THE INTERACTION OF ETHANOL AND MERCURY BODY BURDEN
IN THE MOUSE

by

Jan Dotzler Dunn

Submitted in Partial Fulfillment
of the
Requirements for the Degree

DOCTOR OF PHILOSOPHY

Supervised by Thomas W. Clarkson, Ph.D.

School of Medicine and Dentistry

The University of Rochester
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MASTER MASTER

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VITA

On the [REDACTED], the author was born in Tokyo, Japan where he resided until 1959. Prior to coming to the United States in 1966 he attended various American language schools in the Far East. He graduated from Crossland High School in Camp Springs, Maryland and thereafter entered the University of Maryland at College Park in 1967. After a two year leave of absence, his work towards a Biology-Chemistry degree was completed in 1974 at Skidmore College, Saratoga Springs, New York. He began his graduate study in the Toxicology Program at the University of Rochester School of Medicine and Dentistry in September of that year. His research supervisor was Dr. Thomas W. Clarkson.

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ABSTRACT

The interaction of ethanol with mercury in the body resulting in increased exhalation of the metal was studied in the mouse to gain a further understanding of the phenomenon. Through an apparatus designed to quantitate mercury volatilized by unanesthetized experimental animals, a persistent elimination of the metal in the breath was demonstrated after single, sublethal (<1 mgHg/Kg body weight) exposures to mercury vapor (Hg^0) or mercury II chloride (HgCl_2).

The amount of mercury exhaled per unit time was enhanced by oral or parenteral administration of ethanol solutions. These modifications were investigated in dose-response studies in which the drug was administered in doses ranging from 0.2g to 5.5g/Kg to mice pretreated with mercury. The EC_{50} for blood ethanol with respect to mercury exhalation was determined to be approximately 200 mg/dl corresponding to an output rate of approximately 0.1% of the simultaneous body burden in 30 minutes several days after mercury.

A hypothesis that mercury expired by these animals was proportional to the body burden after mercury administration was addressed in experiments whereby mice given one of several doses of mercuric chloride (0.16-500 $\mu\text{g/Kg}$) were monitored for pulmonary mercury elimination for a fifteen day period. The high correlation obtained between the amount of mercury exhaled in a standard time period and the body burden by group indicated that breath sampling could be applied as an indicator of the mercury body burden which may not be limited to the mouse.

In additional studies the chemical form of the mercury exhaled was detected to be mercury vapor by the use of differential adsorbents and administration of an alkyl-mercury compound.

The question as to the source of the mercury emanated by the mouse was studied in vitro. An apparatus suitable for detecting picogram quantities of volatilized mercury was designed and tissue homogenates assayed for mercury volatilizing activity. Liver and kidney suspensions incubated with a cysteine-mercury complex exhibited heat-labile volatilizing activity in the presence of ethanol in the order of 0.12 and 0.19% of the total mercury in the medium per thirty minutes. Fractionated liver homogenate experiments suggested that the highest activity was associated with the 105,000g supernatant.

These results are consistent with a more dynamic nature of at least a portion of the ionic mercury in the mammalian body than previously shown. That ethanol is a metabolic factor that suppresses reoxidation of, and thus effects a net shift of in vivo mercury equilibria promoting mercury vapor exhalation is proposed in an amended model of inorganic mercury metabolism.

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PART I

GENERAL INTRODUCTION

Unusual powers were vested in metallic mercury by man through the ages due to its remarkable properties and appearance. Aristotle wrote of the unusual liquid quality of the metal and the Romans actively mined cinnabar for mercury. The alchemists named the substance after the mythological messenger of the Roman gods and through it toiled to transmute various base metals into gold. It was their belief that the liquid state and the ability of mercury to form amalgams with other metals was an indication of a magical essence of this material which surely held an important key to the transformation process. In these attempts to overcome natural laws, it might be claimed that mercury had a unique spiritual as well as a physical role in the relationship with man in ancient times.

Medicinal uses of mercury compounds date back to Paracelsus and the utilization of calomel as a cathartic and diuretic. Antiseptic qualities of mercury salts were well known and cleansing of laboratory glassware and disinfection of instruments with solutions of these compounds were routine several decades ago (Krantz and Carr, 1949).

Uses in industry of a variety of mercurials exist and the occupational health hazard associated with some of these substances have long been known (MacGregor and Clarkson, 1974). The worldwide chloralkali industry currently consumes a major portion of the 10,000 tons of mercury produced yearly (Berlin, 1977) which is followed by uses in the manufacturing of electrical control equipment, paint and

agricultural products. Minor uses exist in dental practice, scientific laboratories, paper and cosmetic manufacturing processes.

The greatest impetus for toxicological research into the mercurials resulted however when the ecotoxic effects originating from the mercury discharged to the general environment began to be studied in the 1960's. The Minamata episode was a direct consequence of contamination with mercury of a commercially harvested marine environment and pointed to man's paucity of knowledge concerning not only the transformation processes extant in the hydrosphere, but the mechanisms of toxicity in the body which resulted in this serious health problem. Accidental mercury poisoning in other parts of the world have since been documented (Bakir et al., 1973; Stores et al., 1970).

Although it is agreed that the greatest risk arising from environmental mercury at this time is methylmercury (Clarkson, 1971 and Suzuki, 1977), studies addressing the fate of inorganic mercury in the body is essential in terms of the continuing occupational hazard it presents and more generally, in bringing about a greater understanding of the full scope of mercury toxicology.

INTRODUCTION TO THE THESIS PROBLEM

In the realm of inorganic mercury toxicology, metallic mercury (Hg^0) and its ionic forms are pertinent due to their unique characteristics both out of and within the mammalian body. Mercury vapor, a group IIB element, has an outer electronic configuration represented by $5d^{10}6s^2$ and usually shows no oxidation states above II (Cotton and Wilkinson, 1966). An oxidation state of I or II can occur but in the former case the mercurous ion dimerized to Hg_2^{+2} and through a disproportionation reaction yields Hg^{+2} and Hg^0 . However, most common oxidizing agents react with Hg^0 to form Hg^{+2} instead of Hg^+ (Cotton and Wilkinson, 1966) and additionally, in biological media where various ligands are present, complexation or precipitation of Hg^{+2} vastly minimizes the role in the body of Hg_2^{+2} (Toribara et al., 1970).

It has been shown that the affinity of Hg^{+2} for biological ligands such as carboxyl and amino groups are high (Clarkson, 1977) but sulfhydryl groups exceed binding stabilities shown with other ligands under comparable conditions (Rothstein, 1973). Since sulfhydryl groups are estimated to be at an excess even at toxic levels of mercury in the blood (MacGregor and Clarkson, 1974) the high availability of ligands contributes to the virtually complete binding of mercury that occurs in the plasma (Clarkson et al., 1961).

Under physiological conditions the most common forms expected after administration of mercuric chloride are the parent compound, HgCl_3^- and $\text{HgCl}_4^{=}$. Such chloride complexes of mercury readily penetrate

red cell membranes (Weed et al., 1962) but other mobile forms taken up by tissues may be low molecular weight mercaptides since up to 10% of the total thiols in human plasma have been shown to be composed of this form (MacGregor and Clarkson, 1974). However, the exact chemical nature of the mercury associated with these fractions is not known (Suzuki, 1977).

Through occupational health experiences arising from the workers in contact with mercury, exposure to the metallic form was known to result in accumulation of the metal in the body (Neal, 1941). Hughes (1957) reported on the lipophilicity and diffusibility of mercury vapor and thus established a physico-chemical basis for transport across the alveolar membrane and dissolution into the blood stream. However, the consequences of mercury vapor in the body were not entirely clear until work with red cells showed a rapid accumulation and oxidation to the divalent ion upon exposure to the vapor (Clarkson et al., 1961). The high oxygen tension in this medium and the probability of ready exchange with vapors in the lung presented favorable conditions in the bloodstream for the generation of the ion. It was found that after the oxidation process, approximately half of the total uptake under in vitro conditions could be detected in each of the whole blood components, i.e. plasma and red cells. The time course of uptake and ionization was sufficiently rapid to reach mercury blood levels associated with clinical signs of mercurialism within three hours. This also demonstrated why the clinical picture after mercury vapor exposure appeared similar to poisoning with mercuric chloride,

viz. damage to the kidneys. Blood, by rapid chemical oxidation of mercury vapor, was thus considered the major source of the ion responsible for toxic effects after exposure to the vapor.

In the mid-sixties however, evidence came to light which added new dimensions to the mechanism of mercury ionization. First, a critical difference in tissue distribution was apparent after vapor exposure when compared to equal doses of mercuric chloride (Berlin et al., 1966). The brain mercury levels in experimental animals were an order of magnitude higher after mercury vapor dosing than in animals given mercuric chloride. These findings were not immediately explained by the metabolic scheme proposed by Clarkson et al. (1961). Magos (1968a and b) was able to demonstrate by the direct administration of mercury vapor or mercuric chloride into the bloodstream that the vapor was not instantaneously oxidized as evidenced by the loss of mercury through the lungs immediately after mercury vapor but not after mercuric chloride. This was interpreted as the basis for increased uptake by brain in that the highly diffusible, lipophilic, uncharged element could easily cross the blood-brain barrier whereas the ion would do so to a much lesser extent. Moreover, it also furnished an explanation for the higher degree of neurological involvement in poisonings due to mercury vapor versus inorganic mercury salts. The results of these investigations were compatible with the hypothesis that a saturation of the oxidizing capacity in the blood occurred which allowed unoxidized mercury vapor to persist in the compartment consequently leading to higher accumulation of the metal

in the brain.

A second important development was reported by Nielsen-Kudsk (1965a and b) whereby metabolic factors were shown to be involved in the oxidation of mercury vapor. This came about accidentally during studies on the retention of inhaled mercury vapor by volunteers in his laboratory which suggested that alcoholic beverages reduced by about 40% the amount of mercury taken up. From later in vitro studies with methyl, ethyl, isopropyl and n-butyl alcohol it was demonstrated that blood incubated in the presence of various alcohols and exposed to an atmosphere of mercury vapor showed alcohol-dependent decreases approaching 50% of the uptake of the metal in samples without alcohol (Nielsen-Kudsk, 1969). The structure-activity relationship of the alcohols used in the studies suggested a decreasing effect with the length of the aliphatic chain. These observations were interpreted by Nielsen-Kudsk as steric requirements in what appeared to be an enzymatic role in the oxidation of mercury vapor by blood samples. In red blood cells, the most likely system involving alcohol substrates was catalase. Further investigations led to findings that hydrogen peroxide, methylene blue, menadione and ascorbic acid were able to stimulate mercury uptake by in vitro blood samples with adequate glucose in the suspension (Nielsen-Kudsk, 1973). Ethanol was still able to inhibit uptake under these conditions however. Moreover, it was also demonstrated that an inhibitor of catalase activity, 3-amino-1,2,4-triazole (AT) could inhibit the uptake of mercury by liver catalase much like the effect seen with ethanol.

Confirmation of these findings of the interaction with ethanol in intact animals followed. Magos et al. (1973) found by the mercury vapor injection technique that it was the in vivo oxidation step and not an altered permeability of the alveolar membrane by ethanol which accounted for the depressed retention of the vapor in ethanol-treated animals. Furthermore, oxidation of mercury in tissues other than blood was also demonstrated by the concurrent depression of mercury uptake in the lung and heart. Pre-treatment of animals with AT further sustained the hypothesis of a catalase-mediated oxidation of metallic mercury (Magos et al., 1974) in that tissue uptake was inhibited in vivo after vapor inhalation or injection similar to the results obtained with ethanol pre-treatment.

The interaction of ethanol and mercury oxidation in the variety of tissues reported on above is consistent with the ubiquitous nature of the enzyme thought responsible for the ionization reaction. Catalase (EC 1.11.1.6 $\text{H}_2\text{O}_2:\text{H}_2\text{O}_2$ oxidoreductase) is a hemoprotein present in most mammalian cells in compartments identified as peroxisomes or associated with hemoglobin in the red blood cells (Deisseroth and Dounce, 1970). According to Deisseroth and Dounce, although the enzyme has been under study for decades, the physiological role of catalase is still not entirely clear. It is known to decompose metabolically generated hydrogen peroxide by mechanisms which will be discussed below. The similarity in subcellular location to that of glutathione peroxidase may be taken as evidence of catalase having a role as "protector" of intracellular components from oxidizing agents. In

red blood cells the activity of catalase, as well as methemoglobin reductase activity, may be increased upon stimulation of the hexose monophosphate shunt (Smith, 1975).

The molecular mechanisms associated with this enzyme are varied. In an initial reaction the free enzyme reacts with one mole of hydrogen peroxide to form the enzyme-substrate complex (CAT-I). This conformation is relatively stable and undergoes different reactions depending on the presence and the concentrations of other hydrogen donors. The "catalatic" reaction occurs when CAT-I reacts with another mole of hydrogen peroxide yielding water and oxygen resulting in the net destruction of two moles of hydrogen peroxide. In liver cells, this reaction is believed to depend primarily on the steady-state concentration of hydrogen peroxide in the cytosol which regulates diffusion of the moiety into the peroxisomes (Boveris et al., 1972). Within red blood cells, although normal hydrogen peroxide production has not been directly demonstrated (Nielsen-Kudsk, 1973), the catalatic reaction is thought to be influenced by systems competing for hydrogen peroxide.

