

# Temporal Changes in Metallothionein Gene Transcription in Rat Kidney and Liver: Relationship to Content of Mercury and Metallothionein Protein<sup>1</sup>

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## ABSTRACT

Metallothioneins are encoded by a family of genes that are induced by inorganic mercury. Despite the well-characterized acute response of metallothionein (*MT*) genes in the kidneys and liver after a single exposure to inorganic mercury, relatively little is known about the activity of these genes and the content of *MT* protein during prolonged periods after exposure. Rats treated with inorganic mercury accumulate mercury rapidly in kidneys and liver during the first 24 h after exposure, but only in the kidneys does the content of mercury remain elevated throughout the initial 2 weeks. We report herein that transcription of *MT* genes in response to treatment with inorganic mercury differs dramatically between the kidneys and liver. *MT* gene transcription and levels of *MT* protein remained elevated

in the kidneys throughout 14 days after treatment. In contrast, the initially high rates of *MT* gene transcription and enhanced content of *MT* protein in the liver fell to control levels by 14 days. In the liver, the rates of *MT* gene transcription and levels of *MT* protein were strongly correlated with each other and with the content of mercury. In the kidneys, however, these correlations were very weak or absent. Our data indicate that hepatic levels of *MT* protein are determined primarily by *MT* gene transcription, but that post-transcriptional events are important in determining the renal content of *MT* protein during the initial weeks after exposure. This has important implications in understanding differences in mechanisms controlling *MT* expression in the kidneys and liver.

Within the first 24 h after treating rats i.v. with a non-nephrotoxic dose of mercuric chloride, the greatest fraction (approximately 50%) of the dose ends up in the kidneys (Rothstein and Hayes, 1960; Zalups, 1995, 2000). During the initial week after exposure, very little of the renal burden of mercury is reduced in spite of 30 to 35% of the dose having been excreted in the urine and feces. However, the hepatic burden of mercury decreases by approximately 75% within 24 h after exposure (Zalups, 1995). Much of the reduction of the hepatic burden of mercury is likely related to hepato-biliary excretion of mercury. This is reflected, in part, by the amount of inorganic mercury in feces. It is unclear what factor(s) and events are responsible for the retention of mercury by renal tubular epithelial cells, particularly those lining the proximal tubule (Zalups and Barfuss, 1990; Zalups, 1991a,b).

It is well established that various metal ions (especially group IIB) induce transcription of metallothionein (*MT*) genes, particularly those encoding *MT*-1 and *MT*-2 proteins,

in organs such as the kidneys and liver (Durnam et al., 1981; Koropatnick and Leibbrandt, 1995). We had hypothesized that the increased content of *MT* induced in both kidneys and liver acts as an intracellular ligand to bind mercury, and that the content of induced *MT* persists in the kidneys (consistent with chronically elevated renal content of mercury), whereas it is reduced rapidly in the liver (consistent with the progressive losses of hepatic mercury soon after acute exposure). In addition, we had postulated that rates of transcription of *MT* genes would be the primary mechanism responsible for changes in the cellular content of *MT* protein. Although the physiological roles of the different *MT*s induced by developmental and differentiation signals, and by nonphysiological events, including induction by heavy metals, remains unclear (Palmiter, 1998), there is evidence linking the induction of transcription of these genes and the cellular production of *MT* protein in target cells exposed to exogenous heavy metal ions (Zhu and Thiele, 1996). Based on data from a previous dispositional study (Zalups, 1995), we also had hypothesized that long-term retention of inorganic mercuric ions in the kidneys correlates closely with transcription of *MT* genes and with the cellular content of *MT* protein. High intracellular

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**ABBREVIATIONS:** *MT*, metallothionein; DTT, dithiothreitol; GADPH, glyceraldehyde phosphate dehydrogenase.

levels of MT are capable of sequestering mercury and preventing toxic effects of mercury in different organs (Zalups and Cherian, 1992a,b; Morcillo and Santamaria, 1996; Dameron and Harrison, 1998; Sugawara et al., 1998). Moreover, we postulate that chronically elevated rates of MT gene transcription in kidney, but not in liver, after exposure to mercury, would provide a mechanism for persistent retention of mercury in the kidneys.

One of the primary aims of the present study was to determine the relationships among the disposition of inorganic mercury, the induction of MT gene transcription, and the production of MT protein in the kidneys and liver over a 2-week period after a single exposure to a low, non-nephrotoxic dose of mercuric chloride. We report, for the first time, that there is a strong relationship between the content of inorganic mercury and the concentration of MT or relative rate of transcription of *MT-1* and *MT-2* genes in the liver. However, the relationship between the content of inorganic mercury and the rate of MT gene transcription or content of MT protein in the kidneys is weak. Furthermore, the relationship between MT gene transcription and MT protein is virtually nonexistent, in strong contrast with the situation in liver. These data suggest that post-transcriptional events play a significant role in MT expression in kidney.

## Materials and Methods

**General Experimental Design.** The disposition of injected inorganic mercury, the content of MT protein, and the rates of transcription of *MT-1* plus *MT-2* genes were determined in the kidneys and liver at various times during the course of the initial 2 weeks after a single i.v. injection of a nontoxic dose of mercuric chloride. This allowed us to test the hypothesis that there are relationships between content of mercury and the content of MT protein and/or rates of transcription of MT genes in these organs.

**Animals and Groups Used.** Male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) were fed a commercial laboratory diet and water ad libitum and kept in approved animal quarters at 20°C, on a 12-h light/dark cycle, and at a relative humidity of 50%. Rats were subdivided randomly into two treatment groups. One group received a single i.v. 0.5- $\mu$ mol/kg dose of mercuric chloride in 0.9% saline and the other (control) group was injected i.v. with 0.9% saline alone. All injections were administered by using a volume per body weight ratio of 2 ml/kg.

Each treatment group was subdivided into four groups of five or six animals in a random manner. Paired subgroups of control and mercury-treated rats were housed individually in plastic metabolic cages for 1, 3, 7, or 14 days after treatment.

**Injection of Inorganic Mercury.** All rats given 0.5  $\mu$ mol/kg mercuric chloride were administered the dose into the femoral vein while under light anesthesia induced by diethyl ether. The injection solution was designed to deliver 0.5  $\mu$ mol of Hg<sup>2+</sup>/kg plus 4  $\mu$ Ci of <sup>203</sup>Hg<sup>2+</sup>/kg in 2.0 ml of normal saline (0.2-ml injection volume/100 g b.wt.). Radioactive inorganic mercury was in the form of mercuric chloride (<sup>203</sup>HgCl<sub>2</sub>, specific activity = 26 mCi/mg; Buffalo Materials Corp., Buffalo, NY).

**Collection of Urine and Feces.** Urine and feces were collected continuously from each of the mercury-treated animals during the duration of time that the respective groups of animals were held in the metabolic cages (i.e., 1, 3, 7, or 14 days). The total amounts of urine and feces excreted during each 24-h period were removed from the metabolic cages of all the animals. Each sample was first weighed to determine the amount excreted during the respective 24-h period. One milliliter of each urine sample was sealed in a 12  $\times$  75-mm gamma-counting tube to determine the amount of inorganic

mercury excreted. In contrast, all feces excreted in each 24 h were sealed in multiple 16  $\times$  90-mm polypropylene tubes.

