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System $B^{0,+}$ and the Transport of Thiol-*S*-Conjugates of Methylmercury

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ABSTRACT

Methylmercury (CH_3Hg^+) is a clinically relevant toxicant that is the most abundant form of mercury found in the environment. After exposure, it accumulates in the kidneys, liver, and central nervous system. The mechanisms by which this toxicant is taken up by target cells are only now beginning to be understood. Some experimental data support a hypothesis involving molecular mimicry, whereby thiol conjugates of methylmercury (especially a cysteine *S*-conjugate) mimic one or more amino acids and are transported into target cells by amino acid transporters. In the present study, we tested the hypothesis that Cys and homocysteine (Hcy) *S*-conjugates of methylmercury (CH_3Hg-*S*-Cys and CH_3Hg-*S*-Hcy, respectively) mimic one or more amino acids at the site of the Na^+-dependent amino acid transporter, system $B^{0,+}$. In the kidneys, system $B^{0,+}$ is situated on the luminal plasma membrane of proximal tubular epithelial cells. To test our hypothesis, we measured uptake of CH_3Hg-*S*-Cys and CH_3Hg-*S*-Hcy in *Xenopus laevis* oocytes injected with water or capped RNA encoding mouse $ATB^{0,+}$. Analyses of time course, substrate specificity, and saturation kinetics showed that the uptake of CH_3Hg-*S*-Cys and CH_3Hg-*S*-Hcy was 5- to 10-fold greater in oocytes expressing $ATB^{0,+}$ than in corresponding water-injected controls. Moreover, the transport of CH_3Hg-*S*-Cys and CH_3Hg-*S*-Hcy was inhibited by substrates transported by system $B^{0,+}$. Finally, our data indicate that CH_3Hg-*S*-Cys and CH_3Hg-*S*-Hcy may mimic of one or more amino acids (e.g., methionine) that are normally transported by system $B^{0,+}$. To our knowledge, this is the first report implicating system $B^{0,+}$ in the transport of any mercuric species.

Mercury is a toxic metal that can exist in the environment in several forms: elemental, inorganic (Hg^{2+}), and/or organic [mainly as methylmercury (CH_3Hg^+)]. The release of elemental mercury and Hg^{2+} into the environment results primarily from the burning of fossil fuels. A significant fraction of Hg^{2+} in the environment is biotransformed by microbial methylation to form CH_3Hg^+. This is the principal form of mercury to which humans are most often exposed, primarily through the consumption of contaminated water and/or fish. Following ingestion of CH_3Hg^+, approximately 90% is absorbed by the gastrointestinal tract (Kershaw et al., 1980). Once in systemic circulation, each methylmercuric ion forms a strong bond with the reduced sulfur atom of one of several thiol-containing molecules, such as albumin, Cys, glutathione (GSH), homocysteine (Hcy), and *N*-acetylcysteine (NAC) (Fuhr and Rabenstein, 1973). Some of these mercuric conjugates serve as transportable substrates of certain carrier proteins in target organs such as the kidneys, liver, and brain (Bridges and Zalups, 2005a).

Within the kidney, the epithelial cells of the proximal tubule are the primary cells that take up CH_3Hg^+ and Hg^{2+} (Rodier and Kates, 1988; Rodier et al., 1988; Zalups, 1991, 2000). It has been shown that Hg^{2+} gains access to these cells, in the form of certain thiol *S*-conjugates, via sodium-dependent and -independent transporters present in the luminal plasma membrane (Cannon et al., 2000, 2001). In contrast, there are currently no data indicating specific mechanisms in the uptake of CH_3Hg^+ across the luminal plasma membrane of proximal tubular cells.

Because the chemical structures of CH_3Hg-*S*-Cys and the Hcy *S*-conjugate of CH_3Hg^+ (CH_3Hg-*S*-Hcy) are similar to that of the amino acid methionine, it has been postulated that these two conjugates may act as mimics of methionine to gain access to certain compartments of the body (Clarkson, 1993; Bridges and Zalups, 2005). In recent years, the theory

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ABBREVIATIONS: Hg^{2+}, inorganic mercury; CH_3Hg^+, methylmercury; GSH, glutathione; Hcy, homocysteine; NAC, *N*-acetylcysteine; CH_3Hg-*S*-Cys, cysteine *S*-conjugate of methylmercury; CH_3Hg-*S*-Hcy, homocysteine *S*-conjugate of methylmercury; cRNA, capped RNA; CH_3Hg-*S*-GSH, glutathione *S*-conjugate of methylmercury; CH_3Hg-*S*-NAC, *N*-acetylcysteine *S*-conjugate of methylmercury.

of molecular mimicry has been put forth as a mechanism by which inorganic and methylated forms of mercury gain entry into target cells (Clarkson, 1993; Zalups, 2000; Ballatori, 2002; Zalups and Ahmad, 2003; Bridges and Zalups, 2005a). Indeed, several studies have provided indirect evidence for the involvement of molecular mimicry in the transport of CH_3Hg-*S*-Cys (Aschner, 1989; Aschner and Clarkson, 1989; Kerper et al., 1992; Mokrzan et al., 1995). More direct evidence for this theory comes from a recent study in *Xenopus laevis* oocytes in which CH_3Hg-*S*-Cys was transported by the Na^+-independent amino acid transporter, system L (Simmons-Willis et al., 2002).

Because of these data and the structural similarities among CH_3Hg-*S*-Cys, CH_3Hg-*S*-Hcy, and methionine, we hypothesize that one or more transporters of methionine participate in the transport of these mercuric species. One possible candidate for this transport is the amino acid transporter, system $B^{0,+}$. This carrier mediates the Na^+/Cl^--dependent uptake of a variety of neutral and cationic amino acids, including methionine (Sloan and Mager, 1999; Nakanishi et al., 2001). In addition, system $B^{0,+}$ is localized in the luminal plasma membrane of proximal tubular epithelial cells (Gonska et al., 2000), a location that corresponds to the site of CH_3Hg^+ accumulation and toxicity in the kidney.

Therefore, the purpose of the present study was to test the hypothesis that system $B^{0,+}$ is capable of transporting methyl mercuric ions, as conjugates of Cys, Hcy, GSH, or NAC. This study focused exclusively on the Na^+-dependent component of CH_3Hg^+ transport, although it should be noted the transport of this metal may also be mediated by one or more Na^+-independent carriers. Analyses of time dependence, substrate specificity, and saturation kinetics of CH_3Hg-*S*-Cys and CH_3Hg-*S*-Hcy transport demonstrate that, in the present model, these two mercuric conjugates are transportable substrates of system $B^{0,+}$.

Materials and Methods

Isolation and Microinjection of *X. laevis* Oocytes. Ovaries were isolated from sexually mature, female *X. laevis* (Xenopus One, Ann Arbor, MI) and defolliculated as described previously (Aslamkhan et al., 2003). In brief, connective tissue was digested with 1 mg/ml collagenase A (Roche Molecular Biochemicals, Indianapolis, IN), and follicles were removed by incubating oocytes in 100 mM K_2HPO_4. Oocytes were then placed in oocyte Ringer's 2 [82.5 mM NaCl, 2.5 mM KCl, 1 mM Na_2HPO_4, 3 mM NaOH, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM sodium pyruvate, and 5 mM HEPES, pH 7.6] supplemented with 5% (v/v) horse serum (Atlanta Biological, Atlanta, GA) and 50 μg/ml gentamicin sulfate (Invitrogen, Carlsbad, CA) and stored in a shaking incubator at 18°C.

The construct for mouse $ATB^{0,+}$ (amino acid transporter $B^{0,+}$), the cDNA-encoding system $B^{0,+}$, was obtained from Dr. Vadivel Ganapathy (Medical College of Georgia, Augusta, GA). Capped RNA (cRNA) transcripts encoding $ATB^{0,+}$ were generated using the mMessage mMachine RNA transcription kit (Ambion, Austin, TX). On the day following isolation, stage IV and V oocytes were microinjected with 28.5 ng (23 nl) of cRNA or an equal volume of water. Oocytes were stored in a shaking incubator at 18°C until the time of experimentation.