In the presence of alcohols, CAT-I may react via the "peroxidatic" pathway generating water and the corresponding aldehyde. This is the pathway thought to be involved in the generation of divalent mercury from mercury vapor in the absence of ethanol or other donors. CAT-I decomposition rates in this mechanism have been shown to be dependent on the length of the hydrogen donor carbon chain so that methanol and ethanol are better donors than propanol, butanol and isoamyl alcohol

(Chance, 1948).

There are additional enzyme-substrate complexes described for catalase and the reaction pathways are complex. Catalase compounds II and III have been described which undergo reaction with phenols, sodium nitrite, hydroxylamine or another mole of hydrogen peroxide to yield various products and free catalase or interconvert between the II and II form (Nicholls and Schonbaum, 1963). Additionally, under conditions of a high steady-state level of CAT-I brought about by slowly generated hydrogen peroxide in the absence of a suitable external hydrogen donor, a so-called spontaneous peroxide decomposition can occur by the donation of a hydrogen from an internal apoprotein source (Deisseroth and Dounce, 1970).

The kinetics of the reactions described above are still under investigation (Kremer, 1975; Deisseroth and Dounce, 1970). However, no evidence has been presented that indicates a free-radical involvement in any of the pathways.

Whereas ethanol results in a depletion of CAT-I by the peroxidatic mechanism, AT has been shown to inactivate catalase with respect to mercury vapor metabolism in another way. Tephly et al. (1961) concluded that the free enzyme was not the moiety affected by the presence of the inhibitor. Their studies of the enzyme in rat tissue and crystalline beef liver catalase demonstrated that only when hydrogen peroxide was generated at a sufficient rate, either by administration of methylene blue in erythrocytes or adding xanthine oxidase or

hypoxanthine, could AT lead to an irreversible inhibition of the enzyme activity. Interestingly, various alcohols including methanol and ethanol were shown to antagonize the deactivation of catalase by AT when incubated simultaneously. These investigators concluded that CAT-I formation was a prerequisite step for the AT interaction to occur. Deisseroth and Dounce (1970) have since then claimed formation of a covalent bond between AT and a histidiny residue in the deactivated enzyme complex.

The relationship of ethanol to catalase activity and mercury oxidation is further complicated by the fact that the availability of ethanol for the peroxidatic pathway is normally influenced by other oxidizing systems which compete for alcohol. This is true for most mammalian tissues and especially the liver where the majority of the ethanol is processed via the alcohol dehydrogenase (ADH) pathway (Goldstein et al., 1970).

The possibility of a third system capable of oxidizing ethanol has been reported. Evidence for a NADPH-dependent microsomal ethanol oxidizing system (MEOS) came to light with the observation of smooth endoplasmic reticulum (SER) proliferation after ethanol (Lieber and DeCarli, 1968; Lieber, 1970) and is now considered a general mammalian response to ethanol administration (Lieber, 1973). However, it has been recently claimed that traces of catalase activity contaminating the microsomal preparation may have been responsible for MEOS activity (Teschke et al., 1976). The current equivocal state of the role of

the MEOS pathway can be summarized as follows (Lieber, 1973):

- (1) microsomal ethanol oxidation can be inhibited by carbon monoxide;
- (2) carbon tetrachloride pre-treatment may or may not lead to differences in ethanol metabolism by microsomal preparations;
- (3) SKF525A does not inhibit ethanol oxidation;
- (4) requirement of NADPH for ethanol oxidation can be abolished by adding a hydrogen peroxide generating system;
- (5) AT produces smaller changes in microsomal oxidizing activity than catalase activity; pyrazole in vivo inhibits catalase in addition to ADH;
- (6) substrate-like binding to P-450 has not been shown;
- (7) chronic ethanol leads to hyperplasia of SER in rats and is associated with a 70-80% increase in ethanol oxidation;
- (8) catalase-free microsomes from acatalatic mice exhibit drug metabolism but do not oxidize ethanol;
- (9) microsomal solubilization with deoxycholate followed by separation by column chromatography leads to isolation of a NADPH-dependent ethanol oxidizing system.

Thus it is still unclear at this time whether there is one or two

alternative systems besides ADH for processing ethanol in the liver. However, the product in either case is acetaldehyde and also involves NADPH and oxygen as shown in the summary presented below. The interrelationship of the various factors influencing non-ADH ethanol oxidation is as follows (Lieber, 1973):

Although the proportion of ethanol oxidized by the respective systems have yet to be settled, it has been speculated by Lieber (1973) that very high levels of ethanol may lead to an increased fraction of the ethanol in vivo being processed by the non-ADH pathway.

Further understanding of the interplay of several metabolic factors modifying the oxidation of mercury vapor in the body and additional support for the role of catalase in the biotransformation have accumulated recently. Ogata and Ikeda (1978) have shown that a mutant strain of acatalasemic mice and its tissues took up less Hg^0 in vitro and in vivo compared to the wild-type mice. The only exception was mercury levels in the liver which were markedly elevated after in vivo exposure. Halbach and Clarkson (1978) were able to show that catalase complex I and not the molecular oxygen evolved in the reaction

could account for the high rate of oxidation of mercury vapor. Their work also demonstrated a 1:1 stoichiometry between the molar ratios of mercury oxidized and exogenous hydrogen peroxide consumed in experiments with human red cells in vitro. Inactivation of glutathione peroxidase (GSH-Px) with iodoacetate led to increased uptake of mercury vapor over uninhibited samples. This showed that GSH-Px was not involved in the oxidation process but normally competed with catalase for hydrogen peroxide thereby suppressed mercury vapor uptake.

As briefly mentioned above, a puzzling aspect of studies dealing with the inhibition of the biotransformation of mercury vapor was the apparent inconsistency demonstrated in liver tissues. An increasing uptake by this organ after pre-treatment with an agent lowering mercury levels in other tissues was first reported by Magos and his co-workers (Magos, 1974) and later confirmed by others (Ogata and Ikeda, 1978; Kha t, 1977; Leach et al., 1975; Clarkson et al., unpublished observations). In addressing this point, Magos et al, (1978) demonstrated whereas in most other tissues the rate-limiting step with respect to mercury vapor oxidation was hydrogen peroxide combining with catalase, the situation present in the liver was different. The exogenous addition of hydrogen peroxide to liver homogenates was without effect on the uptake of mercury vapor in their in vitro system. Only when very dilute liver homogenates were exposed did a correlation become apparent between mercury uptake and catalase activity. These workers concluded that the rate-limiting step in this organ was the availability of mercury vapor at the intracellular sites of oxidation. Increased

mercury levels associated with ethanol or AT pre-treatment or acatalasemia could be rationalized as a consequence of biochemical and pharmacokinetic factors extant in vivo thus resolving the paradox.

Critical elements in mercury vapor metabolism in the body were summarized as: (a) the concentration of catalase in the tissue; (b) the availability of hydrogen peroxide; and (c) the availability of mercury vapor.

The general model for the metabolism of metallic mercury thus proposed that mercury vapor was rapidly oxidized in not only the blood but in most tissues of the body. The ionization reaction may not be rapid enough under certain conditions of exposure however to prevent mercury vapor from crossing the blood-brain barrier and undergoing oxidation in that organ. CAT-I mediated the critical step of forming the divalent ion and the reaction was subject to modification by catalase substrate availability and the presence of inhibitors. Specifically, ethanol interacted at this initial point of exposure to inhibit the biotransformation of mercury vapor through competition for the active site of the peroxidatic mechanism. This inhibition by ethanol was suggested as providing dramatic protection against the consequences of administering lethal concentrations of mercury vapor to experimental animals (Leach et al., 1975). It remains to be clarified however, the nature and sequence of the two electron transfer from mercury vapor and the reasons behind the apparent high affinity of the uncharged element for the enzyme complex.

Mercury body burden acquired either by exposure to the vapor or injection of inorganic salts of mercury was known to be eliminated mainly by the urine and feces (Rothstein and Hayes, 1960; Hayes and Rothstein, 1962; Rothstein and Hayes, 1964). However, the loss of mercury from the animals in these studies was not fully recovered in the excreta. This point was investigated by Clarkson and Rothstein (1964) with rats administered mercuric chloride and subsequently assessed for volatilization of mercury. It was shown by these workers that mercury could be eliminated via the pulmonary route but the nature of the relationship of this process to other routes of mercury excretion under their protocol appeared minor. It remained unclear as to whether microbial volatilization of radioactivity from excreta and/or in vivo reduction was responsible for the mass-balance discrepancy in the earlier metabolism studies.

From the viewpoint of future developments these observations by Clarkson and Rothstein were critical in several respects. It was the first report dealing with a possible reduction pathway of ionic mercury in mammals and demonstrated that the metal could be eliminated by the lungs. The results obtained with anesthetized rats suggested that the elimination was a perfusion-limited phenomenon, viz. only a severe depression of the breathing rate led to decreases in the metal eliminated via an endotracheal tube. Although the amounts of mercury involved in this regard were small, it was indicative of a possible re-evaluation of the mercury metabolism model.

Another unpredicted effect was discovered in experiments dealing

with human volunteers. Subjects exposed to low levels of radiolabelled mercury vapor were seen to exhale radioactivity immediately after administration which rapidly declined with time (Hursh et al., 1976). Interestingly, consumption of an alcoholic beverage by one particular volunteer 2 days after the exposure led to a ten-fold increase in the mercury exhalation rate. This unexpected effect was not readily explained in view of the fact that at times as long as 48 hours after exposure, the vapor administered should have been entirely oxidized. It was unclear at that time whether the amounts exhaled with or without ethanol was simply dissolved vapor that escaped oxidation or derived from oxidized mercury. In either case the mechanism of the ethanol effect was puzzling for the metabolic model of mercury does not predict an interaction of ethanol at this relatively late stage after acquisition of a mercury body burden.

The thesis work presented below was initiated to test the accepted metabolic model. Specifically it was designed to determine the reproducibility of the observation made on the single volunteer and to differentiate the ethanol effect on the exhalation rate either as an interaction with a pool of unoxidized mercury vapor or occurring at some other point in the metabolic sequence or both.

PART II: IN VIVO STUDIES

Section A: Characterization of Mercury Exhalation and the Ethanol Effect.

Overview: Utilizing an apparatus designed to detect mercury volatilization, mice exposed once to ^{203}Hg as either mercury vapor, mercuric chloride or methylmercury chloride were determined to persistently exhale mercury after a period of several days. In each case a single treatment of up to 5.5g/kg body weight of ethanol elicited increases in the amount of mercury eliminated by exhalation in a standard period of time and the chemical form was detected as metallic mercury vapor. The dose-response relationship of ethanol was investigated in mice administered a standard dose of mercuric chloride.

To detect possible influences of ethanol on redistribution or elimination of the mercury body burden, ethanol was administered continually in another experiment while whole body burden and excretion of mercury were monitored. Significant differences in these parameters were not observed.

The results obtained in this section indicated that a fraction of the ionized inorganic mercury in the body was subject to reduction to the uncharged elemental form which could then be mobilized to other sites in the body. Ethanol elicited up to 8-fold increases in the amount of mercury exhaled under these experimental conditions but the fraction of the body burden eliminated in this manner was minor compared to other routes of excretion.

MATERIALS AND METHODS

(1) Apparatus. Exhaled mercury was detected with a specially designed apparatus shown in Figure 1. This comprised a polypropylene chamber modified from an ordinary one-liter reagent bottle (Sybron Corp., Rochester, NY) rendered airtight with a No. 15 rubber stopper. Holes were drilled through the stopper for an air intake, exhaust and a manometer probe. Fifty cc disposable syringes (Pharmaseal Co., Glendale, CA) were modified by sawing off the needle seat, enlarging the remaining hole and reducing the overall length of the barrel to approximately 9 cm. Each barrel housed one mouse; this was accomplished by inserting the plunger with the shaft sawn off and gently pushing the mouse through until the head was completely outside this 'capsule'. The diameter of the opening (0.5 cm) was too small to enable the mouse to wriggle out, but in each case the animal and apparatus were carefully examined to ensure unrestricted breathing as well as proper containment of the mouse. Five such capsules at a time were secured in the chamber in each experiment and, before the chamber was sealed with the stopper, distilled water was introduced inside the chamber to a predetermined mark below the opening of the capsules. This reduced the interior volume to approximately 200 cc (with the stopper in place) and had the effect of having only the heads of the mice in the chamber. The entire sealed chamber was then placed in a water bath at 29°C to maintain a constant interior water temperature.

This temperature setting was arbitrary and based on the assumption that 29°C is well within the range of environmental temperatures in

Figure 1. The exhalation chamber and accessory apparatus with arrows indicating direction of airflow. Only two mice are shown for clarity.

^{203}Hg collection apparatus

which the mice could maintain homoiothermy (Bernstein, 1966). Furthermore, as the mice were generally dry throughout the experiment, thermoregulation probably was not a greater problem for them than it would have been under non-experimental conditions with or without ethanol.

When the apparatus was operating, mercury-free air was drawn into the chamber headspace and exhausted into an impinger containing Hopcalite (Mine Safety Appliances, Evans City, PA). Hopcalite has been described as an effective adsorbant of mercury vapor (Clarkson and Rothstein, 1964). The Hopcalite impinger was placed in a sodium iodide crystal well counter (Magos et al., 1973), thus allowing continuous recordings of mercury accumulation. A flowmeter connected to a vacuum pump enabled air flow to be controlled precisely at a rate of one liter per minute and the slight negative pressure created in the chamber (0.02 inches of water) was monitored continuously with the manometer.

