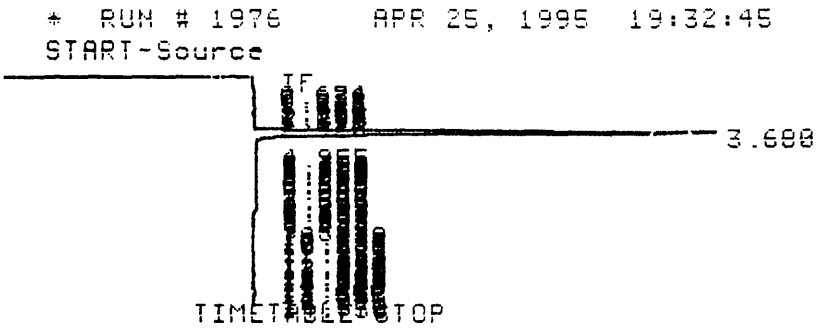


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Figure 5 Sample Chromatogram

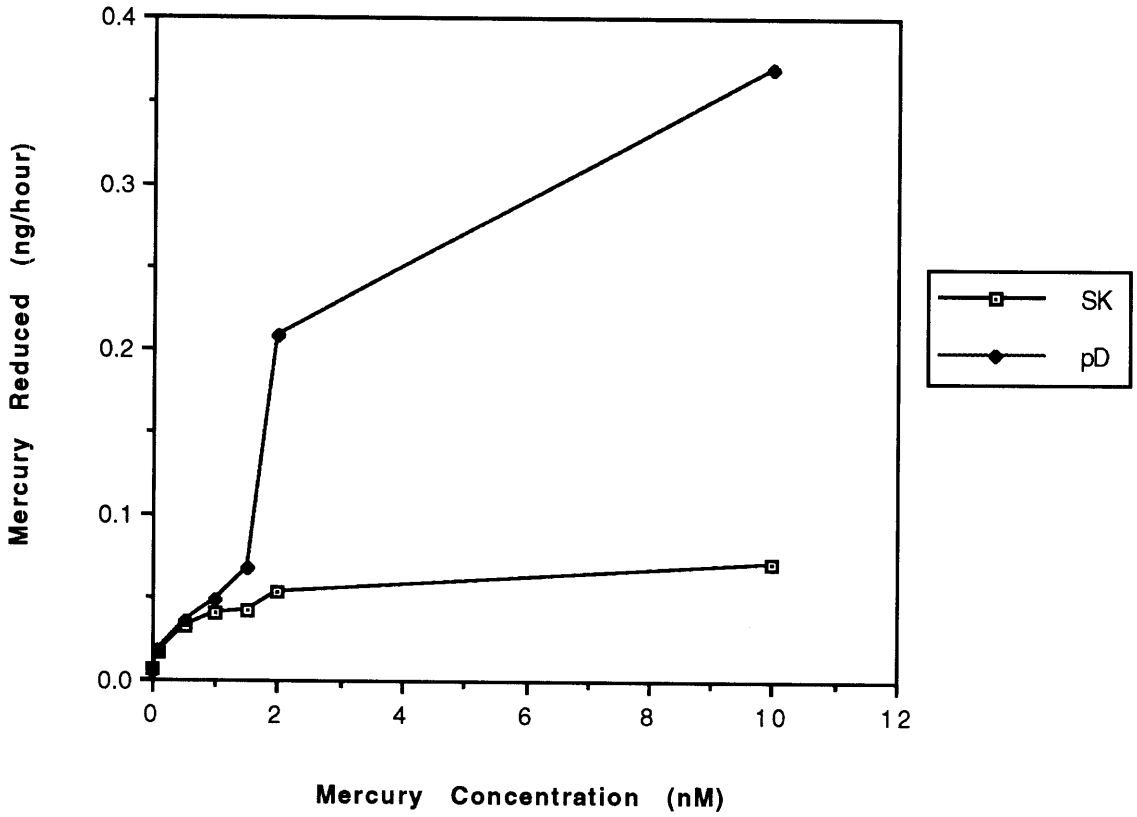

