

## Cystine alters the renal and hepatic disposition of inorganic mercury and plasma thiol status

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Received 10 October 2005; revised 28 November 2005; accepted 5 December 2005

Available online 8 February 2006

### Abstract

In the present study, we determined whether cystine can inhibit, under certain conditions, the renal tubular uptake of inorganic mercury *in vivo*. We co-injected (i.v.) cystine with a non-toxic dose of mercuric chloride to rats and then studied the disposition of inorganic mercury during the next 24 h. We also determined if pretreatment with cystine influences the disposition of administered inorganic mercury. Moreover, plasma thiol status was examined after the intravenous administration of cystine with or without mercuric chloride. During the initial hour after co-injection, the renal tubular uptake of mercuric ions was diminished significantly relative to that in control rats. The inhibitory effects of cystine were evident in both the renal cortex and outer stripe of the outer medulla. In contrast, the renal accumulation of mercury increased significantly between the 1st and 12th hour after co-treatment. Urinary excretion and fecal excretion of mercury were greatly elevated in the rats co-treated with cystine and mercuric chloride. Thus, when cystine and mercury are administered simultaneously, cystine can serve as an inhibitor of the renal tubular uptake of mercury during the initial hour after co-treatment. In rats pretreated with cystine, the renal uptake of inorganic mercury was enhanced significantly relative to that in rats not pretreated with cystine. This enhanced accumulation of inorganic mercury correlated with the increased circulating concentrations of the reduced cysteine and glutathione. Additionally, the present findings indicate that thiol status is an important determinant of renal and hepatic disposition, and urinary and fecal excretion, of inorganic mercury.

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**Keywords:** Kidney; Proximal tubule; Cystine; Amino acid transporters; Hg transport; Molecular mimicry

### Introduction

It is well established that following exposure to inorganic forms of mercury, the greatest fraction of mercuric ions entering systemic circulation accumulates in the kidneys (Zalups, 2000). As much as 50% of the total body burden of mercury can be present in the combined renal mass following the initial hours after exposure (Zalups, 2000). Various lines of evidence indicate that most of the inorganic mercury that becomes localized in the kidneys is due almost exclusively to the uptake and accumulation of mercuric species along the three segments of the proximal tubule (Zalups and Barfuss, 1990; Zalups, 1991a, 1991b).

Interestingly, the uptake of inorganic mercuric ions by proximal tubular epithelial cells can be attributed to mechanisms localized on both luminal and basolateral plasma

membranes (Zalups, 2000). Several lines of evidence indicate that uptake of inorganic mercury at the luminal plasma membrane is at least partly dependent on the activity of the brush-border enzymes  $\gamma$ -glutamyltransferase and cysteinylglycine (Baggett and Berndt, 1986; Berndt et al., 1985; Cannon et al., 2000, 2001; Zalups, 1995; Zalups and Barfuss, 1995b, 1996). These enzymes play a vital role in catalyzing the hydrolytic degradation of filtered and/or secreted glutathione to constituent amino acids (i.e. cysteine, glutamate and glycine), which are then absorbed efficiently by amino acid transporters. Although this process consumes oxygen and ATP, it provides a necessary means by which proximal tubular epithelial cells can recycle glutathione back into circulation. Based on these previous findings, one can assume that much of the inorganic mercury present within the proximal tubular lumen is in the form of a mercuric conjugate of glutathione, but that after the actions of  $\gamma$ -glutamyltransferase and cysteinylglycine, mercuric conjugates of cysteine are the principal species of mercury

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that can be absorbed at the luminal plasma membrane. It is noteworthy to mention that Naganuma et al. (1988) have demonstrated in vitro that mercuric conjugates of glutathione can be degraded to mercuric conjugates of cysteine by the sequential actions of  $\gamma$ -glutamyltransferase and cysteinylglycine. A series of recent studies provide strong evidence indicating that mercuric conjugates of cysteine are indeed important transportable species of inorganic mercury taken up at the luminal plasma membrane of proximal tubular epithelial cells (Bridges et al., 2004; Cannon et al., 2000, 2001; Zalups, 1995; Zalups and Barfuss, 1996, 1998a, 1998b; Zalups and Lash, 1997).

Recently, we hypothesized that the linear II coordinate-covalent complex consisting of a single mercuric ion bonded to two molecules of cysteine (cysteine–Hg–cysteine) is transported across the luminal plasma membrane of proximal tubular epithelial cells by a mechanism involving molecular mimicry. Inasmuch as this mercuric complex is structurally similar to the amino acid cystine, we hypothesized that this mercuric complex can serve as a molecular mimic of cystine at one or more luminal transporters of cystine. Using isolated perfused proximal tubular segments and Madin–Darby canine kidney (MDCK) cells transfected stably with system b<sup>0+</sup> (a sodium-independent transporter of cystine), we recently demonstrated in vitro that cysteine–Hg–cysteine can serve as a transportable substrate of one or more carrier proteins involved in the luminal absorptive transport of cystine along the proximal tubule (Bridges et al., 2004; Cannon et al., 2001).

In the present study, we wanted to determine whether in vivo evidence could be obtained from rats, which support the hypothesis that cystine can serve as a competitive substrate at the site of transporters (presumably amino acid transporters) involved in the absorptive transport of cysteine–Hg–cysteine along the renal proximal tubule. The primary approach used was to assess how systemically administered cystine influences the renal tubular uptake and disposition of a systemically administered, non-toxic dose of inorganic mercury. An additional aim of the present study was to evaluate the effect of cystine administration on plasma thiol status.

## Materials and methods

**General experimental design.** Two experiments (experiments 1 and 2) were carried out to evaluate the effect of co-administration or pretreatment with cystine on the renal, hepatic, and hematological disposition of inorganic mercury. In the first experiment, a 1 mmol/kg dose of cystine was co-administered intravenously with a 0.5  $\mu$ mol/kg dose of mercuric chloride. The renal, hepatic and hematological disposition of inorganic mercury was assessed 1, 3, 12, and 24 h after injection. Urinary and fecal excretion of inorganic mercury was also assessed, but only at 12 and 24 h postinjection.

In the second experiment, the effect of cystine pretreatment on the renal disposition of inorganic mercury was assessed. The rationale for carrying out this experiment was to obtain dispositional data that corresponded in time, after cystine pretreatment, when plasma thiol status was measured in experiment 3. Determination of plasma thiol/disulfide status enabled correlation with cystine-associated changes in the renal disposition of inorganic mercury. In experiment 2, rats were first pretreated with an intravenous, 1 mmol/kg dose of cystine. They were then given an intravenous 0.5  $\mu$ mol/kg dose of mercuric chloride. One hour later, the renal, hepatic, and hematological disposition of inorganic mercury was evaluated.

In a separate experiment (Experiment 3), plasma thiol status was assessed in rats 1 h after they received a 1 mmol/kg intravenous dose of cystine in 2 mL/kg 0.9% saline, which was administered simultaneously with or without 0.5  $\mu$ mol/kg mercuric chloride (in 2 mL/kg 0.9% saline).

**Animals and groups.** Male Sprague–Dawley rats were used in the present study. The animals were purchased from Harlan Sprague–Dawley (Indianapolis, IN) at a weight of 175–200 g. After 3–4 days of acclimation, the animals were separated into groups of four or five in a random manner. During all stages of the present study, the animals were allowed water and a commercial laboratory diet for rats ad libitum.