Evaluation of Transport and Cellular Disposition. Variability in oocyte quality may exist among groups of oocytes isolated from different frogs; therefore, each individual experiment used oocytes isolated from a single frog. Each experiment was repeated (using a different batch of oocytes) three times over the course of 3 consecutive weeks. Obvious seasonal variations in oocyte quality as determined by visual inspection were not noticed during the course of the study.

All experiments were carried out in both water- and $ATB^{0,+}$-injected oocytes in a manner similar to that described previously (Bridges and Zalups, 2005b). In brief, 3 days following injection, the oocytes were separated into groups of five in 24-well plates and were washed with 1 ml of room temperature (25°C) uptake buffer (25 mM HEPES/Tris, 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 5 mM glucose, pH 7.5). Uptake was initiated by adding 350 μl of uptake buffer containing a radiolabeled substrate of interest. The incubation was allowed to proceed for 30 min at 25°C unless otherwise stated. Oocytes were then rinsed twice with 1 ml of ice-cold uptake buffer containing 1 mM sodium 2,3-dimercaptopropane-1-sulfonate (Sigma, St. Louis, MO), an effective chelator of mercuric ions. After washing, oocytes were lysed in 0.5 ml of 1% sodium dodecylsulfate/0.2 N NaOH, and each sample was added to 5 ml of Opti-Fluor scintillation cocktail (Perkin Elmer, Shelton, CT). The radioactivity contained therein was determined by standard isotopic techniques using a Beckman LS6500 scintillation counter (Beckman Instruments, Fullerton, CA).

Confirmation of *X. laevis* Oocytes as a Model for System $B^{0,+}$-Mediated Transport. To confirm that a functional form of system $B^{0,+}$ was being expressed in our *Xenopus* oocyte model, we measured the uptake of amino acids that have been characterized as substrates for this carrier: [^{3}H]L-methionine (78 Ci/mmol; Amersham, Piscataway, NJ), [^{3}H]L-phenylalanine (120 Ci/mmol; Amersham), [^{3}H]L-valine (37 Ci/mmol; Amersham), [^{3}H]L-isoleucine (92 Ci/mmol; Amersham), [^{3}H]L-leucine (160 Ci/mmol; Amersham), [^{3}H]L-alanine (85 Ci/mmol; Perkin Elmer), [^{3}H]L-glycine (60 Ci/mmol; Perkin Elmer), [^{3}H]L-serine (30 Ci/mmol; Amersham), [^{35}S]L-cysteine (100 mCi/mmol; Amersham), [^{3}H]L-threonine (16 Ci/mmol; Amersham), [^{3}H]L-glutamine (52 Ci/mmol; Amersham), and [^{3}H]L-lysine (98.5 Ci/mmol; Perkin Elmer). In addition, we measured the transport of [^{3}H]L-aspartate (16.2 Ci/mmol; Amersham) and [^{35}S]L-cystine (100 mCi/mmol; Amersham), neither of which has been identified as a substrate of system $B^{0,+}$. The transport of each of the aforementioned amino acids (5 μM) was measured for 30 min at 25°C.

Analyses of CH_3Hg^+ Transport Using Biochemical Parameters. CH_3Hg^+ forms a strong bond with thiol-containing molecules. In plasma, the most common nonprotein thiol-containing molecules are Cys, Hcy, NAC, and GSH. Therefore, we studied the uptake of [^{14}C]CH_3HgCl (0.8 μCi/μg; American Radiolabeled Chemicals, St. Louis, MO) as an *S*-conjugate of each of these molecules. CH_3Hg^+ was presented to cells as CH_3Hg-*S*-Cys, CH_3Hg-*S*-Hcy, or as the GSH- or NAC-*S*-conjugate of CH_3Hg^+ (CH_3Hg-*S*-GSH or CH_3Hg-*S*-NAC, respectively). These complexes were formed by incubating 5 μM [^{14}C]CH_3HgCl with 6 μM Cys, Hcy, GSH, or NAC in uptake buffer for 5 min at 25°C. The 1:1.2 ratio of these compounds ensured that every molecule of CH_3Hg^+ bonded stably to a sulfhydryl group in a linear I coordinate manner.

Analysis of time dependence for the cellular uptake of CH_3Hg-*S*-Cys and CH_3Hg-*S*-Hcy was carried out by measuring the uptake of each compound for 5, 10, 15, 20, 30, 45, or 60 min. Saturation kinetics for the transport of these mercuric conjugates were assessed by measuring the transport of radiolabeled CH_3Hg-*S*-Cys or CH_3Hg-*S*-Hcy in the presence of increasing concentrations of unlabeled CH_3Hg-*S*-Cys (1, 5, 10, 15, 25, 50, 75, and 100 μM) or CH_3Hg-*S*-Hcy (5, 10, 25, 50, 75, 100 μM), respectively. Cells were not exposed to higher concentrations of these mercuric compounds because of concerns related to the effect of these compounds on cellular viability. The data were analyzed using the Michaelis-Menten equation: $V = V_{max}[S]/(K_m + [S])$, where V_{max} is the maximal velocity of transport, [S] is the substrate concentration, and K_m is the Michaelis-Menten constant. Substrate specificity analyses were also carried out, where transport of CH_3Hg-*S*-Cys and CH_3Hg-*S*-Hcy was measured in the presence of known substrates (3 mM) of system $B^{0,+}$ (cysteine, me-

thionine, alanine, glycine, leucine, isoleucine, phenylalanine, valine, lysine, glutamine, serine, tryptophan, asparagine, and arginine). In addition, the uptake of CH_3Hg-*S*-Cys and CH_3Hg-*S*-Hcy was measured in the presence of increasing concentrations of methionine (25, 50, 75, 100, 250, 500, 1000, and 2500 μM). Methionine is a high-affinity substrate for system $B^{0,+}$ and is structurally similar to the mercuric conjugates being studied.

Determination of Oocyte Protein Content. Protein content of the oocytes was determined by the method of Bradford (1976). In brief, oocytes were solubilized in 1 ml of 1 N NaOH, and 100 μl of the total solution was placed in a cuvette. Two milliliters of Bradford Reagent (Sigma) was added to the cuvette, and the sample was read in a Shimadzu UV-2101PC spectrophotometer at 595 nm.

Data Analyses. All experiments were performed in triplicate and were repeated at least twice ($n = 6$). Data for each parameter assessed were first analyzed using the Kolmogorov-Smirnov test for Normality and then with Levene's test for homogeneity of variances. Thereafter, the data were analyzed using either a two-tailed Student's t test (Fig. 1) or a two-way analysis of variance. Tukey's multiple comparison procedure was used to assess differences among the means. All data are presented as mean ± S.E. for an n of 3 to 4. $p < 0.05$ was considered statistically significant.

Results

To confirm the expression of system $B^{0,+}$ in oocytes, the uptake of various amino acids (methionine, phenylalanine, valine, isoleucine, leucine, alanine, glycine, serine, cysteine, threonine, glutamine, lysine, cystine, and aspartate) was measured (Fig. 1). With the exception of cystine and aspartate, all of the aforementioned amino acids have been characterized as substrates of system $B^{0,+}$ (Sloan and Mager, 1999; Nakanishi et al., 2001). The transport of all of the amino acids except cystine and aspartate was significantly greater in oocytes expressing system $B^{0,+}$ than in controls. When the uptake of cystine and aspartate was measured, there were no significant differences between water- and $ATB^{0,+}$-injected oocytes. For many of the amino acids tested, the uptake in the oocytes injected with $ATB^{0,+}$ was at least 3-fold greater than that in corresponding controls.

