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LUMINAL TRANSPORT OF THIOL *S*-CONJUGATES OF METHYLMERCURY IN ISOLATED PERFUSED RABBIT RENAL PROXIMAL TUBULES

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Abstract

Lumen-to-cell transport, cellular accumulation, and toxicity of *L*-cysteine (Cys), glutathione (GSH) and *N*-acetylcysteine (NAC) *S*-conjugates of methylmercury (CH_3Hg^+) were evaluated in isolated, perfused rabbit proximal tubular segments. When these conjugates were perfused individually through the lumen of S_2 segments of the proximal tubule it was found that Cys-*S*-CH_3Hg and GSH-*S*-CH_3Hg were transported avidly, while NAC-*S*-CH_3Hg was transported minimally. In addition, 95% of the ^{203}Hg taken up by the tubular cells was associated with precipitable proteins of the tubular extract, while very little was found in the acid-soluble cytosol extract. No visual cellular pathological changes were observed during 30 min of study. Luminal uptake of Cys-*S*-CH_3Hg was temperature-dependent and inhibited significantly by the amino acids *L*-methionine and *L*-cystine. Rates of luminal uptake of GSH-*S*-CH_3Hg were twice as great as that of Cys-*S*-CH_3Hg and uptake was inhibited significantly (74%) by the presence of acivicin. When 2,3-bis(sulfanyl)propane-1-sulfonate (DMPS) was added to the bathing or luminal fluid, luminal uptake of Cys-*S*-CH_3Hg was diminished significantly. Overall, our data indicate that Cys-*S*-CH_3Hg is likely a transportable substrate of one or more amino acid transporters (such as system $B^{0,+}$ and system $b^{0,+}$) involved in luminal absorption of *L*-methionine and *L*-cystine along the renal proximal tubule. In addition, GSH-*S*-CH_3Hg appears to be degraded enzymatically to Cys-*S*-CH_3Hg, which can then be taken up at the luminal membrane. By contrast NAC-*S*-CH_3Hg and Cys-*S*-CH_3Hg (in the presence of DMPS) are not taken up avidly at the luminal membrane of proximal tubular cells, thus promoting the excretion of CH_3Hg^+ into the urine.

Keywords

Thiol *S*-conjugates; Methylmercury; Membrane Transport; Renal Proximal Tubular Segments; Rabbit; Cystine; Cysteine; Methionine; 2, 3-bis(sulfanyl)propane-1-sulfonate (DMPS); amino acid transporters; molecular mimicry

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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

INTRODUCTION

Methylmercury (CH_3Hg^+) is the predominant mercuric species that humans and other mammals are exposed to in various environmental settings. Its formation in the environment occurs following methylation of inorganic mercuric (Hg^{2+}) ions by microorganisms in soil and water. Subsequently, CH_3Hg^+ accumulates widely in numerous species of animals in the food chain, especially in predatory fish. Consumption of animals and/or water contaminated with CH_3Hg^+ by humans can be significantly deleterious to their health, inasmuch as it is absorbed readily by the gastrointestinal tract and is delivered into the circulatory system (Kershaw, Clarkson et al. 1980; Clarkson 2002). Since methylmercuric ions have an extremely high affinity to bond to the reduced sulfur atom of thiol-containing molecules, one can assume that most, if not all, absorbed methylmercuric ions present in blood are in the form of some thiol *S*-conjugate (Fuhr and Rabenstein 1973; Zalups 2000). Some of the mercuric species believed to form in various compartments of the body include *L*-cysteine (Cys), homocysteine (Hcy), glutathione (GSH), *N*-acetylcysteine (NAC), hemoglobin and albumin *S*-conjugates of CH_3Hg^+ (Carty and Malone 1979).

The kidney plays an important role in reducing the body-burden of most heavy metals and their conjugates, including methylmercury. Within the kidney, the proximal tubule is the major site for methylmercury accumulation (Rodier and Kates 1988; Zalups 2000). A few transporters located in the basolateral membrane of the kidney including the organic anion transporter 1 (OAT1) (Zalups and Ahmad 2005; Zalups and Ahmad 2005; Zalups and Ahmad 2005) and system L (Simmons-Willis, Koh et al. 2002; Yin, Jiang et al. 2008) have been reported to transport some thiol *S*-conjugates of methylmercury. By contrast, little is known about the mechanisms involved in the absorptive transport of methylmercuric species at the luminal plasma membrane of epithelial cells lining the length of the proximal tubule.

System $B^{0,+}$, by far, is the sole transporter that has been reported to be involved in the luminal uptake of certain thiol *S*-conjugates of CH_3Hg^+. Using *Xenopus laevis* oocytes, Bridges, *et al.* showed that system $B^{0,+}$ is capable of the transporting the complexes Cys-*S*-CH_3Hg and Hcy-*S*-CH_3Hg, by using competitive inhibition experiments with substrates known to be transported by system $B^{0,+}$ (Bridges and Zalups 2006). System $B^{0,+}$ is a Na^+/Cl^--dependent transporter for a variety of neutral and cationic amino acids including methionine (Met) (Sloan and Mager 1999; Nakanishi, Hatanaka et al. 2001), and is known to be localized in the luminal membrane of proximal tubular epithelial cells (Broer 2008).

Absorptive transport of thiol *S*-conjugates of CH_3Hg^+ has been explained by the putative mechanism involving molecular mimicry or homology (Zalups 2000; Broer 2008). Certain thiol *S*-conjugates of CH_3Hg^+ are similar structurally to some endogenous molecules, such as amino acids and amino acid derivatives, and thus, are believed to behave as mimics at the binding site(s) of selective transporters. Evidence for this theory has been provided by a few groups (Aschner and Clarkson 1988; Mokrzan, Kerper et al. 1995; Simmons-Willis, Koh et al. 2002), who demonstrated that the Cys *S*-conjugates of CH_3Hg^+ (Cys-*S*-CH_3Hg) behave as mimics of *L*-Met at the transporter system L. Additionally, Cys-*S*-CH_3Hg may compete with *L*-Met for transport by System $B^{0,+}$. Moreover, it has also been demonstrated that Cys-*S*-CH_3Hg may serve as a structural and/or functional mimic of *L*-cystine (Zalups 2000). *L*-cystine is known to be transported by system $b^{0,+}$, which like $B^{0,+}$ is located in the luminal membrane, but not the basolateral membrane of the renal proximal tubular epithelial cells (Furriols, Chillaron et al. 1993; Mora, Chillaron et al. 1996). Interestingly, system b^{0+} has been implicated in the luminal absorptive transport of the Cys *S*-conjugates of cadmium (Cys-Cd-Cys) in isolated perfused rabbit proximal tubular segments (Wang, Zalups et al. 2010). Since Hg and cadmium (Cd) are both group IIB metals, and both have a high binding

affinity for sulfhydryl groups, it is possible that Cys-*S*-CH_3Hg may also utilize system b^{0+} to enter proximal tubular cells.

The previous findings from our laboratory indicated that methylmercuric ions are avidly transported across the luminal membrane in isolated perfused proximal tubular segments (Zalups and Barfuss 1993). However, no data for the transport of Cys *S*-conjugates of CH_3Hg^+ by the luminal membrane of isolated perfused proximal tubular cells have been reported to date. The present study examined if methylmercuric ions (CH_3Hg^+) gain entry into proximal tubular epithelial cells at the luminal membrane when co-perfused with Cys, NAC or GSH in isolated perfused rabbit proximal tubular segments. It is presumed that the methylmercuric ions react with the co-perfused thiol compound forming the respective conjugates which are the transportable entities. In addition, we sought to obtain evidence implicating the luminal transporters system B^{0+} and system b^{0+} in the absorption of Cys-*S*-CH_3Hg. Furthermore, the effect of the divalent metal complexing agent, 2,3-bis(sulfanyl)propane-1-sulfonate (known formerly as 2,3-dimercaptopropane-1-sulfonate, DMPS), on the luminal transport of Cys-*S*-CH_3Hg was also evaluated. Our data are apparently the first attempt to characterize the transport of thiol *S*-conjugate of CH_3Hg^+ by the luminal membrane of renal proximal tubules.