**Injections.** All groups of rats in experiments 1 and 2 were administered a 0.5  $\mu$ mol/kg dose of mercuric chloride into the femoral vein while under light anesthesia induced by ether (which lasted no more than 30 seconds). Briefly, immediately prior to administering an injection, the femoral vein was exposed by making a small 1–2 cm incision through the skin and fascia on the ventral side of the thigh with a pair of small surgical scissors. The dose of inorganic mercury, with or without cystine, was administered with a sterile, 1-cm<sup>3</sup> syringe equipped with a 25-gauge needle. After delivering the injection, the incised area was swabbed with cotton gauze saturated with 100% ethanol. The wound was sealed with 2–3 sterile, 9-mm, stainless steel, animal wound clips.

In experiment 1, the experimental group of rats received the 0.5  $\mu$ mol/kg dose of inorganic mercury along with 1 mmol/kg cystine in 2 mL/kg 0.9% saline. The control group received only the 0.5  $\mu$ mol/kg dose of mercuric chloride. In experiment 2, two groups of rats received a saline-based pretreatment intravenously 1 h prior to being treated intravenously with the 0.5  $\mu$ mol/kg dose of mercuric chloride. The experimental group of rats received 1 mmol/kg cystine in 2 mL/kg 0.9% saline, while the control group received 2 mL/kg 0.9% saline. Each pretreatment was administered into the left femoral vein under light anesthesia induced by ether.

Radioactive inorganic mercury, in the form of mercuric chloride (<sup>203</sup>HgCl<sub>2</sub>, specific activity = 20–25 mCi/mg), was added to the injection solution containing non-radioactive mercury. Each injection solution was designed to deliver 0.5  $\mu$ mol Hg<sup>2+</sup>/kg and 4  $\mu$ Ci <sup>203</sup>Hg<sup>2+</sup>/kg in 2.0 mL normal saline (0.2-mL injection volume/100 g body weight).

**Acquisition of tissues and determination of the content of mercury.** At the designated periods after administration of the 0.5  $\mu$ mol/kg dose of mercuric chloride, animals were anesthetized with an intraperitoneal 100 mg/kg dose of sodium pentobarbital. Once the animals were anesthetized, two 1-mL samples of whole blood were obtained from the inferior vena cava. One milliliter of whole blood was placed and sealed in a preweighed 12 × 75 mm, round-bottom, gamma-counting tube. The other 1.0 mL of whole blood was centrifuged at 10,000 × g to separate the cellular fraction of blood from the plasma. Both plasma and cellular fractions were placed individually, and sealed, in gamma-counting tubes. After the blood had been obtained, the kidneys and liver were excised, cleared of fat and connective tissue, and weighed quickly. Each of the two kidneys was cut along the transverse plane. One half of each kidney was placed and sealed in a preweighed gamma-counting tube. A 3-mm section of kidney was sliced away from the mid-region of the remaining half of the left kidney and samples of cortex and outer and inner stripes of the outer medulla and inner medulla were obtained. A 1-g sample of liver was also obtained.

Urine and fecal samples were collected in plastic metabolic cages for the rats studied for 12 or 24 h in experiment 1. The amount of urine and feces excreted was determined by weighing the sample. From each sample of urine, 1 mL was removed and placed and sealed in a 12 × 75 mm gamma counting tube. By contrast, the entire amount of feces excreted was placed and sealed in multiple 16 × 90-mm polypropylene tubes.

The radioactivity of the samples of tissues, urine, feces, and injection solution (standards) was determined by counting the samples in a Wallac gamma spectrometer equipped with a 3-inch sodium iodide crystal (Wallac, Gaithersburg, MD) and that operates at a counting efficiency of approximately 50% for <sup>203</sup>Hg. The content of mercury in the samples was calculated by dividing the activity of <sup>203</sup>Hg (dpm) in the sample by the specific activity of <sup>203</sup>Hg (dpm/nmol) in the injection solution. Concentrations of mercury in the tissues are expressed as percent of the administered dose per gram of tissue and the content

of mercury in organs is expressed simply as a percent of the administered dose. The total volume of blood in rats was estimated to be ~ 6% of body weight.

**Production of  $^{203}\text{Hg}^{++}$ .** There is currently no commercially available source  $^{203}\text{Hg}^{++}$  available. Thus, we had no choice but to generate this isotope ourselves. The first step in manufacturing this isotope involved weighing out 3 mg of mercuric oxide ( $\text{HgO}$ ), containing the stable isotope  $^{200}\text{Hg}^{++}$  and enriched  $^{202}\text{Hg}^{++}$  (target), and doubly-sealing the mercuric oxide in quartz tubing. The actual isotopic composition of the mercuric oxide used was < 0.05%  $^{196}\text{Hg}$ , 1.5%  $^{198}\text{Hg}$ , 2.82%  $^{199}\text{Hg}$ , 4.24%  $^{200}\text{Hg}$ , 3.11%  $^{201}\text{Hg}$ , 86.99%  $^{202}\text{Hg}$ , and 1.34%  $^{204}\text{Hg}$ . The double encapsulated target was sent to the Missouri University Research Reactor (MURR) facility to be irradiated (by neutron activation) for a period of 4 weeks. After receiving the irradiated target from the MURR facility, the target was placed in protected storage for a period of 10 days to allow for the isotopic decay of the newly formed  $^{197}\text{Hg}^{++}$ . Subsequently, the two quartz tubes enclosing the target were broken in half. The target was removed from the quartz tubing with four 50  $\mu\text{L}$  rinses of 1 N HCl. All four rinses were placed and sealed (temporarily) in a single 1.7-mL polypropylene vial. A sample of the solution was then used to determine the precise solid content of Hg in the isotopic solution using plasma-coupled elemental mass spectrometry. The radioactivity of the solution was determined with a Wallac (Gaithersburg, MD), Wizard 3" 1480 Automatic Gamma Counter, which allows one to determine the activities of up to 10 different isotopes simultaneously. The counting-efficiency for  $^{203}\text{Hg}$  in the Wallac gamma counter is approximately 50%. The specific activities of the  $^{203}\text{Hg}^{++}$  used in the present study ranged between 20 and 25 mCi/mg Hg.

**Assessment of thiol-status in plasma.** The content of glutathione, glutathione disulfide, cysteine, and cystine in plasma was assessed in four groups of rats. One group of rats was pretreated with 2 mL/kg 0.9% saline, the second group was pretreated with a 1 mmol/kg dose of cystine in 2 mL/kg 0.9% saline, the third group was pretreated with a 0.5  $\mu\text{mol/kg}$  dose of  $\text{HgCl}_2$  in 2 mL/kg 0.9% saline, and the fourth group was pretreated with a 0.5  $\mu\text{mol/kg}$  dose of  $\text{HgCl}_2$  plus a 1 mmol/kg dose of cystine in 2 mL/kg 0.9% saline. At the time of sacrifice, samples of plasma were obtained from blood extracted from the four groups of rats. Plasma samples were dissolved in a buffer containing 1.0 mM bathophenanthroline disulfonate (used as an antioxidant) and 10% (v/v) perchloric acid. Acid extracts were derivatized with iodoacetic acid and 1-fluoro-2,4-dinitrobenzene for analysis of the aforementioned thiols and disulfides by high-performance liquid chromatography (HPLC). The *S*-carboxymethyl-*N*-dinitrophenyl derivative of thiols and *N,N*-bis-dinitrophenyl derivative of disulfides were separated on a 10- $\mu\text{m}$   $\mu\text{Bondapak}$  amine column with gradient elution (methanol–acetate solvent system) and were detected by absorbance at 365 nm. Quantification was performed by integration. The actual amount of each compound in the plasma is expressed in  $\mu\text{mol/mL}$ .