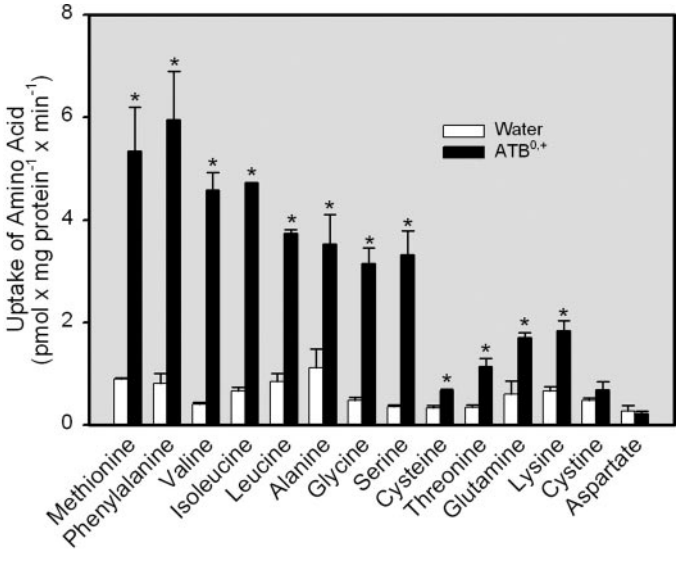

Fig. 1. Uptake of various amino acids in *X. laevis* oocytes injected with either water or mouse $ATB^{0,+}$. Oocytes were exposed to various radiolabeled amino acids (5 μM) for 30 min at 25°C. Results are presented as mean ± S.E. Data represent three experiments performed in triplicate. *, significantly different ($p < 0.05$) from the mean for the corresponding group of water-injected oocytes.

In addition, the saturation kinetics for the uptake of methionine (Fig. 2A) or arginine (Fig. 3A) confirmed that a functional form of system $B^{0,+}$ was being expressed in the $ATB^{0,+}$-injected oocytes. Because methionine is a high-affinity substrate of system $B^{0,+}$ and a molecular homolog of CH_3Hg-*S*-Cys, we chose to study the saturation kinetics of methionine transport as confirmation of the functional insertion of system $B^{0,+}$ in the plasma membrane of the oocytes. Arginine was chosen for study because it is a positively charged substrate of system $B^{0,+}$ at physiologic pH, and this amino acid is an effective substrate that can be used to help characterize the transporting features of this carrier protein. The uptake of both methionine and arginine was significantly greater in the oocytes injected with cRNA encoding system $B^{0,+}$ than in the water-injected controls. To calculate the V_{max} of transport and the Michaelis-Menten constant (K_m) that are specific for the activity of system $B^{0,+}$, the amount of uptake in the water-injected controls was subtracted from that in the $ATB^{0,+}$-injected oocytes. For methi-

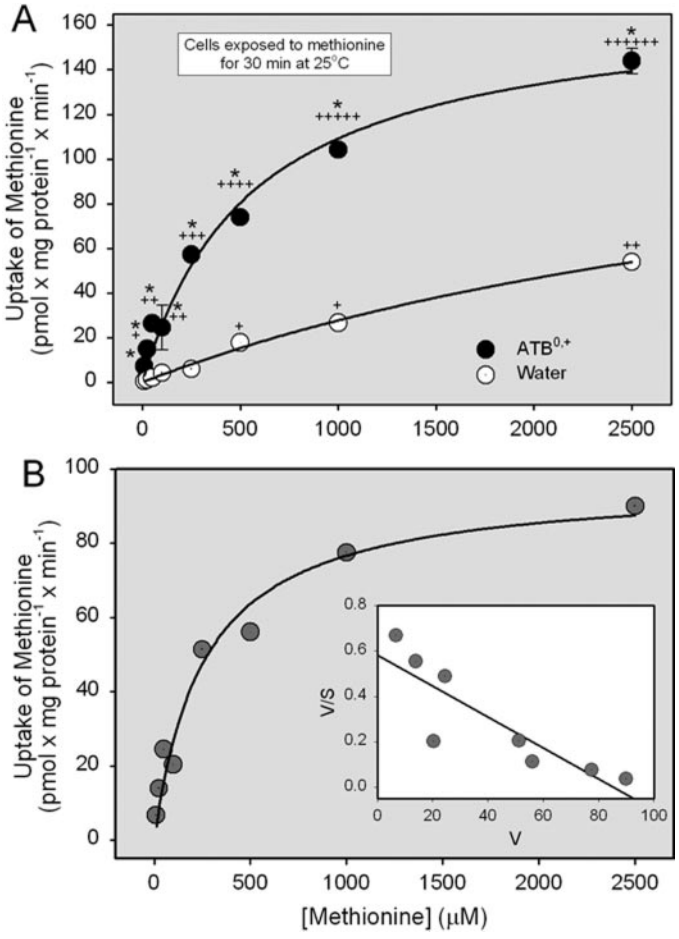

Fig. 2. Analyses of saturation kinetics for the uptake of methionine in *X. laevis* oocytes injected with either water or mouse $ATB^{0,+}$ (A). Oocytes were exposed to 5 μM methionine for 30 min at 25°C in the presence of increasing concentrations of unlabeled methionine. The transport activity of the water-injected oocytes was subtracted from that of the $ATB^{0,+}$-injected oocytes to show the amount of methionine uptake specific to system $B^{0,+}$ (B) (inset, Eadie-Hofstee plot). Results are presented as mean ± S.E. Data represent three experiments performed in triplicate. *, significantly different ($p < 0.05$) from the mean for the corresponding group of water-injected oocytes. +, ++, +++, ++++, +++++, or ++++++, significantly different ($p < 0.05$) from the mean for the same group of oocytes at the previous concentration(s).

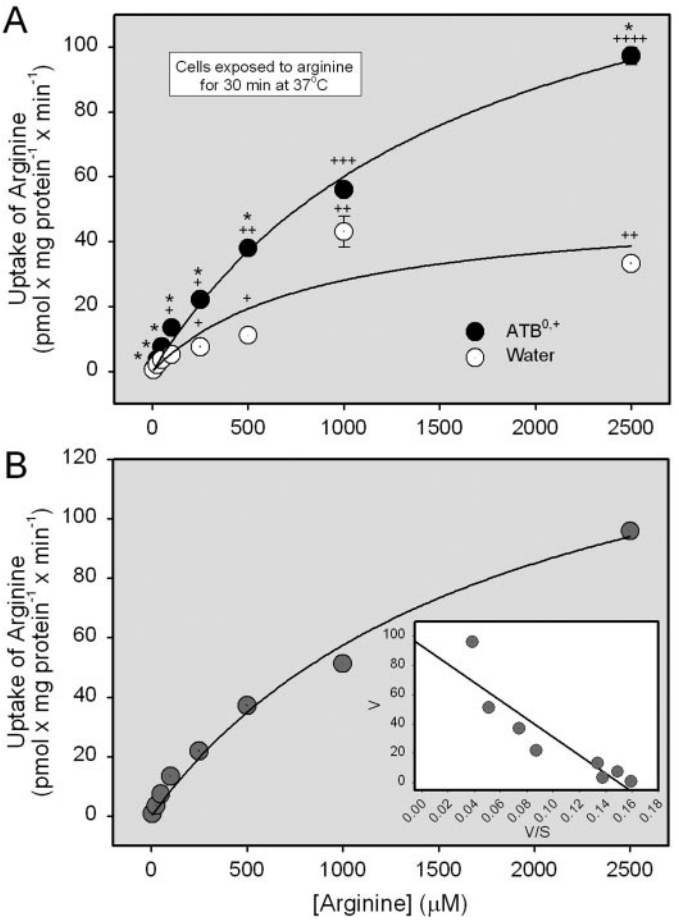

Fig. 3. Analyses of saturation kinetics for the uptake of arginine in *X. laevis* oocytes injected with either water or mouse $ATB^{0,+}$ (A). Oocytes were exposed to 5 μM arginine for 30 min at 25°C in the presence of increasing concentrations of unlabeled arginine. The transport activity of the water-injected oocytes was subtracted from that of the $ATB^{0,+}$-injected oocytes to show the amount of arginine uptake specific to system $B^{0,+}$ (B) (inset, Eadie-Hofstee plot). Results are presented as mean ± S.E. Data represent three experiments performed in triplicate. *, significantly different ($p < 0.05$) from the mean for the corresponding group of water-injected oocytes. +, ++, +++, or ++++, significantly different ($p < 0.05$) from the mean for the same group of oocytes at the previous concentration(s).

onine, the V_{max} was calculated to be 96.4 ± 7.3 pmol/mg protein/min and the K_m was 257 ± 63.0 μM ($r^2 = 0.9887$) (Fig. 2B). For arginine transport, the V_{max} was estimated to be 163 ± 20.0 pmol/mg protein/min, and the K_m was 1.84 ± 0.41 mM ($r^2 = 0.9937$) (Fig. 3B).

