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INORGANIC MERCURY SECRETION INTO BILE AS A LOW MOLECULAR WEIGHT COMPLEX

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Abstract—Previous studies on the biliary secretion of inorganic mercury have concluded that inorganic mercury in bile is bound almost exclusively to substances of high molecular weight (HMW). In contrast, our results showed that inorganic mercury in bile is bound predominantly to a substance of low molecular weight (LMW), which is most likely glutathione (GSH). The previously reported binding of inorganic mercury to bile proteins is now explained as a postsecretory *in vitro* artifact resulting from the rapid oxidation of endogenous GSH which occurs during the collection and storage of bile samples. Gel filtration on Sephadex G-75 of freshly collected bile from rats treated with $^{203}HgCl_2$ or of control bile supplemented *in vitro* with $^{203}HgCl_2$ showed that most of the mercury was in the LMW fraction. On Sephadex G-25, the biliary mercury peak co-eluted with the mercury–GSH standard. However, when bile was allowed to stand at room temperature, there was a time-dependent shift of the mercury towards the HMW fraction. The rate of this shift was proportional to the rate of oxidation of GSH in bile. When GSH oxidation was inhibited by collecting bile in EDTA at 4°, the mercury remained associated with the LMW fraction. At a given GSH concentration in bile, the fraction of mercury bound to the HMW fraction was independent of mercury concentration, in the range of 0.05 to 5.0 μM $HgCl_2$. These results suggest that the inorganic mercury was secreted into bile complexed with a LMW substance. This LMW substance has been tentatively identified as GSH.

Bile is the main route of elimination for many of the essential as well as the toxic metals [1]. The identification of the chemical form of these metal complexes in bile is essential in elucidating the mechanisms of their biliary secretion, as well as in understanding their subsequent metabolism in the intestinal tract.

Glutathione (GSH) has been shown to be of importance in determining the biliary secretion of methylmercury [2–6], cadmium [7], zinc [8, 9], copper [9], and silver [10]. Recently, a biliary transport system for GSH has been identified as the rate-limiting mechanism in the biliary secretion of methylmercury [2, 3]. Indeed, the methylmercury–GSH complex is now recognized as the main form of methylmercury in bile [4]. However, the isolation and identification of the methylmercury–GSH complex in bile has been difficult due to the instability of the complex [4, 11, 12]. GSH in bile is rapidly oxidized to GSSG [13], and, if bile is contaminated with pancreatic juice, GSH and GSSG are subsequently broken down to their constituent amino acids by the enzymes γ-glutamyl transpeptidase and cysteinyl-glycine hydrolase [12]. Unfortunately, in most of the studies that have been performed to identify the chemical forms of metal complexes in bile, no precautions were taken to inhibit GSH oxidation and hydrolysis.

Among all of the heavy metals secreted into bile, inorganic mercury is unusual in that it has been found associated almost exclusively with the high molecular weight (HMW) fraction of bile components [14–18]. In sharp contrast, the organomercurial methylmercury is bound predominantly to the low molecular weight (LMW) fraction (GSH) of bile [4, 11, 12, 14, 16, 18, 19]. This difference is not readily explainable in terms of differences in the physicochemical properties between the two forms of mercury. Since both have high affinity for the reduced sulfhydryl group of GSH, one would expect similar distribution patterns in bile. The present study attempts to resolve the discrepancy by examining the chemical form of the inorganic mercury complex in bile under conditions which prevent the oxidation and hydrolysis of endogenous GSH.

MATERIALS AND METHODS

Male Sprague–Dawley rats (Charles River Laboratories, Boston, MA) weighing 200–300 g were fed *ad lib.* up to the time of the experiment. The trachea, jugular vein and bile duct were cannulated under sodium pentobarbital anesthesia (65 mg/kg, i.p.). Labeled mercuric chloride ($^{203}HgCl_2$, New England Nuclear Corp., Boston, MA) was injected into the jugular vein cannula at a dose of 1.0 μmole/kg in a solution of 0.9% NaCl. The volume of the injection solution was 1 ml/kg, and the specific activity of the mercury was approximately 50 μCi/μmole Hg. Controls were injected with the same volume of saline. Bile obtained from control animals was supplemented with the same amount of mercury which was present in the bile of treated animals, approximately 0.8 nmole/ml. Body temperature was monitored by a Tele-Thermometer connected to a rectal probe (Yellow Springs Instrument Co., Yellow