**Statistical analyses.** Statistical differences among means, of any parameter measured in the mercury disposition experiments, were assessed using a two-way analysis of variance followed by Tukey's multiple comparison procedure. Data expressed as a percentage of total were first normalized using the arcsine transformation before performing any parametric statistical procedure. The arcsine transformation takes the arcsine of the square root of the decimal fraction of the percent score. The level of significance for any of the statistical procedures used was chosen a priori to be  $P < 0.05$ .

## Results

### Experiment 1: effects of cystine co-administration on the disposition of inorganic mercury

#### Renal burden of mercury

By the end of the initial hour after treatment, approximately 46% of the administered dose of mercury was present in the total renal mass in the group of rats administered the non-nephrotoxic intravenous 0.5  $\mu\text{mol/kg}$  dose of  $\text{HgCl}_2$  (Fig. 1). By contrast, the renal burden of mercury in the

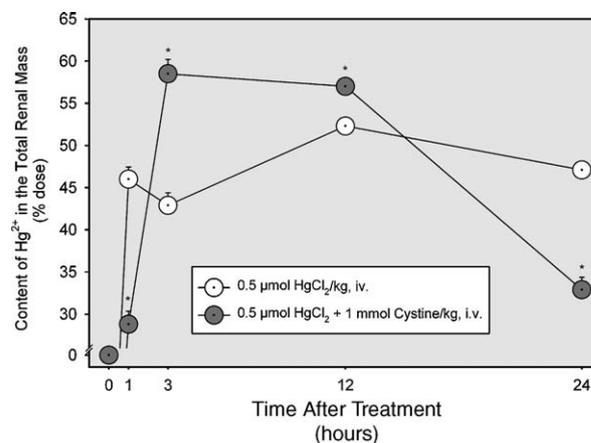


Fig. 1. Content (percent of the administered dose) of inorganic mercury ( $\text{Hg}^{++}$ ) in the total renal mass 1, 3, 12, and 24 h(s) after the intravenous injection of a 0.5  $\mu\text{mol/kg}$  dose of mercuric chloride ( $\text{HgCl}_2$ ), administered with or without a 1 mmol/kg dose of cystine, in 2 mL/kg 0.9% saline. Each value represents the mean  $\pm$  SE obtained from 4–5 animals. \*Significantly different ( $P < 0.05$ ) from the mean for the corresponding control group of rats treated only with the 0.5  $\mu\text{mol/kg}$  dose of  $\text{HgCl}_2$ .

corresponding experimental group of rats co-administered the 0.5  $\mu\text{mol/kg}$  dose of  $\text{HgCl}_2$  and the 1 mmol/kg dose of cystine was only about 29% of the administered dose, which was significantly less than that detected in the control group of rats.

At 3 h after treatment, the renal burden of mercury in the control groups of rats treated only with  $\text{HgCl}_2$  was about 43% of the administered dose. Unlike at 1 h after treatment, the renal burden of mercury in the corresponding experimental group was significantly greater than that in the control group. The renal burden of mercury in this group had increased to about 58% of the administered dose.

The amount of mercury in the total renal mass of the control group of rats at 12 h after treatment was approximately 51% of the administered dose. In the experimental group of rats, the renal burden of mercury was about 57% of the dose, which is significantly greater than that in the control rats.

At the end of 24 h, the renal burden of mercury in the control group was approximately 47% of the dose. By contrast, the renal burden of mercury in the corresponding group of experimental rats treated with  $\text{HgCl}_2$  and cystine was approximately 32% of the administered dose, which is significantly less than that in the corresponding group of control rats.

#### Renal distribution of mercury

**Disposition of mercury in the renal cortex.** During the initial hour after treatment, significant changes occurred in the concentration of mercury in the renal cortex in both groups of rats (Fig. 2A). In the control group, the renal cortical concentration increased to about 38% of the dose/g tissue. However, in the experimental rats treated with  $\text{HgCl}_2$  and cystine, the renal cortical concentration increased to only about 18% of the dose/g tissue, which was significantly less than that in the corresponding group of control rats.

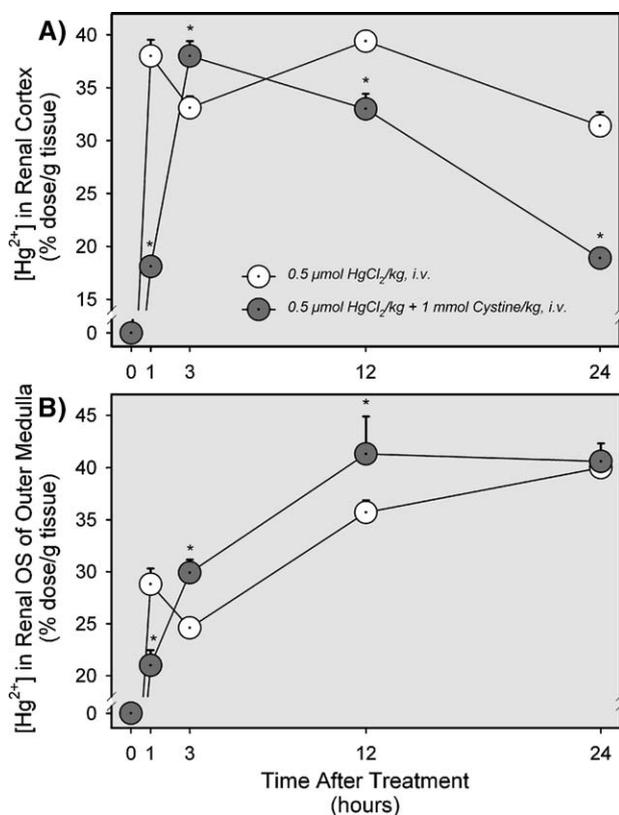


Fig. 2. Concentration (percent of the administered dose per gram tissue) of inorganic mercury ( $\text{Hg}^{2+}$ ) in the renal cortex (A) and outer stripe of the outer medulla (B) 1, 3, 12, and 24 h(s) after the intravenous injection of a 0.5  $\mu\text{mol}/\text{kg}$  dose of mercuric chloride ( $\text{HgCl}_2$ ), administered with or without a 1 mmol/kg dose of cystine, in 2 mL/kg 0.9% saline. Each value represents the mean  $\pm$  SE obtained from 4–5 animals. \*Significantly different ( $P < 0.05$ ) from the mean for the corresponding control group of rats treated only with the 0.5  $\mu\text{mol}/\text{kg}$  dose of  $\text{HgCl}_2$ .

By the end of the initial 3 h posttreatment, the renal cortical concentration in the control group of rats dropped slightly to about 33% of the administered dose/g tissue. In the experimental animals, the renal cortical concentration of mercury increased markedly to about 38% of the dose/g, which was significantly greater than that in the corresponding control group.

At the end of the initial 12 h posttreatment, the concentration of mercury in the renal cortex in the control and experimental groups of rats was about 40% of the dose/g tissue and about 33% of the dose/g tissue, respectively. The renal cortical concentrations of mercury between two groups of rats were significantly different from one another at the time after treatment.

Twenty-four hours after treatment, the renal cortical concentration of mercury in the control group of rats decreased to about 32% of the dose/g tissue. However, in the experimental group of rats, the renal cortical concentration of mercury dropped to about 19% of the dose/g tissue, which was significantly less than that in the corresponding control group.