Inasmuch as Cys, Hcy, NAC, or GSH are the principal nonprotein thiols present in blood, we chose to characterize the potential role of system $B^{0,+}$ in the uptake of CH_3Hg-*S*-Cys, CH_3Hg-*S*-Hcy, CH_3Hg-*S*-NAC, or CH_3Hg-*S*-GSH in oocytes injected with either water or $ATB^{0,+}$ (Fig. 4). The transport of CH_3Hg-*S*-Cys was approximately 10-fold greater in the oocytes injected with $ATB^{0,+}$ than in the controls. Likewise, the uptake of CH_3Hg-*S*-Hcy was also significantly greater in oocytes expressing system $B^{0,+}$. In contrast, the uptake of CH_3Hg-*S*-NAC and CH_3Hg-*S*-GSH was not significantly different between the oocytes expressing system $B^{0,+}$ and the respective controls. Among the water-injected oocytes, there were no significant differences in the uptake of any species of CH_3Hg^+.

Time-course analyses indicate that uptake of CH_3Hg-*S*-Cys (Fig. 5A) and CH_3Hg-*S*-Hcy (Fig. 6A) in both control and system $B^{0,+}$-expressing oocytes was time-dependent. The uptake of each conjugate increased over 60 min of exposure in both $ATB^{0,+}$- and water-injected oocytes. However, at almost every time studied, the transport of CH_3Hg-*S*-Cys and CH_3Hg-*S*-Hcy was significantly greater in the oocytes injected with $ATB^{0,+}$ than in the water-injected controls. To facilitate the analysis of the data, the amount of uptake in the water-injected oocytes was subtracted from that in $ATB^{0,+}$-injected oocytes (Figs. 5B and 6B).

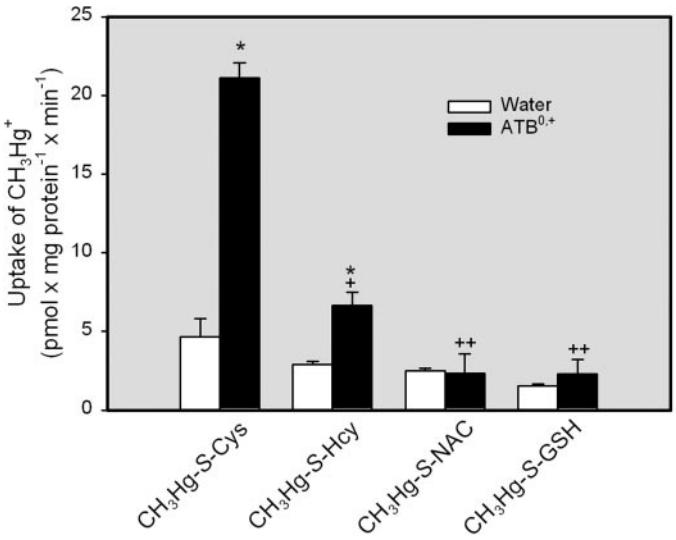

Fig. 4. Uptake of CH_3Hg^+ as a conjugate of Cys (CH_3Hg-*S*-Cys), Hcy (CH_3Hg-*S*-Hcy), NAC (CH_3Hg-*S*-NAC), or GSH (CH_3Hg-*S*-GSH) in *X. laevis* oocytes injected with either water or mouse $ATB^{0,+}$. Oocytes were incubated with 5 μM of each conjugate for 30 min at 25°C. Results are presented as mean ± S.E. Data represent three experiments performed in triplicate. *, significantly different ($p < 0.05$) from the mean for the corresponding group of water-injected oocytes. +, significantly different ($p < 0.05$) from the mean for the corresponding group of oocytes exposed to CH_3Hg-*S*-Cys. ++, significantly different ($p < 0.05$) from the mean for the corresponding group of oocytes exposed to CH_3Hg-*S*-Cys or CH_3Hg-*S*-Hcy.

The saturation kinetics for the uptake of CH_3Hg-*S*-Cys (Fig. 7A) and CH_3Hg-*S*-Hcy (Fig. 8A) were measured in oocytes injected with water or $ATB^{0,+}$. The transport of each conjugate was significantly greater in oocytes expressing system $B^{0,+}$ than in water-injected controls. As before, the amount of CH_3Hg-*S*-Cys and CH_3Hg-*S*-Hcy taken up by the water-injected oocytes was subtracted from that of the $ATB^{0,+}$-injected oocytes. The V_{max} and K_m values for the transport of CH_3Hg-*S*-Cys were estimated to be 42.7 ± 6.86 pmol/mg protein/min and 48.0 ± 16.4 μM, respectively ($r^2 = 0.9850$) (Fig. 7B). The V_{max} for the transport of CH_3Hg-*S*-Hcy was 64.2 ± 25.6 pmol/mg protein/min, whereas the K_m was calculated to be 164 ± 87.4 μM ($r^2 = 0.9963$) (Fig. 8B).

Substrate specificity analyses were carried out in control and $ATB^{0,+}$-injected oocytes to further characterize the transport of CH_3Hg-*S*-Cys (Fig. 9A) and CH_3Hg-*S*-Hcy (Fig. 10A). The transport of CH_3Hg-*S*-Cys and CH_3Hg-*S*-Hcy specific to system $B^{0,+}$ is shown in Figs. 9B and 10B, respectively. All of the neutral amino acids studied significantly inhibited the transport of CH_3Hg-*S*-Cys into oocytes expressing system $B^{0,+}$. As expected, aspartate, a negatively charged amino acid, did not inhibit the transport of CH_3Hg-*S*-Cys, whereas the positive amino acids, arginine and lysine, inhibited transport significantly. Interestingly, many of the amino

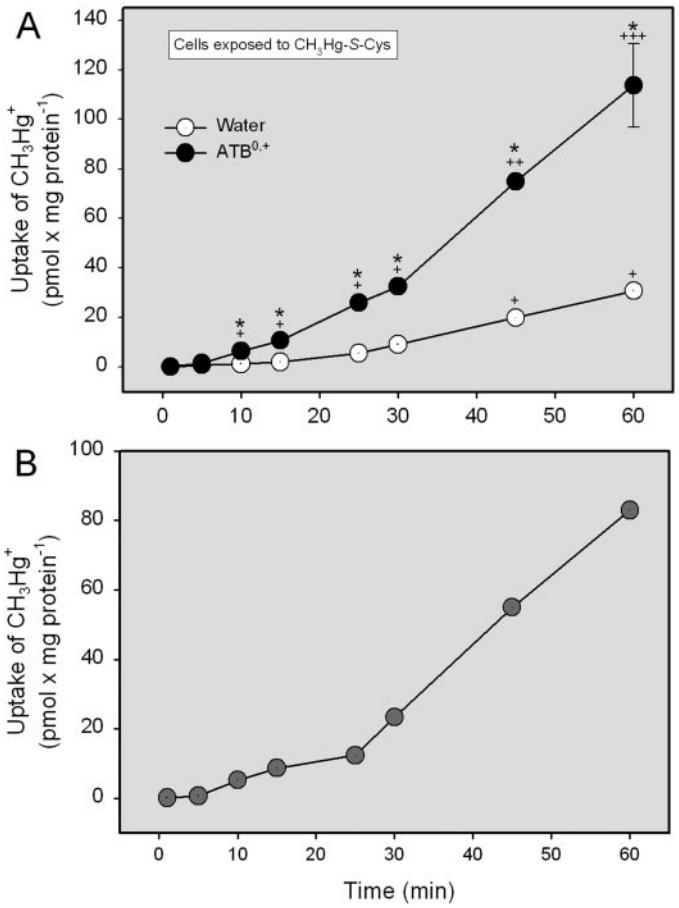

Fig. 5. Time dependence of CH_3Hg^+ transport as a conjugate of Cys (CH_3Hg-*S*-Cys) in *X. laevis* oocytes injected with water or mouse $ATB^{0,+}$ (A). Oocytes were incubated with 5 μM CH_3Hg-*S*-Cys at 25°C, and samples were taken for estimation at indicated times. The transport activity of the water-injected oocytes was subtracted from that of the $ATB^{0,+}$-injected oocytes to show the amount of CH_3Hg-*S*-Cys uptake specific to system $B^{0,+}$ (B). Results are presented as mean ± S.E. Data represent three experiments performed in triplicate. ∗, significantly different ($p < 0.05$) from the mean for the corresponding group of control oocytes. +, ++, or +++, significantly different ($p < 0.05$) from the mean for the same group of oocytes at the previous time(s).