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Springs, OH) and was kept at 37–38° by placing the animals on a support tray above a heated water bath. Bile was collected into ice-chilled, tared tubes for varying periods of time, as described in the text. Bile volume was determined gravimetrically assuming a density of 1.0 g/ml. In some experiments bile was collected directly into 0.2 M disodium salt of EDTA, or into 5% trichloroacetic acid. Acidification of bile during its collection is required to inhibit the oxidation of endogenous GSH to GSSG [4, 5, 13].

Bile samples were subjected to column chromatography on Sephadex G-75 and on Sephadex G-25 fine (Pharmacia Fine Chemicals, Piscataway, NJ). Gel filtration was carried out at 4° with a flow rate of 14 ml/hr, using 0.9 × 60 cm columns. The eluant was 0.1 M Tris–HCl buffer, pH 7.4, containing 1 mM EDTA for the G-75 column and 0.1 M Tris–HCl, pH 7.4, containing 0.5 M NaCl for the G-25 column. Bile samples, 0.3 ml, were added directly to the columns, and 1-ml fractions were collected. The reference compounds, that is, the mercuric derivatives of cysteine and GSH, were prepared by addition of a 10-fold excess of the thiol to a 0.4 mM solution of $^{203}HgCl_2$ in 0.9% NaCl. Radioactivity in each fraction was determined in a Packard model 3002 gamma scintillation spectrometer. Absorbance at 254 nm was monitored with an LKB Uvicord S flow-through detector. The void volume was defined by the elution of Blue Dextran, and the elution volume of GSH from the Sephadex G-75 columns was determined by measuring nonprotein sulfhydryl groups [20], in individual fractions.

Trichloroacetic acid precipitation of proteins in tissue homogenates has been used previously as an estimate of the relative amount of mercury in the non-diffusable, protein-bound form [21, 22]. In this study, trichloroacetic acid precipitation was used to estimate the amount of mercury bound to the HMW constituents of bile. Separation of HMW and LMW constituents with trichloroacetic acid was performed by adding 4 vol. of 5% trichloroacetic acid in 1 mM EDTA to the bile samples, centrifuging at 10,000 *g*