*Disposition of mercury in the renal outer stripe of the outer medulla.* Significant changes also occurred in the concentra-

tion of mercury in the renal outer stripe of the outer medulla in both groups of rats during the initial hour after treatment (Fig. 2B). The concentration of mercury in the renal outer stripe of the outer medulla in the control group of rats increased to approximately 28% dose/g tissue. In the experimental group of rats, however, the concentration of mercury in the renal outer stripe increased to only about 21% of the dose/g tissue, which was significantly less than that in the corresponding group of control rats.

By 3 h posttreatment, the concentration of mercury in the outer stripe of the outer medulla in the control group of rats had decreased to about 25% of the administered dose/g tissue. In the experimental animals, however, the concentration of mercury in the renal outer stripe increased to about 30% of the dose/g tissue, which was significantly greater than that in the corresponding control group.

Twelve hours after treatment, the concentration of mercury in the renal outer stripe of the outer medulla increased in both the control and experimental groups of rats to about 36% of the dose/g tissue and about 41% of the dose/g tissue, respectively. These two concentrations of mercury were significantly different from one another.

At the end of the initial 24 h after treatment, the concentration of mercury in the renal outer stripe of the outer medulla in the control group of rats increased slightly to about 40% of the dose/g tissue. However, in the experimental group of rats, the concentration of mercury in the renal outer stripe of the outer medulla was similar to that detected at 12 h. The renal concentrations of mercury in the renal outer stripe between the control and experimental groups of rats were not significantly different at 24 h posttreatment.

#### *Hepatic burden of mercury*

At all times studied during the initial 24 h posttreatment, the hepatic burden of mercury was significantly greater in the experimental group of rats injected intravenously with  $\text{HgCl}_2$  and cystine than in the control group of rats treated with only  $\text{HgCl}_2$  (Fig. 3A). In the groups of control animals, the hepatic burden of mercury fell in the range of 6.5 to 8.5% of the administered dose during the period between 1 and 24 h after treatment. By contrast, the hepatic burden of mercury in the experimental animals fell in the range of 10.5 to 14% of the administered dose over the same period of time.

#### *Disposition of mercury in the blood*

By the end of the first hour after treatment with the 0.5  $\mu\text{mol}/\text{kg}$  dose of  $\text{HgCl}_2$ , the content of mercury in the estimated volume of blood in the control rats was approximately 16% of the administered dose (Fig. 3B). In contrast, only about 2.5% of the administered dose of mercury was present in the blood in the experimental group of rats treated with both  $\text{HgCl}_2$  and cystine. In fact, the content of mercury in blood in the groups of rats treated with both  $\text{HgCl}_2$  and cystine did not change significantly during the period between 1 and 24 h posttreatment. At 3 h posttreatment, the content of mercury in blood in the control rats decreased to slightly more than 10% of the

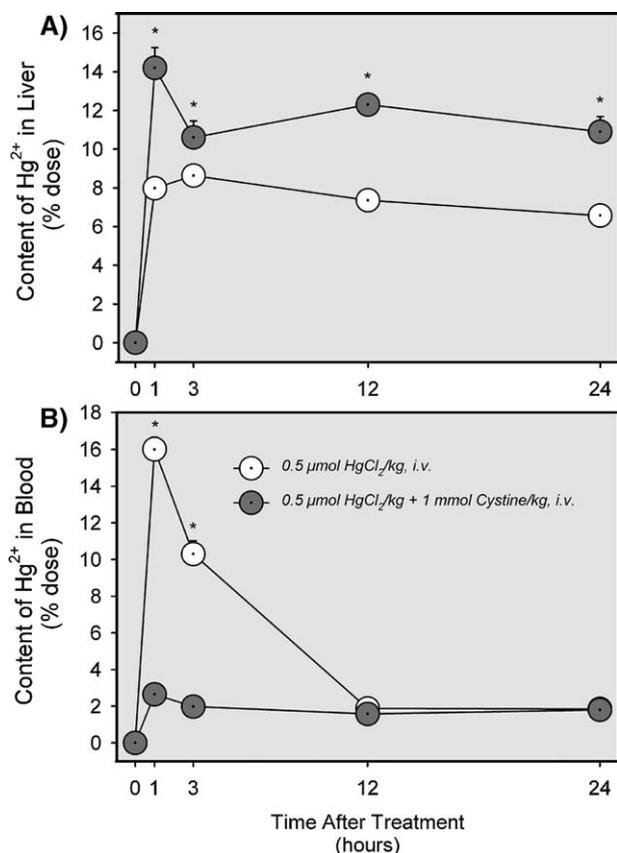


Fig. 3. Content (percent of the administered dose) of inorganic mercury (Hg<sup>2+</sup>) in the liver (A) and blood (B) 1, 3, 12, and 24 h(s) after the intravenous injection of a 0.5 μmol/kg dose of mercuric chloride (HgCl<sub>2</sub>), administered with or without a 1 mmol/kg dose of cystine, in 2 mL/kg 0.9% saline. Each value represents the mean ± SE obtained from 4–5 animals. \*Significantly different ( $P < 0.05$ ) from the mean for the corresponding control group of rats treated only with the 0.5 μmol/kg dose of HgCl<sub>2</sub>.

administered dose. By the end of 12 h posttreatment, the content of mercury in blood between the control and experimental groups of rats was not significantly different, and remained that way through the initial 24 h posttreatment.

#### Urinary excretion of mercury

Urinary excretion of mercury during the 12- and 24-h periods studied was greater in the groups of experimental animals treated with both HgCl<sub>2</sub> and cystine than in the corresponding groups of control rats treated with only HgCl<sub>2</sub> (Fig. 4A). In the control groups of rats, the amount of mercury excreted in 12 and 24 h was approximately 4.5% and 5.5% of the administered dose, respectively. By contrast, the experimental animals excreted 14% and 24.5% of the administered dose in the urine in 12 and 24 h, respectively.

#### Fecal excretion of mercury

As with the urinary excretion of mercury, the fecal excretion of mercury during the 12- and 24-h periods studied was greater in the groups of experimental animals treated with both HgCl<sub>2</sub> and cystine than in the corresponding groups of control rats treated with only HgCl<sub>2</sub> (Fig. 4B).

After the initial 12 h posttreatment, approximately 9% of the administered dose of mercury was excreted in the feces by the control rats. The level of fecal excretion of mercury in the control animals at 24 h posttreatment was not much different from that at 12 h posttreatment. In the experimental animals, approximately 11% of the dose of mercury was excreted during the initial 12 h after treatment. By the end of the first 24 h after treatment, the experimental rats had excreted approximately 11.5% of the administered dose.

#### Experiment 2: comparison of effects of cystine pretreatment and simultaneous exposure on disposition of inorganic mercury

##### Renal burden of mercury

In the rats treated with a 0.5 μmol/kg dose of HgCl<sub>2</sub>, the renal burden of mercury 1 h after treatment with HgCl<sub>2</sub> was approximately 46% of the administered dose (Fig. 5). By contrast, the renal burden of mercury in the corresponding group of rats treated with the intravenous 0.5 μmol/kg dose of HgCl<sub>2</sub> plus the 1 mmol/kg dose of cystine was significantly less, at approximately 29.5% of the administered dose.