Figure 6 Bacterial Mercury Reduction

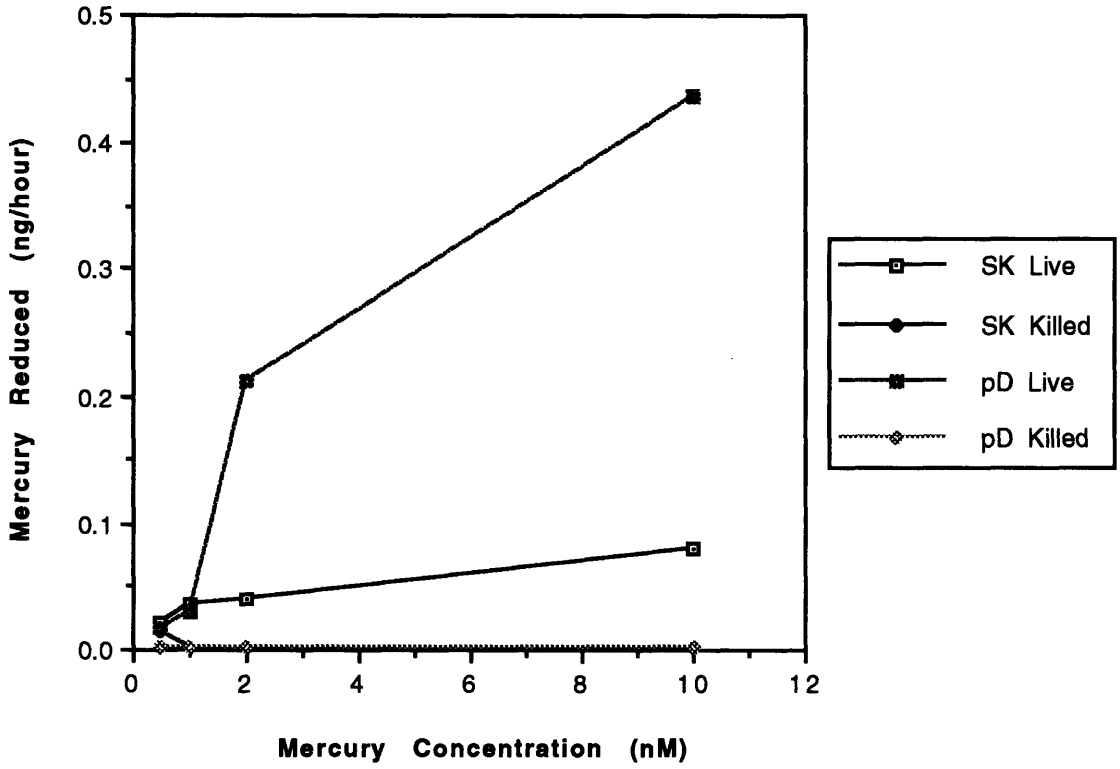

Figure 7 Reduction by Live and Killed Cells

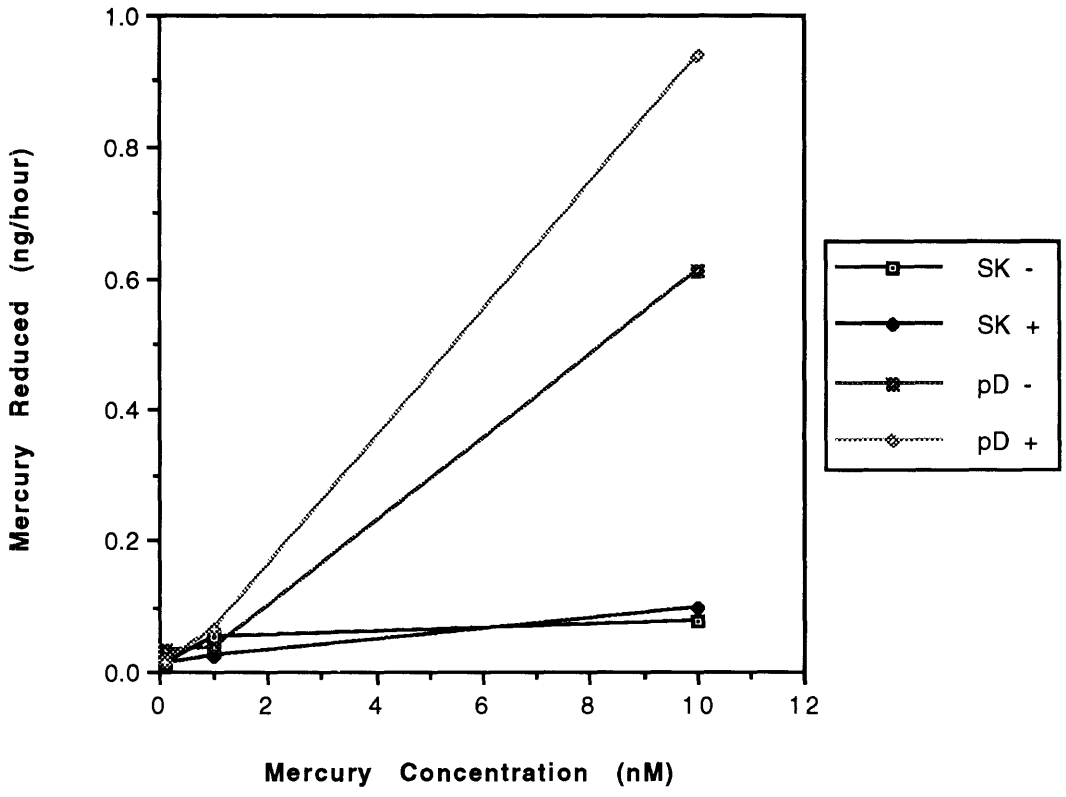