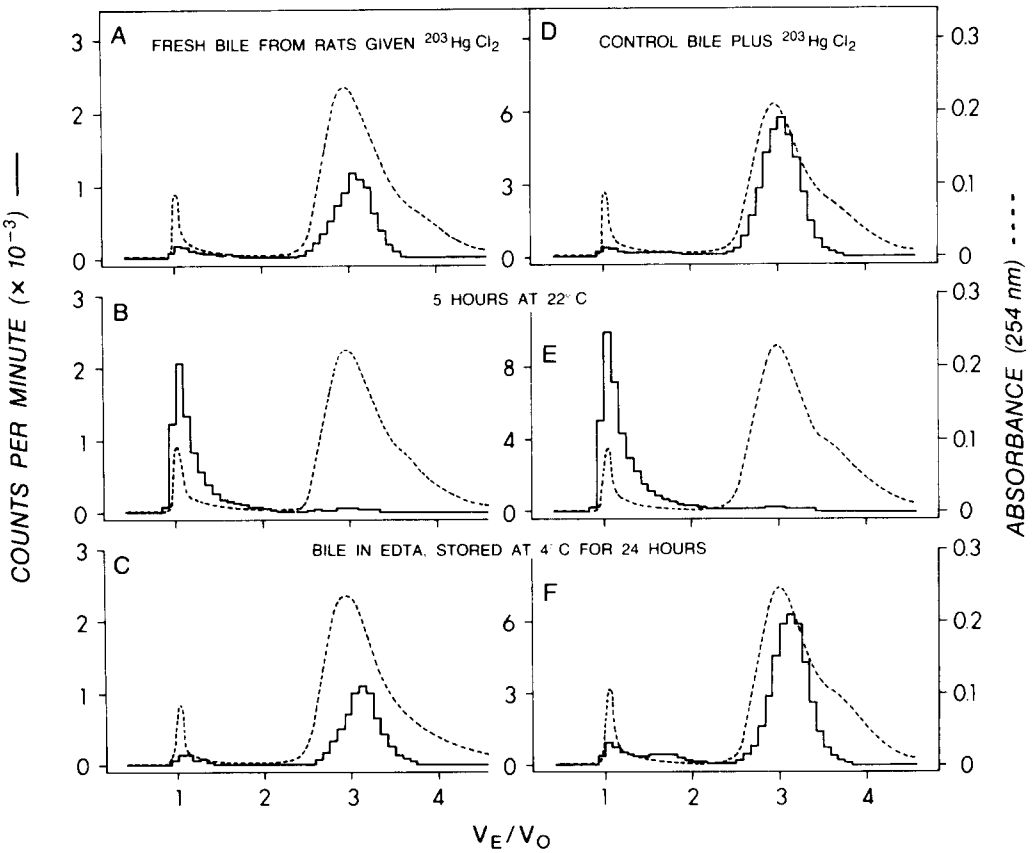

Fig. 1. Distribution of inorganic mercury and of absorbance at 254 nm in Sephadex G-75 fractions of rat bile. Panels A, B and C refer to bile collected 15–90 min after the intravenous administration of 1 μmole $HgCl_2$/kg. (A) Bile collected in ice-chilled tubes and applied immediately to the column, (B) bile incubated at room temperature for 5 hr prior to gel filtration, and (C) bile collected at 4° in 0.2 M EDTA (1 : 4 v/v) and stored at 4° for 24 hr prior to gel filtration. Panels D, E and F are from bile from control animals supplemented *in vitro* with the same amount of mercury which was present in the bile of treated animals, approximately 0.8 nmole/ml. (D) Freshly collected bile (4°) to which $^{203}HgCl_2$ had been added 10 min prior to gel filtration, (E) an aliquot of the same bile sample after incubation at 22° for 5 hr, and (F) another aliquot of the same bile sample to which EDTA was added along with $^{203}HgCl_2$ and then incubated at 4° for 24 hr. The glutathione (GSH) in bile eluted at a V_E/V_0 of 2.8 to 3.5.

for 10 min, and removing the supernatant fraction using a Pasteur pipette.

This trichloroacetic acid precipitation technique was used to study the role of GSH oxidation on the mercury distribution among the bile constituents. Bile was collected at room temperature for 3 hr after bile duct cannulation in untreated animals. The endogenous GSH in bile was allowed to oxidize completely by storing the bile at room temperature for at least 2 hr after collection [13]. One-half of the bile (A) was chilled to 4° for 10 min and the other half (B) was maintained at 22°. Aliquots of 0.8 ml of both A and B were added to tubes containing a 0.2 ml mixture of $^{203}HgCl_2$ and GSH (0.5 nmole Hg; 2.2 μmoles GSH), dissolved in either 0.2 M EDTA, or in saline (pH adjusted to 7.0). The tubes were mixed thoroughly and incubated at 4° and 22°. Aliquots were removed from the tubes at various times to determine the distribution of mercury among the bile constituents using the trichloroacetic acid precipitation technique. GSH content of bile was estimated by measurement of nonprotein sulfhydryl content by the method of Sedlak and Lindsay [20]. Proteins were measured by the method of Lowry *et al.* [23], using bovine serum albumin as the standard.

A study of the effects of varying concentrations of mercury and of GSH on the distribution of mercury in bile was also performed using the trichloroacetic acid precipitation technique. Bile was collected from control animals for 3 hr after bile duct cannulation, and the endogenous GSH was allowed to oxidize. Aliquots of 160 μl of bile were added to tubes containing a 40 μl mixture of various concentrations of GSH and of mercury, in a solution of 0.2 M EDTA. The tubes were stirred and allowed to incubate at room temperature for 10–15 min prior to the addition of trichloroacetic acid.