**Acquisition and Handling of Tissues.** Paired groups of animals injected with inorganic mercury or saline were anesthetized heavily with a 100-mg/kg dose of sodium pentobarbital (i.p.) at 1, 3, 7, or 14 days after treatment and two 1-ml samples of whole blood obtained from the inferior vena cava. One milliliter of whole blood was sealed in a preweighed 12  $\times$  75-mm, round bottom, gamma-counting tube and another 1.0 ml was centrifuged at 10,000g to separate the cellular and plasma fractions of blood. Both fractions were sealed separately in gamma-counting tubes. The kidneys and liver were then removed, cleared of fat and connective tissue, and weighed quickly. The left kidney was cut along the transverse plane and one-half was sealed in a preweighed gamma-counting tube. A 1-g sample of liver was obtained similarly and sealed for gamma counting. The right kidney and an additional 1-g sample of liver from each animal were snap frozen in polypropylene tubes for isolation of nuclei for analysis of MT gene transcription, and for measurement of MT protein content.

**Determination of Content of Mercury in Samples.** Radioactivity of <sup>203</sup>Hg<sup>2+</sup> in tissues, urine, feces, and injection solution (standards) was determined by counting samples in a 1282 Compugamma CS deep-well gamma spectrometer equipped with a 3-inch sodium iodide crystal (Wallac, Gaithersburg, MD), with a counting efficiency of approximately 50%. The content of inorganic mercury in each sample was calculated by dividing the activity of <sup>203</sup>Hg<sup>2+</sup> (dpm) in the sample by the specific activity of <sup>203</sup>Hg<sup>2+</sup> (dpm/nmol) in the injection solution. Concentrations of mercury in the tissues are expressed as percentage of the administered dose per gram of tissue and the content of mercury in organs is expressed as a percentage of the administered dose. The total volume of blood in rats was estimated to be ~6% of body weight.

**Run-On Transcription.** Snap-frozen samples of kidney or liver were homogenized in 10 volumes of buffer 1 [0.32 M sucrose, 3 mM CaCl<sub>2</sub>, 2 mM Mg(OAc)<sub>2</sub>, 0.1 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol (DTT), 10 mM Tris-HCl, pH 8.0] by using a motor-driven Teflon-glass homogenizer. The homogenate was filtered through Miracloth, homogenized again, and combined with 2 volumes of buffer II [2 M sucrose, 5 mM Mg(OAc)<sub>2</sub>, 0.1 mM EDTA, 1 mM DTT, 10 mM Tris-HCl, pH 8.0]. The crude nuclear suspension was layered over a 10-ml cushion (2 M sucrose, 5 mM magnesium acetate, 0.1 mM EDTA, 10 mM Tris-HCl, pH 8.0) and pelleted by ultracentrifugation at 24,000 rpm for 60 min in an SW-28 rotor at 4°C. The pellet of nuclei was suspended in nuclei storage buffer (40% glycerol, 5 mM MgCl<sub>2</sub>, 50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA), counted by hemacytometer, and stored at a concentration of 2  $\times$  10<sup>7</sup> nuclei/400  $\mu$ l in liquid nitrogen until use. The yield of intact nuclei was approximately 50 to 60% of that obtained by using fresh, unfrozen tissue. RNA elongation reactions were performed for 30 min at 30°C by using 2  $\times$  10<sup>7</sup> nuclei/400- $\mu$ l reaction. Reaction mixtures were composed of 20  $\mu$ l of nuclei storage buffer [plus 200  $\mu$ l of sterile 2 $\times$  reaction buffer (10 mM Tris-HCl, pH 8.0, 5 mM MgCl<sub>2</sub>, 0.3 M KCl, 1 mM ATP, 1 mM CTP, 1 mM GTP, 5 mM DTT, 2  $\mu$ l [ $\alpha$ -<sup>32</sup>P]CTP (~3000 Ci/mmol, 10 mCi/ml)]. Nucleotides, radionucleotides, and DTT were added immediately before use. Nascent RNA transcripts were allowed to elongate for 30 min at 30°C on a shaking platform, followed by addition of 600  $\mu$ l of RNase-free DNase 1 [0.04 U of RQ1 DNase 1 (RNase-free; Promega, Madison WI), 0.5 M NaCl, 50 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 10 mM Tris-HCl, pH 7.4]. The <sup>32</sup>P-labeled RNA was isolated by using Trizol (Life Technologies, Inc., Grand Island, NY), and the final precipitated RNA was dissolved in Church hybridization buffer (1 mM EDTA, 0.5 M NaHPO<sub>4</sub>, pH 7.2, 7% sodium lauryl sulfate), to a final concentration of 4  $\times$  10<sup>6</sup> cpm/ml.

**Hybridization of Radiolabeled RNA to Immobilized Unlabeled Probes.** Target DNA (immobilized on nylon filters in triplate dots, 2  $\mu$ g/dot) consisted of the following: 1) a single-stranded synthetic oligonucleotide (5'-ATGGACCCCACTGCTCCTG-3') complementary to a sequence at the 5' end of both *MT-1* and *MT-2*

mRNA, and 2) an unlabeled cDNA probe complementary to glyceraldehyde phosphate dehydrogenase (*GAPDH*) mRNA (Denhardt et al., 1988), denatured and immobilized on the same nylon filters by using a previously described protocol (Koropatnick, 1980). Hybridization of radiolabeled RNA to these dots assessed transcription of *GAPDH* genes and acted as internal standards against which to measure changes in *MT-1* + *MT-2* gene transcription. Nylon filters containing triplicate dots of unlabeled target DNA were prehybridized in Church buffer for 20 min at 65°C in a Hybaid hybridization chamber. The prehybridization buffer was then removed, 2 ml of radiolabeled RNA resulting from 30 min of run-on transcription in isolated nuclei (in Church hybridization buffer,  $4 \times 10^6$  cpm/ml) was added, and the filters were hybridized for 48 h at 65°C. The filters were then washed twice at 65°C in posthybridization buffer (40 mM  $\text{Na}_2\text{HPO}_4$ , 1% SDS; 20 min/wash). Posthybridization buffer was removed and 8 ml of RNase A (1  $\mu\text{g}/\text{ml}$  in  $6\times$  standard saline citrate) was added and incubated for 30 min at 37°C to reduce signal from unhybridized radiolabeled RNA. After a final wash in posthybridization buffer (10 min, 37°C) filters were blotted dry, dots were individually cut out, and the radioactivity of bound  $^{32}\text{P}$  was measured by scintillation spectrophotometry with an LKB Rackbeta 1211 liquid scintillation counter. This was distinguishable from  $^{203}\text{Hg}$  present in samples by appropriate choice of energy windows to detect decay particles. Phosphorimage visualization of hybridization was not performed because  $^{203}\text{Hg}$  present in samples introduced an unacceptably high background that was not possible to exclude with that technology. The ratio of *MT-1* + *MT-2* gene transcription to the transcription of the housekeeping *GAPDH* gene was used as a measure of relative rate of *MT* gene transcription.