MATERIALS AND METHODS

Animals

Female New Zealand rabbits (1–2kg) were used in the present study. All animals were allowed at least two days of acclimation prior to any experimentation. Water and a commercial laboratory diet for rabbits were provided *ad libitum* during all phases of the study.

Tubule Perfusion

Proximal tubular segments were dissected from isolated kidneys from female rabbits, mounted on a system of glass pipettes, and perfused and bathed in solutions similar to those described previously (Zalups and Barfuss 1993; Wang, Zalups et al. 2010) except for a few differences that are pointed out below. In general, 15 min after warming a tubular segment to 37°C, three collections of 30–50nL, at the flow rate of 7–10nL min^{-1}, were made. Each perfused tubular segment was harvested and placed in 10μL of 3% trichloroacetic acid (TCA) solution for 20 sec to extract the accumulated ^{203}Hg into acid-precipitable and non-precipitable fractions.

To evaluate the net absorption of each thiol *S*-conjugate of CH_3Hg^+ studied, a fresh perfusing solution (perfusate) was made, containing a 1:1 ratio of the thiol-containing molecule (*L*-Cys, *L*-glutathione, *N*-acetylcysteine or DMPS) to each molecular ion of CH_3Hg^+. The 1:1 ratio of thiol to CH_3Hg^+ was used to ensure the formation of a linear I coordinate covalent bond between each molecule of CH_3Hg^+ and the corresponding thiol. The vital dye FD&C Green 3 (809Da) was placed in the perfusate at a concentration of 250nM to visually determine any toxic effects of the thiol *S*-conjugates of CH_3Hg^+. *L*-[^{3}H]-glucose (14.6Ci/mmol; American Radiolabeled Chemicals, Inc., St. Louis, MO, USA) was added to the perfusate as a volume marker.

In some experiments, *L*-methionine and *L*-cystine were added to the perfusate at the ratio of at least 10:1 (with respect to CH_3Hg^+) to promote potential inhibition of putative amino acid transporters possibly involved in the luminal uptake of CH_3Hg^+. All variants of perfusates were made fresh on the day of experiments.

The perfusate was identical to the bathing solution except that 2mM NaH_2PO_4 was replaced by 2mM HEPES because it was found that HPO_4^- or HPO_4^{2-} caused precipitation of CH_3Hg^+ in aqueous solutions.

Generation of $^{203}Hg^{2+}$ and $CH_3{}^{203}Hg^+$

$^{203}Hg^{2+}$ was generated by the method described previously (Belanger, Westin et al. 2001; Bridges, Bauch et al. 2004). Briefly, 3 mg of mercuric oxide (HgO) were sealed in two quartz tubes, one inside the other, with an acetylene torch. Subsequently, the doubly-sealed sample of HgO was sent to the Missouri University Research Reactor (MURR) facility to be irradiated via neutron activation for four weeks. After we received the irradiated sample from the MURR facility, the solid content of mercury was dissolved in 1 N HCl and the radioactivity of $^{203}Hg^{2+}$ was determined by standard isotopic methods using a Wallac Wizard 3 automatic gamma counter (Perkin Elmer, Gaithersburg, MD). The specific activities of the $^{203}Hg^{2+}$ ranged, on average, between 6 to12 mCi/mg.

$CH_3{}^{203}Hg^+$ was generated later by following a protocol adapted from Rouleau and Block (Rouleau 1997). Two mCi of $^{203}Hg^{2+}$ were diluted in 40μL of deionized water. Subsequently, 670μL of 2 M acetate buffer and 2 mL of methylcobalamin were added to the solution. Methylcobalamin served as the methyl donor. This mixture was incubated for 24 h at room temperature in a fume hood. Following incubation, 16.7mL of 30% potassium chloride (KCl_2) in 4% hydrochloric acid (HCl) were added. $CH_3{}^{203}Hg^+$ was extracted with five washes of 8.3 mL of dichloromethane (DCM). The collected DCM was evaporated by bubbling nitrogen gas into the solution. Afterwards, the $CH_3{}^{203}Hg^+$ was collected and stored at −20°C. The purity of the extracted $CH_3{}^{203}Hg^+$ has been confirmed previously by thin layer chromatography (Rouleau 1997).

Calculations

Lumen-to-Cell Flux of ^{203}Hg—Transport of ^{203}Hg in lumen-to-cell transport experiments was determined by measuring the rate at which ^{203}Hg disappeared from the luminal fluid. This disappearance flux (J_D) (fmol min^{-1} (mm tubule length)$^{-1}$) measurement was calculated by equation #1:

$$J_D=(V_P[^{203}Hg]_P-V_C[^{203}Hg]_C)/L \quad 1)$$

Where $[^{203}Hg]_P$ and $[^{203}Hg]_C$ are the concentrations (fmol nL^{-1}) of *^{203}Hg* in the perfusate and collectate, respectively. $[^{203}Hg]_P$ and $[^{203}Hg]_C$ were determined from the specific activity of ^{203}Hg. L is the length (mm) of the perfused tubular segment. V_C is the collectate collection rate (nL min^{-1}), which was measured from the time required to fill the constant volume pipette. V_P is the perfusion rate (nl min^{-1}) and was calculated by equation #2:

$$V_P=V_C([VM]_C/[VM]_P) \quad 2)$$

Where $[VM]_C$ and $[VM]_P$ are the concentrations (cpm nL^{-1}) of the Volume Marker (^{3}H-*L*-glucose) in the collectate and perfusate respectively. The final $[VM]_C$ was determined by adding the amount of [^{3}H]-*L*-glucose (cpm min^{-1} x collectate collection time, min) that leaked into the bathing solution during the collection period to the collectate [^{3}H]-*L*-glucose (cpm) then divided by the volume of the constant volume pipette, nL.

Tubular ^{203}Hg Content—^{203}Hg extracted from the tubule was divided into two fractions: precipitable and non-precipitable. The precipitable fraction is defined as the ^{203}Hg that

remained in the tubular structure that became rigid, white and opaque when place in the TCA solution. Presumably, the ^{203}Hg is bound to cellular structures. The non-precipitable fraction is defined as the ^{203}Hg that is extracted in the TCA fluid. Presumably, this ^{203}Hg was free in the cell cytoplasm. Equation 3 was used to calculate both the precipitable fraction and the non-precipitable fraction.

$$\mathrm{Hg}_{\text{Precipitalbe or Non-precipitable}} = \mathrm{cpm}_{\mathrm{Hg}} \times \text{fmol cpm}^{-1} \div \mathrm{L} \qquad 3)$$

Where $\mathrm{Hg}_{\text{Precipitable or Non-precipitable}}$ (fmol (mm tubular length)$^{-1}$) is the precipitable or non-precipitable amounts reported; $\mathrm{cpm}_{\mathrm{Hg}}$ is the amount (cpm) of ^{203}Hg in the precipitable or the non-precipitable fraction, respectively; while fmol cpm^{-1} is the specific activity of the luminal fluid ^{203}Hg (perfusate). The total tubular content is the sum of the precipitable and the non-precipitable fractions, fmol min^{-1} (mm tubular length)$^{-1}$.

Statistical Analyses

A minimum of five tubules were perfused under each experimental condition, with three samples per tubule. Data for each variable assessed were obtained from tubular segments isolated from at least two animals. For each perfused tubular segment, three measurements of disappearance flux (J_D) for CH_3Hg^+ were averaged. The mean values for J_D were used to compute the overall mean and standard error of the mean for each experimental condition. The same analytical sequence was used on the data for determining cellular concentration of mercuric ions in the perfused tubules.

Each set of data was first analyzed with the Smirnov-Kolmogorov test to assess normality and Levene's test to assess for homogeneity of variance. Then, a one-way analysis of variance (ANOVA) was applied to all relevant data for each parameter being evaluated. When statistically significant (P<0.05) F-values were obtained by ANOVA, Tukey's *post-hoc* test was used to determine statistically significant differences among all relevant pairs of means for each parameter. Values were assumed to be significantly different at P<0.05.