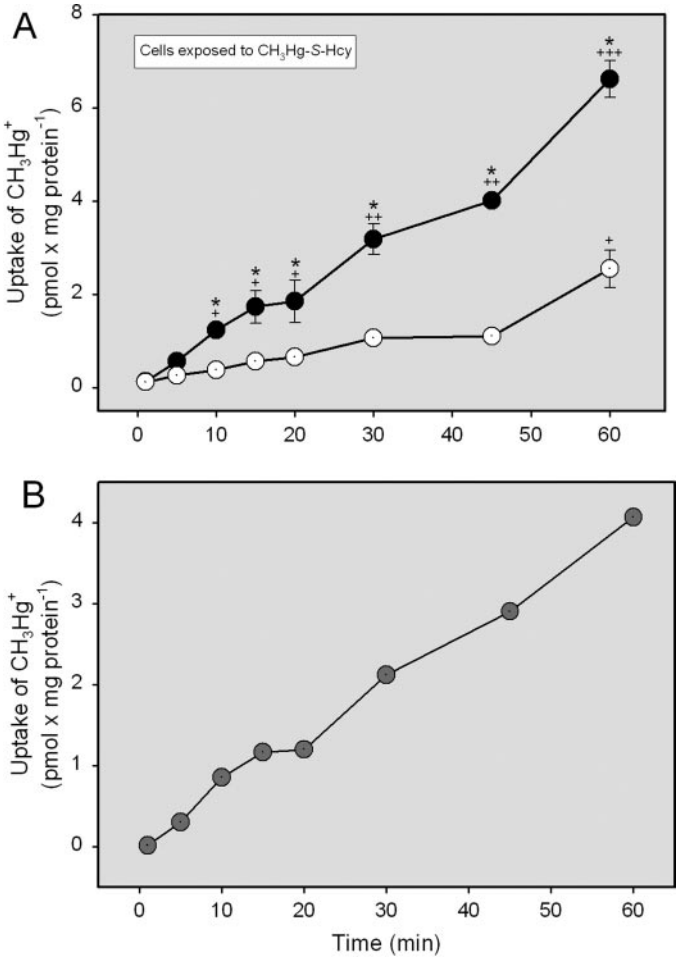

Fig. 6. Time dependence of CH_3Hg^+ transport as a conjugate of Hcy (CH_3Hg-*S*-Hcy) in *X. laevis* oocytes injected with water or mouse $ATB^{0,+}$ (A). Oocytes were incubated with 5 μM CH_3Hg-*S*-Hcy at 25°C, and samples were taken for estimation at indicated times. The transport activity of the water-injected oocytes was subtracted from that of the $ATB^{0,+}$-injected oocytes to show the amount of CH_3Hg-*S*-Hcy uptake specific to system $B^{0,+}$ (B). Results are presented as mean ± S.E. Data represent three experiments performed in triplicate. ∗, significantly different ($p < 0.05$) from the mean for the corresponding group of control oocytes. +, ++, or +++, significantly different ($p < 0.05$) from the mean for the same group of oocytes at the previous time(s).

acids also inhibited the uptake of CH_3Hg-*S*-Cys in the water-injected oocytes (Fig. 9A). When the uptake of CH_3Hg-*S*-Hcy was measured in the presence of the same amino acids, all of the amino acids examined were capable of inhibiting the uptake of this metal complex to some extent (Fig. 10A). The amino acids alanine, isoleucine, phenylalanine, valine, lysine, and serine completely blocked the $ATB^{0,+}$-mediated component of CH_3Hg-*S*-Hcy uptake (Fig. 10B).

The ability of methionine to inhibit the transport of CH_3Hg-*S*-Cys and CH_3Hg-*S*-Hcy was measured in control and $ATB^{0,+}$-injected oocytes. Methionine inhibited the uptake of CH_3Hg-*S*-Cys (Fig. 11) and CH_3Hg-*S*-Hcy (Fig. 12) in a dose-dependent manner. The $ATB^{0,+}$-specific uptake of CH_3Hg-*S*-Cys and CH_3Hg-*S*-Hcy is shown in Figs. 11B and 12B, respectively.

Discussion

CH_3Hg^+ is ubiquitous in our environment and presents a significant risk to human health. This toxicant has gained prominence in recent years because of its detrimental effects on fetal development. Also of clinical importance, but not as well publicized, are its nephrotoxic effects. Indeed, a primary site for the accumulation of methylmercuric ions is the renal proximal tubule (Rodier and Kates, 1988; Rodier et al., 1988; Zalups and Barfuss, 1993); however, the mechanisms by which this accumulation occurs remain unclear. One of the logical candidates for the uptake of CH_3Hg^+ into proximal tubular cells is system $B^{0,+}$, which is located in the luminal plasma membrane of these cells (Gonska et al., 2000). The expression of this carrier is probably greatest in the proximal tubule because 99% of filtered amino acids are absorbed by this region of the nephron (Giebisch and Windhager, 2003). Not surprisingly, the proximal tubular location of system $B^{0,+}$ corresponds with the site of CH_3Hg^+ accumulation and toxicity. In addition, because methionine is a major substrate of system $B^{0,+}$, and Cys *S*-conjugates of CH_3Hg^+ have been implicated as potential mimics of methionine, we sought to test the hypothesis that CH_3Hg-*S*-Cys, as well as CH_3Hg-*S*-Hcy, CH_3Hg-*S*-NAC, and CH_3Hg-*S*-GSH, are taken up from

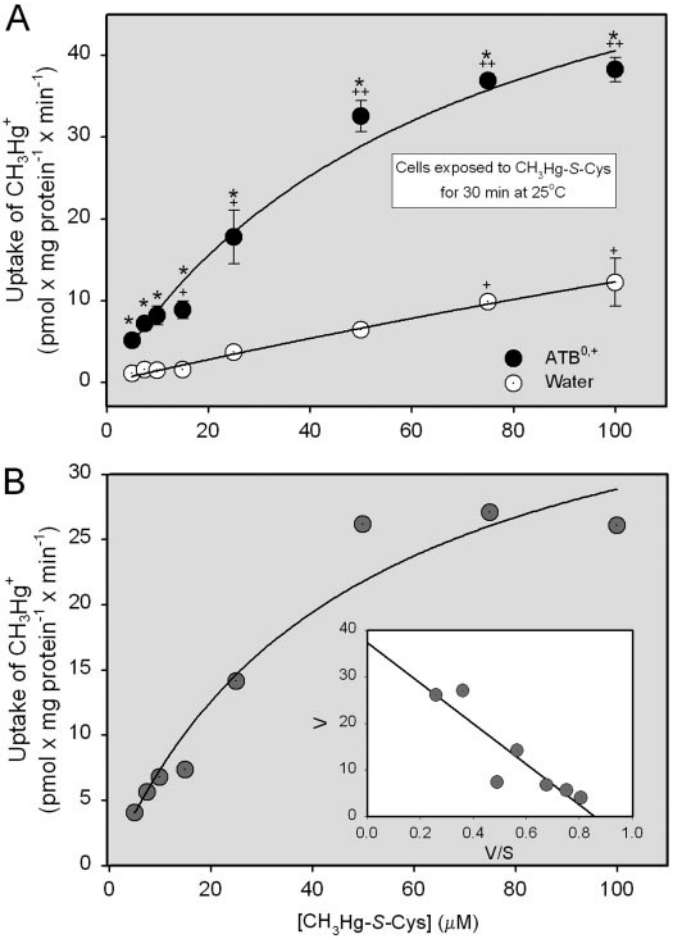

Fig. 7. Analyses of saturation kinetics of CH_3Hg^+ transport as a conjugate of Cys (CH_3Hg-*S*-Cys) in *X. laevis* oocytes injected with water or mouse $ATB^{0,+}$ (A). Oocytes were incubated with 5 μM CH_3Hg-*S*-Cys in the presence of unlabeled CH_3Hg-*S*-Cys for 30 min at 25°C. The transport activity of the water-injected oocytes was subtracted from that of the $ATB^{0,+}$-injected oocytes to show the amount of CH_3Hg-*S*-Cys uptake specific to system $B^{0,+}$ (B) (inset, Eadie-Hofstee plot). Results are presented as mean ± S.E. Data represent three experiments performed in triplicate. *, significantly different ($p < 0.05$) from the mean for the corresponding group of water-injected oocytes. + or ++, significantly different ($p < 0.05$) from the mean for the same group of oocytes at the previous concentration(s).