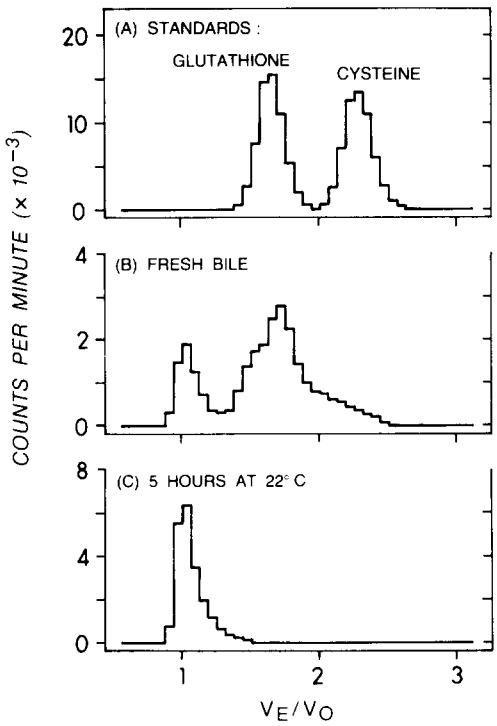

Fig. 2. Gel filtration on Sephadex G-25 of bile collected 15–90 min after the intravenous administration of $^{203}HgCl_2$, and of reference compounds. (A) Solutions of the mercuric derivatives of glutathione and cysteine were applied separately to the column, (B) bile was collected at 4° and applied immediately to the column, and (C) bile, was incubated at 22° for 5 hr prior to gel filtration. When a solution of $^{203}HgCl_2$ in saline was added to the column, the radioactivity eluted at a V_E/V_0 of 2.4 to 2.8.

RESULTS

When bile from rats given $^{203}HgCl_2$ intravenously was applied to a Sephadex G-75 column immediately after collection, most of the mercury eluted in the LMW (less than approximately 10,000 daltons; $V_E/V_0 > 2$) fraction (Fig. 1A). However, when the bile was allowed to stand at room temperature, there was a time-dependent shift of the mercury towards the HMW fraction. After 5 hr at 22° most of the mercury eluted in the void volume (Fig. 1B). Storing the bile at 4° slowed the rate of this shift, but it did not inhibit it. However, when the oxidation of endogenous biliary GSH was prevented by collection of bile in EDTA ([4, 5, 13] and Fig. 3), the mercury shift was inhibited completely (Fig. 1C). Most of the mercury was in the LMW fraction even as long as 24 hr after bile collection, when the bile was collected in EDTA. The effects of EDTA are not due to the direct chelation of the mercury ions by the EDTA molecules since (1) EDTA was unable to reverse the shift, (2) the shift occurred even in the presence of EDTA, after either extended storage of the bile samples, or storage at room temperature, and (3) EDTA is a relatively poor chelator of mercury ions.

Similar results were obtained when bile from control rats was supplemented *in vitro* with $^{203}HgCl_2$ (Fig. 1, panels D, E, and F). The amount of mercury associated with the HMW fraction (V_E/V_0 = 1–2) was 7.1 ± 1.8% (N = 5) for fresh bile, 91.5 ± 2.9% (N = 5) for bile incubated at 22° and 14.9 ± 4.1% (N = 5) for bile stored in EDTA.

Gel filtration of freshly collected bile on Sephadex G-25 revealed that most of the mercury co-eluted with the mercury-GSH standard (Fig. 2, panels A and B). Once again, prolonged storage resulted in the appearance of the mercury in the void volume (Fig. 2C). Also, the *in vitro* addition of $^{203}HgCl_2$ to control bile provided results similar to those obtained after the intravenous administration of mercury (not shown).

Aliquots of some of the same bile samples used in the gel filtration experiments were subjected to trichloroacetic acid protein precipitation to determine whether this procedure would give similar distribution patterns between HMW (proteins) and LMW fractions. Quantitatively similar distribution patterns were obtained: only 13.1 ± 1.8% (N = 5) of the mercury was in the acid precipitable fraction of freshly collected bile, while 94.1 ± 3.1% (N = 5) of the mercury was in the acid precipitate when bile was incubated at room temperature for 5 hr. For bile collected in EDTA and stored at 4° for 24 hr, only 17.6 ± 6.8% (N = 4) of the mercury was in the acid precipitate.