As a means to assess potential toxic effects of treatment with inorganic mercury, overall transcription ( $^{32}\text{P}$ ]CTP incorporation into total cellular RNA) and *GAPDH* gene transcription ( $^{32}\text{P}$ ]CTP incorporated in RNA hybridized to a *GAPDH* cDNA probe, per  $8 \times 10^6$  total radiolabeled RNA) were calculated for each sample of kidney and liver (for all animals) at all times studied after exposure to mercury. Lack of change in overall transcription, or *GAPDH* transcription only, would indicate a lack of cellular toxicity induced by inorganic mercury.

**Measurement of MT Protein.** MT protein in tissue samples was measured, in triplicate, by solid-phase radioimmunoassay as described previously (Leibbrandt et al., 1991) with a rabbit polyclonal antiserum to rat MT-2, which has affinity for both MT-1 and MT-2 isoforms. Briefly, samples of renal and hepatic tissues were homogenized in 4 volumes of 0.25 M sucrose and centrifuged at 20,000g for 10 min at 4°C. The supernatants were heated to 70°C for 10 min to precipitate heat-denaturable proteins, cooled on ice, and then centrifuged at 20,000g for 20 min at 4°C. The amount of MT-1 + MT-2 protein in the supernatant was expressed relative to total tissue weight.

**Numerical and Statistical Analysis.** Data expressed as a percentage of a total were first normalized with the arcsine transformation before applying any parametric statistical analysis. This transformation takes the arcsine of the square root of the decimal fraction of the percentage score.

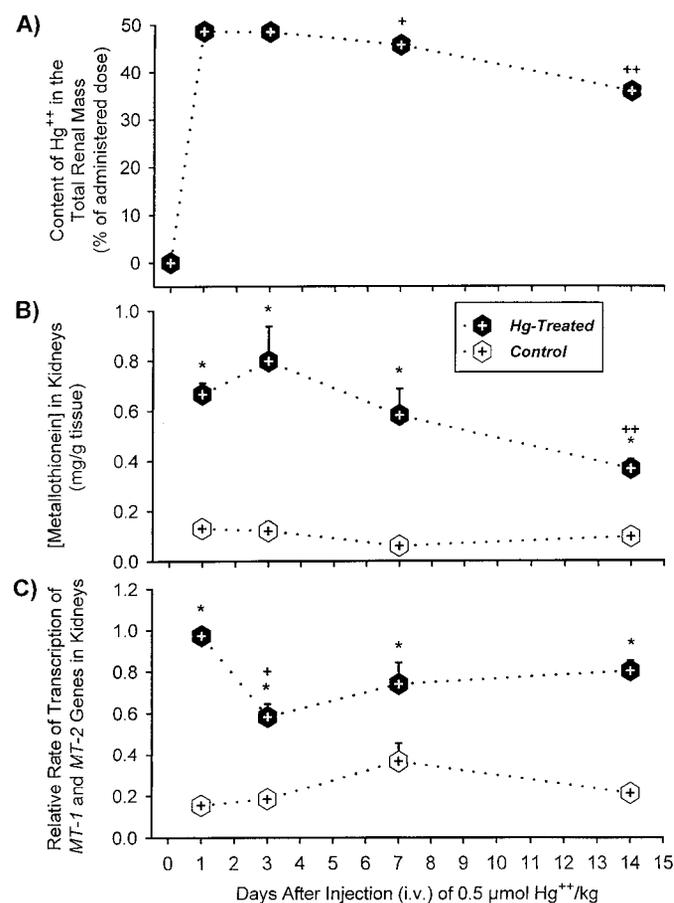
Data for the disposition of inorganic mercury, content of MT protein, and rates of transcription of *MT-1* and *MT-2* genes in the kidneys and liver are presented as mean  $\pm$  S.E. for an  $n$  of five or six animals. For the cumulative excretion of inorganic mercury in the urine and feces, the data for day 1, 3, 7, and 14 represent the mean  $\pm$  S.E. for  $n = 23, 17, 12,$  and  $6$  animals, respectively. Assessment of differences among means for the renal and hepatic disposition of inorganic mercury was carried out with a one-way ANOVA followed by Tukey's protected  $t$  test post hoc procedure. Differences among means for the data for MT protein or rates of transcription of *MT-1* and *MT-2* genes were carried out with a two-way ANOVA, followed by Tukey's protected  $t$  test post hoc procedure. The two variables assessed with these analyses were time and treatment. In all three studies, evaluation of differences among means for any set of data

was carried out by applying a two-way ANOVA followed by Tukey's protected  $t$  test. The level of significance for all statistical analyses was chosen a priori to be  $P < .05$ . Determination of the mathematical function that best describes the cumulative urinary excretion of mercury in the urine and feces was done with nonlinear regression, best-fit, least-squares analysis.

To better understand the potential relationships between the concentration of mercury and the concentration of MT protein or the rate of transcription of *MT-1* and *MT-2* genes in the kidneys and liver, linear regression analysis was applied to plots of individual data derived from each animal studied. The relationships between the rate of transcription of *MT-1* and *MT-2* genes and the concentration of MT in the kidneys and liver was also carried out using linear regression analysis.

## Results

**Renal Burden of Mercury.** Approximately 50% of the administered dose of inorganic mercury was present in the total renal mass 1 day after the i.v. injection of the 0.5- $\mu\text{mol}/\text{kg}$  dose of mercuric chloride (Fig. 1A). There was no significant difference in the renal burden of mercury between



**Fig. 1.** Content of inorganic mercury (percentage of the administered dose) (A), concentration of MT (B), and rate of transcription of *MT-1* and *MT-2* genes (relative to the rate of transcription of the *GAPDH* gene) in the kidneys of rats at 1, 3, 7, and 14 days after the i.v. injection of 0.5  $\mu\text{mol}/\text{kg}$  mercuric chloride or saline. Each value represents the mean  $\pm$  S.E. obtained from five or six animals. \*, significantly different ( $P < .05$ ) from the mean for the corresponding control group (treated with normal saline). +, significantly different ( $P < .05$ ) from the mean for the corresponding group (treated in the same manner) that was studied 1 day after treatment. ++, significantly different ( $P < .05$ ) from the means for the corresponding groups (treated in the same manner) that were studied 1 and 3 days after treatment.

the animals studied on the 1st day after treatment and the animals studied on the 3rd day after treatment. At 7 days after treatment, the renal burden of mercury was approximately 6% lower than that in the animals studied at 1 day after treatment. The greatest decrease in renal mercury was detected in the animals studied at 14 days after treatment, and was approximately 26% less than that in the animals studied 1 day after treatment.