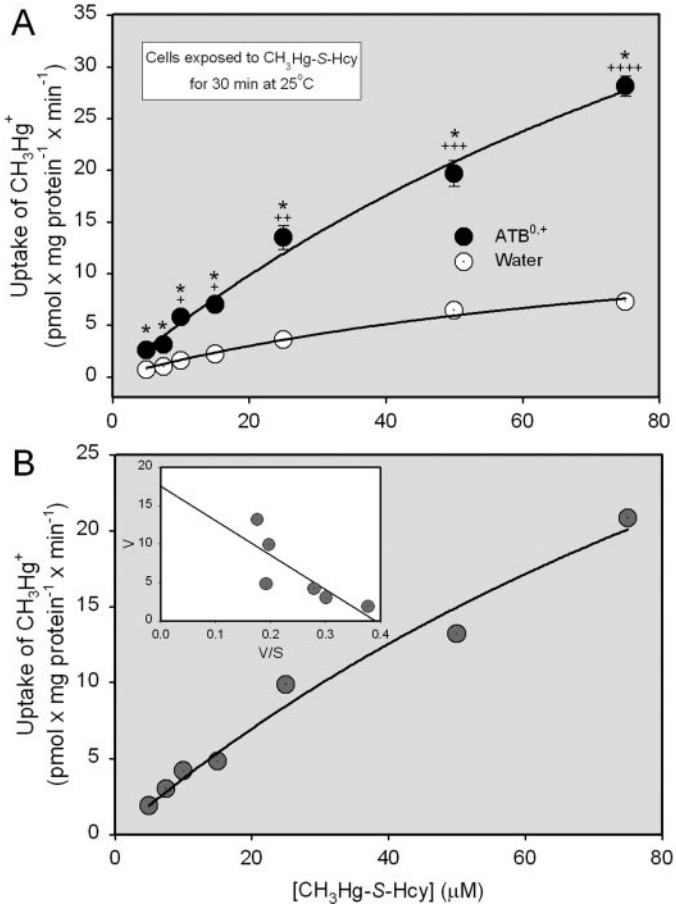

Fig. 8. Analyses of saturation kinetics of CH_3Hg^+ transport as a conjugate of Hcy (CH_3Hg-*S*-Hcy) in *X. laevis* oocytes injected with water or mouse $ATB^{0,+}$ (A). Oocytes were incubated with 5 μM CH_3Hg-*S*-Hcy in the presence of unlabeled CH_3Hg-*S*-Hcy for 30 min at 25°C. The transport activity of the water-injected oocytes was subtracted from that of the $ATB^{0,+}$-injected oocytes to show the amount of CH_3Hg-*S*-Hcy uptake specific to system $B^{0,+}$ (B) (inset, Eadie-Hofstee plot). Results are presented as mean ± S.E. Data represent three experiments performed in triplicate. *, significantly different ($p < 0.05$) from the mean for the corresponding group of water-injected oocytes. +, ++, +++, or ++++, significantly different ($p < 0.05$) from the mean for the same group of oocytes at the previous concentration(s).

the tubular lumen by the Na^+-dependent monomeric amino acid carrier, system $B^{0,+}$.

To test our hypothesis, we used *Xenopus* oocytes microinjected with the cRNA for $ATB^{0,+}$ (which encodes system $B^{0,+}$). To ensure that this transporter was functioning properly in the current oocyte model, we measured the uptake of various amino acids in water- and $ATB^{0,+}$-injected oocytes. All of the amino acids tested, except cystine and aspartate, have been identified as substrates of system $B^{0,+}$ (Sloan and Mager, 1999; Nakanishi et al., 2001). The transport of these substrates was significantly greater in oocytes injected with $ATB^{0,+}$ than in controls, indicating the presence of a functional system $B^{0,+}$ transporter. To further confirm the functionality of system $B^{0,+}$ in our model, we measured the saturation kinetics for two known substrates of this carrier, methionine and arginine. Given the number of endogenous amino acid transporters in *X. laevis* oocytes (i.e., systems $B^{0,+}$, ASC, X^-_{AG}, asc, L, y^+, $b^{0,+}$, and x_c^-), it is possible that some of the measured uptake is mediated by transporters other than system $B^{0,+}$ (Campa and Kilberg, 1989; Taylor et al., 1989, 1996; Van Winkle, 1993). However, when the uptake in water-injected oocytes was subtracted from that in $ATB^{0,+}$-injected oocytes, it became obvious that system $B^{0,+}$ mediates a large fraction of methionine and arginine transport. Collectively, the analyses of amino acid uptake and saturation kinetics support the validity of our in vitro model (i.e., *X. laevis* oocytes injected with $ATB^{0,+}$) as an appropriate and useful model in which to study the activity of system $B^{0,+}$.

In plasma, the most common nonprotein thiols that may come in contact with CH_3Hg^+ include Cys, Hcy, NAC, and GSH. Therefore, we evaluated the transport of CH_3Hg^+ as a conjugate of each of these compounds. Only CH_3Hg-*S*-Cys and CH_3Hg-*S*-Hcy seem to be transported by system $B^{0,+}$. CH_3Hg-*S*-NAC and CH_3Hg-*S*-GSH were not taken up efficiently by this carrier; a phenomenon that may be related to the steric nature and negative charge of each complex. System $B^{0,+}$ recognizes neutral and cationic, but not anionic, amino acids as substrates (Sloan and Mager, 1999; Nakan-

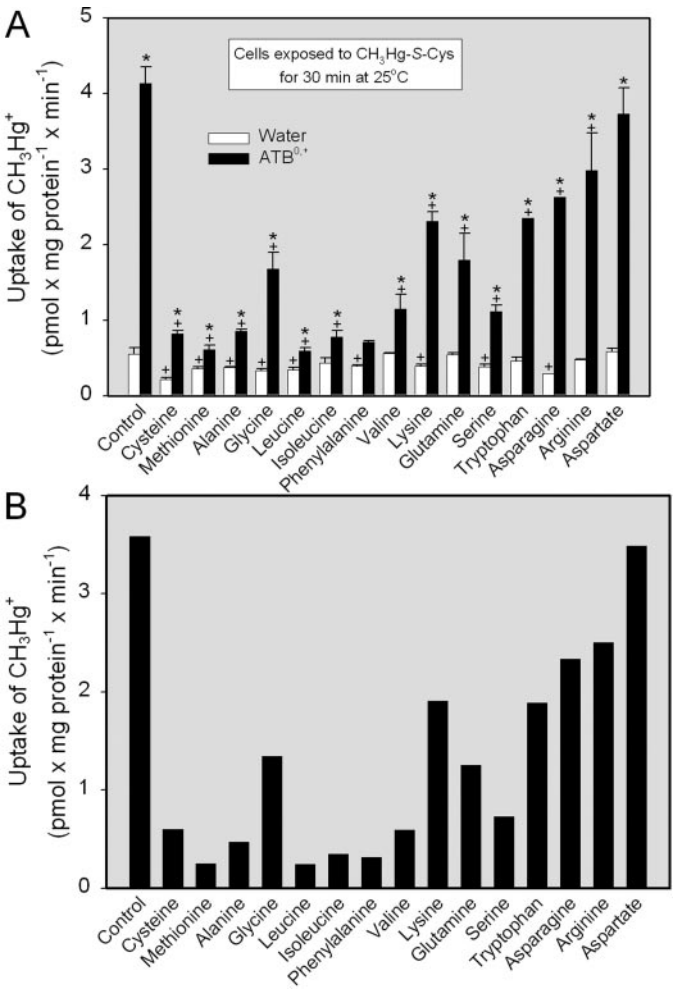

Fig. 9. Substrate specificity of the uptake of cysteine conjugates of methylmercury, CH_3Hg-*S*-Cys, in *X. laevis* oocytes injected with either water or mouse $ATB^{0,+}$ (A). Oocytes were incubated with 5 μM CH_3Hg-*S*-Cys in the presence of 3 mM unlabeled amino acids for 30 min at 25°C. The transport activity of the water-injected oocytes was subtracted from that of the $ATB^{0,+}$-injected oocytes to show the amount of CH_3Hg-*S*-Cys uptake specific to system $B^{0,+}$ (B). Results are presented as mean ± S.E. Data represent three experiments performed in triplicate. ∗, significantly different ($p < 0.05$) from the mean for the corresponding group of control oocytes. +, significantly different from the mean for the control group of the corresponding group of oocytes.