Since the trichloroacetic acid precipitation tech-

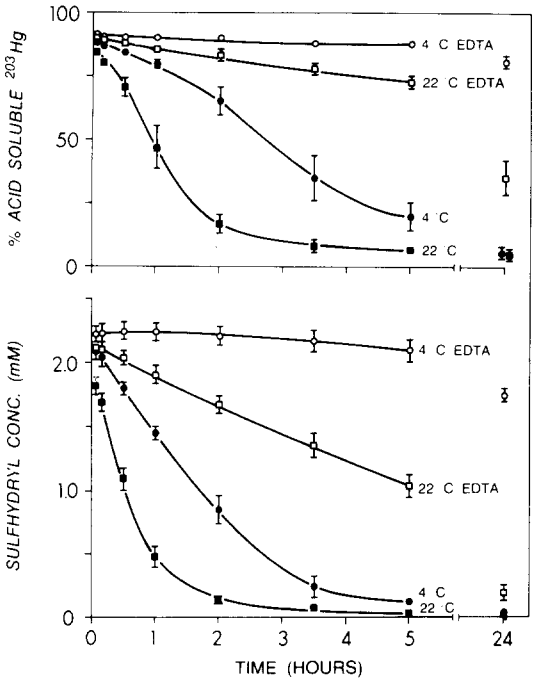

Fig. 3. Effects of temperature and of EDTA on the time course of mercury binding to trichloroacetic acid precipitable components and on the rate of glutathione disappearance in rat bile. Bile was incubated at either 4° (circles) or at 22° (squares), in the presence (open symbols) and in the absence (closed symbols) of EDTA. Each point represents the mean ± S.E. of eight separate experiments. See Materials and Methods for more details.

nique was found to give valid estimates of mercury distribution, and since it is a simple and rapid procedure, it was used to study the role of GSH oxidation on the kinetics of the mercury shift towards the HMW fraction. The effects of temperature and of EDTA on the time course of mercury binding to trichloroacetic acid precipitable components, as well as on the rate of GSH oxidation, are shown in Fig. 3. Incubation of bile at 22° resulted in the rapid disappearance of GSH and a concomitant shift of the mercury towards the HMW components. Incubation at 4° slowed the rate of disappearance of GSH as well as the rate of the mercury shift. However, after 5 hr of incubation at either temperature, little GSH remained, and most of the mercury was in the acid precipitable fraction. By 24 hr, GSH was not detectable and 95% of the mercury was in the acid precipitate.

Incubation of bile at 4° in the presence of EDTA completely prevented the oxidation of GSH as well as the mercury shift for at least 5 hr (Fig. 3). A substantial amount of GSH oxidation occurred even in the presence of EDTA at an incubation temperature of 22°. Figure 3 also shows that for a given GSH concentration in bile the fraction of mercury bound to the HMW components was constant, regardless of the experimental conditions. At a GSH concentration of 0.5 mM approximately 50% of the mercury was in the acid precipitate and at a GSH concentration of 1.25 mM approximately 25% was in the acid precipitate. The mercury concentration in these bile samples was 0.5 μM, and the mean protein concentration was 3.5 ± 0.8 mg/ml of bile.