**Renal Concentration of MT Protein.** At all times studied, the renal concentration of MT (mg/g of tissue) was significantly greater in the rats treated with the 0.5- $\mu$ mol/kg dose of mercuric chloride than in the corresponding control rats treated with normal saline (Fig. 1B). Among the four groups of rats treated with inorganic mercury, only in the rats studied at 14 days after treatment were significant differences in the renal concentration of MT detected. The renal concentration of MT in these animals was significantly lower than that in the corresponding rats studied 1 or 3 day(s) after the injection of mercuric chloride.

**Relative Rates of Transcription of *MT-1* and *MT-2* Genes in Kidney.** The rate of transcription of *MT-1* and *MT-2* genes (relative to the rate of transcription of the gene for GAPDH) in the kidneys was significantly greater in the rats treated with mercuric chloride than in the corresponding rats treated with normal saline at each time studied (Fig. 1C).

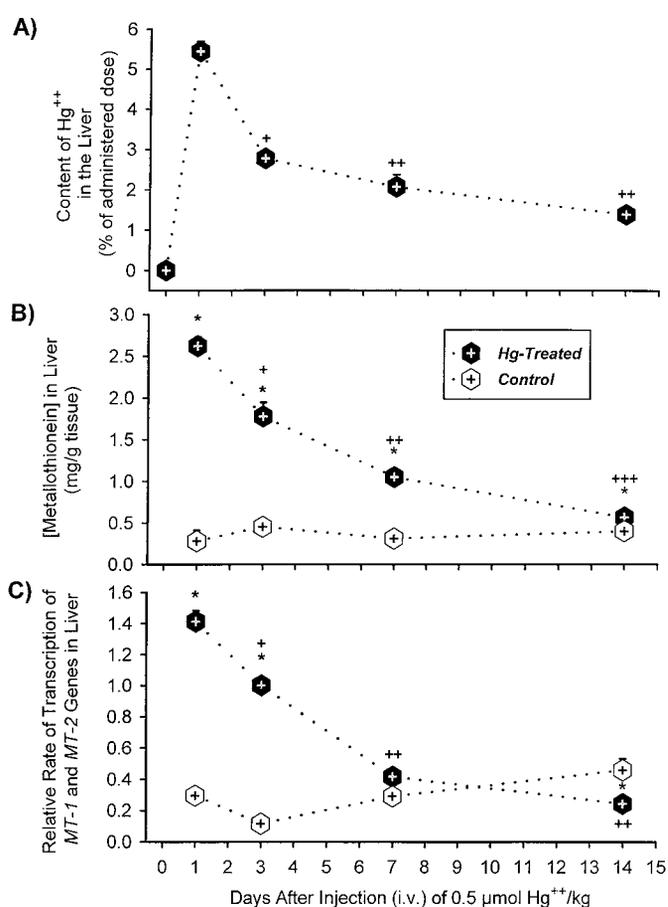
With one exception, the rates of transcription of *MT* genes in the kidneys remained relatively constant among the groups of rats treated with mercuric chloride. Only in the rats studied at 3 days after treatment was the relative rate of transcription of *MT* genes decreased, compared with that in the group studied 24 h after treatment. In contrast, there was no significant difference in the relative rate of transcription of renal *MT* genes between the group studied 24 h after treatment and the group studied 7 or 14 days after treatment.

**Hepatic Burden of Mercury.** Approximately 5.5% of the administered dose of inorganic mercury was present in liver 24 h after treatment (Fig. 2A). Fifty percent of the hepatic burden mercury was eliminated by 3 days post-treatment, and 62 and 74% by 7 and 14 days after treatment, respectively.

**Hepatic Concentration of MT Protein.** Hepatic concentrations of MT were significantly greater in the rats treated with 0.5  $\mu$ mol/kg mercuric chloride than control rats at all times studied (Fig. 2B). The hepatic concentration of MT decreased with time in parallel with the content of inorganic mercury (Fig. 2A). At 3, 7, and 14 days after the injection of mercuric chloride, the hepatic concentration of MT was approximately 32, 60, and 78% lower, respectively, than that at 1 day after treatment.

**Relative Rates of Transcription of *MT-1* and *MT-2* Genes in Liver.** The relative rates of transcription of *MT-1* and *MT-2* genes in the liver were highest in the group studied 1 day after treatment, and were lowest (at almost baseline levels) in the group studied at 14 days after treatment (Fig. 2C). The temporal pattern of transcription of *MT-1* and *MT-2* genes in the liver was clearly different from that detected in the kidneys (Fig. 1C).

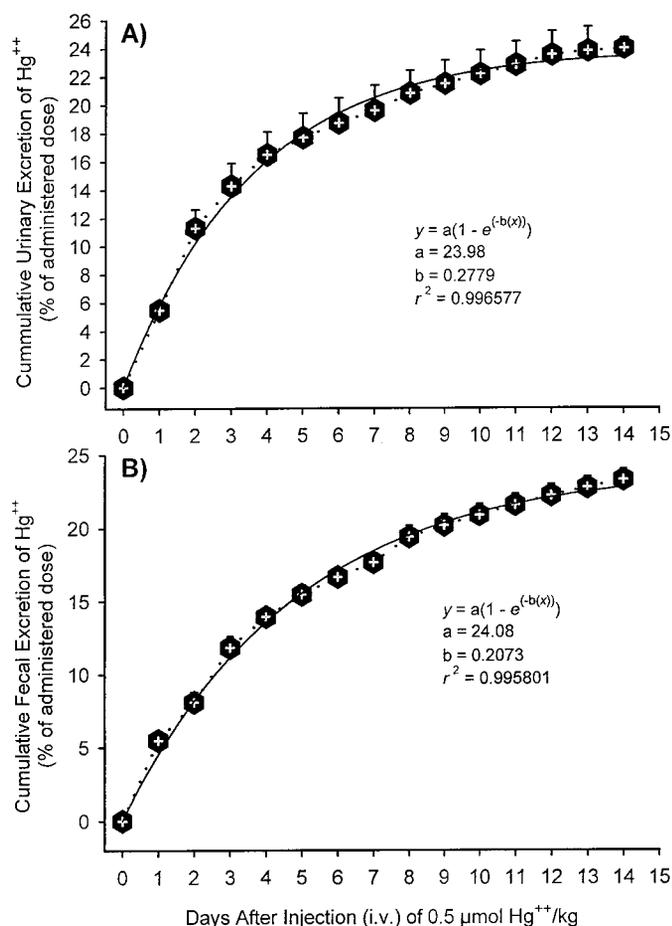
**Urinary Excretion of Mercury.** Rats injected with 0.5  $\mu$ mol/kg mercuric chloride excreted approximately 5.5% of the dose during the first 24 h, and 11% by 48 h, after treat-