Figure 8 Effects of Competency Prep. on Reduction

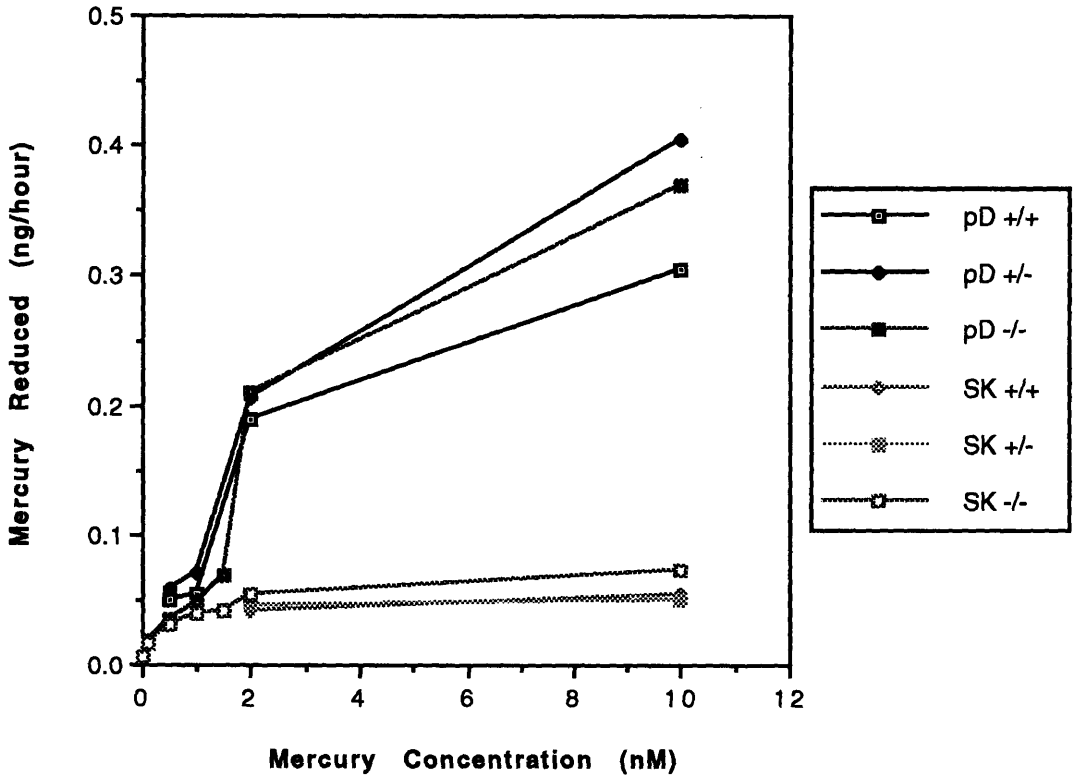

Figure 9 Effects of Antisense Oligo on Reduction

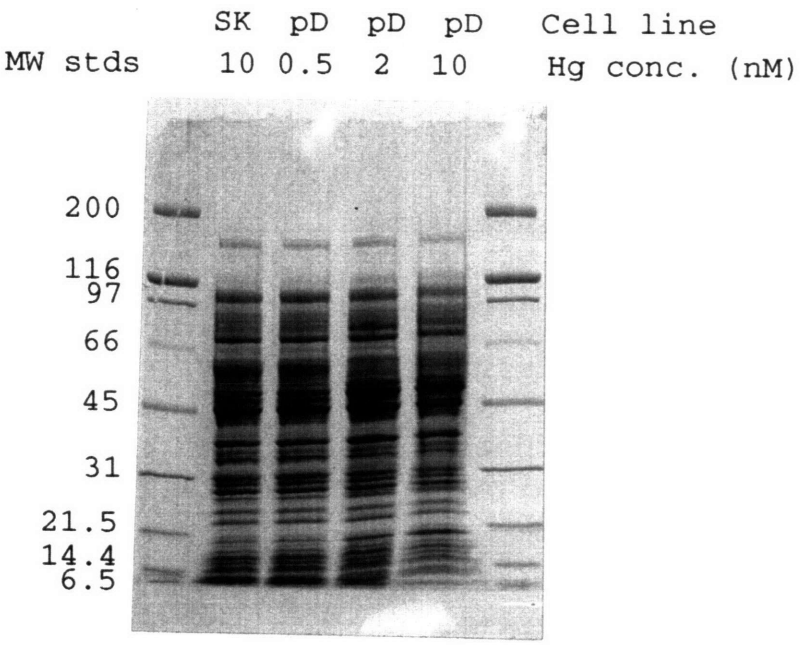