Before the experiment, this mercury collection system was checked for recoveries of known amounts of radioactive mercury vapor generated from $^{203}\text{HgCl}_2$ (Magos, 1968a). The average recovery was $87.4 \pm 6.5\%$ (mean $\pm$ SD, $n=11$).

(2) General Protocol. Male and female mice in these studies were of the CBA/J strain (Jackson Laboratories, Bar Harbor, ME) with body weights ranging from 25-32 grams. Experimental animals were whole-body counted for radioactivity using a spectrometer with an adequate well size for this purpose (Model 3002 TriCarb Spectrometer, Packard Instruments, La Grange, IL). On several occasions lead shields were used

to decrease the efficiency of this counter and appropriate standards were also counted in this configuration at these times. The whole body counts obtained just prior to exhalation tests were defined as the instantaneous body burdens (BB_i). In calculating the percent body burden exhaled per time, the sum of the body burden of the mice in the chamber was utilized in the following formula:

$$\frac{\overline{BB}_i \text{ Exhaled}}{\text{Time}} = \frac{1}{\text{Time}} \left[\frac{M}{\sum_{k=1}^n (BB_i)_k} \times 100 \right] \text{ for } 1 < n \leq 5 \text{ mice}$$

where BB_i = amount of mercury in a mouse

M = amount of mercury detected on filter

$\overline{BB}_i$ = mean percent instantaneous body burden

Immediately after dosing with ethanol or water, the mice were usually placed in the exhalation chamber for a thirty minute period although some tests were of 6 hours duration. Some groups of mice were sacrificed in the chamber by ether (Mallinckrodt Inc., St. Louis, MO) introduced through the air intake hose. Other groups of mice receiving continual, single or no ethanol treatments were sacrificed by cervical dislocation at the end of the experiments and selected tissues taken for mercury determination. Tissue samples were counted using an automated spectrometer (Auto-Gamma Model 578, Packard Instruments, La Grange, IL).

Radioactive decay was taken into account in all calculations and

standards were used to cross-calibrate between gamma counters where necessary. All isotopes were obtained from New England Nuclear (Boston, MA) and specific activities were adjusted with cold mercury compounds (Sigma Chemical Co., St. Louis, MO). Mercury injection volumes were 0.2 to 0.4cc/per mouse intraperitoneally. Food and water were available ad libitum throughout these experimental periods except for the time the animals were undergoing exhalation tests. Ethanol solutions (EtOH) administered in these experiments were either of 12% or 25% (v/v) concentration diluted from a 95% stock solution (Pharmacy Grade) with distilled water or normal saline and injected on the basis of the weight of ethanol (density = 0.79g/ml, Handbook of Chemistry and Physics, 1974-75). No differences in vivo of the vehicle used in conjunction with ethanol administration or the two concentrations of ethanol used were observed with respect to mercury exhalation.

Blood samples drawn at times other than sacrifice were obtained by capillary tubes carefully inserted into the post-orbital venous plexus. When serial samples were needed blood was drawn from both plexi alternately utilizing disposable 20 microliter heparinized tubes (Drummond Scientific Co., Broomall, PA).

(3) Inorganic Mercury Experiments. Either mercury vapor or mercuric chloride labelled with ^{203}Hg was administered to the experimental animals. Mercury vapor was generated according to the method of Sugata et al. (1976) and ten mice were exposed* on day 0 for approximately

*Average chamber concentration over the exposure period was 0.22 mgHg/m^3 .

30 minutes in the apparatus described therein. The mercury dosage received was estimated to be $9.7 \pm 0.5 \mu\text{g/kg}$ using the formula for retained dose. Four hours later and once daily thereafter each mouse was whole body counted. Two days after mercury, half of the animals were selected randomly and given 5.5 gm/kg ethanol p.o. and tested for mercury exhalation. The remaining animals served as controls receiving a comparable volume of distilled water before the exhalation test. On day 5 the control group was tested for exhalation with ethanol after serving as its own control.

In parallel experiments, mice given 0.1, 0.5, or 1.0 mgHg/kg radiolabelled mercuric chloride on day 0 were treated several days later with none or one of several ethanol dosages. For comparison to vapor experiments, twenty mice were dosed with ethanol or water p.o.. The usual route of administration of ethanol or saline was via interperitoneal injections. For studies dealing with continual dosing of ethanol, injections were at 0, 2.0, or 4.0 gm/kg/day for 7 days with urine and feces collected over 24 hour periods. Excretions were collected by placing absorbent paper beneath wire-mesh cages and separating feces prior to counting strips of the paper in a spectrometer. This enabled estimation of the mercury eliminated in the urine apart from fecal matter for each treatment group.

(4) Organic Mercury Experiment. ^{203}Hg -labelled methylmercuric chloride was administered at a dosage of 0.5 mg Hg/kg to five mice on day 0. Prior to injection, the isotope was purified by acidification

and extraction into benzene (Norseth and Clarkson, 1970). The isotope in the benzene phase was back-extracted into the aqueous phase and an aliquot taken for making up the injection solutions. This solution containing the isotope and unlabelled-methylmercury was checked for inorganic mercury contamination by the cold atomic absorption method described by Magos and Clarkson (1972). The inorganic mercury level in the sample did not exceed blank values for this method.

At various times up to day 7 after injection, these mice were assayed for exhalation with or without 2 gm/kg ethanol i.p.. Tissues were taken after serial sacrifices during the experiment and total mercury determined. Aliquots were then analyzed for inorganic mercury content using the method of Clarkson and Greenwood (1970).

(5) Breathing Rate Determinations. Mice treated according to standard exhalation testing procedures as described above but not placed in the exhalation chamber were observed in order to determine breathing rates after 0, 2.0, or 5.5 gm/kg ethanol up to two hours post dosing. Using a stopwatch, the number of breaths taken by a mouse was counted for one minute at various times after ethanol administration. These animals were not administered mercury.

(6) Blood Ethanol Determinations. Serial whole blood samples were taken at various times after ethanol injection and analyzed for ethanol according to the gas chromatographic method of Wilkinson et al. (1975). A Perkin-Elmer Model 900 gas chromatograph (Perkin-Elmer Instruments, Norwalk, CT) equipped with a flame ionization detector

was used. Details of this method are presented in Appendix I.

(7) Differential Adsorbents of Volatile Mercury. The exhaust air line from the exhalation chamber was modified on several occasions (as shown in Figure 1) to permit the chemical characterization of the exhaled mercury. A cellulose acetate filter (Model HA-045, Millipore Corp., Bedford, MA) was included to detect particulates $>0.47 \mu\text{m}$ (rigid diameter) in the airstream. A special absorbent system consisting of gold-plated glass beads and silver-covered diatomaceous earth* in series was used to supplement the Hopcalite filters used normally. According to Braman and Johnson (1974), this configuration allowed separation of organic mercury of the dimethylmercury class from the free metal, Hg^0 . After each exhalation determination, the filters were individually counted for radioactivity.

* These special adsorbents were kindly supplied by Dr. T. Mercer, Department of Radiation Biology and Biophysics, University of Rochester.

RESULTS

(1) Mercury Volatilization in Mice Given Inorganic Mercury.

(a) Single Ethanol Administrations. Volatilized mercury was detected two and five days after a single exposure to mercury vapor. Ethanol administration led to increases approaching eight-fold the amount of mercury eliminated after treatment with water as shown in Figure 2. Injection of mercury chloride led to similar results in regard to volatilization. Figure 3 shows the effect of ethanol on mercury exhalation three days after injection of 0.1 mg or 1.0 mg/kg mercury where again more than an eight-fold increase over control (water-treated) values in four hours were seen. Also shown in the illustration is the cessation of mercury accumulation on the filter after in situ etherization of the mice in the apparatus. This demonstrates two important points. First, volatilization required live animals because the emanation of mercury which may possibly occur from the fur or skin could not be detected from the radioactive carcass. Secondly, if significant amounts of mercury were being volatilized from the excreta, continued accumulation of mercury might be expected following the death of these animals. This was not observed even after one hour of continuous airflow.

The possible contribution of volatilization from excreta was rechecked in another way. Mice given 1.0 mg/kg mercuric chloride and ethanol as described above were kept in the chamber for six hours at which time the mice were removed and the capsules containing accumulated

Figure 2. Mercury accumulation on the filter after comparable times spent by mice in the chamber with or without ethanol. The shaded figures represent ethanol-modified rates at times after mercury vapor as shown on the abscissa. The ordinate scale gives the ratio of post-ethanol to control (water) exhalation of radioactivity.

EFFECT OF ETHANOL ON MERCURY EXHALATION

Figure 3. Accumulation of radioactivity from the exhalation chamber on the Hopcalite filter, plotted with time for different treatment groups consisting of five mice each. ■ ; 1.0 mg/kg mercury and 5.5 gm/kg 25% ethanol; ▲ ; 0.1 mg/kg mercury and 5.5 gm/kg 12% ethanol; □ ; 1.0 mg/kg mercury and 0.25 ml water; △ ; 0.1 mg/kg mercury and 0.25 ml water. Arrows indicate time when ether was introduced into the chamber through the air intake hose.

excreta were quickly replaced in the apparatus for one hour. As presented in Figure 4, no further increase in the filter counts were detected.

These observations indicated that the radioactivity released by the experimental animals reflected pulmonary elimination of mercury. This conclusion was consistent with reports of mercury in air sampled via endotracheal tube from rats given HgCl_2 (Clarkson and Rothstein, 1964) and from mercury exposed volunteers exhaling into a collection device (Hursh et al., 1976). Although the saliva and/or the buccopharyngeal tissue could not be rejected as possible contributory sources of volatile mercury in these experiments, published evidence supported a major role of the lung in this phenomenon.

A simple explanation for the increased output of mercury from the lung was the possible influence of ethanol on the breathing rates of these animals. The results of visual estimates made of the post-treatment breathing rates are given in Figure 5 and showed no large differences to account for the amounts of mercury eliminated. These estimates of breathing rates were within the range of values given in the literature for mice (Schaffer, 1965). The assumption that the tidal volume remained unchanged after the various treatments was not experimentally verified so that it was not possible to completely rule out changes in pulmonary function being responsible for the ethanol effect. However, it was unlikely that only changes in this parameter would explain the many-fold increases detected in the elimination of mercury.

Figure 4. Recording of mercury accumulation on the filter originating from the apparatus with and without mice. The arrow at six hours indicates the point when the airflow was stopped and the mice removed from the capsules. These capsules containing excreta were then replaced in the apparatus and the airflow resumed for approximately one hour (shown on expanded time-scale).

RECORDING OF Hg ACCUMULATED ON FILTER WITH
AND WITHOUT MICE.

Figure 5. Breathing rates after treatment with ethanol or saline.

The mean breathing rates $\pm$ SD of four mice in each treatment group are plotted with time after dosing.

Control animals received saline. , 2g/kg EtOH;

, 5.5 g/kg EtOH; , Controls.

COMPARISON OF BREATHING RATES AFTER TREATMENT

The ethanol dose-effect relationship was studied in mice given 0.5 mg/kg mercuric chloride. Increasing doses of ethanol led to graded increases in the mercury exhaled as presented in Figure 6. Significant increases above controls were observed with 0.5 gm EtOH/kg up to 5.5 gm/kg. With the highest doses of ethanol administered the variability increased and more than doubling the dose from 2 gm/kg produced smaller increases than at lower doses.

From recordings of the accumulation of mercury from the exhalation apparatus, the kinetics of mercury elimination were obtained from several ethanol treatment groups and summarized in Figure 7. Generally, the intermediate ethanol doses (0.2 and 0.5 gm/kg) elicited exhalation rates that were not constant over the experimental period whereas the rates for control mice and those administered 2 gm/kg ethanol or more were linear with time. These increased rates were apparent immediately after placing ethanol animals in the chamber (less than five minutes after injection) but diminished with time even with the highest doses used in these studies. No elevated mercury exhalation rates were detected 8 hours after 5.5 gm/kg ethanol.

Analysis of the blood taken for ethanol determination after various doses gave the family of curves shown in Figure 8. Time up to 60 minutes post-intraperitoneal administration of ethanol was included to aid in the evaluation of the slope over experimental time periods. With 5.5 gm/kg, the data suggest that the absorption phase was not complete until ten to twenty minutes after administration although the exhalation rates were in the accelerated state almost

Figure 6. Ethanol dose-response results. Doses are given on the abscissa with percent instantaneous body burden exhaled in thirty minutes on the ordinate. Control mice received saline. The results are expressed as mean $\pm$ SE of at least four experimental observations consisting of five mice per experiment. These mercury exhalation tests were performed approximately five days after 0.5 mg Hg/kg injection of HgCl_2 .

* Significant increase over control values ($P < 0.05$) Student's t-test.

DOSE-EFFECT RELATIONSHIP OF ETHANOL AND MERCURY EXHALATION

Figure 7. Accumulation with time of radioactivity on Hopcalite after different ethanol treatments. Either saline or one of the ethanol doses listed next to the symbol was administered immediately before mercury exhalation tests. The average amounts ($\pm$ SE) of mercury exhaled with time in three experiments are represented for the various ethanol groups. Mercury was administered approximately five days prior to these determinations.

KINETICS OF MERCURY EXHALATION

Figure 8. Blood ethanol with time after various doses. Blood concentrations are given as mg ethanol/dl of whole blood. Symbols and error bars represent mean $\pm$ SD of three to eight animals unless otherwise noted. $\blacklozenge$, 5.5 g/kg; $\blacktriangledown$, 2.0 g/kg; $\blacksquare$, 1.0 g/kg; $\bullet$, 0.5 g/kg; $\blacktriangle$, 0.2 g/kg. Earliest determination was at ten minutes after injection.

immediately after ethanol.