The renal burden of mercury in the group of rats pretreated with saline 1 h prior to receiving the intravenous 0.5 μmol/kg dose of HgCl<sub>2</sub> was approximately 43% of the dose 1 h after

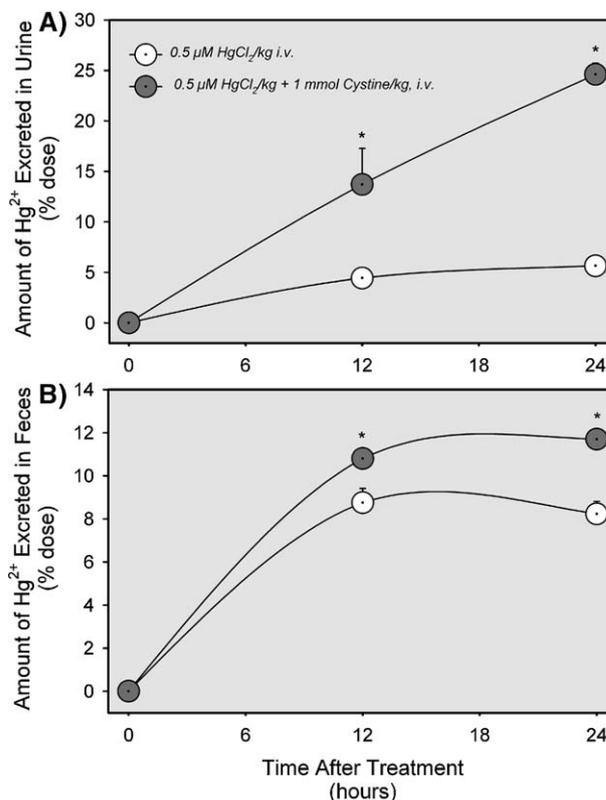


Fig. 4. Urinary (A) and fecal (B) excretion (percent of the administered dose) of inorganic mercury (Hg<sup>2+</sup>) 12 and 24 h(s) after the intravenous injection of a 0.5 μmol/kg dose of mercuric chloride (HgCl<sub>2</sub>), administered with or without a 1 mmol/kg dose of cystine, in 2 mL/kg 0.9% saline. Each value represents the mean ± SE obtained from 4–5 animals. \*Significantly different ( $P < 0.05$ ) from the mean for the corresponding control group of rats treated only with the 0.5 μmol/kg dose of HgCl<sub>2</sub>.

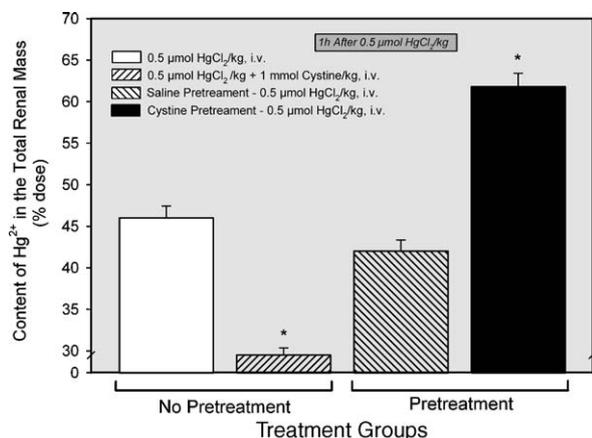


Fig. 5. Content (percent of the administered dose) of inorganic mercury ( $\text{Hg}^{2+}$ ) in the total renal mass. The renal disposition of  $\text{Hg}^{2+}$  was assessed in all groups 1 h after the intravenous injection of a  $0.5 \mu\text{mol/kg}$  dose of mercuric chloride ( $\text{HgCl}_2$ ). Two groups of rats were administered this dose of  $\text{Hg}^{2+}$  with or without a  $1 \text{ mmol/kg}$  dose of cystine, in  $2 \text{ mL/kg}$   $0.9\%$  saline. Two additional groups of rats received an intravenous pretreatment with cystine ( $1 \text{ mmol/kg}$ ) 1 h prior to receiving the intravenous dose of  $\text{Hg}^{2+}$ . Each value represents the mean  $\pm$  SE obtained from 4–5 animals. \*Significantly different ( $P < 0.05$ ) from the mean for the corresponding control group of rats treated only with the  $0.5 \mu\text{mol/kg}$  dose of  $\text{HgCl}_2$ .

treatment (Fig. 5). Interestingly, the renal burden of mercury in the group of rats pretreated with the  $1 \text{ mmol/kg}$  dose of cystine 1 h prior to receiving the intravenous  $0.5 \mu\text{mol/kg}$  dose of  $\text{HgCl}_2$  was significantly greater than that in the corresponding control group pretreated with saline. The actual content of mercury in the total renal mass in these rats was approximately 63% of the administered dose.

#### Renal distribution of mercury

**Disposition of mercury in the renal cortex.** Concentrations of mercury in the renal cortex of the rats treated with a  $0.5 \mu\text{mol/kg}$  dose of  $\text{HgCl}_2$  averaged approximately 37% of the administered dose/g tissue (Fig. 6A). In the corresponding group of rats treated intravenously with  $0.5 \mu\text{mol HgCl}_2/\text{kg}$  plus  $1 \text{ mmol}$  cystine/kg, the concentrations of mercury in the renal cortex 1 h after treatment were significantly lower than those in the corresponding animals treated with only  $\text{HgCl}_2$ , averaging about 18% of the dose/g tissue.

In the group of rats pretreated with saline 1 h prior to receiving the intravenous  $0.5 \mu\text{mol/kg}$  dose of  $\text{HgCl}_2$ , the concentration of mercury in the renal cortex was approximately 34% of the dose/g tissue 1 h after treatment with mercury (Fig. 6A). The renal cortical concentrations of mercury in the corresponding group of rats pretreated with the  $1 \text{ mmol/kg}$  dose of cystine 1 h prior to receiving the intravenous  $0.5 \mu\text{mol/kg}$  dose of  $\text{HgCl}_2$  were significantly greater than those in the corresponding control group pretreated with saline, averaging approximately 43% of the administered dose/g tissue.

**Disposition of mercury in the renal outer stripe of the outer medulla.** At 1 h after treatment, the concentrations of mercury

in the renal outer stripe of the outer medulla of the rats treated with a  $0.5 \mu\text{mol/kg}$  dose of  $\text{HgCl}_2$  averaged approximately 29% of the administered dose/g tissue (Fig. 6B). By contrast, the concentrations of mercury in the renal outer stripe of the outer medulla in the corresponding group of rats treated intravenously with  $0.5 \mu\text{mol HgCl}_2/\text{kg}$  plus  $1 \text{ mmol}$  cystine/kg were also significantly lower than those in the corresponding animals treated with only  $\text{HgCl}_2$ , averaging about 21% of the dose/g tissue.

In the rats pretreated with saline 1 h prior to receiving the intravenous  $0.5 \mu\text{mol/kg}$  dose of  $\text{HgCl}_2$ , the concentrations of mercury in the renal outer stripe of the outer medulla were approximately 30% of the dose/g tissue 1 h after treatment (Fig. 6B). By contrast, the concentrations of mercury in the renal outer stripe of the outer medulla in the corresponding group of rats pretreated with the  $1 \text{ mmol/kg}$  dose of cystine 1 h prior to receiving the intravenous  $0.5 \mu\text{mol/kg}$  dose of  $\text{HgCl}_2$  were significantly greater than those in the corresponding control group pretreated with saline. These concentrations average approximately 39% of the administered dose/g tissue.