Figure 10 Laemmli Protein Gel

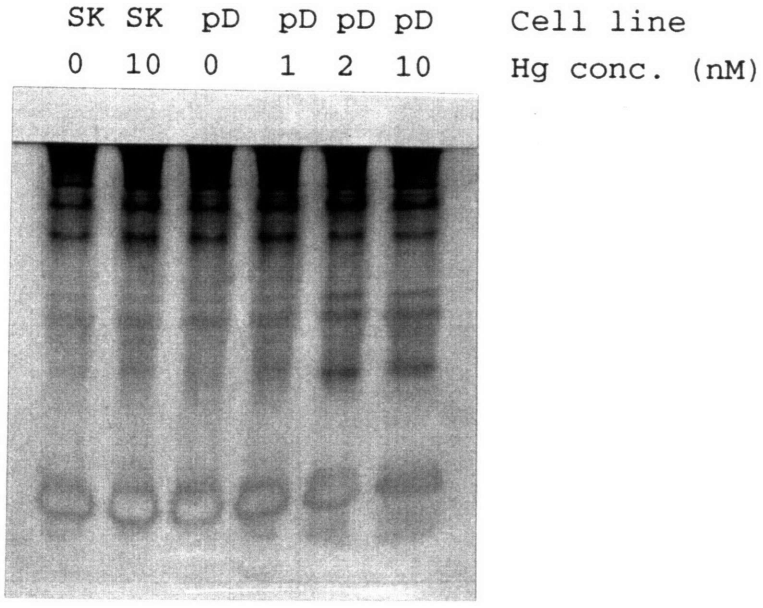

Figure 11 Jovin Protein Gel

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