**Fig. 2.** Content of inorganic mercury (percentage of the administered dose) (A), concentration of MT (B), and rate of transcription of *MT-1* and *MT-2* genes (relative to the rate of transcription of the *GAPDH* gene) in the liver of rats at 1, 3, 7, and 14 days after i.v. injection of 0.5  $\mu$ mol/kg mercuric chloride or saline. Each value represents the mean  $\pm$  S.E. obtained from five or six animals. \*, significantly different ( $P < .05$ ) from the mean for the corresponding control group (treated with normal saline). +, significantly different ( $P < .05$ ) from the mean for the corresponding group (treated in the same manner) that was studied 1 day after treatment. ++, significantly different ( $P < .05$ ) from the means for the corresponding groups (treated in the same manner) that were studied 1 and 3 days after treatment. +++, significantly different ( $P < .05$ ) from the means for the all of the other corresponding groups (treated in the same manner).

ment (Fig. 3A). By the end of the 1st and 2nd week after treatment, approximately 20 and 24% of the dose, respectively, had been excreted in the urine. Thus, about 83% of the cumulative urinary excretion of mercury occurred during the 1st week after treatment. With nonlinear regression analysis, the data for the urinary excretion of mercury had a best fit with the cumulative function  $y = a(1 - e^{-b(x)})$ , with an  $r^2 = 0.9966$  when coefficient  $a = 23.98$  and coefficient  $b = 0.2779$ .

**Fecal Excretion of Mercury.** Approximately 5.5% of the administered dose was excreted in feces during the 1st day after the injection of mercuric chloride (Fig. 3B). By the end of the 2nd day, approximately 8% of the dose had been excreted. Approximately 18 and 23% of the dose had been excreted in the feces by the end of the 1st week and 2nd week post-treatment, respectively. Similar to the cumulative urinary excretion of mercury, about 78% of the cumulative fecal excretion of mercury occurred during the 1st week after treatment. Interestingly, the data for the fecal excretion of



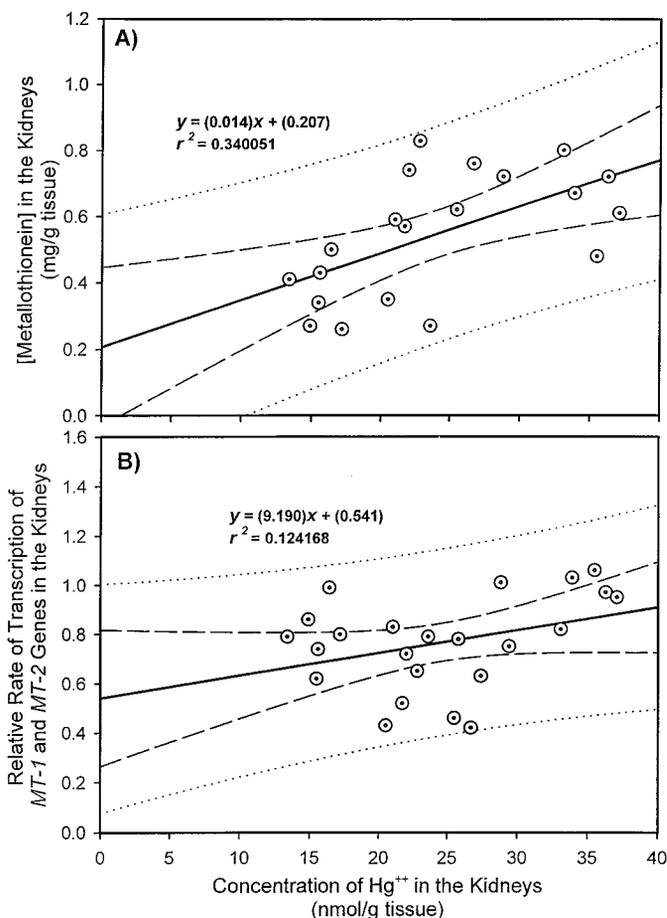
**Fig. 3.** Cumulative urinary (A) and fecal (B) excretion of mercury (percentage of the administered dose) over 14 days after i.v. injection of 0.5  $\mu\text{mol/kg}$  mercuric chloride. The values for day 1 represents the mean  $\pm$  S.E. for 23 animals, whereas the value for day 2 and day 3 represents the mean  $\pm$  S.E. for 17 animals. Each value for day 4 through day 7 represents the mean  $\pm$  S.E. for 12 animals and each value for day 8 through day 14 represents the mean  $\pm$  S.E. for 6 animals. Using nonlinear regression analysis, both the pattern for cumulative urinary and fecal excretion of mercury, over the 14 days of the study, were found to fit the cumulative function  $y = a(1 - e^{-b(x)})$  with an  $r^2$  of better than 0.99.

mercury also fit the cumulative function  $y = a(1 - e^{-b(x)})$  very well. An  $r^2 = 0.9966$  is obtained by using coefficient  $a = 24.08$  and coefficient  $b = 0.2073$ .

**Relationships among Concentration of MT Protein, Rate of MT Gene Transcription, and Concentration of Mercury in Kidneys.** Linear regression analysis of the plot of renal concentration of MT (mg/g of tissue) versus the renal concentration of mercury (nmol/g of tissue) for each individual animal revealed that the equation  $y = 0.014x + 0.207$  is the best-fit linear equation characterizing the relationship between these two parameters (Fig. 4A). The  $r^2$  of 0.340051, however, indicates that the relationship is a poor one.

Analysis of the plot of relative rate of transcription of MT genes in the kidneys versus the renal concentration of mercury revealed a relationship between these parameters that is defined by the equation  $y = 9.190x + 0.541$  (Fig. 4B). Unfortunately, the  $r^2$  of 0.124168 indicates a very poor relationship between these two parameters.

**Relationships among Concentration of MT Protein, Rate of MT Gene Transcription, and Concentration of Mercury in Liver.** Assessment of the plot of hepatic con-



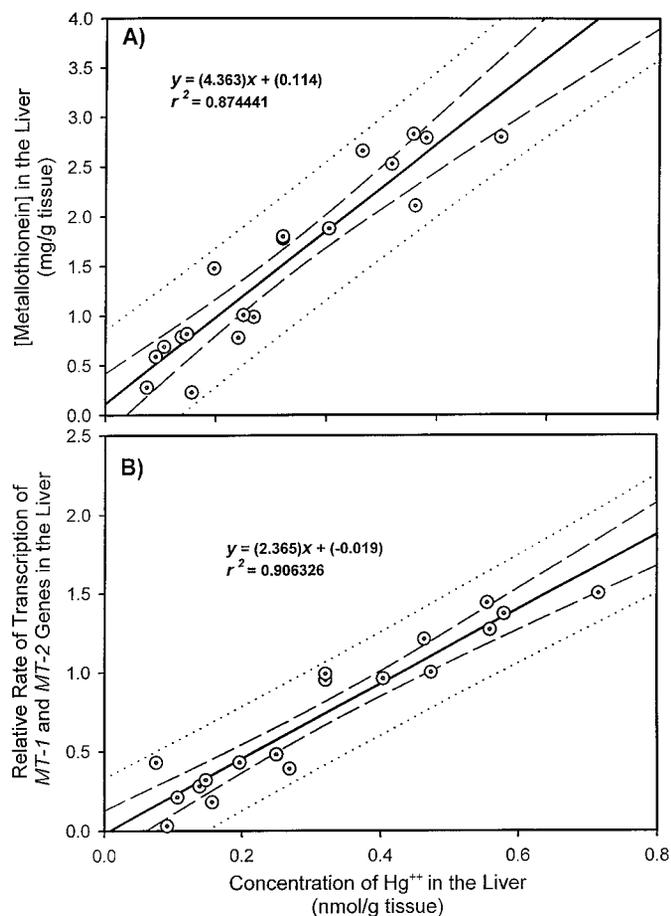
**Fig. 4.** Relationships, in the kidneys, between the concentration of mercury [ $Hg^{2+}$ ], and the concentration of MT protein (mg/g of tissue) (A), and the relative rate of transcription of MT-1 and MT-2 genes in the 19 individual rats at 1, 3, 7, or 14 days after i.v. injection of 0.5  $\mu\text{mol/kg}$  mercuric chloride (B). Linear regression analysis of the data indicates a poor relationship between the renal concentration of inorganic mercury, and the renal concentration of MT protein, and the relative rate of transcription of MT-1 and MT-2 genes in the liver.