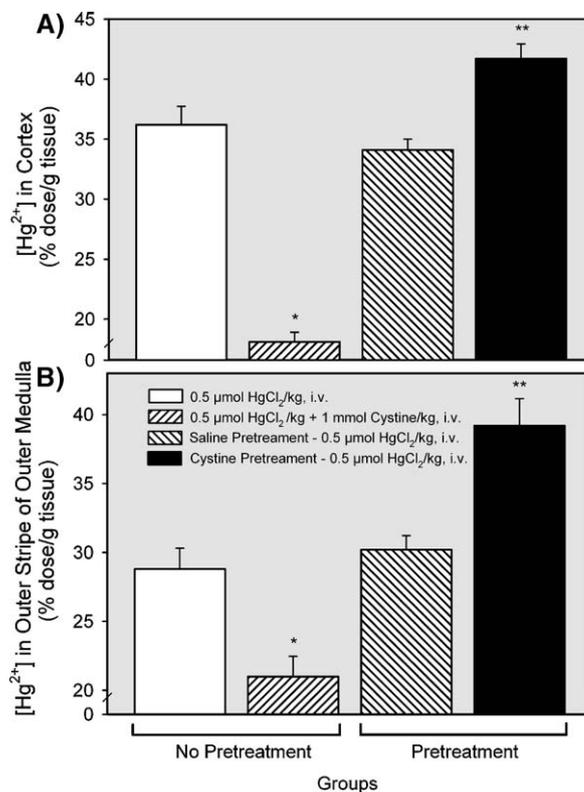


Fig. 6. Concentration (percent of the administered dose per gram tissue) of inorganic mercury ( $\text{Hg}^{2+}$ ) in the renal cortex (A) and outer stripe of the outer medulla (B). The renal disposition of  $\text{Hg}^{2+}$  was assessed in all groups 1 h after the intravenous injection of a  $0.5 \mu\text{mol/kg}$  dose of mercuric chloride ( $\text{HgCl}_2$ ). Two groups of rats were administered this dose of  $\text{Hg}^{2+}$  with or without a  $1 \text{ mmol/kg}$  dose of cystine, in  $2 \text{ mL/kg}$   $0.9\%$  saline. Two additional groups of rats received an intravenous pretreatment with cystine ( $1 \text{ mmol/kg}$ ) 1 h prior to receiving the intravenous dose of  $\text{Hg}^{2+}$ . Each value represents the mean  $\pm$  SE obtained from 4–5 animals. \*Significantly different ( $P < 0.05$ ) from the mean for the corresponding control group of rats treated only with the  $0.5 \mu\text{mol/kg}$  dose of  $\text{HgCl}_2$ . \*\*Significantly different ( $P < 0.05$ ) from all of the other groups of rats.

### Hepatic burden of mercury

The hepatic burden of mercury in the rats treated with a 0.5  $\mu\text{mol/kg}$  dose of  $\text{HgCl}_2$  averaged approximately 8% of the administered dose (Fig. 7A). By contrast, the hepatic burden of mercury in the corresponding group of rats treated intravenously with 0.5  $\mu\text{mol HgCl}_2/\text{kg}$  plus 1 mmol cystine/kg was significantly greater than that in the corresponding animals treated with only  $\text{HgCl}_2$ , averaging about 14% of the administered dose.

Hepatic content of mercury in the rats pretreated with saline 1 h prior to receiving the intravenous 0.5  $\mu\text{mol/kg}$  dose of  $\text{HgCl}_2$  was approximately 30% of the administered dose 1 h after treatment (Fig. 7A). The average hepatic content of mercury in the corresponding group of rats pretreated with the 1 mmol/kg dose of cystine 1 h prior to receiving the intravenous 0.5  $\mu\text{mol/kg}$  dose of  $\text{HgCl}_2$  was not significantly different from that in the corresponding control group pretreated with saline.

### Disposition of mercury in the blood

Approximately 16% of the administered dose of mercury was present in the estimated total volume of blood in the rats

treated with a 0.5  $\mu\text{mol/kg}$  dose of  $\text{HgCl}_2$  (Fig. 7B). By contrast, the amount of mercury in the blood of the corresponding group of rats treated intravenously with 0.5  $\mu\text{mol HgCl}_2/\text{kg}$  plus 1 mmol cystine/kg was significantly less than that in the corresponding animals treated with only  $\text{HgCl}_2$ , averaging about 3% of the administered dose.

The content of mercury in the blood of the rats pretreated with saline 1 h prior to receiving the intravenous 0.5  $\mu\text{mol/kg}$  dose of  $\text{HgCl}_2$  was approximately 14.5% of the administered dose 1 h after treatment (Fig. 7B). In the corresponding group of rats pretreated with the 1 mmol/kg dose of cystine 1 h prior to receiving the intravenous 0.5  $\mu\text{mol/kg}$  dose of  $\text{HgCl}_2$ , the average content of mercury in the blood (7.5% of the dose) was significantly lower than that in the corresponding control group pretreated with saline.

### Experiment 3: effects of cystine pretreatment on plasma thiol/disulfide status

#### Thiol status in plasma

**Cysteine.** Concentrations of cysteine in plasma averaged about 10  $\mu\text{M}$  in the rats 1 h after they were pretreated with 0.9% saline (Fig. 8). In the rats pretreated with the 1 mmol/kg dose of cystine, the plasma levels of cysteine were 50-fold greater than those in the rats pretreated with saline. In the rats pretreated with 0.5  $\mu\text{mol HgCl}_2/\text{kg}$ , the concentration of cysteine in plasma also averaged about 10  $\mu\text{M}$ . In addition, the concentration of cysteine in plasma of the rats pretreated with both  $\text{HgCl}_2$  and cystine averaged about 39-fold greater than that in the rats pretreated with only  $\text{HgCl}_2$ .

**Cystine.** In the rats pretreated with 0.9% saline, plasma concentrations of cystine averaged about 150  $\mu\text{M}$  1 h after pretreatment (Fig. 8). The plasma levels of cystine in the rats pretreated with the 1 mmol/kg dose of cystine were significantly greater than those in the corresponding rats pretreated with saline, averaging about 1.43 mM. Cystine concentrations in the rats pretreated with 0.5  $\mu\text{mol HgCl}_2/\text{kg}$  also averaged about 150  $\mu\text{M}$ . By contrast, in the group of rats pretreated with both  $\text{HgCl}_2$  and cystine, the concentration of cystine in plasma averaged about 1.5 mM.

**Glutathione.** The concentrations of glutathione in the plasma of the rats pretreated with saline averaged about 5  $\mu\text{M}$  1 h after pretreatment (Fig. 8). Concentrations of glutathione in the plasma of rats pretreated with the 1 mmol/kg dose of cystine were approximately 11-fold greater than those in the rats pretreated with saline. In the rats pretreated with only  $\text{HgCl}_2$ , the concentration of glutathione in plasma averaged approximately 3  $\mu\text{M}$ . However, in the rats pretreated with both  $\text{HgCl}_2$  and cystine, the plasma concentration of glutathione was 41  $\mu\text{M}$ .