An upper limit to the ethanol exhalation rate can be approximated from the data presented above. Both the kinetics of mercury exhalation and ethanol in the blood have been evaluated at ten minute intervals. Thus the ten minute change in the cumulative percent body burden exhaled can be plotted for the corresponding mean blood alcohol concentrations after various doses. This is shown in Figure 9 where the reciprocals of the exhalation rates were plotted against reciprocal blood ethanol concentrations. The estimated maximum mercury exhalation rate in vivo under these experimental conditions was 0.06 percent/ten minutes, or, extrapolated to the standard exhalation period, 0.18% per 30 minutes. Taking this value as 100% response, the log EtOH concentration-response graph shown in Figure 10 illustrated that the data were consistent with the usual sigmoid shape of drug interactions in the body (Goldstein et al., 1974). The half-maximal effect under these conditions was shown to be elicited at a blood ethanol level of 215 mg/dl.

(b) Continual Ethanol Administration. The transient nature of the effect of ethanol on the increased rate of mercury elimination was observed in single dose studies. It was of some interest at this point to test the effect of a multiple dose regimen in order to determine the extent of the influence of ethanol on the pharmacokinetics of the metal. Mice pretreated with 0.5 mg Hg/kg as mercuric chloride were monitored with respect to whole body burden, fecal and urinary mercury elimination, in addition to tissue levels of mercury to detect

Figure 9. Mean blood ethanol concentrations were related to mercury exhalation rates with ethanol for 10 minute intervals after injection of the alcohol doses. The reciprocals were plotted and the best fit for the line was obtained by least-squares regression. Maximum exhalation rate was calculated to be $0.06 \frac{\%BB_1}{\text{per ten minutes}}$.

DOUBLE RECIPROCAL PLOT OF Hg EXHALATION
AND BLOOD ETHANOL CONCENTRATION

Figure 10. Blood ethanol concentration - response relationship.
The 100% response value was estimated from the curve in Figure 9. Blood ethanol levels are plotted on a logarithmic scale. $EC_{50} = 215$ mg/dl under these experimental conditions.

ETHANOL CONCENTRATION-PERCENT RESPONSE RELATIONSHIP

possible differences under various ethanol treatments. Exhalation was checked in these groups only occasionally since the main purpose was to determine effects of ethanol on parameters other than volatilization.

The whole body burden curves obtained from these experimental animals are shown in Figure 11 for mice receiving saline or 2 gm/kg/day ethanol for seven days starting on day two after mercury. No significant differences could be shown at the end of the experimental period despite the fact that the ethanol-dosed animals were transiently exhaling mercury at a higher rate than controls.

As regards differences in fecal or urinary output of mercury under these conditions, no clear dose-related effects could be shown for animals receiving zero, 2.0 gm or 4.0 gm/kg ethanol. This is summarized in Figure 12 where 24-hour excreta were determined for total mercury content over the period of continual ethanol dosing two to eight days after mercury. An initial effect of higher amounts of mercury eliminated via the urine with ethanol was seen but not sustained throughout the experimental period.

In Figures 13 A and B, the distribution of mercury in selected organs from control and ethanol-treated mice are shown. With single administrations of 5.5 gm/kg ethanol prior to sacrifice, no difference in the fraction of the body burden found in the organs from control (saline) values could be seen five days after mercuric chloride. In mice given 2 gm/kg ethanol per day for 15 days, the mean body burden

Figure 11. Whole body counts of animals given ethanol or saline once a day for seven days starting two days after a single administration of mercuric chloride. Each symbol represents the mean ($\pm$ SD where given) daily CPM on a logarithmic scale for each group of six mice. Arrow indicates start of the treatment regimen. No differences by group could be shown using the Student's t-test. $\bigcirc$, saline injections; $\blacksquare$, 2gm/kg EtOH.

EFFECT OF CONTINUAL EtOH ON BODY BURDEN Hg

Figure 12. Mercury excretion with continual ethanol treatment. 24-hour excretions for six mice are shown for different ethanol treatment groups. Daily saline or ethanol administrations began on day 2 (arrow) and continued for one week. Urine volumes were not measured. ○, Saline; ◇, 2g/kg EtOH, ■, 4g/kg EtOH.

URINARY AND FECAL MERCURY OF MICE RECEIVING ETHANOL

Figure 13. Tissue mercury levels with and without ethanol 5 or 15 days after mercuric chloride. All mercury in tissues are expressed as percent body burden at sacrifice. In graph A, the shaded bar represents tissue mercury from mice receiving a single 5.5 gm/kg injection of ethanol; the open bar represents saline-injected controls. In graph B, tissues from mice administered multiple ethanol treatments (2 gm/kg/day for 15 days) are compared to tissues from mice given a single administration of ethanol at that dose as represented by open figures. The mean $\pm$ SE of four mice per treatment group is given. No significant differences could be shown for the tissue pairs in either graph using the Student's t-test.

EFFECT OF ETHANOL ON TISSUE MERCURY

found in brain and liver suggested an increase over cohorts receiving only a single 2 gm/kg ethanol injection over the same time period. However, differences were not statistically significant.

The tissue data from continual ethanol mice are consistent with a hypothesis that mobilization of small amounts of mercury occurs at some internal loci and eventually exhaled. It is compatible with suggested increases in mercury levels to theorize the entry of metallic mercury into the bloodstream where it may be transported to liver and become ion-trapped. The increase suggested for brain mercury after multiple ethanol treatments is also consistent in this respect. It leads to an interesting speculation that the mechanism of long half-times associated with this organ as a whole with respect to mercury elimination could be the continuous uptake of diffusible mercury in the blood released at some distal point in the circulatory system. This "scavenging" activity could conceivably establish a mercury flux at the blood-brain barrier resulting in net lower elimination rates for this tissue. Several studies have shown non-uniform mercury distribution in various parts of the brain (Berlin et al., 1966; Berlin et al., 1969; Cassano et al., 1969) which may be indications of on-going mercury uptake from the blood.

Figure 14 summarizes the occasional exhalation tests performed during this series of experiments primarily to confirm that the ethanol effect was still observable. Interestingly the exhaled fraction of the body burden did not seem to vary greatly at day four

Figure 14. Results of exhalation tests with animals receiving continual treatments of either 2 g/kg ethanol or saline. Five mice out of a treatment group of six were selected randomly on these occasions without determining individual identities. Estimates of the amount exhaled were made from recordings of the accumulation of mercury with time. Shaded bars, exhalation with saline; open bars, exhalation with ethanol.

EXHALATION OF MERCURY WITH MULTIPLE ETOH TREATMENTS

compared to day nine whereas the whole body burden had dropped in that time by a factor of nearly two. These unexpected observations were checked and follow-up studies are reported in detail below (Part II, Section B).

(2) Mercury Volatilization After Organic Mercury. This experiment was performed to determine whether or not organic mercury could be eliminated in expired air. When mice were given methylmercury at comparable doses to that administered in the inorganic mercury experiments described above, virtually no exhalation of the metal was detected at times when inorganic Hg-mice were observed to volatilize mercury. Table 1 gives the results of exhalation tests done on methylmercury mice with or without ethanol. It can be seen that although the percent instantaneous body burden exhaled generally increased with time after the single mercury administration, the quantity differed from inorganic-treated mice. This is best illustrated by comparing Table 1 to Figure 6. On day 4 or 5 after methylmercury, the exhalation rate brought about with 2 gm/kg ethanol was about 0.8 to 1.0×10^{-2} percent of the total mercury body burden. For inorganic mercury mice, that same dose of alcohol elicited an average rate of 9.0×10^{-2} percent body burden. On the last day shown (day 7) the remaining methylmercury mouse exhaled only 15% of the rate exhaled by inorganic animals despite the fact that due to the longer biological half-time of methylmercury, the whole body content of mercury was higher at this time. These findings did not support the hypothesis that methylmercury chloride was the identity of the mercurial being exhaled.

TABLE 1

MERCURY EXHALATION AFTER METHYLMERCURY

Time after CH_3HgCl (Days)	Mean Bodyburden ^(a) (μg total Hg)	EXHALATION (b)	
		No Ethanol	With Ethanol ^(c)
0	13.5	----	----
0.1	13.5	<0.01	----
0.7	13.0	<0.01	----
1.0	----	<0.01	----
2.0	12.2	0.02	0.28
3.0	11.2	0.05	0.43
4.0	10.4	0.06	0.82
5.0	9.3	0.10	0.95
6.0	8.6	0.22	0.94
7.0	7.9	0.43	1.51

(a) Initial dose to each mouse = $13.5 \mu\text{g Hg}$ ($\approx 2.6 \times 10^6$ CPM at efficiency of exhalation spectrometer)

(b) $\frac{\% \text{BB}_t}{100} \times 10^2$; thirty-minute exhalation tests.

(c) 2.0 gm/kg , i.p.

However, since methylmercury is known to be slowly degraded to inorganic mercury (MacGregor and Clarkson, 1974; Suzuki, 1977) the observations of the exhalation rates might be consistent with the release of inorganic mercury from the parent compound which then may be volatilized. To check this possibility tissues taken from the methylmercury mice were analyzed for inorganic mercury content by determining the stannous chloride-reducible fraction in the homogenates. The results are presented in Table 2 where it can be seen that there is a general trend toward higher inorganic fraction being found in the samples with time. It might be expected from this that the inorganic mercury content of whole animals passed through a maximum within the seven day experimental period as a result of elimination and bio-transformation of methylmercury. However, the exhalation data suggest no such peak in exhalation; rather it is consistent with the increasing inorganic mercury content of the kidneys which may have increased in total mercury content over this time. Available information regarding the biotransformation kinetics (Norseth and Clarkson, 1970; Landry, 1978) generally supports the contention that conversion of methylmercury to inorganic mercury precedes volatilization.

(3) Identity of Exhaled Mercury. When particulate and differential adsorption filters were utilized in characterizing the form of mercury exhaled, radioactivity was trapped only on the material selecting for mercury vapor in exhalation tests after mercuric chloride or methylmercury. No attempt was made to detect the form after mercury vapor administration. Table 3 summarizes the findings when various

TABLE 2

PERCENT INORGANIC MERCURY IN TISSUES AFTER METHYLMERCURY^(a)

TIME TISSUE	DAY 1		DAY 3		DAY 7	
	% Inorg.	Total Hg ^(b)	% Inorg.	Total Hg	% Inorg.	Total Hg
Liver	12.9	1.22	19.0	1.26	40.4	0.89
Kidney	3.4	0.72	7.6	0.66	14.0	0.75
Muscle	2.6	0.27	1.7	0.19	4.1	0.15
Stomach	3.8	0.11	2.7	0.09	10.2	0.02
Brain	3.3	0.07	3.9	0.06	7.0	0.05
Lung	2.2	0.22	3.7	0.18	7.3	0.06

(a) Determinations on tissue samples obtained from one to two mice. Recoveries = $89\% \pm 6$ ($\bar{X} \pm \text{SD}$) with 10-20% homogenates spiked with $^{203}\text{HgCl}_2$.

(b) Total Hg expressed as $\mu\text{g Hg/gm}$ sample (wet wt.).

TABLE 3

RADIOACTIVITY IN DIFFERENTIAL ADSORBENTS^(a)

→ DIRECTION OF AIRFLOW FROM CHAMBER →

Mice treated with:	Particulate	Silver	Gold	Hopcalite
HgCl ₂ + Saline	-	+	-	-
HgCl ₂ + EtOH	-	+	-	-
MeHg + None	-	+	-	-
MeHg + EtOH	-	+	-	-
HgCl ₂ + SnCl ₂ (chemical reduction)	-	+	-	-

(a) Counts obtained when filters were counted are indicated by +, >> background; -, background radioactivity.

treatment groups were tested under different conditions. No counts were ever trapped on the particle filter. Ethanol treatments had no effect on the site of mercury adsorption and since no counts above background were detected downstream from the silver adsorbent, the radioactivity was shown to be entirely trapped in a single pass.

Braman and Johnson (1974) have used similar configurations to differentiate between various volatile mercurials in simultaneous samples. The silver filter, placed upstream from the gold or Hopcalite filter, was shown in their studies to have a very low capacity to extract dimethylmercury in the airstream. When present in the air, this form of mercury was leached off downstream within 5 minutes of sampling at ambient temperatures. Under the conditions described in Table 3 where sampling was continuous for thirty minutes, dimethylmercury would be expected to pass downstream in that time.

Evidence was also presented by Braman and Johnson for the low efficiency of silver to trap monomethylmercury chloride. The previous section of this report indicated that a high methylmercury body burden did not elicit as high an exhalation response as inorganic mice. Blood mercury levels after the organomercurial are at least an order of magnitude greater than after inorganic mercury at comparable times (MacGregor and Clarkson, 1974) and organomercurials exhibit less stability than the divalent ion when complexed to biological ligands. Despite these conditions the estimated exhalation rate was an order of magnitude less with methylmercury. Thus considering the results

obtained with the differential adsorbents it was concluded that the intact methylmercury compound was not the form of radioactivity volatilized from these mice.