centration of MT (mg/g of tissue) versus the hepatic concentration of mercury (nmol/g of tissue) by simple linear regression analysis indicates that the best-fit linear equation defining the relationships between these parameters is  $y = 4.363x + 0.114$  (Fig. 5A). The  $r^2$  of 0.874441 indicates that there is a predictable direct relationship between these two parameters.

Regression analysis of the plot of relative rate of transcription of MT genes in the liver versus hepatic concentration of mercury revealed a relationship between these parameters defined by the equation  $y = 2.365x + (-0.019)$  (Fig. 5B). The  $r^2$  of 0.906326 indicates a strong direct relationship between these two parameters.

**Relationship between Rates of Transcription of MT-1 and MT-2 Genes and Concentration of MT Protein in Kidneys and Liver.** The linear equation that best describes the relationship between the concentration of MT protein and the relative rate of transcription of MT genes in the kidneys is  $y = 0.041x + (0.532)$  (Fig. 6A). However, the regression analysis indicates that there is essentially no correlation between these two renal parameters ( $r^2 = 0.001581$ ).

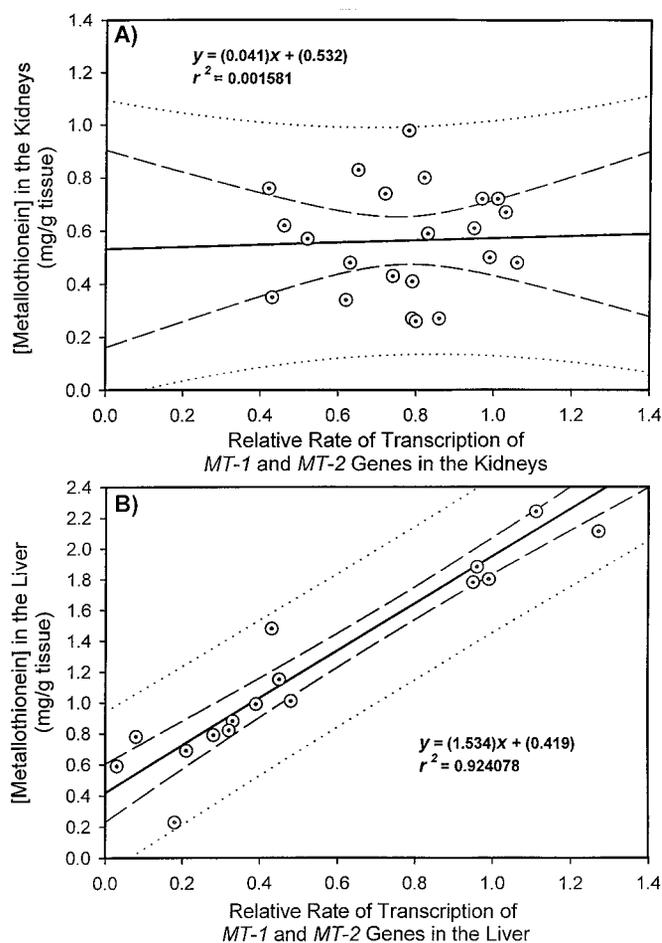
In liver, however, the concentration of MT protein was strongly and directly correlated with the rate of transcription



**Fig. 5.** Relationships, in the liver, between the concentration of mercury [Hg<sup>2+</sup>], and the concentration of MT protein (mg/g of tissue) (A), and the relative rate of transcription of *MT-1* and *MT-2* genes in the liver of 19 individual rats at 1, 3, 7, or 14 days after i.v. injection of 0.5  $\mu$ mol/kg mercuric chloride (B). Linear regression analysis of the data indicates, that in contrast to the situation in kidney (Fig. 4), there is a strong direct relationship between the renal concentration of inorganic mercury and the hepatic concentration of MT protein, and the relative rate of transcription of *MT-1* and *MT-2* genes in the liver.

of *MT* genes ( $r^2 = 0.924078$ ) (Fig. 6B). The linear equation  $y = 2.365x + (-0.019)$  best defined the relationship between these hepatic parameters.

**Evaluation of Potential Toxic Effects of 0.5  $\mu$ mol of HgCl<sub>2</sub>/kg on Transcription in Kidneys and Liver.** To rule out the possibility that the dose of inorganic mercury used might suppress transcription in target tissues and confound results, we assessed overall transcription and transcription of *GAPDH* in the kidneys and liver over the 14 days of study. Incorporation of radiolabeled precursor into total tissue RNA (per 10<sup>5</sup> nuclei) was assessed in the kidneys and liver of animals treated and not treated with the 0.5- $\mu$ mol/kg dose of mercuric chloride. No significant changes in overall transcription in the kidneys or liver were detected between the animals not treated with inorganic mercury and those treated with inorganic mercury (Fig. 7). Moreover, there were no significant changes in overall transcription in the kidneys and liver from day 1 after treatment through day 14 after treatment (Fig. 7). Transcription of the housekeeping gene *GAPDH* (as assessed by the amount of radiolabeled *GAPDH* RNA hybridized to immobilized *GAPDH* cDNA probe, per  $8 \times 10^8$  cpm radiolabeled RNA resulting from 30