RESULTS

Effect of *L*-cystine and *L*-methionine on the Lumen-to-Cell Transport of Cys-S-CH_3Hg

When 2 μM Cys-*S*-CH_3Hg was perfused through the lumen of S_2 segments of the rabbit proximal tubule for 30 minutes, the mean disappearance flux rate (J_D) for Cys-S-CH_3Hg was 8.6 ± 0.7 fmol min^{-1} (mm tubular length)$^{-1}$) (Figure 1A). Addition of 100 μM *L*-cystine to the perfusate caused the mean J_D in perfused S_2 segments to be significantly lower (3.9 ± 0.6 fmol min^{-1} (mm tubular length)$^{-1}$) than that in the control S_2 segments perfused through the lumen with only 2 μM Cys-*S*-CH_3Hg. On average, the J_D was 54% less than that in the control S_2 segments (Figure 1A). Addition of 100μM *L*-methionine to the perfusate containing 2μM Cys-*S*-CH_3Hg also had a significantly effect on the net uptake of Cys-S-CH_3Hg in S_2 segments of the rabbit proximal tubule. On average, the J_D for Cys-S-CH_3Hg in the presence of 100μM methionine was approximately 22.7% less (8.6 ± 0.7 to 6.7 ± 0.2 fmol min^{-1} (mm tubular length)$^{-1}$) than that in the S_2 segments perfused with 2μM Cys-*S*-CH_3Hg (Figure 1A).

The mean total tubular content of Cys-S-CH_3Hg that accumulated in the perfused S_2 segments over the course of 30 minutes being perfused through the lumen with 2 μM Cys-*S*-CH_3Hg was 140.6 ± 13.5 fmol (mm tubular length)$^{-1}$ (Figure 1B). Addition of 100μM *L*-cystine to the perfusate caused a significant decrease in net accumulation of Cys-S-CH_3Hg (a decrease to 64.9 ± 10.11 fmol (mm tubular length)$^{-1}$). Interestingly, the net decrease in

accumulation of 2 μM Cys-S-CH_3Hg was also about 54% less than that in the control S_2 segments perfused with Cys-*S*-CH_3Hg (Figure 1B). In the S_2 segments perfused with 100 μM *L*-methionine and 2 μM Cys-*S*-CH_3Hg, the total tubular content of Cys-S-CH_3Hg after 30 minutes of perfusion was 90.29 ± 7.4 fmol (mm tubular length)$^{-1}$. This amount of accumulation of Cys-S-CH_3Hg was about 35.8% less than that in the control S_2 segments (Figure 1B).

Comparison of Transport of Cys-*S*-CH_3Hg Among S_1, S_2 and S_3 Segments of the Proximal Tubule

When a perfusate containing 20μM Cys-*S*-CH_3Hg was perfused through the lumen of S_1, S_2, and S_3 proximal tubular segments for 30 minutes, the following transport data were obtained: In S_1 segments, the J_D of Cys-*S*-CH_3Hg was 38.2 ± 3.2 fmol min^{-1} (mm tubular length)$^{-1}$, the total tubular content of Cys-*S*-CH_3Hg was 730.5 ± 90.8 fmol (mm tubular length)$^{-1}$, the TCA precipitable fraction of Cys-*S*-CH_3Hg was 678.2 ± 93.3 fmol (mm tubular length)$^{-1}$ and the TCA non-precipitable fraction of Cys-*S*-CH_3Hg was 52.3 ± 10.3 fmol (mm tubular length)$^{-1}$. In S_2 segments, the J_D of Cys-*S*-CH_3Hg was 45.2 ± 4.2 fmol min^{-1} (mm tubular length)$^{-1}$, the total tubular content of Cys-*S*-CH_3Hg was 806.7 ± 120.5 fmol (mm tubular length)$^{-1}$, the TCA precipitable fraction of Cys-*S*-CH_3Hg was 778.9 ± 108.1 fmol (mm tubular length)$^{-1}$ and the TCA non-precipitable fraction of Cys-*S*-CH_3Hg was 27.8 ± 13.0 fmol (mm tubular length)$^{-1}$. In S_3 segments, the J_D of Cys-*S*-CH_3Hg was 49.6 ± 4.6 fmol min^{-1} (mm tubular length)$^{-1}$, the total tubular content of Cys-*S*-CH_3Hg was 864.3 ± 40.8 fmol (mm tubular length)$^{-1}$, the TCA precipitable fraction is 857.8 ± 44.9 fmol (mm tubular length)$^{-1}$ and the TCA non-precipitable fraction was 6.5 ± 5.4 fmol (mm tubular length)$^{-1}$.

There were no significant differences in J_D among the perfused S_1, S_2, and S_3 segments (Figure 2A). Greater than 95% of the $^{203}Hg^+$ were found in the TCA-precipitable fraction of the tubular extract in all experiments, shown in Figure 2B.

Effect of Acivicin on the Lumen-to-Cell Transport of the GSH-*S*-CH_3Hg Conjugate

Addition of 750μM acivicin (a specific inhibitor of the luminal plasma membrane enzyme γ-glutamyltransferase) to a perfusate containing 20 μM of the GSH-*S*-conjugate of CH_3Hg^+ (GSH-*S*-CH_3Hg) resulted in significant changes in transport of GSH-*S*-CH_3Hg (Figure 3). More specifically, the J_D of GSH-*S*-CH_3Hg in tubules perfused through the lumen with 20 μM GSH-*S*-CH_3Hg in the presence of 750 μM acivicin, was 73% lower (25.9 ± 5.4 fmol min^{-1} (mm tubular length)$^{-1}$) than that (96.1 ± 12.2 fmol min^{-1} (mm tubular length)$^{-1}$) in corresponding tubular segments not exposed to acivicin. Tubular accumulation of GSH-*S*-CH_3Hg corresponded to the relative levels of J_D under the two experimental conditions (2552.4 ± 519.5 fmol (mm tubular length)$^{-1}$ in the absence of acivicin and 978.7 ± 113.6 fmol (mm tubular length)$^{-1}$ in the presence of acivicin (Figure 3).

Comparison of Lumen-to-Cell Transport of the GSH-S-CH_3Hg and Cys-S-CH_3Hg Conjugates

The J_D of GSH-*S*-CH_3Hg averaged 96.1 ± 12.2 fmol min^{-1} (mm tubular length)$^{-1}$ in the tubular segments perfused with 20 μM GSH-*S*-CH_3Hg. Interestingly, this value for J_D of GSH-*S*-CH_3Hg was 113% greater than that for that in the tubules perfused with Cys-*S*-CH_3Hg. Moreover, the total tubular content of GSH-*S*-CH_3Hg in the tubular segments perfused with GSH-*S*-CH_3Hg only was 216% greater than that in the tubular segments perfused with Cys-*S*-CH_3Hg only (Figure 4).

Lumen-to-Cell Transport of the NAC-S-CH_3Hg Conjugate

By contrast, when 2μM of the *N*-acetylcysteine (NAC) *S*-conjugate of CH_3Hg^+ (NAC-*S*-CH_3Hg) was perfused through the lumen of S_2 segments of the rabbit proximal tubule, the J_D of NAC-*S*-CH_3Hg averaged 1.9 ± 0.2 fmol min^{-1} (mm tubular length)$^{-1}$ and the total tubular content of NAC-*S*- CH_3Hg was 22.6 ± 2.7 fmol (mm tubular length)$^{-1}$ (Figure 5). This J_D of NAC-*S*-CH_3Hg was significantly lower (by approximately 77%) than those perfused with 2μM Cys-*S*-CH_3Hg only. The total tubular content of NAC-*S*-CH_3Hg was also significantly (84%) lower than the Cys-*S*-CH_3Hg control tubules (Figure 5).

Effect of DMPS on the Lumen-to-Cell Transport of Cys-S-CH_3Hg Conjugate

Flux experiments with DMPS were carried out under three conditions. Under control conditions, S_2 segments of the proximal tubule were perfused through the lumen with 20 μM Cys-*S*-CH_3Hg, with no DMPS in either the perfusate or bath. Under the second set of experimental conditions, 200 μM DMPS was added only to the solution bathing the basolateral surface of the perfused tubular segments. In the third experimental condition, 200 μM DMPS was added to the perfusate which also contained 20 μM Cys-*S*-CH_3Hg.