Mercury vapor is efficiently trapped by silver, gold or Hopcalite. Chloride complexes of mercury expected in the serum ($\text{HgCl}_4^{=}$ and HgCl_3^{-}) probably will not diffuse readily across the alveolar septum from the blood due to the intrinsic charge on the compound. Mercuric chloride may be present in the bloodstream but consideration of the relative partial pressures of this compound compared to mercury vapor in aqueous solutions (0.1 μbar vs. 2.66 μbar respectively at 26°C ; Toribara et al., 1970) indicate a much higher probability of mercury vapor diffusing out into the lung airspace. Therefore, it was reasonable to conclude that mercury vapor was exhaled in these experiments after administration of mercurials in mice. In the body, physico-chemical properties of the uncharged metallic form would probably allow the highest mobility of all the possible chemical forms of mercury in biological material.

DISCUSSION

Exhalation is a persistent but minor route of mercury elimination in mice. Without ethanol the "basal" rate of volatilization can be calculated to be roughly 1% of the mercury in the body per day. For this approximation, a static body burden over a 24 hour period was used. The dynamic relationship between the mercury exhalation rate and the change in body burden as a function of time and initial dose of mercury is addressed in detail in Section B, Part II. Whole body elimination rates in these experiments can be derived from the biological half-times (≈ 6 days) observed from day 3 or 4 to day 10 where a single exponential term best describes the mercury retained in the mice with time. This loss rate, 11.6 percent of the mercury per day, includes the contribution to the loss by the exhalation route. Thus as a fraction of the total eliminative process, exhalation accounts for a little over 8 percent per day.

Similar calculations can be performed for the mean exhalation rates observed with 2 gm/kg ethanol. Should this rate be sustained over a 24 hour period it can be expected to lead to a decrease in the biological half-time of HgCl_2 in these animals from 6 to 4.5 days for the interval under discussion. However, the transience of the ethanol effect precludes this potential; virtually continuous administration (infusion) would be required for therapeutic application.

Moreover, the experimental evidence suggests that although such radical procedures could be of theoretical interest, it may lead to significant redistribution of mercury within the body and cause a higher accumulation of the metal in the brain. Of more practical interest in the interaction of these agents is that exhaled mercury provides a useful indication of mercury in the body; the role of ethanol would be to amplify the output of mercury per unit time.

A reduction pathway must exist because the free metal is released after ionic mercury injection and thus confirms the findings of Clarkson and Rothstein (1964). Whereas the release from depot stores of unoxidized mercury vapor is a likely explanation for exhalation of the metal in vapor exposed animals, this mechanism does not explain exhalation after mercuric chloride or methylmercury treatment. Moreover, the kinetics of mercury released by the animals in these experiments are not consistent with a preformed bolus of mercury vapor being liberated; it is seen rather as a constant generation of vapor with time. Mercury exhalation has been detected only minutes after injection of mercuric chloride (Section B) and in vitro studies (Part III) have shown sequestered vapor need not be invoked to explain increased volatilization with ethanol.

The apparent increase in the rate of Hg^{+2} reduction due to ethanol finds support in studies dealing with the metabolism of ethanol. Ethanol

oxidation is known to shift the redox state of hepatocytes through accumulation of reducing equivalents in the cytosol (Lieber, 1973). It may be that under electron-rich conditions in the microenvironment, the mercuric ion increasingly acts as the electron acceptor for cytosolic NADH generated via the alcohol dehydrogenase (ADH) pathway.

Ethanol has been discussed as a substrate of the catalase peroxidatic reaction. It may be that through competition for active sites ethanol is able to prevent reduced Hg^{+2} from undergoing re-oxidation. The net effect may be manifested in the increased elimination of mercury by exhalation.

The methylmercury experiments reported here suggest that an inorganic mercury "pool" can be formed from organomercurials and may be subject to exhalation as shown in the schematic below:

Where R-Hg^+ is an organomercurial such as methyl- or phenylmercury.

Thus exhalation of mercury vapor might be detected after administration of a variety of mercury compounds if analytical methods are not limiting. Calibration of exhalation rates and known rates of biotransformation may possibly yield quantitative information on total and inorganic mercury burden as well as initial dose.

Some of the mechanisms and loci of mercury vapor generated in vivo will be discussed in a later section (Part II). At this point however, studies on tissue distribution and content after mercury have indicated several candidates. The kidney, because of the high affinity of mercury to this organ, is considered a likely source. The liver cannot be ruled out because of the tissue mass and variety of xenobiotic transformations that are known to occur in this organ, and should be considered. Kinetics of mercury uptake in the blood following i.p. injections show that whereas the mercury concentration in the blood reaches a peak in approximately 10-15 minutes after administration, exhalation does not reach a maximum for 2 or 3 days. Blood therefore, is probably not a major contributor to the pool of exhaled mercury.

Section B. Application of Exhaled Mercury as an Indicator of Body Burden.

Overview. Historically, there existed considerable interest in obtaining a reliable, non-invasive index of inorganic mercury exposure (Neal et al., 1941; Bidstrup et al., 1951). Recent reports dealing with animals and human studies confirm the limited usefulness of urinary mercury for diagnostic purposes (Nakayama et al., 1977; Friberg and Nordberg, 1973; and El-Sadik and El-Dakhakhny, 1970). Rothstein and Hayes (1960) concluded that even under carefully controlled laboratory conditions urine and fecal elimination rates of mercury from their experimental animals did not show a good correlation. After a single mercuric chloride injection, the excretion was seen to be dependent on dose, time after dose and route of administration.

Novel approaches to quantitating exposures to a number of metals have been reported which utilize samples of saliva (El-Sadik and El-Dakhakhny, 1970), nails (Hislop et al., 1977; Suzuki, 1977), milk (Bakir et al., 1973), hair (Berglund et al., 1971; Birke et al., 1972) and sweat (Lovejoy et al., 1973; Putman, 1972). Breath sampling may be another source of information in regard to mercury exposure that is available quickly and routinely without the need of invasive techniques.

In the preceding studies (Section A) a persistent exhalation of mercury in mice after a single exposure to either mercury vapor, ionic mercury (Hg^{+2}) or methylmercury chloride was demonstrated and ethanol

administration after mercury led to graded increases in the amount of mercury eliminated via this route. Those experiments were designed to elucidate the ethanol interaction within several days after mercury but in long-term multiple-dose experiments, it was discovered that mice given mercuric chloride seemed to exhale a constant fraction of their body burden. Ethanol administration at these times elicited many-fold increases in mercury exhalation similar to the effects seen several days after mercury. It was of interest to determine specifically if mercury exhalation with or without ethanol was proportional to the instantaneous body burden. Clarkson and Rothstein (1964) found after mercuric chloride injection that no correlation of exhaled amounts to the body burden could be shown after the first day nor was there a clear relationship of exhaled metal to the amount excreted by their experimental animals.

The hypothesis of proportional exhalation was tested by administering a wide range of initial mercuric chloride doses and then monitoring exhaled mercury in addition to whole body burdens. Ethanol was administered once near the endpoint of the experiment at a standard dose to all animals. Tissue samples were taken of various organs to determine mercury content and the relation of the concentration to mercury exhalation.

MATERIALS AND METHODS

Twenty-five mice were divided into five groups. On day zero each group received ^{203}Hg -labelled mercuric chloride in doses over a thirty-fold range listed in Table 4. One of the groups receiving 500 μg Hg/kg (Group 5) was administered 2 gm/kg ethanol (25% solution) per day intraperitoneally. Other groups received no treatment after the mercury injection until the final day of the experiment. All mice were assayed for mercury exhalation by group and individually whole-body counted at least once daily for the duration of the experiment starting immediately after the mercury administration. On the last day, all groups were given saline injections prior to their daily exhalation determinations. No more than 1.5 hours later, the procedure was repeated with ethanol whereby each mouse received a single injection of 2.0 gm/kg from a 25% EtOH solution and immediately placed in the exhalation apparatus.

Following the final exhalation measurement, blood samples were taken for ethanol determination by gas chromatography. Mice were then sacrificed by cardiac puncture under ether anesthesia and various organs and tissues were excised for gamma-counting.

TABLE 4

EXPERIMENTAL PROTOCOL FOR SECTION B

Group #	1	2	3	4	5 ^(a)
Hg Dose (μ g/Kg)	17	125	250	500	500

(a) Group 5 received continual ethanol (2 gm/kg/day).

RESULTS

(1) Single Ethanol Dose Groups. The mercury burden decline for each of the dose groups except Group 5 is presented in Figure 15. The elimination curves when analyzed* gave two exponential components: an initial rapid phase with an average half-time of 1.36 days (range 1.09 to 1.52 days) and a slower component characterized by a 5.90 day half-time (range 5.70 to 6.07 days). The variations observed between groups showed no trends related to initial mercury doses.

Minutes after the injection of mercury, some radioactivity was detected to be exhaled by all dose groups. The kinetics of the mercury eliminated at these times was of a mixed nature in that some groups exhibited linear accumulation of radioactivity in the filter while others showed increasing slopes over the 30 minute test period. However, all groups exhaled less at this time than on day one. All basal exhalations from this point forward in time were linear with respect to the accumulation of mercury during test periods. In Figure 16, the amounts of mercury detected on the filters by group are shown as $\overline{\%BB}_i$ respectively for the entire experimental period. Despite large differences among the groups in the body burden over time, the basal exhalation rates expressed in this manner showed approximately a two-fold

* Individual mercury retention curves were plotted and data for the slow component was fitted by a least-squares regression [linearized form of $y = a(\exp [bx])$]. The fast component was likewise computed from values derived from the observed whole body burden at time t and y for $x = t$. A general solution to the equations describing the mercury retention with time was: Body burden $(t) = C_1 \exp(-\lambda_1 t) + C_2 \exp(-\lambda_2 t)$.

Figure 15. Whole-body retention of mercury as fraction initial dose for groups receiving single ethanol injections. To obtain the curves drawn here, semi-logarithmic plots of daily whole-body mercury (time on linear scale) were resolved into exponential components using the "curve stripping" method. Best fits for the curves were computed by regression (see footnote p. 74) and the half-time values were used to generate the lines shown (normalized to fraction of mercury dose). The three points schematically represent the fast and slow components derived in this manner for each dose group. ○, Group 1; ●, Group 2; □, Group 3; ■, Group 4.

WHOLE BODY MERCURY ELIMINATION WITH TIME

Figure 16. Exhalation of mercury as percent body burden for Groups 1 through 4. The amounts exhaled in 30 minutes by different dose groups are plotted as percent of their respective instantaneous body burden. The earliest time of assay was approximately five minutes after injection of mercuric chloride.

DAILY MERCURY EXHALATION

difference. Non-parametric analysis of $\overline{\%BB}_i$ from day zero to day 15 (without ethanol) showed no evidence of discontinuous populations in these observations.* Averaged rates for the groups are presented in Table 5.

The relationship of the absolute amount of mercury exhaled in 30 minutes to the absolute amount retained in the body at respective times can be approximated linearly over most of the experimental period as shown in Figure 17. The selection of the time points (day 4 to day 15) used in this analysis was subjective. A straight line regression was not a good estimate for all of the data points obtained from day zero onward. However, the time after mercury that these divergent points represented were all early determinations made within four days after injection. The rationale for omitting these points was that an initial lag-time existed for mercury exhalation to reach terminal rates as shown in Figure 16. The low rate of exhalation at these times may be reflecting the distribution phase of mercury in the body and/or the rapid phase of clearance of the metal. The first possibility is supported by reports on the kinetics of mercuric chloride distribution in mice (Berlin and Ulberg, 1963) and rats (Rothstein and Hayes, 1960). They suggest rapid shifts in the relative mercury content of organs occurred up to two to three days post-dosing with the kidneys accumulating a higher proportion of the mercury left in the body after this time. In conjunction with these events was the simultaneous rapid phase of mercury elimination seen in these mice. This component

* Kruskal-Wallis one-way analysis of variance by ranks.

TABLE 5
COMBINED BASAL EXHALATION RATES

Days after HgCl ₂	$\overline{\%BB}_i \times 10^2$ (a)
0	0.27 ± 0.11
1	1.74 ± 0.71
2	2.16 ± 0.66
3	2.51 ± 0.61
4	2.60 ± 0.66
5	2.80 ± 0.47
6	3.06 ± 0.32
7	2.96 ± 0.44
8	2.84 ± 0.44
9	2.81 ± 0.50
10	2.58 ± 0.32
11	2.46 ± 0.37
12	2.27 ± 0.11
13	2.15 ± 0.23
14	2.20 ± 0.34
15	2.31 ± 0.34

(a) Values given are the means ± SD of Groups 1, 2, 3 and 4.

Figure 17. Correlation of mercury exhalation with body burden mercury. The linear relationship between the absolute amount of mercury (ng) exhaled in thirty minutes by different dose groups on the respective body burden (ng Hg) over the experimental period day 4 to day 15 was obtained by a least-squares regression. The correlation indicated significance at the 1% level (df = 44) for the data. For further explanations see text.

RELATIONSHIP OF MERCURY EXHALATION TO GROUP BODY BURDEN

has cycled through at least two half-times and the influence on the whole body elimination rate is less prominent by day four. It is entirely possible that the exhalation rates more accurately reflect whole body burden in the slower phase of mercury elimination seen in these experiments. If this assumption is made, deletion of data up to the arbitrarily chosen fourth day after mercury reduces the observations to a simple linear relationship. Deletion of data after this day does not dramatically change the correlation.