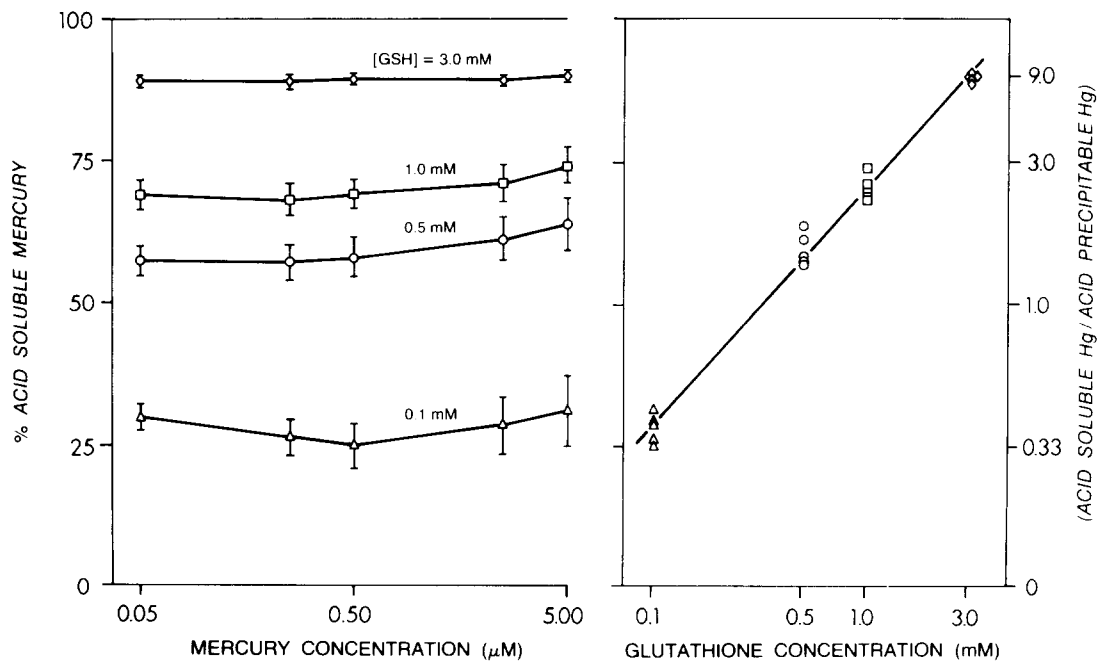

Fig. 4. Effects of varying the concentrations of mercury and of glutathione on the distribution of mercury in bile. The trichloroacetic acid precipitation technique was used to separate the high molecular weight and the low molecular weight bile constituents. Each point represents the mean ± S.E. of six separate experiments. The slope of the linear regression for the log-log plot is 0.89. See Materials and Methods for more details.

The experiments reported in Fig. 4 were designed to explore the effects of varying the concentration of mercury and of GSH on the distribution of mercury in bile. Once again, trichloroacetic acid precipitation was used to estimate the distribution of mercury between HMW and LMW components. At all concentrations of GSH used, the fraction of mercury bound to the acid precipitate was independent of mercury concentration, in the range from 0.05 to 5 nmoles Hg/ml of bile. A concentration of 5 nmoles Hg/ml of bile is achieved after a mercury dose of 10 μmoles/kg body weight, which is close to the LD_{50} value of 15 μmoles $HgCl_2$/kg body weight [24]. The mean protein concentration in these bile samples was 5.5 ± 0.6 mg/ml, or approximately 92 μM if one assumes a mean molecular weight of 60,000 daltons for biliary proteins. Thus, the data in Fig. 4 show the mercury distribution among bile constituents at concentrations of mercury which are not sufficient to saturate either protein or GSH sulfhydryl groups. The relation between GSH concentration and the ratio of acid soluble to acid precipitable mercury, which is shown in Fig. 4 on a log scale, was also linear when plotted on a linear scale ($y = 2.76x - 0.03$, $r = 0.99$). This linear relation suggests a simple equilibrium distribution of mercury between proteins and GSH in bile.

DISCUSSION

In contrast to previous observations [14–18], the results presented here show that inorganic mercury in bile is associated with a substance of LMW. Although the data cannot conclusively identify the nature of the complex, all of the evidence indicates that it is a mercury–GSH complex. First, mercury has a high affinity for reduced sulfhydryl groups, including those of GSH [25, 26]. GSH is found in bile in unusually high concentrations (1–4 mM) [2–5, 13], and it accounts for at least 90% of the total reduced sulfhydryl groups in bile [4, 5, 13]. The rest of the sulfhydryl groups in bile are either protein bound, approximately 5% or in the form of cysteine [4], which is, in turn, believed to originate from the hydrolysis of GSH within the biliary tree [27]. The metal binding protein metallothionein has not been found in bile [7, 28]. In view of the abundance of GSH in bile and view of its affinity for mercury, one would expect most of the mercury in bile to be complexed with GSH. Second, gel filtration of bile from mercury-treated animals showed that most of the mercury co-eluted with the mercury–GSH standard (Fig. 2). Third, the rapid *in vitro* oxidation of endogenous GSH to GSSG, which occurs during the collection and subsequent storage of bile samples [13], resulted in a parallel shift in the mercury distribution towards the HMW components of bile. Importantly, the rate of this shift was directly proportional to the rate of GSH oxidation in bile (Fig. 3). Fourth, a subsequent study [29] has shown that the rate of inorganic mercury secretion in bile is closely coupled to the rate of GSH secretion, suggesting that mercury may be secreted into bile as a GSH complex.