When 200 μM DMPS was added to the bath only, the J_D of Cys-S-CH_3Hg was about 92% less (dropping from 45.2 ± 4.2 fmol min^{-1} (mm tubular length)$^{-1}$ to 3.5 ± 1.3 fmol min^{-1} (mm tubular length)$^{-1}$) than that in the tubular segments not exposed to DMPS (Control) (Figure 6A). In the corresponding tubular segments exposed to DMPS in the bath only, the total net tubular content of Cys-S-CH_3Hg was 158.4 ± 11.8 fmol (mm tubular length)$^{-1}$, which was 80% less than that in the control tubules (806.7 ± 120.5 fmol (mm tubular length)$^{-1}$) (Figure 6B).

When 200 μM DMPS was co-perfused with Cys-S-CH_3Hg in the lumen, the J_D of Cys-S-CH_3Hg (3.25 ± 1.99 fmol min^{-1} (mm tubular length)$^{-1}$) was very low, and it averaged about 93% less than that in control tubules. Accordingly, the total tubular content of Cys-S-CH_3Hg was also very low (99.1 ± 16.5 fmol (mm tubular length)$^{-1}$), averaging about 87% less than that in control tubules, Cys-S-CH_3Hg only (Figure 6B).

Effect of Temperature on the Transport of Cys-S-CH_3Hg Conjugate

To assess whether the transport of Cys-S-CH_3Hg (when presented to the luminal compartment as Cys-*S*-CH_3Hg) is modulated by temperature, S_2 segments of proximal tubules were perfused with 20μM Cys-*S*-CH_3Hg at 37°C and 13°C. The J_D of Cys-S-CH_3Hg at these temperatures was 45.2 ± 4.2 and 2.8 ± 1.2 fmol min^{-1} (mm tubular length)$^{-1}$, respectively. The tubular content of Cys-S-CH_3Hg in the corresponding S_2 segments was 806.7 ± 120.5 and 119.2 ± 29.1 fmol (mm tubular length)$^{-1}$. Significant decreases in J_D (94% reduction at 13°C), tubular content (85.2% reduction at 13°C) of Cys-S-CH_3Hg were observed in response to these reductions in temperature (Figure 7).

No Acute Cellular Intoxicated of Tubular Segments

No visual evidence of acute cellular toxicity, such as cellular swelling, blebbing of the luminal membrane, or uptake of the cellular vital dye (FD&C green), was noted in any of the S_1, S_2 and S_3 segments used in the experiments of this study. In addition, intercellular leak of the volume marker ([^{3}H]-*L*-glucose) did not increase under any of the experimental conditions studied.

DISCUSSION

Findings from previous studies have suggested that thiol *S*-conjugates of CH_3Hg^+ may be the primary transportable forms of CH_3Hg^+ in the kidney (Carty and Malone 1979; Zalups

2000). The present study tested indirectly the hypothesis that luminal membrane transport of three thiol *S*-conjugates of CH_3Hg^+, namely Cys-*S*-CH_3Hg, GSH-*S*-CH_3Hg, and NAC-*S*-CH_3Hg, are absorbed along the three segments (S_1, S_2 and S_3) of the proximal tubule via carrier-mediated processes. The data indicate that Cys-*S*-CH_3Hg is likely absorbed avidly by the S_2 segments of the proximal tubule, while NAC-*S*-CH_3Hg is not as avidly transported.

Absorptive transport of Cys-*S*-CH_3Hg has been demonstrated recently to be mediated by *Xenopus Laevis* oocytes expressing B^{0+} (Bridges and Zalups 2006). Moreover, it was demonstrated that this transport could be inhibited by *L*-methionine, suggesting that system B^{0+} may participate in the luminal absorptive transport of Cys-*S*-CH_3Hg in renal proximal tubules (Bridges and Zalups 2006). To provide additional support for this hypothesis, we studied the luminal transport of Cys-*S*-CH_3Hg in perfused proximal tubular segments in the presence of *L*-methionine. We also demonstrate that *L*-methionine can inhibit significantly the absorptive transport of Cys-*S*-CH_3Hg in *pars recta* segments of the proximal tubule (Figure. 1), indicating that an amino acid transporter involved in the uptake of methionine likely participates in the luminal uptake of Cys-*S*-CH_3Hg.

Based on the hypothesis that Cys-*S*-CH_3Hg may have chemical morphology similar to the amino acid, *L*-cystine, we measured the absorptive transport of Cys-*S*-CH_3Hg in the presence of *L*-cystine. The *L*-cystine transporter, system $b^{0,+}$ like system B^{0+}, is localized almost exclusively in the luminal membrane of proximal tubular epithelial cells (Furriols, Chillaron et al. 1993; Mora, Chillaron et al. 1996), and has been implicated in the renal cellular uptake of the Cys S-conjugate of the divalent forms of inorganic mercury (Hg^{2+}), Cys-*S*-Hg-*S*-Cys (Bridges, Bauch et al. 2004), and cadmium (Cd^{2+}), Cys-*S*-Cd-*S*-Cys (Wang, Zalups et al. 2010). Interestingly, we demonstrate in the present study that *L*-cystine does indeed inhibit significantly the transport of Cys-*S*-CH_3Hg (Figure 1). This finding lends support to the hypothesis that system $b^{0,+}$ may also be involved in the luminal uptake of Cys-*S*-CH_3Hg in proximal tubular cells.

Possible axial heterogeneity in the transport of Cys-*S*-CH_3Hg among all three segments of the proximal tubule, i.e. S_1, S_2, and S_3 segments, was also evaluated in this study. Our data show, however, that there are no significant differences in disappearance flux (J_D) of Cys-*S*-CH_3Hg and the cellular distribution among these three segments (Figure 2). The absence of a difference in luminal uptake and cellular accumulation of Cys-S-CH_3Hg may relate to the distribution of transporters capable of transporting Cys-*S*-CH_3Hg along the length of the proximal tubule. For example, expression of system $B^{0,+}$ occurs primarily in the S_1 segment and decreases gradually from S_1 to S_3 segments (Fleck, Schwertfeger et al. 2003). In contrast, the expression of system b^{0+} is minimal in the S_1 segment, but increases progressively from the S_1 to the S_3 segments (Fleck, Schwertfeger et al. 2003). Due to this reciprocal axial distribution of these two transporters along the proximal tubule, the combined transport of these two transporters may result in similar levels of absorptive transport of Cys-*S*-CH_3Hg along the length of the entire proximal tubule. These observations also support our hypotheses that Cys-*S*-CH_3Hg may utilize more than one transport system in its luminal uptake.

GSH-*S*-CH_3Hg has also been suggested to be a transportable form of CH_3Hg^+ in tissues and organs such as brain and liver (Dutczak and Ballatori 1994). GSH is a metabolic reservoir for *L*-cysteine and is more abundant than free *L*-cysteine in mammalian cells. γ-Glutamyltransferase and cysteinylglycinase, enzymes that metabolize GSH to *L*-cysteine are present in great abundance in the luminal plasma membrane of proximal tubular cells (Zalups 2000). When these enzymes act on GSH-*S*-CH_3Hg, the methyl mercuric ion remains bonded to *L*-Cys during the catabolism of GSH to Cys (Naganuma, Oda-Urano et al. 1988). Therefore, Cys-*S*-CH_3Hg is likely the primary transportable form of CH_3Hg^+, and

not GSH-*S*-CH_3Hg. Use of the inhibitory alkylator of γ-glutamyltransferase, acivicin, provides additional support for this notion (Figure 3). Our findings are consistent with those of previous studies showing that pretreatment with acivicin decreases renal accumulation and increases urinary excretion of CH_3Hg^+ (Berndt, Baggett et al. 1985; Gregus, Stein et al. 1987; Naganuma, Oda-Urano et al. 1988; de Ceaurriz and Ban 1990). Interestingly, rates of GSH-*S*-CH_3Hg transport were about twice that of Cys-*S*-CH_3Hg (98 vs 45 fmol min^{-1} (mm tubular length)$^{-1}$) at a luminal concentration 20 μM (Figure 4) and there was not 100% inhibition of GSH-*S*-CH_3Hg transport by acivicin (98 to 25 fmol min^{-1} (mm tubular length)$^{-1}$). Both of these observations indicate that perhaps some GSH-*S*-CH_3Hg may be transported across the luminal membrane intact.