Saline given on day 15 as control injections for subsequent comparison to post-ethanol exhalation rates had no obvious effects on the basal exhalation rates. The administration of ethanol however, led to increases in all mercury dose groups.* In Table 6, the findings on day 15 are summarized. The basal exhalation rates exhibited by the groups ranged from 1.9 to $2.4 \times 10^{-2} \overline{\%BB_i}$. With 2 gm/kg ethanol, net rates increased from 8.8 to $18.1 \times 10^{-2} \overline{\%BB_i}$. Group 1 yielded the highest increases under these conditions (radiochemical parameters for this day are given in Table A-1, Appendix). It is noteworthy that although the variability in the exhalation rate among the groups increased after administration of ethanol compared to basal values, it was still less than the differences in the body burdens detected for that time.

No significant differences between groups could be shown for

* $P < 0.001$, Student's paired t-statistic.

TABLE 6

MERCURY EXHALATION ON DAY 15

Group	Mercury Exhalation Rates ($\overline{\%BB_i} \times 30 \text{ minutes}^{-1}$) $\times 10^2$		Mercury Instantaneous Body Burden ($\bar{X}$ ng Hg)	Blood Ethanol ^(a) Concentration (mg/dl)
	Without Ethanol	Net With Ethanol		
1	2.4	18.1	23.9	290 $\pm$ 36 (n = 4)
2	2.3	8.9	329.3	245 $\pm$ 32 (n = 4)
3	2.7	9.0	555.0	254 $\pm$ 20 (n = 5)
4	1.9	8.8	1065.0	263 $\pm$ 12 (n = 4)
Range of Values	1.9 - 2.7	8.8 - 18.1	23.9 - 1065.0	245 - 290 ^(b) (± 10) , (± 18)

(a) $\bar{X} \pm$ SD of blood ethanol approximately 40 minutes after injection

(b) NS. Fisher's One Way Analysis of Variance

blood ethanol levels listed in Table 6*. However, Group 1 had the highest mean concentration of ethanol detected and although no explanation for the higher blood level can be given, it is likely that this was the basis for the higher ethanol exhalation rate in this group.

(2) Continual Ethanol: Group 5. Mice receiving 2 gm/kg ethanol on a daily basis were determined to have whole body burden with time as illustrated in Figure 18. The estimated half-times were 0.8 days and 5.0 days for the rapid and slow phases respectively. The variability in this parameter was higher in comparison to their counterparts receiving the identical mercury dose but only a single ethanol injection. It suggests an interaction may have occurred upon virtual simultaneous administration of mercury chloride and ethanol which possibly affected absorption and/or distribution of the injected mercury.

Exhalation for this group, shown in Figure 19, was not as expected. Whereas a response to ethanol was seen from day zero, there was an increase with time, or cumulative ethanol dose, of the exhalation rate with ethanol over the experimental period. Several observations made of basal exhalation rates prior to the daily ethanol doses showed no large deviation from Group 4 however.

At sacrifice, the body weights of these mice were not significantly different from those in Group 4 as shown in Table 7. However, liver weights appeared to be less in Group 5 and yet contained a slightly

* $P > 0.05$, F-test

Figure 18. Whole body burden with time found in mice receiving ethanol from day zero are shown as the mean $\pm$ SD. The initial mercury dose was 500 μ g/kg which was administered several minutes before 2 gm/kg EtOH. For details see Materials and Methods, Section B.

MERCURY BODY BURDEN CURVE FOR GROUP FIVE

Figure 19. Exhalation rates with and without ethanol in Group 5. Ethanol at 2 gm/kg per day was started immediately after mercuric chloride. Basal rates, where determined, were taken prior to the ethanol injection for that day. (●) ethanol rate; (▲) basal rate.

DAILY MERCURY EXHALATION BY GROUP FIVE

TABLE 7

COMPARISON OF GROUP 5 TO GROUP 4 AT SACRIFICE^a

PARAMETER	GROUP 5	GROUP 4
Body Wt. (g)	26.6 ± 1.71 ^{NS}	27.8 ± 1.27
Liver as Percent of Body Weight	5.55 ± 0.32 ^{NS}	6.02 ± 0.35
Percent Whole Body Burden in Liver	20.3 ± 4.62 ^{NS}	15.0 ± 3.71
Percent Body Burden in Brain	1.51 ± 0.30 ^{NS}	1.25 ± 0.10
Percent Body Burden in Lungs	0.57 ± 0.05 ^{NS}	0.54 ± 0.13
Percent Body Burden in Kidneys	33.0 ± 10.61 ^{NS}	31.93 ± 5.05

^a Each group was comprised of 4 mice; values given are means ± SD.

^{NS} No significant difference between groups by Student's t-test ($P > 0.05$).

larger proportion of the body burden mercury detected at sacrifice.

The mercury exhalation rate with or without ethanol on the last day was seen to be 15.8 and $2.3 \times 10^{-2} \overline{\%BB}_i$ respectively. The net increase with ethanol (13.5×10^{-2}) was not dramatically different from those of the rest of the experimental groups (Groups 1 through 4). The high variability associated with the body burdens observed within this particular group renders difficult concrete conclusions in regard to real differences in exhalation with continual versus a single ethanol administration. Further studies into the nature of very early interactions of mercuric chloride and ethanol in addition to continual administrations of ethanol are indicated.

DISCUSSION

Following different mercury doses, the elimination of the metal was observed to be biphasic in all dose groups. The exhalation rates at very early times (<2 to 3 days) after injection were still rising to the exhalation rates seen from day 4 through 15. The similarity in the elimination kinetics of the wide range of doses administered is also reflected in the proportion of the body burden eliminated via exhalation in these groups. Despite the large differences in the absolute body burden, the mean basal rates of exhalation in Groups 1, 2, 3, and 4 from day 4 ranged from approximately 2.15×10^{-2} percent (Day 13) to 3.06×10^{-2} percent BB_i (Day 6). The overall estimate taken for days 4 through 15 gives the value 2.59×10^{-2} percent as the approximate 30 minute rate of the simultaneous body burden exhaled by the experimental animals under these conditions.

The results suggest that a consistent relationship can be shown between the body burden and the exhalation of mercury. Although only a minor amount of the mercury in the body is volatilized, it generally represented an uniform fraction over most of the experimental period which was independent of the initial mercury doses administered. Thus, exhalation can be characterized (within the limitations of the experiment) as obeying first-order kinetics on a group basis. Further work including testing for individual variations and/or measurements in other species will be required to further characterize the utility of breath sampling as an indicator, but the consistent relationship of this process to the body content of mercury shows some promise.

Mathematically, the relationship can be described as follows: from approximately day 4 through day 15, the mice in this experiment, regardless of the dose, lost a constant fraction of the remaining mercury in the body so that:

$$(eq.1) \quad \frac{d(BB)}{dt} = -K_e(BB) \quad \text{where } (BB) = \text{body burden}$$

K_e = total fractional loss of mercury from the animal per time

However, it was shown that the loss rate constant (K_e) reflected the sum of two processes eliminating mercury:

$$(eq.2) \quad K_e = k_e + k_x \quad \text{where } k_e = \text{urinary and fecal routes of mercury elimination per time}$$

k_x = pulmonary route of mercury elimination per time

Assuming no other elimination routes exist, eq.1 is more accurately presented as:

$$(eq.3) \quad \frac{d(BB)}{dt} = -(k_e + k_x)(BB)$$

Although the quantity $-k_x \int_0^t (BB)dt$ is very much less than $-k_e \int_0^t (BB)dt$, k_x is associated with a process uniquely amenable to sampling.

Whereas gaseous exchange in the lung occurs in a temporally regular fashion with subjects at rest, other excretions of mercury cannot be sampled at will under the same conditions and cannot reflect continuous, "real time" elimination as does exhaled mercury. Moreover, it can be speculated that fecal and urinary routes of mercury elimination are

sensitive to qualitative and quantitative aspects of solid and liquid food intake and, with the notable exception of ethanol, pulmonary elimination might be less prone to influence by common foodstuffs.

Mercury exhalation detected with ethanol indicates that the increase in rate can be elicited two weeks after a single exposure to mercury regardless of the initial doses used. It has been shown to be effective up to 23 days after mercury (unreported preliminary observations) and may be a general response independent of time. Therefore, although mercury exhaled by mice under these conditions showed an increased variability in terms of percent body burden, ethanol may not be without value in approximating the mercury in the body by the exhalation method. If the results obtained are applicable to man, this method may have use as a supplemental non-invasive test for estimating body burdens.

PART III: IN VITRO STUDIES

Overview: Previous work on the volatilization of mercury was done on intact mice. The question as to the source of the vapor eliminated under these experimental conditions was still unclear. One approach to this problem was to examine isolated tissues for mercury volatilization activity (MVA). Quantitative contribution to in vivo exhalation rates may then be assessed and information regarding the molecular events involved may be derived.

Studies on the mechanism of volatilization from biological media have been published by several investigators. Magos and co-workers investigated the loss of activity from homogenates containing radiolabeled mercury over time periods up to 48 hours after incubation (Magos, Tuffery and Clarkson, 1964). Their study concluded that microorganisms commonly present in laboratories could be responsible for volatilization and also that a lag period of around 16 hours was necessary for rapid losses to occur. Subsequent reports (Tonomura et al., 1968; Komura, et al., 1971a; Komura et al., 1971b) confirmed that procaryotes possess the ability to liberate Hg^0 from several forms of mercurials. A recent report (Rowland, et al., 1978) has shown that methylmercury-treated rats can liberate Hg^0 in the gastrointestinal tract through the activity of normal gut flora.

That microbial activity was not essential for mammalian degradation of organomercurials was shown by workers identifying mercury

metabolites in tissue homogenate systems (Daniel, et al., 1972; Gage, 1975). These studies concluded that the liberated mercuric ion was not released as Hg^0 in homogenates. No studies have been reported with ethanol as an agent involved in the reduction of mercurials.

In this section, fresh or heat-deactivated mouse tissue homogenates were incubated with approximately 0.16 μM mercury as ^{203}Hg -labelled Hg II-cysteine complex. The volatilization of radioactivity from liver or kidney suspensions in 30 minute assay periods was increased by ethanol in a dose-response manner with concentrations of ethanol ranging from 30 to 613 mg/dl (6.5 to 133mM) initially. Tetracycline in the medium had no effect on volatilization. Only background volatilization was obtained with heated homogenate samples and the effect of ethanol was abolished. Aminotriazole pre-incubation also led to significant increases in mercury volatilization over control values. Liver fractionation studies showed the highest mercury volatilizing capacity was associated with the soluble component of the liver homogenates under these experimental conditions.

In vivo administration of mercury and subsequent assay of volatilization from tissue homogenates gave results consistent with those obtained after exogenous mercury. These experiments suggest that liver and kidney tissues of mice are capable of partially volatilizing mercury in the medium and therefore probably contributes to the pool of mercury exhaled in vivo.

MATERIALS AND METHODS

(1) Apparatus. Single side-arm Warburg flasks of 15 ml internal volume were modified to allow detection of mercury volatilization from tissue preparations. These modifications enabled air to be swept through the flask, over the incubation medium and carry volatilized material through an exit connected to a filter. Figure 20 is a diagram of the system used in these studies. As indicated in the illustration, the entire system was driven by a vacuum pump whereby laboratory air, metered by a flowmeter (Dwyer Corp., Michigan City, IN) passed through the system at 100 cc/min. into a 3cc syringe (Pharmaseal Co., Glendale, CA) containing approximately 0.5 gm of Hopcalite (Mine Safety Appliances Co., Evans City, PA). The sidearm opening was sealed with a rubber septum through which an 18 gauge needle was inserted. The main well was likewise sealed with a #2 rubber stopper with an 18 gauge needle driven through so that flowmeters and filters were thus easily connected or disconnected via the needles. Five such units were placed in a shaking Dubnoff metabolic water bath at 37°C (Precision Scientific Co., Chicago, IL) and operated simultaneously in these studies. To assure comparable air flow through each flask, the individual flowmeters were calibrated with another reference flowmeter.

Prior to experimentation the systems were checked for recoveries on the filter of known amounts of mercury volatilized within the flasks. Table 8 shows the results obtained under normal operating procedures

Figure 20. Modified Warburg flasks used to detect mercury volatilization by homogenates. Lab air was metered through the flask equipped with a Hopcalite filter. These filters were counted for radioactivity to determine mercury volatilized at the end of assays. Usually, five flasks were run simultaneously. A, air flow meter; B, Hopcalite. Arrows show direction of air flow.

IN VIVO APPARATUS

TABLE 8

IN VITRO SYSTEM RECOVERIES

N	Range of Initial Mercury in Main Compartment (ng)	Range of Incubation ^(a) Times (minutes)	% Recovery ^(b)
26	68 - 226	15 - 120	97.8 ± 5.1 ^(c)

(a) 37°C water bath with shaking at approximately 80 cycles/minute.

(b) Flasks were counted before and after introduction of stannous chloride to the main compartment during normal operating mode. The loss of radioactivity from flasks was assumed to be volatilized mercury. This difference divided into counts found on filter and multiplying the quotient by 100 was defined as percent recovery.

(c) Mean ± SD.

when radiolabeled mercury was chemically reduced with stannous chloride (Magos, 1968).

(2) Tissue Homogenates. Untreated mice were usually sacrificed by cervical dislocation and tissues quickly excised and kept over ice until assayed for mercury volatilization. Weighed and minced tissues were homogenized using a Polytron (Model PT 10-35 with a PT 10 Generator, Kinematica GmbH, Luzern, Switzerland) after the addition of appropriate volumes of phosphate buffer (0.1M, pH 7.4, oxygenated with 100% O₂). Aliquots of the suspension were pipetted into the main compartment of the Warburgs and kept over ice until commencement of assays. Additions to the homogenate (described below) were made directly into the main compartments.