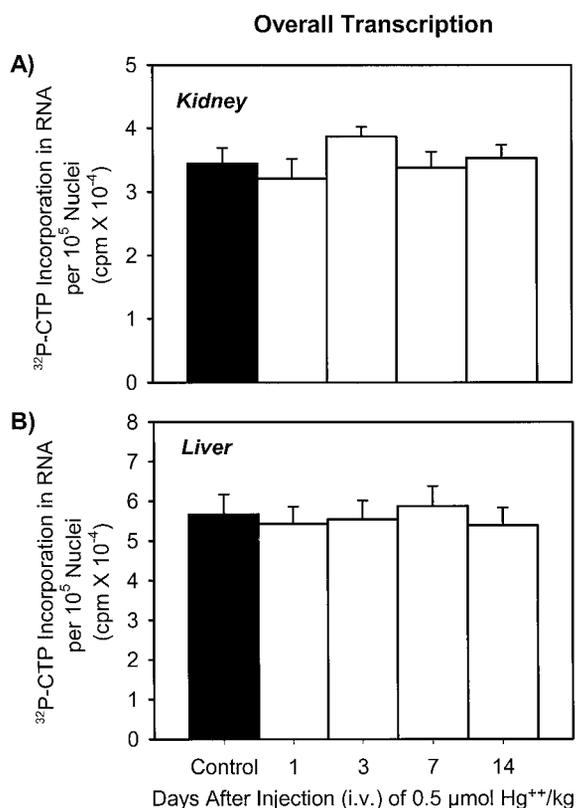
**Fig. 6.** Relationship between the tissue concentration of MT protein (mg/g of tissue) and the relative rate of transcription of *MT-1* and *MT-2* genes in the kidneys (A) and liver (B) in individual rats ( $n = 22$  for kidney;  $n = 16$  for liver) at 1, 3, 7, and 14 days after i.v. injection of 0.5  $\mu$ mol/kg mercuric chloride. Linear regression analysis revealed that there is essentially no relationship between tissue concentration of MT protein and the relative rate of transcription of *MT-1* and *MT-2* genes in the kidneys. In the liver, however, there is a strong, positive, direct relationship between these parameters.

min of run-on transcription in isolated nuclei) also was unaffected in either the kidneys or liver at all times studied (Fig. 8). These findings confirm that the dose of mercuric chloride used in the present study does not elicit demonstrable toxic effects.

## Discussion

Numerous studies (Rothstein and Hayes, 1960; Zalups and Cherian, 1992a,b; Zalups, 1995, 2000), including the present one, indicate that the kidneys of rats exposed to a low dose of inorganic mercury accumulate approximately one-half the dose during the initial 24 h after exposure. Approximately 75% of the mercury accumulated in the kidneys is retained by the tubular epithelial cells over the subsequent weeks after exposure.

In contrast, only about 5 to 6% of the dose is present in the liver by the end of the 1st day after exposure (Zalups and Cherian, 1992a,b; Zalups, 1995). However, the hepatic burden of mercury is as great as 7 to 8% of the dose 1 h after exposure (Zalups et al., 1999). Thus, the maximal hepatic burden of mercury is achieved rapidly after exposure, and

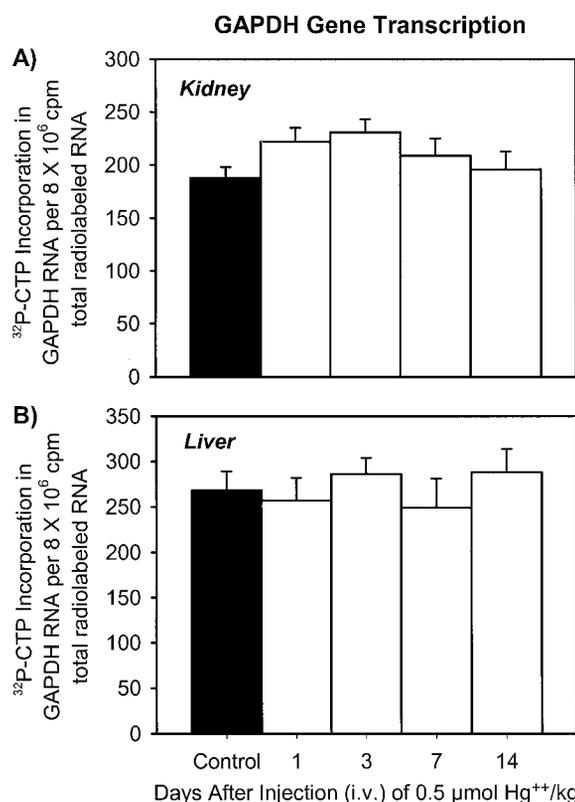


**Fig. 7.** Absence of effect of treatment with 0.5 μmol of HgCl<sub>2</sub>/kg (i.v.) on overall rates of transcription in the kidneys and liver of rats. Nuclei were isolated from tissues obtained 1, 3, 7, and 14 days post-treatment with inorganic mercury and were allowed to incorporate [<sup>32</sup>P]CTP into RNA for 30 min as described under *Materials and Methods*. Each value represents the mean ± S.E. (*n* = 5 or 6) for the amount of radiolabel incorporated in the total RNA isolated from 1 × 10<sup>5</sup> nuclei. "Control" denotes nuclei from control animals treated with saline. No significant differences in the overall rates of transcription in either the kidneys or liver were detected among the groups of animals treated with inorganic mercury.

hepatic elimination of mercury begins within hours. The relatively efficient elimination of mercury results in the hepatic burden of mercury being reduced to less than 1.5% of the dose, which is an 80% reduction of the initial hepatic burden, by the end of the initial 2 weeks after treatment. Only about 25% of the renal burden of mercury is eliminated during the same period of time. It is not clear why there are such great differences in the efficiency of elimination of accumulated mercury between the kidneys and liver.

MT has been shown to play a significant role in protecting against the cytotoxic effects of various forms of mercury, including mercury vapor (Hg<sup>0</sup>) (Yoshida et al., 1999) and mercuric chloride (Zalups and Cherian, 1992a,b; Morcillo and Santamaria, 1996). In mice with genetically ablated *MT-1* and *MT-2* genes (*MT*-null mice), lack of MT results in increased susceptibility to renal damage induced by inorganic mercury, even though the renal accumulation of inorganic mercury in the *MT*-null mice is less than that in the wild-type control mice (Satoh et al., 1997). The importance of MT in the kidneys in providing resistance to the toxic effects of a wide variety of transition metals (Klaassen and Liu, 1998; Nordberg et al., 1992) has directed attention to processes that regulate the cellular content of MT protein in the kidneys, as well as other organs and tissues.

The mechanisms involved in controlling transcription of



**Fig. 8.** Absence of effect of treatment with 0.5 μmol of HgCl<sub>2</sub>/kg (i.v.) on *GAPDH* gene transcription in the kidneys and liver of rats. Nuclei were assessed as described in the legend for Fig. 7. Each value represents the mean ± S.E. (*n* = 5 or 6) for the amount of radiolabel RNA that hybridized to immobilized, unlabeled *GAPDH* cDNA probe isolated. "Control" denotes nuclei from control animals treated with saline. No significant differences in the overall rates of transcription in either the kidneys or liver were detected among the groups of animals treated with inorganic mercury, indicating that inorganic mercury did not elicit significant toxic effects in these organs.

members of the MT gene family have been studied extensively. Transcription of *MT-1* and *MT-2* genes is induced rapidly in various cell types by oxidative stress, inflammation, and exposure to numerous metals, including zinc, cadmium (Andrews, 2000), and mercury (Murata et al., 1999). Increased transcription of these genes in response to acute exposure to metals appears to correlate well with increased production of MT protein (Durnam et al., 1981), suggesting that transcription is a major point of control in the induction and maintenance MT protein within certain target cells. However, it is not clear whether there is a direct relationship between the transcription of *MT* genes and the consequent production of MT protein in all target cells that take up and accumulate toxic metals. In addition, it is not clear whether the relationships that exist between the rates of transcription of *MT* genes and the content of MT protein in certain target cell populations persist during more chronic periods of time after the exposure to toxic metals, such as mercury.