We also examined the transport of the NAC *S*-conjugate of CH_3Hg^+ (NAC-*S*-CH_3Hg), which is a polar negatively charged species. Our data are consistent with previous findings showing that NAC-*S*-CH_3Hg is transported poorly by *Xenopus Laevis* oocytes expressing the luminal transporter system B^{0+} (Bridges and Zalups 2006). The very low rate of uptake of NAC-*S*-CH_3Hg at the luminal plasma membrane of S_2 segments is likely related to the net negative charge of this molecule. Interestingly, luminal secretion of NAC-*S*-CH_3Hg was first noted when NAC was observed to cause transient increases in urinary excretion of methylmercury (Aremu, Madejczyk et al. 2008). Current literature indicates that the organic anion transporter 1 (OAT1) in the basolateral plasma membrane and multidrug resistance protein 2 (MRP2) in the luminal plasma membrane are involved in the enhanced urinary excretion of CH_3Hg^+ mediated by administration of NAC. Accordingly, NAC-*S*-CH_3Hg in blood appears to be a substrate taken up into the proximal tubular epithelial cells by OAT1 at the basolateral membrane (Koh, Simmons-Willis et al. 2002; Zalups and Ahmad 2005). Subsequently, intracellular NAC-*S*-CH_3Hg is extracted or eliminated by the epithelial cells by secretion into tubular lumen via the actions of the ATP-binding cassette protein, MRP2, (Madejczyk, Aremu et al. 2007). It is possible that the NAC-*S*-CH_3Hg which gained access into the epithelial cells lining the S_2 segments of the proximal tubule in the present study would have been made available for secretion back into the tubular lumen by the actions of MRP2 (Ballatori 2002). This could also serve as an explanation for the low rate in net absorption and tubular content of ^{203}Hg *that we* observed (Figure 5).

DMPS is a metal complexing agent known to increase the renal clearance and urinary excretion of CH_3Hg^+ (Aposhian 1983; Aposhian, Maiorino et al. 1992). These effects of DMPS have been assumed to be due to unimpeded filtration of DMPS *S*-conjugates of CH_3Hg^+ at renal glomeruli and secretion of CH_3Hg^+ from within proximal tubular epithelial cells, subsequent to the formation of transportable DMPS *S*-conjugates of CH_3Hg^+. A series of recent studies have demonstrated the proximal tubular extraction of DMPS *S*-conjugates of CH_3Hg^+ is mediated by the multi-drug resistance protein, MRP2 (Zalups and Bridges 2009). It had been reported that DMPS *S*-conjugates of CH_3Hg^+ may be substrates for the organic anion transporter 1 (OAT1) (Koh, Simmons-Willis et al. 2002), also definitive proof of this transport remains lacking. Overall, there is sufficient evidence indicating that there are likely transport-mechanisms involved in the secretion of DMPS *S*-conjugates of CH_3Hg^+ by the epithetical cells of the proximal tubule. In the present study, when DMPS was added to the luminal fluid, absorptive transport and accumulation of Cys-*S*-CH_3Hg was reduced to very low to negligible levels (Figure 6). These findings suggest that once formed in the lumen, the DMPS *S*-conjugate of Cys-*S*-CH_3Hg or CH_3-Hg^+ is not transported into proximal tubular epithelial cells at the luminal membrane. Similar findings were obtained when DMPS was placed in the basolateral compartment while tubules were perfused with Cys-*S*-CH_3Hg. In this case, the low levels of net absorptive transport are likely due to avid basolateral uptake of DMPS and subsequent binding to Cys-*S*-CH_3Hg or CH_3-Hg^+ intracellularly and/or in the lumen. Once the conjugate enters or is formed in the lumen, it becomes a molecular species not readily taken up by proximal tubular epithelial cells. It can

only be speculated as to what conjugate is formed in these circumstances, DMPS-$HgCH_3$-S-Cys or DMPS-S-$HgCH_3$.

It is note-worthy to mention that greater than 95% of the mercuric ions of conjugated-S-$CH_3{}^{203}Hg$ associated with the perfused proximal tubular segments were present in a TCA-precipitable fraction of the tubular extract in all experiments (Figure 2B). This is in contrast to our findings from proximal tubular segments tubules perfused with non-conjugated CH_3Hg^+ (Zalups and Barfuss 1993) where the majority of the metal ions was found in the TCA- or acid-soluble fraction of the tubule extract. It is presumed that TCA extracts and precipitates large cellular proteins because the tubule retains its overall shape, and becomes rigid, white, and opaque after exposure to TCA. The non-precipitable fraction is presumed to be acid-soluble cytosolic contents. There is no current explanation as to why CH_3Hg^+ conjugates and free CH_3Hg^+ are distributed differently between these extracts.

The temperature-dependent experiments indicate strongly that the luminal uptake of Cys-*S*-CH_3Hg involves specific carrier proteins, and that the uptake is not due to non-specific binding and/or paracellular leakage (Figure 7). The leak properties of tight junctions remained unchanged since the leak rate of the volume marker was not altered when temperature was altered.

In conclusion, the current studies show that Cys-*S*-CH_3Hg and GSH-*S*-CH_3Hg appear to be transportable forms of CH_3Hg^+ conjugates at the luminal membrane of proximal tubular epithelial cells. Cys-*S*-CH_3Hg appears to utilize amino acid transporters involved in the transport of *L*-methionine and *L*-cystine. GSH-*S*-CH_3Hg appears to be degraded to Cys-*S*-CH_3Hg by exo-enzymes in the luminal membrane, which is subsequently transported into proximal tubular epithelial cells, and transported intact. NAC-*S*-CH_3Hg and the DMPS conjugate of Cys-*S*-CH_3Hg (or DMPS-$HgCH_3$?) are not transported efficiently, if at all, at the luminal membrane of proximal tubular epithelial cells.

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- Luminal transport of thiol conjugates of CH_3Hg^+ were studied for the first time
- CH_3Hg^+ conjugates of Cys and GSH are transported avidly
- CH_3Hg^+ conjugates of NAC and DMPS are transported minimally
- Transport of Cys-*S*-CH_3Hg is inhibited by *L*-methionine and *L*-cystine
- Transport of GSH-*S*-CH_3Hg was only partially inhibited by acivicin