Thirty-minute incubations were started by placing the flasks in the waterbath and initiating the airflow. At the end of the timed period the filters were removed from the system and counted for accumulated radioactivity in a well-type automated scintillation spectrometer (Model 203, Packard Instr. Co., Inc., Downers Grove, IL). The pH of the homogenates after the assays were routinely checked by test paper (Gallard-Schlesinger Chemical Manufacturing Corporation, Long Island, NY).

Heat-deactivated tissue samples were obtained by treating fresh samples in a 75°C waterbath for 10-15 minutes. Flocculated tissue was resuspended by vortexing prior to incubations which were otherwise identical to fresh tissue procedures.

In several experiments the kinetics of mercury volatilization was characterized by modifying the in vitro system by placing the in-line filter in a well-type scintillation detector as described in the in vivo section. Recordings were obtained of the accumulation of mercury on the filter with time.

Except for assays of tissues from mice injected with mercury in vivo, testing for MVA's of homogenates were carried out in the presence of $^{203}\text{HgCl}_2$ in excess cysteine. The preparation of the isotope solution was as follows:

- (a) aliquots of $^{203}\text{HgCl}_2$ in 0.5 M HCl (New England Nuclear, Boston, MA) were placed in a 25ml midget impinger (Bendix Corp., Baltimore, MD) and the exit arm was connected by vacuum line to a Hopcalite filter in the crystal-well scintillation detector (described above);
- (b) with laboratory air flowing through the impinger and the spectrometer recorder on, aliquots of a fresh 2 μM solution of cysteine hydrochloride (Sigma Chemical Co., St. Louis, MO) in distilled deionized water were added to the isotope in the impinger to yield a cysteine (cys) to mercury molar ratio of 200:1;
- (c) 0.5 M NaOH was added to neutralize the solution in the impinger;
- (d) distilled, deionized water was added to yield 100 ng of mercury per ml of solution;

- (e) nitrogen was bubbled through the isotope solution at approximately 1 liter per minute for several minutes
- (f) 1 ml aliquots of the isotope solution were added to each homogenate preparation and assays begun.

This procedure was repeated for each set of five incubations to minimize the chances of adding dissolved mercury vapor to the homogenates. The recording of the accumulation of radioactivity during these preparations served to indicate that the isotope solutions were initially free of volatile mercury. A typical profile is shown in Figure 21.

(3) Addition of Ethanol and Aminotriazole to the Incubation Medium. Ethanol (EtOH) used in these experiments was diluted with saline or water to yield various concentrations from a 95% (v/v) stock solution. One hundred microliter aliquots were added to the main compartment to generate the initial EtOH concentrations desired.

The change in concentration of ethanol with time after the start of the incubation was determined for several levels of ethanol. Twenty microliter aliquots at timed intervals were withdrawn from the incubation medium during normal assay procedures and analysis of ethanol was done by the gas chromatographic procedure described in Part II.

Aminotriazole (Sigma Chem. Co., St. Louis, MO) an inhibitor of catalase activity (see p. 6) was made in a stock solution containing 250 mg per ml saline. Aliquots were preincubated with liver homogenate

Figure 21. Isotope solutions were flushed with N_2 before adding to Warburg flasks. The figure shows a typical procedure where small amounts of volatile radioactivity in the solution were removed by purging. Arrow indicates start of gas flow.

N₂ FLUSH OF ISOTOPE SOLUTIONS

samples one hour prior to mercury volatilization assays at which time the isotope and ethanol or saline was introduced into the flask.

(4) In Vivo Injection and In Vitro Volatilization of Mercury.

Two mice were injected i.p. with 0.5 mg Hg/kg as ^{203}Hg -labeled mercury II chloride and 48 hours later were checked for in vivo mercury exhalation rates according to the techniques described in Part II. Following this procedure, the animals were sacrificed and 25% (w/v) homogenates in phosphate buffer were made of pooled liver and kidney tissues. Aliquots were taken for radioactivity counting and subsequently assayed with or without EtOH as described above but without exogenously added mercury.

(5) Fractionation Experiments - Low Speed. Homogenates prepared from untreated mice livers were centrifuged in several experiments. A refrigerated Sorvall centrifuge equipped with a Type SS-34 rotor was used (Sorvall Inc., Newtown, CT) and the temperature during centrifugation was maintained at $0-5^{\circ}\text{C}$. Ten percent homogenates in phosphate buffer were treated according to the flow chart shown in Figure 22. First, a $600g \times 10$ minute spin was performed to sediment unbroken cells, connective tissue, erythrocytes and debris (Reid, 1972). The supernatant (S_1) was subjected to $8000g$ for ten to twenty minutes to sediment the majority of mitochondria in the suspension (Tata, 1972). The pellet derived from this procedure (P_2) was resuspended in phosphate buffer to yield a 10% (w/v) suspension and centrifuged at $2000g$ for ten minutes. This step was repeated once more and the final 10% suspension was assayed for MVA. The supernatants generated from these washings (S_1P_2' and S_2P_2'') were also assayed.

Figure 22. Schematic of centrifugation procedures used to obtain crude fractions of liver homogenates.

FRACTIONATION PROCEDURE

10% (w/v) Mouse Liver Homogenate, 0.1M PO_4

The supernatant (S_2) was centrifuged at 5000g for the same length of time to yield (S_2') or was divided into two parts whereby one part was precipitated with 10% (v/v) trichloroacetic acid (Sigma Chemical Co., St. Louis, MO) and compared to the corresponding non-TCA fraction diluted with distilled water. Some final fractions were heat-treated.

(6) Ultracentrifugation Experiments. The S_2' fraction generated from low-speed centrifugations were subjected to 105,000g for one to two hours and the resulting fractions were assayed for MVA. According to Tata (1972) the pellet is rich in smooth endoplasmic reticulum and the supernatant virtually free of subcellular organelles. No attempt was made to assay for marker enzymes in these studies. The weight of microsomes obtained averaged 250 mg per gram of liver (wet weight).

(7) Protein Determination. The method of Lowry (Lowry et al., 1951) was used.

(8) Tetracycline Additions. To determine if tetracycline-sensitive microorganisms affected the results obtained in whole homogenate experiments, a series of incubations were performed with fresh liver homogenates in which half of the flasks contained 0.96 mg tetracycline HCl (Charles Pfizer & Co., Inc., New York, NY) per ml of the incubation medium*. Control flask volumes were adjusted with phosphate buffer and ethanol was added to all flasks at an initial concentration of 613 mg/dl. Assays were performed with the addition of mercury isotope solution as described above.

*The antibiotic was added as a bacteriostatic agent. No effect on microbes possibly present in the homogenate.

RESULTS

A. Whole Homogenate Experiments

Changes in the concentration of ethanol within the incubation medium were not linear with time under these experimental conditions. This is shown in Figure 23 as fraction of the initial ethanol concentration remaining after start of the 30 minute incubation with fresh or heat-denatured homogenates. Since a similarity of the kinetics of ethanol loss from these incubations were seen whether biological oxidation of the compound was present or not, the majority of the loss (~30% in 15 minutes) was assumed to be evaporation from the system. With the highest concentration used (613 mg/dl initially) ethanol was seen to be in excess of 100 mM at the end of the incubation.

Thirty-minute mercury volatilizations from 5% (w/v)* liver and kidney homogenates showed ethanol-dependent increases in the amount of radioactivity detected on the filters. Figures 24 and 25 show the effect of initial ethanol concentrations up to 613 mg/dl with standard mercury concentrations in the media. It can be seen that increasing ethanol above 123 mg/dl led to diminished increases in the amount of mercury volatilized. The amounts volatilized in 30 minutes with the highest ethanol concentration under these experimental conditions were 0.19% and 0.12% of the total mercury present in the incubations for liver and kidney homogenates respectively.

* Final concentration of tissue in the flasks after additions were 3.3% (w/v).

Figure 23. Ethanol levels as percent initial concentration after start of incubation with fresh (open symbols) or heat-treated (solid symbols) homogenates. Squares, 613 mg/dl; triangles, 307 mg/dl; circles, 31 mg/dl initial concentrations. Averages of duplicate determinations at each timepoint are plotted.

ETHANOL CONCENTRATIONS IN FLASKS WITH TIME

Figure 24. Mercury released from liver homogenates. 2 ml aliquots of the 5% (w/v) homogenates were assayed for mercury volatilization in the presence of ^{203}Hg and ethanol. Saline was added for control rates. Values are the means $\pm$ SE from 5 determinations unless otherwise noted.

(a) $n = 4$

* Significantly different from control values, $P < 0.05$.

MERCURY VOLATILIZATION FROM LIVER HOMOGENATES

Figure 25. Results of kidney homogenate incubations with different ethanol concentrations in the flask. Assay conditions were identical to that of liver procedures. The mean $\pm$ SE of 5 determinations are reported unless otherwise noted.

(a) $n = 4$

* Significantly different from control (saline) values, $P < 0.05$ by Student's t -test

MERCURY VOLATILIZATION FROM KIDNEY HOMOGENATES

Aminotriazole alone or with ethanol also elicited significant increases in the amount of mercury volatilized. Figure 26 shows results obtained with liver homogenates pre-incubated with aminotriazole and then assayed with or without ethanol in the medium. The amount volatilized with aminotriazole plus ethanol was significantly higher than volatilizations with either of those compounds alone. The mean net increase in the amount of mercury volatilized with only aminotriazole or EtOH for this experiment was 78.6 pg and 94.2 pg respectively. It might be expected that these increases would be additive in the sample containing both, but the results obtained were slightly lower (~33pg) than that calculated for strictly additive effects. It may be that the maximal MVA for these tissue samples was reached, since the protocol for the experiment included a one hour pre-incubation procedure.

An example of the time course of mercury release from liver suspensions are shown in Figure 27 for two ethanol levels and a control flask. With ethanol the rate of mercury accumulation on the filter at 30 minutes was still elevated over the control rate.

No difference in mercury volatilization was observed between groups of liver homogenates incubated with or without tetracycline in the medium. The results are given in Table 9.

The effect of heat-treatment on MVA of liver homogenates is shown in Figure 28. The ethanol effect was completely abolished so that comparable amounts were volatilized with or without ethanol in

Figure 26. The effect of 3-amino-1,2,4-triazole (AT) and/or ethanol on mercury volatilized from liver homogenates. Homogenates were pre-incubated with 93 mM AT for one hour prior to assay with or without ethanol. Ethanol was administered at 123 mg/dl (27 mM). Control homogenates received saline. All final volumes were 3.2 ml. The mean of 5 determinations $\pm$ SD are shown for each treatment.

* Significantly different from controls by Student's t-test, $P < 0.05$.

** Significantly different from either AT or alcohol alone by Student's t-test, $P < 0.05$.

EFFECT OF AT AND EtOH ON MERCURY
VOLATILIZATION

Figure 27. Time course of mercury released from liver homogenates with different ethanol concentrations. Cumulative amounts of mercury volatilized was estimated from curves obtained on spectrometer recorder. Control flask received saline.

TIME COURSE OF Hg VOLATILIZATION WITH LIVER HOMOGENATES

TABLE 9

EFFECT OF AN ANTIBIOTIC ON LIVER MVA

	CONTROLS	TETRACYCLINE
Hg Volatilized (pg x 30 min ⁻¹)(a)	128.4 $\pm$ 8.2 n=5	139.4 $\pm$ 9.2 n=5 ^(b)

(a) Mean + SD

(b) N.S. Student's t-test

Figure 28. Mercury volatilization after heat-deactivation. Livers were homogenized, heated in a 75°C waterbath and otherwise treated as fresh tissue. Control incubations were with saline. The mean $\pm$ SD of five determinations are shown.

MVA AFTER HEAT TREATMENT

the flasks. However, adding aliquots of fresh liver homogenates to flasks containing heat-deactivated homogenate led to resumption of volatilizations. This is shown in Figure 29 where equivolume additions of different homogenate concentrations made under standard mercury and ethanol conditions led to fresh tissue weight-dependent volatilizations. The addition of the vehicle used for these tissue suspensions, i.e., phosphate buffer, showed only background volatilization. Since MVA can only be exhibited in the presence of fresh tissue in the medium, the results strongly suggest a heat-labile, biological mechanism responsible for MVA.

B. Tissues from In Vivo-Injected Mice

Tissues from mice injected with mercury were assayed for MVA's under similar experimental conditions used for exogenous cys-Hg incubations. Mercury volatilization and sensitivity to added ethanol were detected as illustrated in Figure 30. The absolute amounts and percent total mercury volatilized in 30 minutes are shown for liver and kidney homogenates under different conditions of ethanol in the media. Interestingly, with high ethanol both tissue homogenates volatilized comparable fractions (~0.046%) of total mercury associated with the aliquots.

C. Tissue Fractionation Studies

Crude Fractions. It was of interest to determine which subcellular components were involved in mercury volatilization. The liver homogenate was centrifuged and the fractions obtained were assayed to detect

Figure 29. Effect of adding fresh tissue to heat-deactivated homogenates. Five different concentrations of fresh liver homogenates were prepared in phosphate buffer and equivolume additions from each were made to flasks containing heat-deactivated homogenate plus ethanol and mercury. The volatilizations in 30 minutes after addition are shown as mean $\pm$ SE of five determinations. Controls received buffer only. All final volumes were 3.3 ml.