Since in previous investigations on the biliary secretion of inorganic mercury no precautions were taken to inhibit GSH oxidation, one may assume that most, if not all, of the GSH in bile was oxidized. Thus, the preponderance of mercury bound to the HMW fraction as reported by others represents a post-secretory *in vitro* artifact.

The instability of metal complexes in bile has proved to be a major obstacle to their identification. Methylmercury in bile, for example, was first identified as a cysteine complex, with only 20% bound to biliary proteins [14]. The fraction of methylmercury bound to biliary proteins was subsequently reported to be dependent on the dose and the time elapsed after administration [16]. Other investigators have reported that methylmercury is complexed with a compound with a molecular size between cysteine and GSH [19]. Further, Norseth [11] observed that, upon storage of the bile samples, there is a redistribution of the methylmercury to the protein fraction. This redistribution is also dependent on the dose of mercury. In 1975 Refsvik and Norseth [4] reported that most of the methylmercury in bile is complexed with GSH, but that this complex is degraded to the methylmercury–cysteine complex upon storage. These observations have been confirmed recently by Hirata and Takahashi [12], who also showed that the conversion of the methylmercury–GSH complex to the methylmercury–cysteine complex in bile is accomplished by the pancreatic enzymes γ-glutamyl transpeptidase and cysteinyl-glycine hydrolase. However, the role of GSH oxidation in mediating some of the observed effects has not been investigated.

The recent identification of a GSH transport system as the rate-limiting step in the biliary secretion of methylmercury suggests that methylmercury is secreted into bile as a GSH complex [2, 3]. Any modification of this complex must be attributed to postsecretory events. It is likely, therefore, that the shift of methylmercury to the protein fraction observed by Norseth [11] was due to the oxidation of GSH, upon storage of the bile samples.

There are also contradictory reports on the chemical form of cadmium complexes in bile. Cherian and Vostal [7] reported that most of the cadmium in bile is complexed with GSH. Havrdova *et al.* [17], on the other hand, found 50% of the cadmium bound to the HMW fraction of bile. This discrepancy is probably also related to the *in vitro* oxidation of GSH: the former investigators used freshly collected bile, while the latter stored their bile samples for extended periods prior to analysis.

GSH has also been shown to be an important determinant of the biliary secretion of zinc [8, 9], copper [9], and silver [10]. Indeed, it has been speculated that the biliary transport system for GSH may be the rate-limiting mechanism for the biliary secretion of all metals that have a selective affinity for reduced sulfhydryl groups [2, 3]. A crucial test of the validity of this hypothesis is the identification of the metal–GSH complexes in bile. This has now been performed for inorganic mercury, suggesting that the GSH transport system may play a role in its secretion. A subsequent study has confirmed a role for the GSH transport system in regulating the biliary secretion of inorganic mercury [29].

A previous report from this laboratory demonstrated that methylmercury undergoes extensive

enterohepatic circulation whereas inorganic mercury does not [14]. This difference had been explained as due to the difference in the biliary forms of mercury, i.e. that the LMW complex of methylmercury was reabsorbed in the intestine, while the protein form of inorganic mercury was not. The observation that inorganic mercury is also present in bile as a LMW complex requires that this explanation be revised.

In fact, methylmercury is well absorbed from the gut (~95% of the ingested dose) irrespective of the chemical form in which it is presented: as a salt [30], protein bound in liver homogenates [31], or in fish muscle [32]. Compounds of inorganic divalent mercury are poorly absorbed (<15% of the oral dose) irrespective of the type of compound administered [33, 34]. The chemical transformations of the GSH complexes as the bile enters the intestine are not known except that some of the mercury, whether methyl or inorganic, is present in the gut contents as protein (i.e. HMW) complexes. We need to know more about the chemical fate of mercury in the gut and the mechanisms of reabsorption across the gut wall before we can explain the large difference in enterohepatic circulation between methyl and inorganic mercury.

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