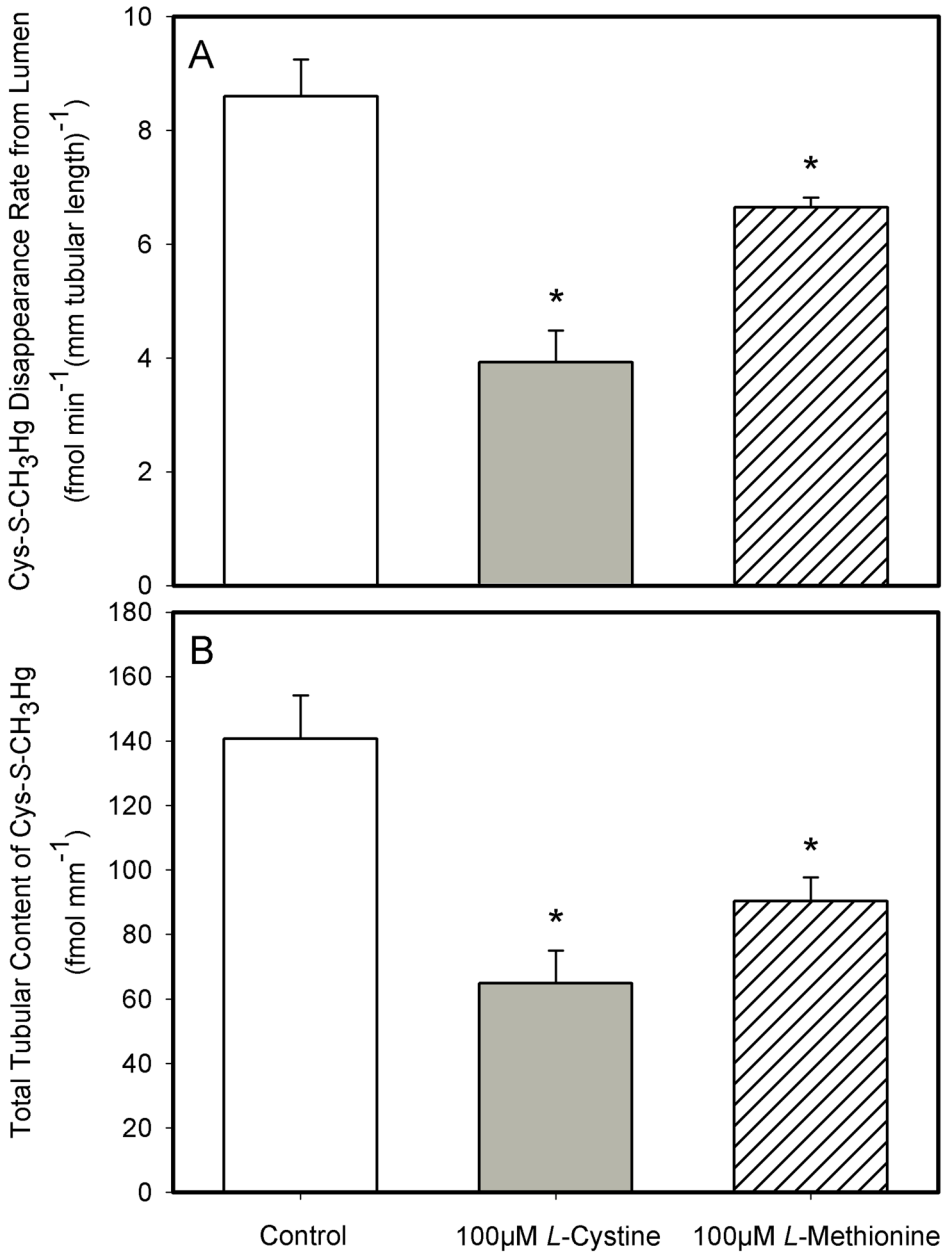

Figure 1.

The effect of 100μM *L*-cystine and 100 μM *L*-methionine on the lumen-to-cell transport (A) and total tubular content (B) of Cys-S-CH_3Hg in isolated S_2 segments of the proximal tubule perfused through the lumen with 2 μM Cys-S-CH_3Hg (at 37°C). Each value represents the mean ± S.E.M for a sample size of five or six. The "*" indicates a significant difference (P<0.05) from control S_2 segments.

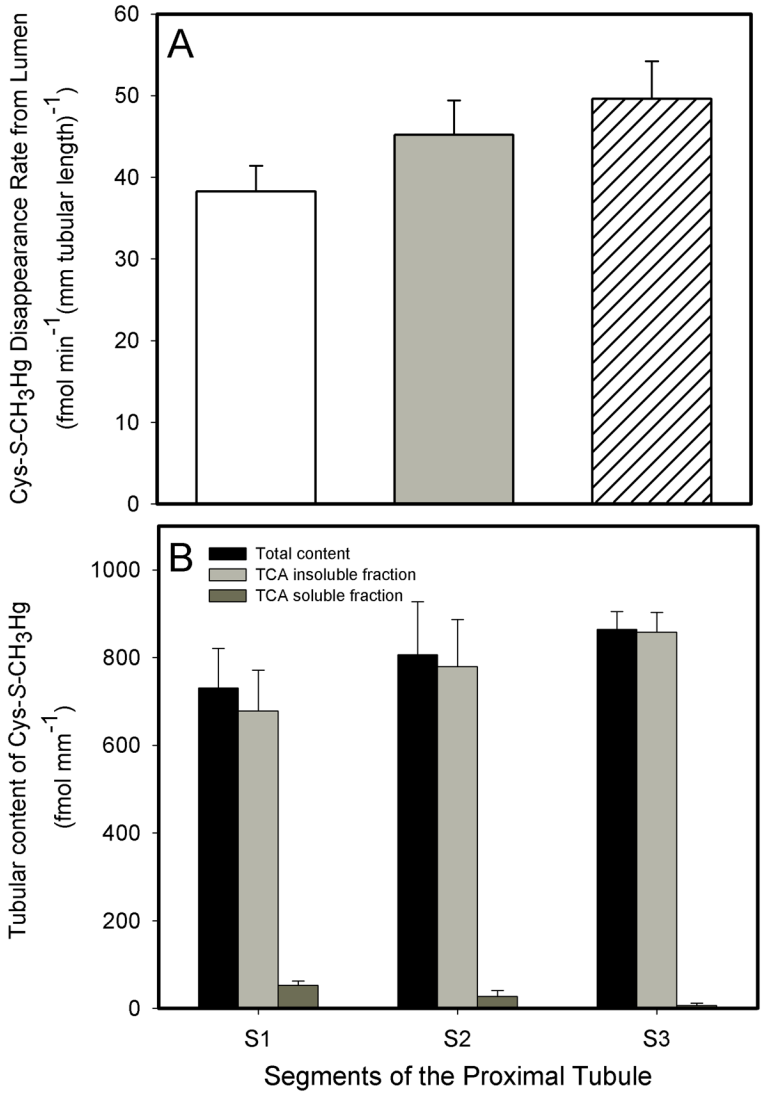

Figure 2.

Rate of the luminal disappearance (A) and total tubular content, TCA precipitable & TCA non-precipitable fractions (B) of Cys-S-CH_3Hg (20 μM) in S_1, S_2, and S_3 segments of the proximal tubule of the rabbit. Each value represents the mean ± S.E.M for a sample size of five or six.

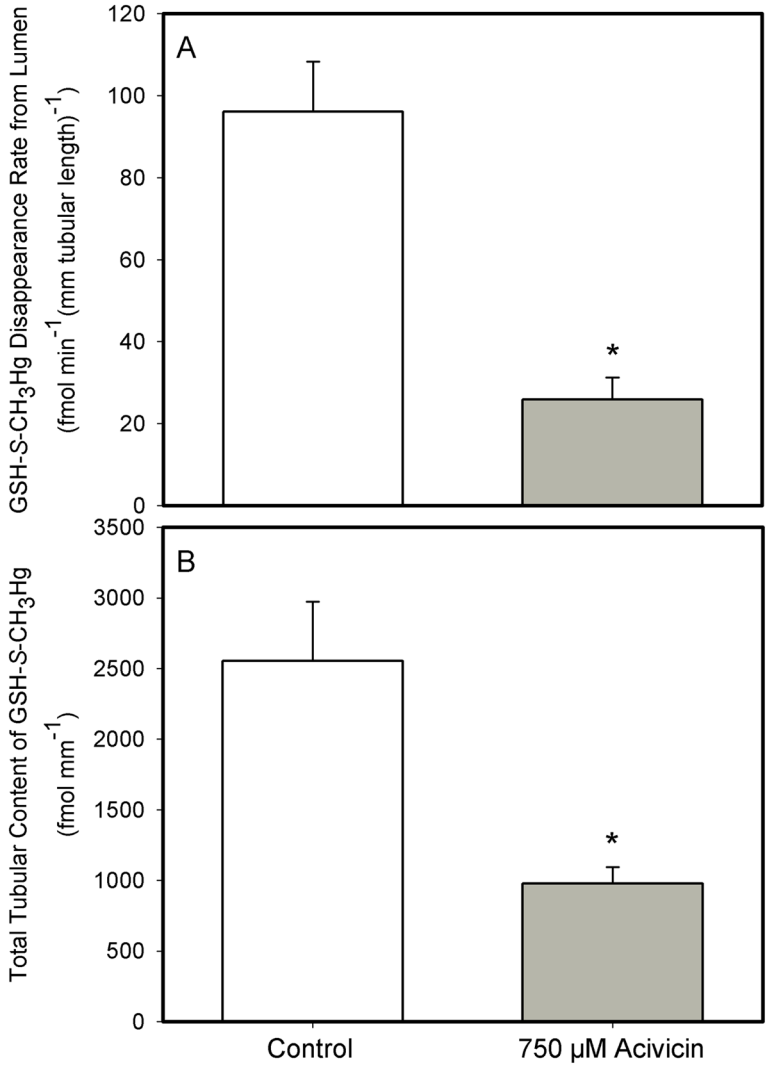

Figure 3.