**Glutathione disulfide.** Glutathione disulfide levels in the plasma of the rats pretreated with saline averaged about 0.4  $\mu\text{M}$  1 h after pretreatment (Fig. 8). By contrast, the

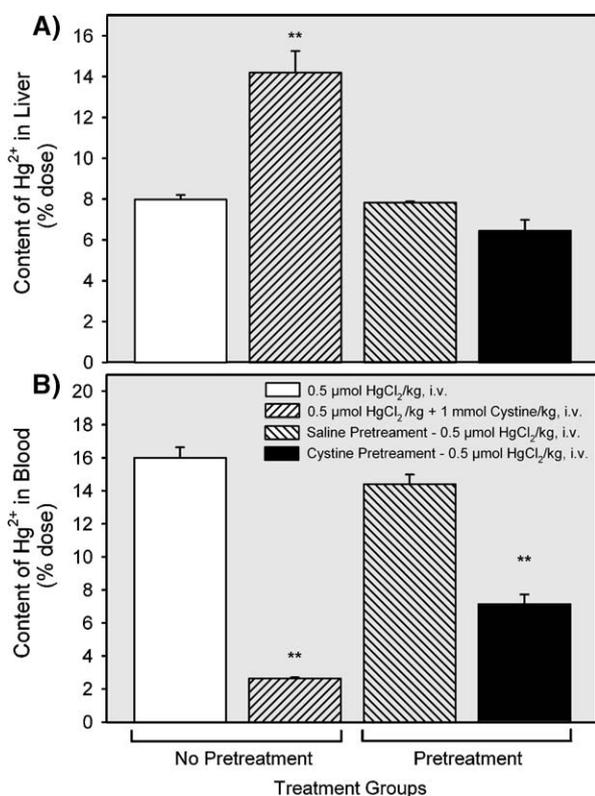


Fig. 7. Content (percent of the administered dose per gram tissue) of inorganic mercury ( $\text{Hg}^{2+}$ ) in the liver (A) and blood (B). The hepatic and hematological disposition of  $\text{Hg}^{2+}$  was assessed in all groups 1 h after the intravenous injection of a 0.5  $\mu\text{mol/kg}$  dose of mercuric chloride ( $\text{HgCl}_2$ ). Two groups of rats were administered this dose of  $\text{Hg}^{2+}$  with or without a 1 mmol/kg dose of cystine, in 2 mL/kg 0.9% saline. Two additional groups of rats received an intravenous pretreatment with cystine (1 mmol/kg) 1 h prior to receiving the intravenous dose of  $\text{Hg}^{2+}$ . Each value represents the mean  $\pm$  SE obtained from 4–5 animals. \*Significantly different ( $P < 0.05$ ) from the mean for the corresponding control group of rats treated only with the 0.5  $\mu\text{mol/kg}$  dose of  $\text{HgCl}_2$ . \*\*Significantly different ( $P < 0.05$ ) from all of the other groups of rats.

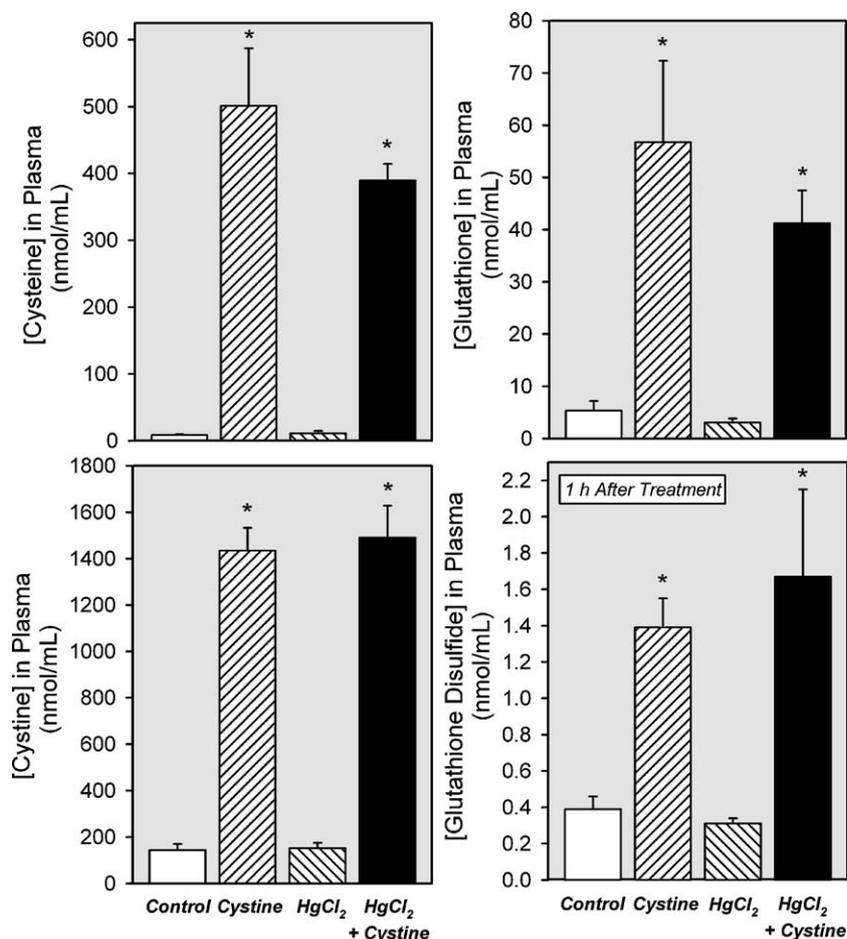


Fig. 8. Concentration (nmol/mL) of cysteine, cystine, glutathione, and glutathione disulfide in the plasma 1 h after pretreatment with or without a 1 mmol/kg intravenous dose of cystine in the presence or absence of a 0.5  $\mu\text{mol/kg}$  dose of mercuric chloride ( $\text{HgCl}_2$ ). Each value represents the mean  $\pm$  SE obtained from 4–5 animals. \*Significantly different ( $P < 0.05$ ) from the mean for the corresponding control group of rats.

concentrations of glutathione disulfide in the plasma of rats pretreated with the 1 mmol/kg dose of cystine were approximately 1.4  $\mu\text{M}$ . In the rats pretreated with  $\text{HgCl}_2$ , the concentrations of glutathione disulfide in plasma averaged approximately 0.3  $\mu\text{M}$ . Finally, the plasma concentrations of glutathione disulfide in the rats pretreated with both  $\text{HgCl}_2$  and cystine were approximately 1.7  $\mu\text{M}$ .

## Discussion

Prior to the present investigation, several sets of recent *in vitro* data have implicated the formation of the mercuric conjugate, cysteine–Hg–cysteine, and the transport of this complex by one or more cystine transporters in the luminal plasma membrane as components of the mechanisms involved in the renal proximal tubular uptake of inorganic mercury (Bridges et al., 2004; Cannon et al., 2000, 2001; Zalups, 1995; Zalups and Barfuss, 1996; Zalups and Lash, 1997; Zalups and Barfuss, 1998a, 1998b). In particular, recent data from MDCK cells transfected stably with system  $\text{b}^{0,+}$  (which, in the kidneys, is a sodium-independent amino acid transporter inserted primarily in the luminal plasma membrane of proximal tubular epithelial cells) provide strong evidence supporting the

hypothesis that cysteine–Hg–cysteine can behave chemically as a molecular homolog and mimic of the amino acid cystine at system  $\text{b}^{0,+}$  as a means to gain entry into the cytosolic compartment of proximal tubular epithelial cells (Bridges et al., 2004). Additional recent data from MDCK cells transfected stably with OAT1 and from *Xenopus laevis* oocytes micro-injected with the cRNA for OAT1 indicate that cysteine–Hg–cysteine is also a transportable substrate of OAT1 and OAT3 (which are expressed exclusively on the basolateral membrane of proximal tubular epithelial cells). However, it is not clear at present whether the OAT1- or OAT3-dependent uptake of cysteine–Hg–cysteine involves molecular mimicry. Further studies are clearly needed for this determination.

Although the *in vitro* data mentioned above provide strong evidence that cystine can serve as a competitive inhibitor for the luminal uptake of cysteine–Hg–cysteine, there are no data from whole animals supporting this hypothesis. Thus, the primary purpose of the present study was to test the hypothesis that cystine can inhibit the renal tubular uptake of inorganic mercury *in vivo*. To accomplish this aim, we simultaneously co-injected (i.v.) a 1 mmol/kg dose of cystine with a 0.5  $\mu\text{mol/kg}$  dose of mercuric chloride. An additional aim was to determine if pretreatment with cystine influences the subsequent disposition

of intravenously administered inorganic mercury, especially when it administered as mercuric chloride. The status of various thiols in plasma was also examined after the intravenous administration of a 1 mmol/kg dose of cystine in order to correlate the disposition (especially the renal disposition) of mercuric ions with plasma thiol status.