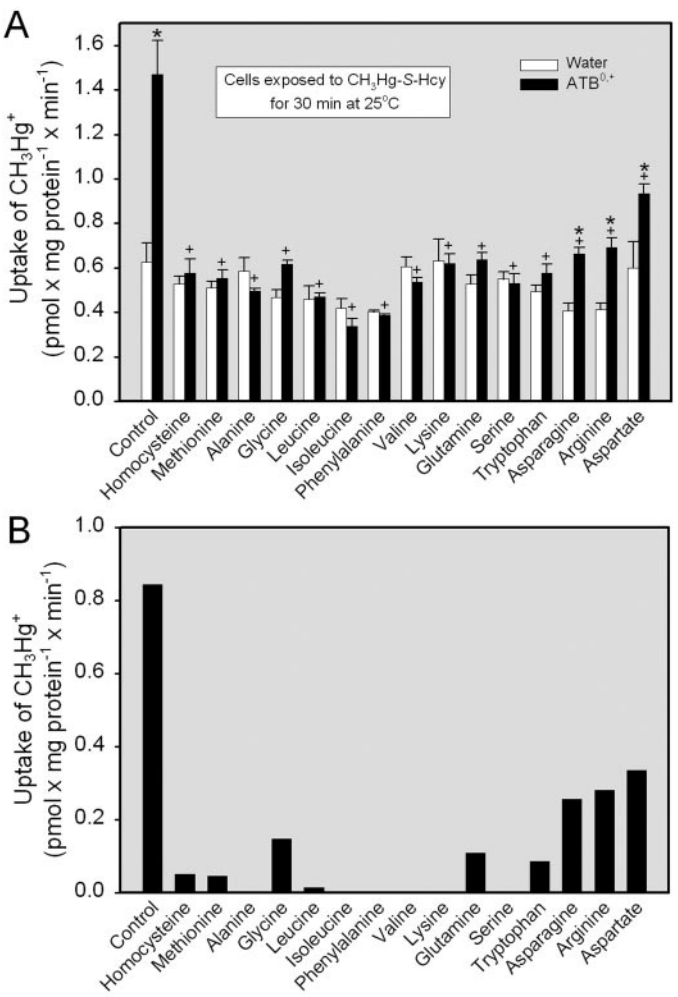

Fig. 10. Analysis of the substrate specificity of the uptake of homocysteine conjugates of methylmercury, CH_3Hg-*S*-Hcy, in *X. laevis* oocytes injected with either water or mouse $ATB^{0,+}$ (A). Oocytes were exposed to 5 μM CH_3Hg-*S*-Hcy in the presence of unlabeled amino acids for 30 min at 25°C. The transport activity of the water-injected oocytes was subtracted from that of the $ATB^{0,+}$-injected oocytes to show the amount of CH_3Hg-*S*-Hcy uptake specific to system $B^{0,+}$ (B). Results are presented as mean ± S.E. Data represent three experiments performed in triplicate. ∗, significantly different ($p < 0.05$) from the mean for the corresponding group of control oocytes. +, significantly different from the mean for the control group of the corresponding group of oocytes.

ishi et al., 2001; Hatanaka et al., 2004); thus, it is not surprising that it does not accept the negatively charged molecules, CH_3Hg-*S*-NAC and CH_3Hg-*S*-GSH, as substrates. Likewise, these mercuric conjugates were not transported by the neutral amino acid carrier, system L, when it was expressed in *Xenopus* oocytes (Simmons-Willis et al., 2002). In addition, GSH and NAC conjugates of Hg^{2+} were not identified as substrates of the Na^+-independent neutral and cationic amino acid transporter, system $b^{0,+}$ (Bridges and Zalups, 2004; Bridges et al., 2004). Furthermore, the transport of NAC *S*-conjugates of Hg^{2+} and CH_3Hg^+ appears to occur almost exclusively at the basolateral plasma membrane of the proximal tubule by means of one or more multispecific carrier proteins (Zalups, 1998; Zalups and Barfuss, 1998, 2002; Koh et al., 2002; Zalups and Ahmad, 2005). The localization of system $B^{0,+}$ on the luminal plasma membrane, therefore, eliminates this carrier as a potential mechanism for the transport of these conjugates.

The transport of CH_3Hg-*S*-Cys and CH_3Hg-*S*-Hcy via system $B^{0,+}$ was further characterized by multiple biochemical analyses. Time course analyses of the transport of these conjugates demonstrated that uptake of each conjugate increased over 60 min of study without reaching a plateau. It seems that the transport of CH_3Hg-*S*-Cys and CH_3Hg-*S*-Hcy may plateau only after a much longer time period, possibly because of the large intracellular volume of the oocyte. The transport of the two mercuric species was not measured for time periods longer than 60 min because of potential toxicity issues. A similar trend in transport was observed in oocytes expressing system L where the transport of CH_3Hg-*S*-Cys continued to increase after 180 min of uptake (Simmons-Willis et al., 2002).

Assessment of the saturation kinetics for the transport of CH_3Hg-*S*-Cys and CH_3Hg-*S*-Hcy also suggests that system $B^{0,+}$ is capable of transporting these forms of mercury. CH_3Hg-*S*-Cys and CH_3Hg-*S*-Hcy transport was higher in the

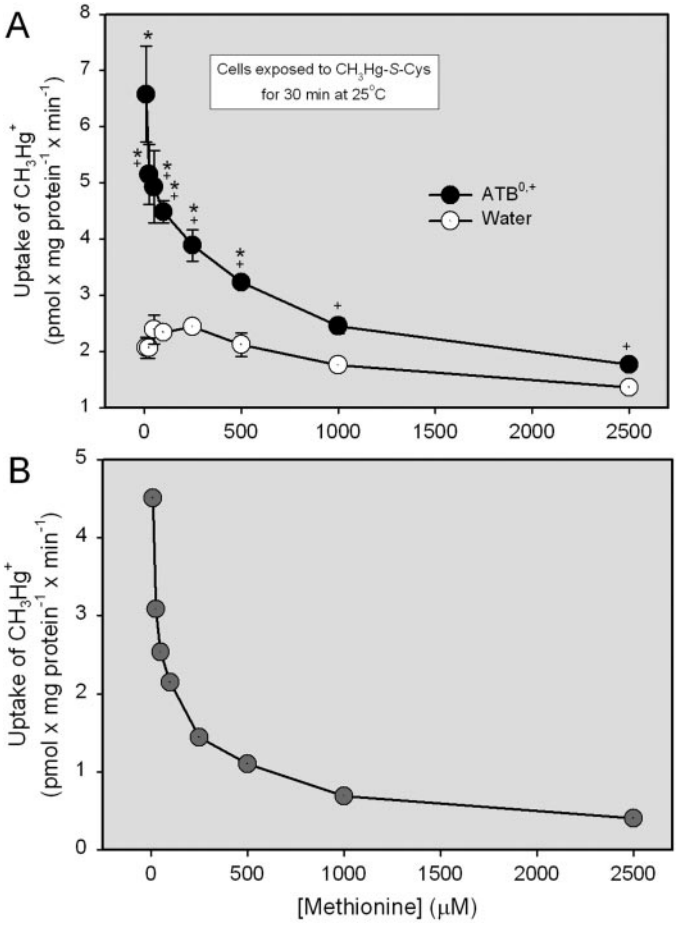

Fig. 11. Inhibition of the transport of CH_3Hg^+ as a conjugate of Cys (CH_3Hg-*S*-Cys) in *X. laevis* oocytes injected with water or mouse $ATB^{0,+}$ (A). Oocytes were incubated with 5 μM CH_3Hg-*S*-Cys in the presence of increasing concentrations of methionine for 30 min at 25°C. The transport activity of the water-injected oocytes was subtracted from that of the $ATB^{0,+}$-injected oocytes to show the amount of CH_3Hg-*S*-Cys uptake specific to system $B^{0,+}$ (B). Results are presented as mean ± S.E. Data represent two experiments performed in triplicate. *, significantly different ($p < 0.05$) from the mean for the corresponding group of control oocytes. +, significantly different ($p < 0.05$) from the mean for the same group of oocytes at the previous concentration(s).