The effect of 750μM acivicin on the lumen-to-cell transport (A) and total tubular content (B) of GSH-S-CH_3Hg in isolated S_2 segment of the proximal tubule of the rabbit perfused with 20 μM GSH-S-CH_3Hg (at 37°C). Each value represents the mean ± S.E.M for a sample size of five or six. The "*" indicates a significant difference (P<0.05) from control S_2 segments.

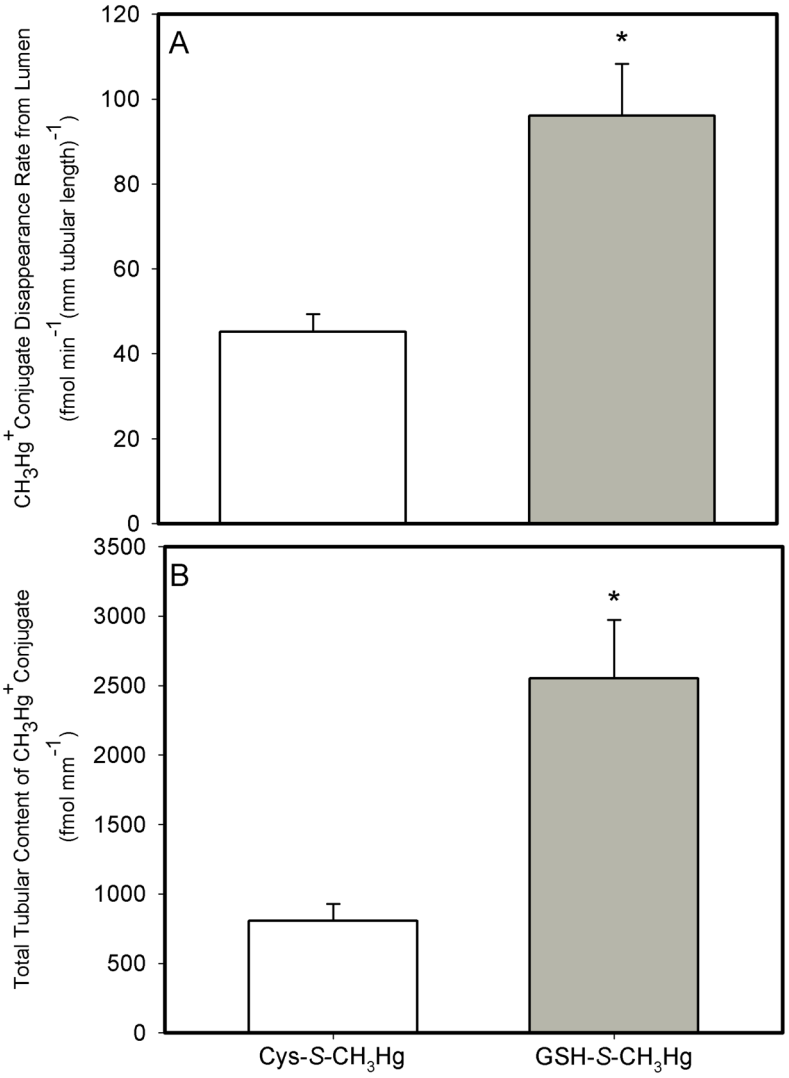

Figure 4.

Rate of luminal disappearance of both conjugates (A) and total tubular content (B) of when perfused with Cys-S-CH_3Hg and GSH-S-CH_3Hg in isolated S_2 segments of the proximal tubule of the rabbit. The perfusate concentration was 20 μM for both solutes. Each value represents the mean ± S.E.M for a sample size of five or six.

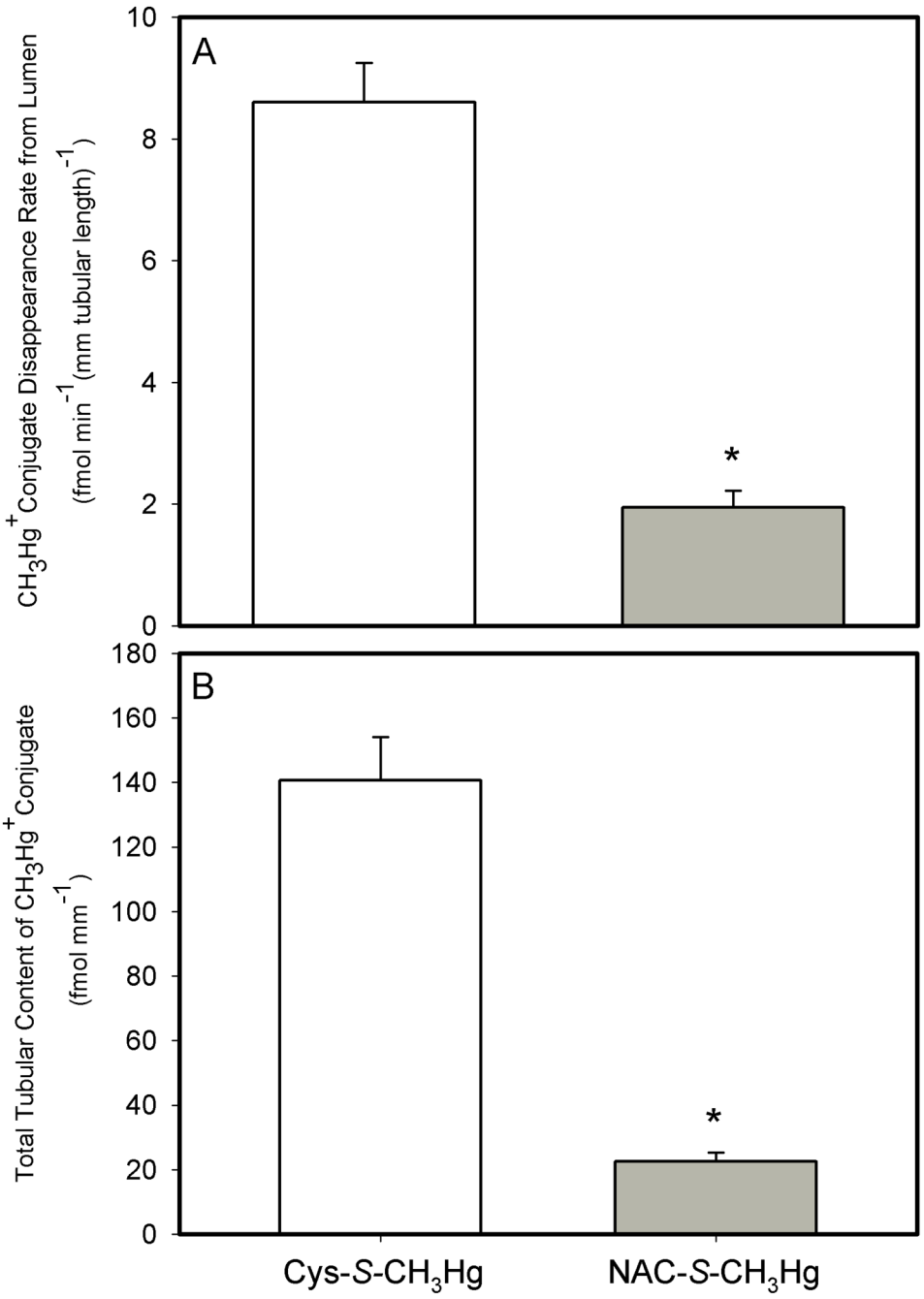

Figure 5.

Rate of luminal disappearance of the conjugates (A) and total tubular content (B) of Cys-S-CH_3Hg and GSH-S-CH_3Hg in isolated S_2 segments of the proximal tubule of the rabbit. The perfusate concentration was 2 μM for both solutes. Each value represents the mean ± S.E.M for a sample size of five or six.