During the initial hour after the intravenous administration of the 1 mmol/kg dose of cystine and the 0.5  $\mu\text{mol/kg}$  dose of mercuric chloride, the renal tubular uptake and net accumulation of mercuric ions was diminished significantly relative to that in control rats treated only with the 0.5  $\mu\text{mol/kg}$  dose of mercuric chloride. The inhibitory effects of cystine were evident in both the renal cortex and outer stripe of the outer medulla, which are the renal zones containing the three segments of the proximal tubule. In the control rats treated only with mercuric chloride, approximately 45% of the administered dose of mercury was present in the combined renal mass by the end of the initial hour after treatment. This level in the renal burden of mercury is consistent with that detected typically after the initial hour following the intravenous treatment with the 0.5  $\mu\text{mol/kg}$  dose of mercuric chloride. In general, approximately 40–45% of the administered dose of inorganic mercury is present in the total renal mass of a rat one hour after treatment (Zalups, 1993, 1995; Zalups and Barfuss, 1995b, 1998a).

The dispositional findings obtained during the initial hour after the simultaneous exposure to cystine and inorganic mercury are consistent with recent *in vitro* findings from MDCK cells transfected stably with the heavy (rBAT) and light ( $b^{0,+}$  AT) chain subunits of system  $b^{0,+}$  (Bridges et al., 2004). These findings demonstrate that both cystine and cysteine–Hg–cysteine are transportable substrates of system  $b^{0,+}$ . Thus, based on the dispositional data obtained during the initial hour after co-exposure to cystine and mercuric chloride, and the data on thiol status obtained in rats treated with the 1 mmol/kg dose of cystine with or without the 0.5  $\mu\text{mol/kg}$  dose of mercuric chloride, there is sufficient justification to postulate that cystine can serve as a competitive substrate (or molecular mimic) at the sites of transporters (such as system  $b^{0,+}$ ) responsible for the proximal tubular uptake of cysteine–Hg–cysteine and cystine.

Interestingly, when the 0.5  $\mu\text{mol/kg}$  dose of mercuric chloride was administered 1 h after pretreatment with the 1 mmol/kg dose of cystine, the renal accumulation and content of mercuric ions (especially in the renal cortex and outer stripe of the outer medulla) were significantly greater than those in corresponding control rats treated only with the 0.5  $\mu\text{mol/kg}$  dose of mercuric chloride. This enhanced net accumulation of inorganic mercury is likely related to the fact that the circulating concentrations of the reduced thiols cysteine and glutathione were greatly increased (likely as the result of both hepatic and renal conversion of cystine to these thiols), which provided additional thiol substrates to bind and interact with the injected mercuric ions. It has been demonstrated previously in our laboratories that co-administration of mercuric ions with either cysteine or glutathione enhances the renal accumulation and toxicity of mercury (Zalups and Barfuss, 1995a, 1995b, 1996). There is strong *in vivo* and *in vitro* evidence indicating that the

mercuric conjugates glutathione–Hg–glutathione and cysteine–Hg–cysteine are biologically relevant species of inorganic mercury that are handled by renal tubular epithelial cells (Zalups, 2000). Experimental evidence also indicates that mercuric conjugates of glutathione are converted in the lumen of the proximal tubule to mercuric conjugates of cysteine by the sequential actions of  $\gamma$ -glutamyltransferase and cysteinylglycine, which are present in the luminal plasma membrane. Thus, the primary species of inorganic mercury that are taken up from the luminal compartment of the proximal tubule are mercuric conjugates of cysteine, in particular, cysteine–Hg–cysteine (Zalups, 2000).

Unlike the renal disposition of mercuric ions detected 1 h after the simultaneous co-exposure to cystine and mercuric chloride, the renal accumulation of inorganic mercury between the first and 12th hour posttreatment increased to a level that was significantly greater in the rats treated with both cystine and mercuric chloride than in corresponding rats treated only with mercuric chloride. Although the precise mechanisms for the change in the renal disposition of inorganic mercury during this period of time are not known, it is likely that alterations in thiol status, particularly the increases in the plasma concentration of reduced thiols, played an important role in promoting the uptake of inorganic mercury in the renal cortex and outer stripe of the outer medulla.

Interestingly, at 24 h posttreatment, the renal content of inorganic mercury in the rats treated with both cystine and mercuric chloride was again significantly lower than that in the rats treated only with inorganic mercury. This diminished renal accumulation of inorganic mercury in the rats treated with cystine and mercuric chloride was likely related to the decreased accumulation of inorganic mercury in the renal cortex.

The animals treated with both cystine and mercuric chloride also excreted significantly more inorganic mercury in the urine throughout the 24 h after treatment than the corresponding control rats. This enhanced urinary excretion of inorganic mercury correlates with the detected patterns in the renal accumulation/retention of inorganic mercury and is consistent with cystine playing an inhibitory role in the uptake of inorganic mercury during the initial 24 h after treatment.

During all periods studied, hepatic content of inorganic mercury and fecal excretion of mercury were significantly greater in the rats co-treated with cystine and mercuric chloride than in the rats treated with just mercuric chloride. These data suggest that enhanced biliary secretion of mercury was a primary mechanism responsible for the increased fecal excretion of inorganic mercury in the rats treated with both cystine and mercuric chloride (relative to that in the control rats).

Plasma levels of mercury were also greatly diminished during the initial hour in the rats treated with both cystine and mercuric chloride relative to those in the control rats. Interestingly, the hepatic content of mercury was similar in the rats whether or not they were pretreated with cystine prior to being exposed to a non-toxic dose of inorganic mercury. The mechanisms underlying the plasma disposition, the enhanced hepatic uptake, and enhanced fecal excretion of mercury are not

fully understood. Clearly, additional studies are warranted to better define the mechanisms underlying the dispositional findings detected in the present investigation.

Overall, the present findings indicate that when cystine and a non-toxic dose of inorganic mercury are administered simultaneously, cystine functions as an inhibitor of the renal tubular uptake of inorganic mercury during the initial hours after co-administration. However, when rats are subjected to conditions promoting increases in the concentrations of non-protein thiols in blood prior to exposure of inorganic mercury (such as that generated by pretreatment with cystine), conditions are generated in vivo that lead to marked increases in the renal uptake and accumulation of inorganic mercury (relative to that in rats not pretreated with cystine). Overall, the present findings indicate that thiol status is an important determinant in the renal and hepatic disposition, and the urinary and fecal excretion, of inorganic mercury. The present findings also have significant toxicological implications. Enhanced renal uptake and accumulation of inorganic mercury promoted by extracellular thiols (especially by cysteine) have been shown to be associated with increased levels of cellular injury in renal proximal tubules both in vivo (Zalups and Barfuss, 1996) and in vitro (Lash et al., 1999). Moreover, the effects of cystine detected in the present study also have physiological implications in understanding the mechanisms involved in the disposition and handling of inorganic mercury because plasma contains relatively high concentrations of free cystine (present study and Lash and Jones, 1985).

### Acknowledgments

Support for the present study was provided by grants awarded to RKZ by the National Institute of Environmental Health Sciences (ES05157, ES05980, and ES11288).

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