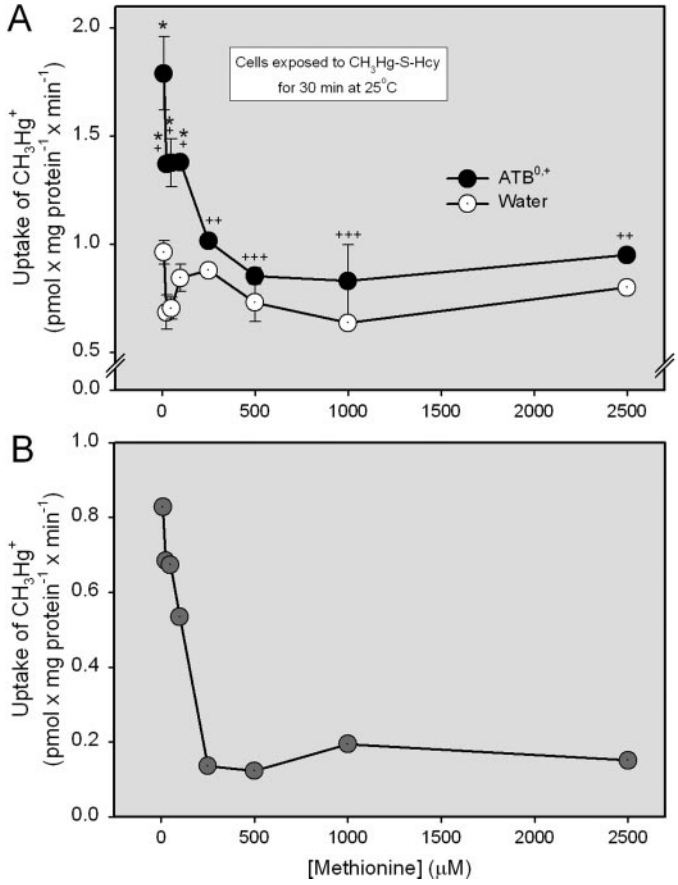

Fig. 12. Inhibition of the transport of CH_3Hg^+ as a conjugate of Hcy (CH_3Hg-*S*-Hcy) in *X. laevis* oocytes injected with water or mouse $ATB^{0,+}$ (A). Oocytes were incubated with 5 μM CH_3Hg-*S*-Hcy in the presence of increasing concentrations of methionine for 30 min at 25°C. The transport activity of the water-injected oocytes was subtracted from that of the $ATB^{0,+}$-injected oocytes to show the amount of CH_3Hg-*S*-Hcy uptake specific to system $B^{0,+}$ (B). Results are presented as mean ± S.E. Data represent three experiments performed in triplicate. *, significantly different ($p < 0.05$) from the mean for the corresponding group of control oocytes. +, ++, or +++, significantly different ($p < 0.05$) from the mean for the same group of oocytes at the previous concentration(s).

$ATB^{0,+}$-injected oocytes than in the controls; thus, we can conclude that system $B^{0,+}$ does indeed play a role in the transport of these conjugates. At the concentrations tested, we did not observe complete transporter saturation with either conjugate, possibly because the carriers responsible for this uptake have a low affinity for CH_3Hg-*S*-Cys and CH_3Hg-*S*-Hcy. It is likely that saturation may be reached at concentrations greater than those examined; however, they were not included in this study due to concerns related to toxicity. Regardless, these data provide strong support for the hypothesis that system $B^{0,+}$ is involved in the transport of CH_3Hg-*S*-Cys and CH_3Hg-*S*-Hcy in oocytes injected with $ATB^{0,+}$.

Substrate specificity experiments provide additional support for the notion that CH_3Hg-*S*-Cys and CH_3Hg-*S*-Hcy are potential transportable substrates of system $B^{0,+}$. The uptake of these mercuric species into oocytes injected with $ATB^{0,+}$ was inhibited by neutral and cationic amino acids. Interestingly, in the water-injected oocytes, some amino acids were able to significantly inhibit the uptake of CH_3Hg-*S*-Cys. This reduction in transport is most likely due to the inhibition of endogenous amino acid transporters. The presence of these transporters, however, does not alter the significance of the current findings. Our initial experiments indicate that the uptake of individual amino acids is much greater in $ATB^{0,+}$-injected oocytes than in water-injected controls. Therefore, the activity of system $B^{0,+}$ can be studied easily by comparing the transport of various substrates in both sets of oocytes.

In contrast to the uptake of CH_3Hg-*S*-Cys, the transport of CH_3Hg-*S*-Hcy in the water-injected oocytes was not affected by the presence of additional amino acids. Given this, we can postulate that CH_3Hg-*S*-Cys is a preferred substrate for one or more of the endogenous transporters. These results are similar to those obtained from studies on the transport of Cys and Hcy *S*-conjugates of Hg^{2+} in MDCK cells transfected stably with the Na^+-independent amino acid transporter, system $b^{0,+}$, wherein the transporter affinity for the Cys conjugate was greater than that of the Hcy conjugate (Bridges and Zalups, 2004; Bridges et al., 2004).

In the present study, the uptake of CH_3Hg-*S*-Cys by system $B^{0,+}$ was significantly greater than the uptake of CH_3Hg-*S*-Hcy in all of the experiments carried out. Likewise, in oocytes ex-

pressing system L, the uptake of CH_3Hg-*S*-Cys appeared to be greater than the uptake of CH_3Hg-*S*-Hcy (Simmons-Willis et al., 2002). These differences in transport may be due, in part, to the size of the molecule. CH_3Hg-*S*-Hcy possesses an additional methyl group not present on CH_3Hg-*S*-Cys. Therefore, the differences in transport may be due partially to the differences in size between the two mercuric species.

Transport of CH_3Hg-*S*-Cys and CH_3Hg-*S*-Hcy was also measured in the presence of increasing concentrations of methionine. In addition to being a high-affinity substrate of system $B^{0,+}$, methionine is chemically and structurally similar to CH_3Hg-*S*-Cys and CH_3Hg-*S*-Hcy. Our data demonstrate that in oocytes expressing system $B^{0,+}$, methionine is able to inhibit the uptake of Cys and Hcy *S*-conjugates of CH_3Hg^+ in a concentration-dependent manner. These data indicate that CH_3Hg-*S*-Cys, CH_3Hg-*S*-Hcy, and methionine share a common transport mechanism, i.e., system $B^{0,+}$. It is important to note that the implications for this finding may spread beyond the scope of system $B^{0,+}$. Because methionine is a neutral amino acid, it may be transported by a number of amino acid carriers, including systems L, $b^{0,+}$, B^0, y^+L, and A (Ganapathy et al., 2004). These transporters are found in numerous organ systems and may serve as mechanisms for the entry of CH_3Hg-*S*-Cys and CH_3Hg-*S*-Hcy. It is possible that the distribution of these transporters in the various organs contribute to the detrimental effects observed following exposure to CH_3Hg^+.

In conclusion, the identification of system $B^{0,+}$ as a mechanism for the transport of CH_3Hg-*S*-Cys and CH_3Hg-*S*-Hcy is an important step in understanding the way in which CH_3Hg^+ is handled by the human body. More specifically, because of its location in the luminal plasma membrane of proximal tubular epithelial cells, system $B^{0,+}$ may play a role in the uptake of thiol conjugates of CH_3Hg^+ from the tubular fluid. To our knowledge, this study represents the first time that a Na^+-dependent amino acid transporter has been implicated in the transport of any form of mercury.

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