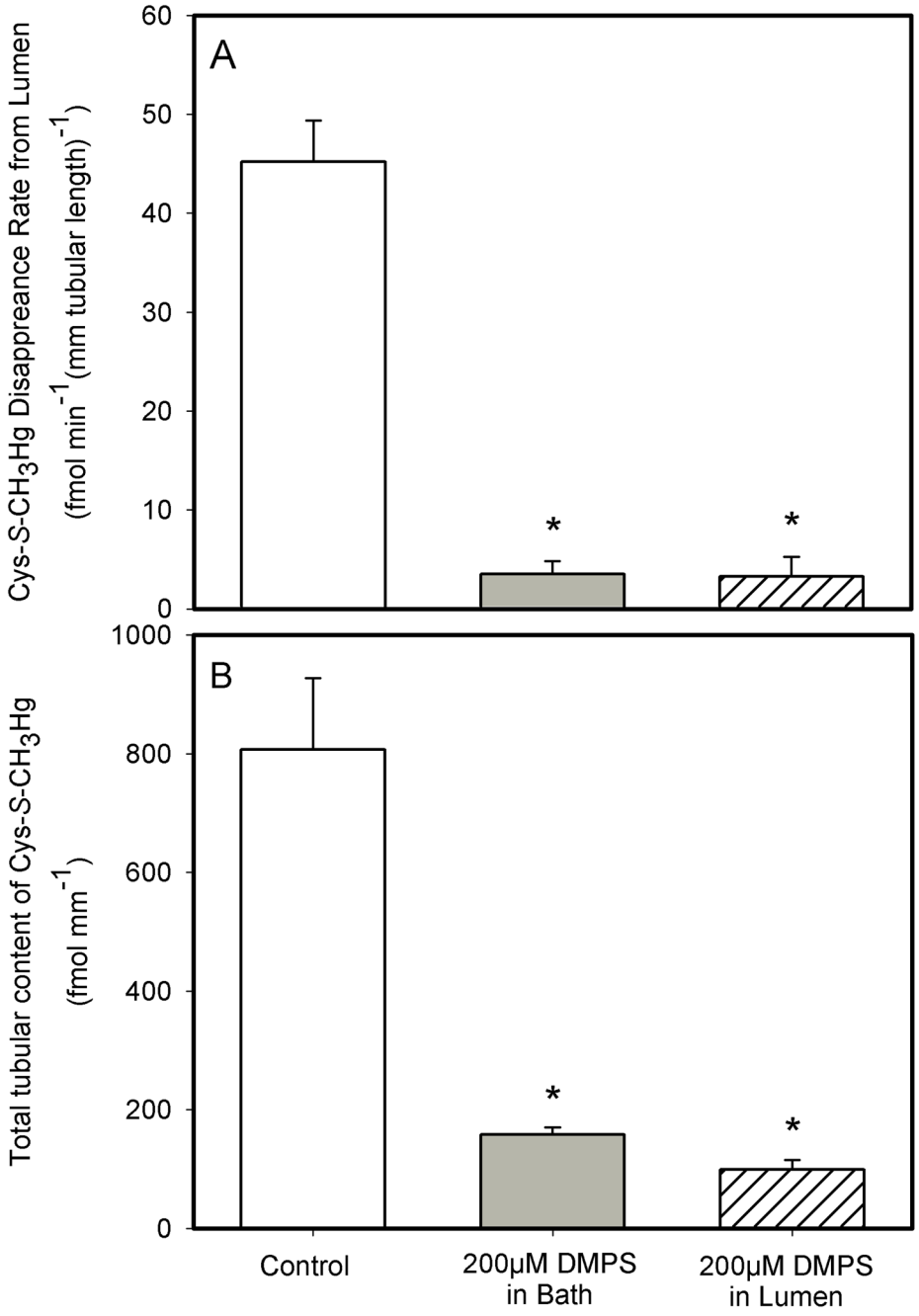

Figure 6.

The effect of 200 μM DMPS on the luminal rate of disappearance (A) and total tubular content (B) of Cys-S-CH_3Hg (20 μM) in isolated S_2 segments of the rabbit proximal tubule perfused with DMPS in the bathing solution only or co-perfused with Cys-S-CH_3Hg (at 37°C). Each value represents the mean ± S.E.M for a sample size of five or six. The "*" indicates a significant difference (P<0.05) from control S_2 segments.

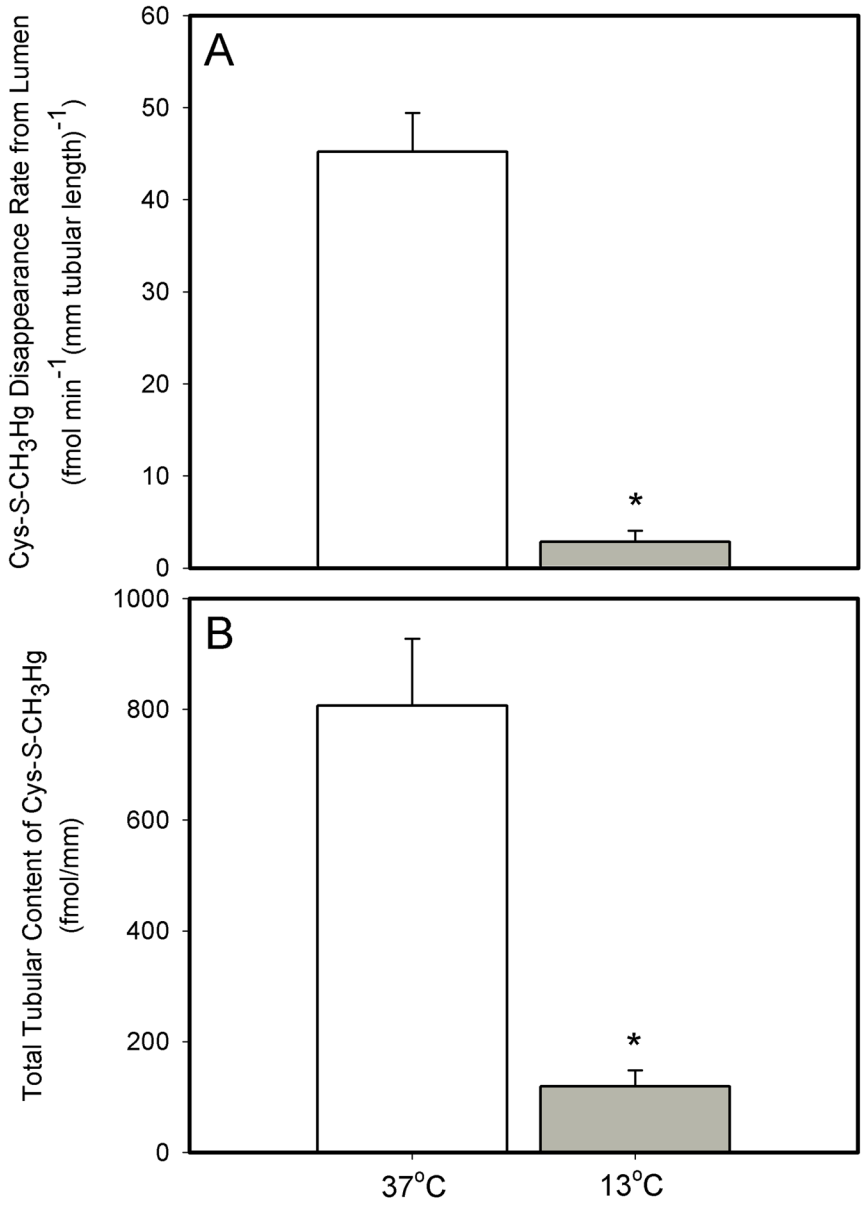

Figure 7.

The effect of temperature on the lumen-to-cell transport (A) and total tubular content (B) of Cys-S-CH_3Hg in isolated S_2 segments of the rabbit proximal tubule perfused with 20 μM CH_3Hg-Cys (at 37°C). Each value represents the mean ± S.E.M for a sample size of five or six. The "*" indicates a significant difference (P<0.05) from control S_2 segments.