Of relevance to these issues, post-transcriptional events are involved in cellular accumulation MT protein under some circumstances (Andrews, 1990). Rats treated with cadmium or copper salts recruit less *MT-2* mRNA into hepatic cell polyribosomes than the amount of total *MT-2* mRNA induced by the metal (Vasconcelos et al., 1996). In transgenic mice overexpressing *interleukin-6* genes, *MT-1* and *MT-2* protein

are elevated in brain tissue in spite of minimal increases and even decreases in the levels of *MT-1* mRNA (Hernandez et al., 1997). The stability *MT* mRNA in embryonic chick hepatocytes has been reported to be significantly increased during inhibition of protein synthesis induced by treatment with cycloheximide, but not puromycin (McCormick et al., 1991). The inferred capacity of specific proteins to degrade *MT* mRNA indicates possible post-transcriptional regulation. Hepatic *MT* protein with enhanced stability has been reported in mutant mice that overaccumulate copper-associated *MT*, indicating a post-translational mechanism regulating *MT* protein levels under some circumstances (Koropatnick and Cherian, 1993). The potential of post-transcriptional events to play a role in the accumulation of *MT* protein in renal and hepatic tissues exposed to inorganic mercury (as well as other tissues exposed to heavy metals in general) has been largely unexplored. Therefore, it is important to explore physiological events other than gene transcription that may regulate accumulation of heavy metals, and resistance to their toxic effects, in different organs.

We studied changes in the disposition of inorganic mercury and *MT* protein, and rates of transcription of *MT-1* and *MT-2* genes, in kidneys and liver during the initial 2 weeks after treatment with mercuric chloride. Our aim was to determine whether transcription of *MT* genes alone was the most likely factor responsible for the accumulation of *MT* protein in response to exposure to inorganic mercury in those tissues. As mentioned above, the net retention of mercury was greater in kidneys than in liver. Differences, between kidney and liver, with respect to the amount of induced *MT* that persisted over time were even more dramatic: The renal content of *MT* did not change significantly during the initial 7 days after exposure to inorganic mercury, whereas the content *MT* in the liver dropped by more than 50%.

By 14 days post-treatment, the content of *MT* protein in liver was near to basal levels, which paralleled closely the relative rate of *MT-1* and *MT-2* gene transcription (Fig. 2). These data support the hypothesis that *MT* gene transcription in the liver is indeed a primary factor regulating hepatic levels of *MT* after exposure to inorganic mercury, a concept that is strengthened further by the very strong direct relationship ( $r^2 = 0.924$ ) between the concentration of *MT* and the relative rate of *MT* gene transcription (Fig. 6). The strong correlation ( $r^2 = 0.90$ ) between the content of inorganic mercury and *MT* gene transcription strongly suggests that inorganic mercury was the primary agent inducing hepatic *MT* gene transcription (Fig. 4). In addition, the strong correlation ( $r^2 = 0.87$ ) between the content of mercury and the concentration of *MT* suggests the transcription of *MT* genes alone, and not post-transcriptional events mediated by mercuric ions, plays the primary role in regulating the content of *MT* in the liver.

In contrast, the close relationships observed in liver are extremely weak or absent in kidney. Over a period of 7 days after treatment with mercuric chloride, the renal content of inorganic mercury remained elevated compared with the content of inorganic mercury in the liver (Fig. 1). This is consistent with reports indicating that exposure to mercury results in relatively high renal levels compared with concentrations in other tissues (Rothstein and Hayes, 1960, Zalups 1995; Tanaka-Kagawa et al., 1998). By 14 days, renal mercury and *MT* levels were substantively decreased compared with 7-day

levels. However, unlike the situation in liver, the correlation between the renal concentrations of *MT* and inorganic mercury was weak ( $r^2 = 0.34$ ) over the 14 days of study. This suggested that other factors, independent of the presence of inorganic mercury, contributed to regulating the amount of *MT* present in kidney. Those factors had a more significant effect in the kidneys than in the liver. Furthermore, *MT* gene transcription and *MT* protein levels were extremely poorly associated with content of inorganic mercury. The  $r^2$  between the renal concentration of inorganic mercury and the rate of transcription of *MT-1* and *MT-2* genes in the kidneys was only 0.12 (Fig. 5), and the association between the rate of transcription of *MT* genes and the tissue concentration of *MT* protein in the kidneys was even worse. In kidneys isolated from 22 rats, with *MT* gene transcription levels that varied over a nearly 3-fold range, the  $r^2$  with *MT* protein levels was virtually zero (Fig. 6). These data strongly support the hypothesis that, in kidney, events other than *MT* gene transcription play a significant role in mediating the production and/or retention of *MT*.

At early times after mercuric chloride treatment, both kidney and liver responded by accumulating inorganic mercury, increasing *MT* gene transcription, and accumulating *MT* protein (Figs. 1 and 2). Clearly, *MT* gene transcription in response to inorganic mercury was the preeminent factor responsible for *MT* protein accumulation at early stages (1 to 3 days) after metal ion exposure. However, between 7 and 14 days post-treatment, the persistence of *MT* gene transcription in kidney contrasted with the loss in transcription in liver and contributed to the increasingly poor relationship between transcription and protein accumulation. Therefore, these data suggest that post-transcriptional events in kidney may contribute to regulation of *MT* protein levels more strongly during long-term exposure to metal ions than in situations of short-term acute exposure. This situation may reflect more accurately the pattern of exposure in humans exposed to environmental metal ions.

The post-transcriptional events mediating *MT* levels in kidney are not known at the present time, and are under investigation in our laboratories. Although differences in the stability of *MT* mRNAs will be difficult to assess in tissues, assessment of differences in the stability of *MT* protein in the kidneys and liver of rats treated with mercuric chloride is possible by using a combined post-labeling/Western blot technique (Koropatnick and Cherian, 1993). This strategy, in combination with analysis of the metal ligands associated with renal *MT* at varying times after mercuric chloride treatment, is being pursued to determine whether increased stability of renal *MT* protein contributes to *MT* levels.

Overall, our data indicate that, in addition to initial induction of *MT* gene expression, post-transcriptional events contribute to *MT* protein accumulation in the kidney in response to exposure to mercuric chloride. These factors become increasingly significant at longer periods of time between the initial inducing event and the measurement of the levels of *MT* protein. The importance of *MT* in mediating resistance to toxic metals, and in retaining metals in different cells and tissues, contributes to the importance of understanding multiple factors (in addition to gene transcription) that regulate *MT* protein levels in vivo.

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