Transport of the Glutathione-Methylmercury Complex across Liver Canalicular Membranes on Reduced Glutathione Carriers*

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Methylmercury transport across liver canalicular membranes into bile, a major route of excretion of this toxic compound, is dependent upon intracellular GSH, and a glutathione-methylmercury complex (CH₃Hg-SG) has been detected in liver tissue and bile. To examine whether the CH₃Hg-SG complex is itself transported across the canalicular membrane and to identify the transport system involved, studies were performed in isolated rat liver canalicular plasma membrane vesicles. Uptake of CH₃Hg-SG (10 μM) into an osmotically active space was temperature-sensitive and unaffected by either ATP or an inward-directed Na⁺ gradient (100 mM); however, CH₃Hg-SG uptake was enhanced by a valinomycin-induced K⁺ diffusion potential (inside-positive) indicating that its transport was electrogenic. Transport of CH₃Hg-SG exhibited saturation kinetics with both high affinity (Kₘ = 12 ± 2 μM, Vₘₐₓ = 0.23 ± 0.02 nmol·mg⁻¹·s⁻¹) and low affinity (Kₘ = 1.47 ± 0.22 mM, Vₘₐₓ = 1.23 ± 0.14 nmol·mg⁻¹·s⁻¹) components. Uptake of this complex was inhibited by, the GSH analog ophthalmic acid, S-methyl, S-ethyl, S-butyl, S-hexyl, S-octyl, and S-dinitrophenyl glutathione, but not by GSSG, bile acids, amino acids, and P-glycoprotein inhibitors. Furthermore, GSH competitively inhibited (Kᵢ = 83 μM) and trans-stimulated CH₃Hg-SG uptake into the canalicular vesicles. These studies provide the first kinetic characterization of a transport system for glutathione-mercaptides and indicate that CH₃Hg-SG is not a substrate for the ATP-dependent, canalicular GSSG or glutathione S-conjugate carriers, but appears to be a substrate for canalicular carriers that also transport GSH. Because efflux systems for GSH are found in all mammalian cells, transport of glutathione-metal complexes by such carriers may be a common mechanism for the removal of methylmercury and possibly other metals from cells.

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Biliary excretion is a major pathway for the elimination of metals, including methylmercury; however, the mechanisms by which metals traverse the canalicular plasma membrane have not been identified (Norseth and Clarkson, 1971; Ballatori and Clarkson, 1985b; Ballatori, 1991). For methylmercury, canalicular transport appears to be dependent on the availability of intracellular GSH. Methylmercury has a high affinity for reduced sulfhydryl groups and will readily bind to the sulfhydryl group of GSH forming a coordinate covalent bond (Simpson, 1961). The CH₃Hg-SG complex has been detected in a variety of biologic fluids and tissues, including the liver (Omata et al., 1978) and bile (Refvik and Norseth, 1975).

Under a variety of experimental conditions, a close correlation is observed between the rates of GSH and methylmercury excretion into bile, providing evidence for methylmercury transport into bile as a GSH complex. Refvik (1978) showed that depletion of hepatic GSH levels with diethylmaleate, cyclohexene oxide, or acrylamide produces a dramatic decrease in the excretion of GSH and methylmercury into bile, suggesting a close coupling between the biliary excretion of these two substances. As intracellular GSH concentrations are lowered, methylmercury redistributes to other competing ligands, such that the intracellular concentration of CH₃Hg-SG remains roughly proportional to that of GSH. Conversely, studies by Magos et al. (1978) demonstrate that increasing hepatic GSH (and therefore CH₃Hg-SG) levels leads to an increase in the biliary excretion of both GSH and methylmercury. Sex and individual differences in the biliary excretion of methylmercury are also correlated with differing rates of GSH excretion (Ballatori and Clarkson, 1983). Furthermore, changes in the rates of GSH excretion into bile induced by the exogenous agents sulfobromophthalein, phenol-3,6-dibromophthalein disulfonate, indocyanine green, and phenobarbital are followed by parallel changes in methylmercury excretion (Ballatori and Clarkson, 1983, 1985a). Additional evidence that methylmercury is transported into bile as a GSH complex comes from studies in neonatal rats (Ballatori and Clarkson, 1982). Neonatal rats are inefficient at excreting GSH or methylmercury into bile, even though their hepatic GSH and methylmercury levels are comparable to those of adults. Development of the ability to excrete GSH into bile parallels their ability to excrete methylmercury.

Although these studies provide valuable information into the overall process by which methylmercury enters bile, more definitive experiments are needed to critically assess the transport mechanism by which methylmercury traverses the canalicular membrane. Canalicular transport systems for GSSG and glutathione S-conjugates (Akerboom et al., 1984, 1991; Inoue et al., 1984; Kobayashi, et al., 1990), as well as for GSH itself (Inoue et al., 1983; Fernandez-Checa et al., 1992, 1993; Garcia-Ruiz et al., 1992) have been described, and these systems may be involved in biliary transport of CH₃Hg-SG. To test this hypothesis, the present study utilized membrane vesicles derived from the canalicular domain of rat hepatocytes to characterize the transport of CH₃Hg-SG. The findings indicate that...
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FIG. 1. Time course and temperature dependence of CH$_2$Hg-SG uptake in canalicular liver plasma membrane (cLPM) vesicles. Effects of adding a chelating agent (DMPS) to the stop solution. Canalicular membrane vesicles containing 250 mM sucrose, 10 mM Hepes/Tris, pH 7.5, and 0.25 mM acivicin were incubated in media containing 10 μM CH$_2$Hg-SG, 150 mM sucrose, 10 mM Hepes/Tris, pH 7.5, 100 mM NaCl, and 90 μM GSH. The stop solution contained 150 mM sucrose, 125 mM tetramethylammonium chloride, 10 mM Hepes/Tris, pH 7.5, 0.2 mM CaCl$_2$, and DMPS at concentrations of zero (A), 250 (B), 500 (C), or 1000 μM (D). Uptake values at 60 min (25°C) for 0, 250, 500, and 1000 μM DMPS were 1235 ± 99, 614 ± 50, 554 ± 20, and 456 ± 17 pmol/mg of protein, respectively. Data are means ± S.E. of three experiments, each performed in triplicate.