FRESH TISSUE ADDITION TO HEAT-DEACTIVATED
HOMOGENATES

Figure 30. Results of homogenate assays when mercury was injected in vivo. The amount of mercury volatilized is given for three conditions of assay where the ethanol concentration was varied. Control incubations received saline. All final volumes were 3.2 ml. Initial mercury concentrations in the incubations were 24 ng and 488 ng for liver and kidney homogenates respectively, as detected by counting aliquots. Means of duplicate determinations shown except for triplicate results of 31 mg/dl incubations.

MVA OF TISSUES FROM MICE INJECTED WITH MERCURY

the subcellular locus of the ethanol-mercury interaction. It had been shown (Fig. 29) that fresh whole homogenates of liver could volatilize mercury in the presence of ethanol when added to heat-deactivated liver suspensions and background volatilization under these conditions were specially suitable. For MVA assays, aliquots of fractions were tested in this system.

The results indicated that the majority of the activity was associated with the 8000g supernatant as shown in Figure 31. Certain fractions exhibited background volatilizations, that is, no increase over control flasks containing only heat-deactivated homogenate plus equivolume addition of phosphate buffer. Such fractions are also listed in Figure 31*. Table 10 gives the total activity associated with either the 8000g pellet or supernatant fractions taking into account the volumes on hand for each fraction. For comparison, the results are also expressed as percent whole homogenate additions under similar assay conditions. The summations accounted for roughly 60% of volatilizing activity expected by adding unfractionated homogenates.

Ultracentrifugation Fractions. Utilizing the same assay procedures as that for low-speed fractions the products of 105,000g spins were also determined for MVA. No volatilization above background was

* Also shown in the Figure are the results normalized to the amount of protein detected in the tissue fraction aliquots. There appeared to be no general relationship between the fractions exhibiting high MVA and protein concentrations under these conditions.

Figure 31. Mercury volatilization observed after addition of 500 μ l aliquots of fresh liver fractions to heat-deactivated homogenates. Shaded bars show the absolute volatilizations observed whereas open bars are volatilizations expressed as per mg protein in the fraction aliquot. Control incubations consisted of deactivated homogenate and equivolume addition of buffer. Fractions not volatilizing above control amounts under these conditions are also listed. The values in parentheses give the number of observations used to obtain the means.

MERCURY VOLATILIZATION WITH CRUDE HOMOGENATE FRACTIONS

TABLE 10

MVA OF 8000g FRACTIONS WITH ETHANOL^(a)

	FRACTIONS	
	P ₂	S ₂
Hg Volatilization (per 30 minutes)	65.8 pg	195.3 pg
Whole Homogenate Activity ^(b)	15%	45%

(a) Net activities of 8000g fractions were summed for total sample on hand and expressed as per gram liver (wet weight). Initial ethanol concentration for all assays were 562 mg/dl, total volumes were 3.6 ml.

(b) From data presented in Figure 29 where the mean net volatilization with 100 mg addition was extrapolated to gm fresh tissue.

detected when the fractions were heat-treated or when ethanol was not added to the medium. Results are given in Table 11 which show a high volatilization activity associated with the cytosolic fraction obtained in this procedure. This unexpected activity was sufficient to exceed MVA's detected with the S_2 low-speed fraction by approximately 388% on a tissue weight basis.

TABLE 11

MVA OF LIVER MICROSOMAL FRACTIONS WITH ETHANOL^(a)

PELLET			SUPERNATANT		
Raw Data		Total Activity ^(b) (pg Hg/gm liver)	Raw Data		Total Activity ^(b) (pg Hg/gm liver)
pg Hg	Average Net Count		pg Hg	Average Net Count	
13.0 + 3.6 (n=5)	5788	19.0	59.1 + 7.0 (n=6)	26,284	756.8

(a) Identical conditions to low-speed assays. Ethanol was 562 mg/dl initially; 500 μ l aliquots of fractions in a total volume of 3.6 ml per flask. Concentration of protein in the re-suspended pellet and supernatant aliquots were 13.5 and 9.6 mg/ml respectively (duplicate determinations).

(b) Estimated by correcting the net activity obtained with aliquots to total volume of fraction and expressing as per gram wet weight of liver at start. Background volatilization (buffer addition only) was 8.1 pg per 30 minutes.

DISCUSSION

Transformation of the mercury was necessary because without fresh tissue or tissue fractions only background volatilizations were detected. This transformation reaction was probably reduction to the free metal because the physico-chemical properties of Hg^0 (discussed in Part I, Introduction) make this form the most likely product to be released into the gas phase. Since the results could not be explained by the possibility of chemical contaminants in the suspension medium and heating homogenates at 75°C would not be expected to destroy endogenous inorganic reducing agents, volatilization with ethanol was concluded to be of biological origin. Tetracycline-sensitive microorganisms apparently were not involved in the reduction process.

The subcellular locus of the mercury-ethanol interaction was studied with liver tissue because of the more homogenous nature of the parenchymal cells in comparison to the kidney in addition to the high tissue mass available. The separate assay of cytosol aliquots which resulted in higher volatilization of mercury than seen in the non-ultracentrifuged fractions suggested that components isolated in the microsomal fraction may somehow inhibit the biotransformation and/or subsequent transport of volatile mercury. These ultracentrifuged preparations were thought to be reasonably free of peroxisomes. This organelle is sedimented with the 8000g pellet though there is no general agreement in the literature as to centrifugation conditions required (Eriksson et al., 1978; Reid, 1972).

However, even if contamination with peroxisomes existed in these preparations the high level of ethanol would have inhibited oxidation of Hg^0 by CAT-I so that no difference stemming from catalase in the MVA of S_2 ' and summed MVA's of ultracentrifuge fractions should have been observed. Unlike catalase, this mechanism was insensitive to ethanol; only by physical separation through ultracentrifugation was the inhibition removed. Assessment of purities of the fractions which would shed more light on the subcellular dynamics involved in mercury volatilization are left to future studies.

Even under the limitations of the fractionation experiments reported here however, the extremely high MVA detected in the cytosol fraction implies that the majority of MVA resides in this cellular location. The cytosol contains most notably the enzymes of the glycolytic pathway including alcohol dehydrogenase. It is very possible that the biotransformation process leading to the net $2e^-$ transfer to the mercuric ion is in equilibrium with glucose metabolism either via the Embden-Meyerhof or the glucose-6-phosphate dehydrogenase pathway. In addition, the cytosol of the kidney would probably contain metallothionein (Cherian and Clarkson, 1976) which may be another factor modifying the events leading to volatilization.

The kidney may be the main source of the mercury exhaled by intact animals. Although the fraction of mercury volatilized in cys-Hg incubations with high ethanol was generally comparable between liver and kidney homogenates at 0.15 and 0.10% (net) respectively, in vivo

mercury administration showed that the kidney homogenates were able to volatilize greater absolute amounts of Hg^0 due to higher tissue levels. Thus, in vivo exhalation may be more a matter of distribution of the mercury body burden rather than a question of what tissues are capable of MVA, and kidneys may increasingly generate a higher fraction of the mercury exhaled with time. Methylmercury conversion to inorganic mercury occurred in the kidney as reported in an earlier section (Part II) and was proposed at that point to be related to exhalation rates detected after organomercury injection. The results obtained in this section provide further support for this hypothesis in that ionic mercury, once it is generated, can be further converted to the free metal.

It is interesting in this light to consider the proposal by Henderson (1974) that the detection of mercury vapor in urine from occupationally exposed workers serves as a biological standard. It may not be necessary to invoke the saturation of Hg^0 oxidation mechanisms to explain metallic mercury in this fluid since the results reported here indicate that mercury vapor generated along the nephron can conceivably diffuse into the lumen. Additionally, the known variability of mercury in the urine may be a result of MVA's associated with the kidney tissue and the possible sensitivity of it to physiological and/or pharmacological status of the animal.

Daniel et al. (1972) reported phenylmercury was degraded in the soluble and not the microsomal fraction of rat liver homogenates. These workers concluded that inorganic mercury liberated in this process was

not volatilized as mercury vapor at phenylmercury concentrations of 3.6 to 33.3 $\mu\text{g/ml}$, but probably became bound to unidentified components in this fraction. Neither NADH nor NADPH was necessary for fission of the carbon-mercury bond nor did the addition of either of these reduced co-factors elicit metallic mercury volatilization*. Hamdy and Noyes (1977) also found NADH did not lead to metallic mercury release from HgCl_2 in vitro. The apparent disagreement of results between these workers and others finding NADPH-sensitive MVA in pseudomonads (Tonomura and Kanzaki, 1969) was explained on the basis of totally different mechanisms within the two systems. However, the in vitro experiments reported here show that mercury vapor can in fact be liberated from inorganic mercury to an extent, but there is a requirement of ethanol.

Finally, a simple summation of tissue fraction MVA's may not reflect the entire dynamic situation present in an intact organ. Obviously there were indications of cellular or subcellular regulation of mercury reduction which make extrapolation to higher levels of biological organization difficult. However, the fraction of total mercury volatilized by kidney and liver homogenates in the presence of ethanol is in reasonable agreement with the in vivo rates of total body burden exhalation after ethanol.

* In preliminary experiments not reported here, NADH added to buffered cys-Hg solutions did not elicit volatilization up to a molar ratio of 40:1 NADH to Hg. Also inactive in this respect were ethanol, reduced glutathione and ascorbic acid.

SUMMARY

These studies were undertaken to gain further understanding of the ethanol effect on mercury exhalation rates. In mice, ethanol elicited an increase of mercury eliminated via the pulmonary route after administration of several forms of mercury. In vivo and in vitro findings suggested that small amounts of mercury vapor can be biologically generated in mammals exposed to mercury and that the source of the volatile mercury was likely to be, but not limited to, liver and kidney tissue. An expanded role for catalase was indicated in that not only the primary oxidation reaction of the metal but also the reoxidation of reduced ionic mercury was mediated by the enzyme. Evidence of a heat-labile cytosolic component evolving mercury vapor in situ from ionic mercury complexes was presented and the possible utilization of breath sampling as a means of estimating mercury body burden investigated.

An amendment of the general model of mammalian mercury vapor metabolism is proposed to include reverse reactions that take into account the persistent elimination of vapor and under specific conditions, lead to an acceleration of the mercury exhaled. An overall scheme of mercury exhalation is shown in Figure 32.

Figure 32. Some mechanisms involved in mercury exhalation are summarized in a simplified diagram. Inorganic mercury, which may be derived from organomercurials ($R-Hg^+$) or low molecular weight mercury complexes ($LMW-Hg^+$) undergo reduction to mercury vapor (Hg^0). Transport of mercury vapor is subject to inhibition by microsomal component(s) which may oxidize or physically impede diffusion (shaded area). Mercury vapor able to escape this regulation and also able to circumvent oxidation by the peroxisomal catalase system (C), may diffuse into the blood compartment. Depending on hydrogen peroxide availability, blood catalase may oxidize mercury. Circulation through the lungs allows unoxidized mercury to diffuse across the alveolar membrane and be exhaled. The vapor in the blood may persist under certain conditions to enter the brain. Ethanol interacts at various points in the diagram (indicated by triangles) to promote the net transfer of mercury out of the body.

SOME MECHANISMS INVOLVED IN MERCURY EXHALATION

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Appendix to Part II, Section A

Analytical Method for Ethanol

Gas chromatographic technique utilizing head space gas and propanol as internal standard (Wilkinson, et al., 1975) was used to detect ethanol in biological samples.

Gas Chromatograph: Model 900, (Perkin-Elmer, Norwalk, CT)

Detector: hydrogen flame

Column: six-foot glass tube packed with Carbowax 1500 - coated Chromosorb-W 60/80 mesh beads (Applied Science Laboratories, Inc., State College, PA)

Temperature Settings: injection port 200°C
column oven 90°C
manifold 150°C

Recorder : Speedomax, (Leeds and Northrup, North Wales, PA)

Sample Preparation: Twenty microliters of samples to be analyzed were pre-diluted or directly combined with an ice-cold equivolume solution containing propanol (Fisher Chemicals, Fairlawn, NJ), NaNO_2 and NaF (Sigma Chemical Co., St. Louis, MO) in a 2 ml glass serum bottle (Wheaton Scientific, Millville, NJ). Rubber septums were used to seal the bottle. Standards were prepared from an ethanol stock used for injection solutions and twenty microliter aliquots were combined with the internal standard solutions. All unknown, standard and blank bottles were vortexed and kept over ice or refrigerated until analyzed.

Analysis Technique: Sealed bottles were incubated in a 60°C waterbath for 3-5 minutes. Subsequently a 300 to 500 microliter head space gas sample was withdrawn via a gas-tight

syringe (PS Corp., Baton Rouge, LA) and quickly injected into the sample port. Retention times were approximately 45 seconds for ethanol and 60 seconds for propanol.

Calculations: Standard curves were plotted from the ratio of ethanol peak height to that of propanol obtained from ethanol standards. The peak height ratios of unknowns were compared and ethanol concentrations read from the scale calibrated to mg ethanol/ml.

Appendix to Part II, Section B

TABLE A-1

MINIMUM RADIOCHEMICAL PARAMETERS (DAY 15)

Group	WHOLE BODY DATA		EXHALATION DATA (a)	
	$\bar{X}$ Body Burden (ng Hg)	$\bar{X}$ Activity (CPM x 10^{-4})	Without EtOH (ng Hg)	Net Activity ₃ (Counts x 10^{-3})
1	23.9	2.58	0.023	2.4
2	329.3	7.24	0.30	3.1
3	555	7.92	0.74	4.8
4	1065	7.99	0.79	2.4
5	394	2.96	0.36	1.3

(a) Amount of mercury trapped on Hopcalite in 30 minutes.