CH$_2$Hg-SG transport into bile is carrier-mediated and occurs via GSH transport systems.

**EXPERIMENTAL PROCEDURES**

**Preparation of Rat Canaliculal Liver Plasma Membrane (cLPM) Vesicles**—Male Sprague-Dawley rats (~250 g) were obtained from Charles River Laboratories and fed Purina rodent chow (formula 5001) ad libitum. The methods for isolating cLPM subfractions are as described by Meier et al. (1984a) and previously performed in this laboratory (Ballatori al., 1986, Simmons et al. 1992). In brief, a mixed liver plasma membrane subfraction was first separated out of a "crude nuclear pellet" by rate zonal flotation (44-36.5, w/w, sucrose density interface) using a T-28 (Sorvall) zonal rotor. After high homogenization (type B Dounce homogenizer), 75 up and down strokes, the vesiculated cLPM subfraction was separated by high speed centrifugation (195,200 × g, for 3 h) of the mixed LPM through a three-step sucrose gradient (31, 34, and 38%, w/w). The membranes were collected at 105,000 × g, for 60 min and, except where stated otherwise, were suspended in 250 mM sucrose and 10 mM Hepes/Tris, pH 7.5 (suspension solutions also contained 20 mM KCl under voltage-clamped conditions) and stored at −70°C.

**Characteristics of the cLPM Vesicles**—The degree of purification of the cLPM has been extensively analyzed previously by intracellular and plasma membrane marker enzyme activities (Meier et al., 1984a). These studies indicate minor contamination of the cLPM subfraction with intracellular organelles and virtually complete separation of cLPM from basolateral membranes as reflected by minimal NaK-ATPase activity (Meier et al., 1984a). In the present study, the purity of each membrane preparation was routinely analyzed by measurement of NaK-ATPase and Mg2+ATPase activities (Scharschmidt et al., 1979) and γ-glutamyltransferase activity (Orlowski and Meister, 1963). Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard. Intravesicular volumes are approximately 2 μl/mg of protein as calculated from equilibrium uptakes of either L-alanine, L-glutamate, or D-glucose; substrates that do not bind to any appreciable extent to the isolated membrane vesicles (Ballatori et al., 1986; Meier et al., 1984b).

**Transport Measurements**—Frozen membrane suspensions were quickly thawed by immersion in a 37°C water bath, diluted to the desired protein concentration, and passed repeatedly (10x) through a 25-gauge needle. Acivicin (250 μM), an irreversible inhibitor of γ-glutamyltransferase, was added to all vesicle preparations to inhibit the catabolism of CH$_2$Hg-SG and GSH. To prevent the membrane potential from influencing the transport of substrates, the appropriate experiments were voltage-clamped using the potassium ionophore valinomycin (10 μg/mg protein) in the presence of 20 mM KCl in the incubation and vesicle suspension media. In all experiments, an excess of GSH was used to ensure that methylmercury was present as the GSH complex. Specific details regarding the GSH to methylmercury ratio are given in the legends.

Uptake of radiolabeled substrates into cLPM vesicles was measured by a rapid Millipore filtration technique (Ballatori et al., 1986; Meier et al., 1984b). Membrane suspensions (50-120 μg of protein in 20 μl) were preincubated at 25 or 37°C for 15 min. Uptake studies were initiated by the addition of 80 μl of incubation medium, also prewarmed, containing the desired protein concentration and the appropriate incubation media. The membranes were collected at 105,000 × g, for 60 min (25°C) for 0, 250, 500, and 1000 μM DMPS. DMPS, a water-soluble compound containing two sulphydryl groups, was used to remove methylmercury nonspecifically bound to the outside of the vesicles. The stop solution used for voltage-clamped experiments contained 250 mM sucrose, 10 mM Hepes/Tris, pH 7.5, 0.2 mM CaCl$_2$, and 500 μM DMPS. Membrane vesicle-associated ligand was separated from free ligand by rapid filtration through a 0.45-μm filter (Millipore, HAWP). Vesicles were then washed with an additional 4 ml of ice-cold stop solution. Filters containing 3H were dissolved in 4.5 ml of Ecoscint A (National Diagnostics, Mannville, NJ) and counted in a Packard Tri-Carb scintillation counter (model 4530), whereas 35S-labeled compounds were assayed with a Packard Auto Gamma scintillation counter (model 5650).

**Chemicals**—Methylmercury chloride was obtained from ICN. 203HgCl$_2$ and [3H]taurocholate (2.1 Ci/mmol) were purchased from Du-Pont NEN. CH$_2$HgCl$_2$ was synthesized from 203HgCl$_2$, by the method of Toribara (1985). This method yields a product of high specific activity, with less than 0.5% inorganic impurity. The dinitrophenyl conjugates of glutathione and N-acetylcysteine were synthesized as previously described (Hinchman et al., 1991). The R-(+)-verapamil enantiomer was purchased from RBI (Natick, MA). Other chemicals and reagents were purchased from either Sigma, Bachem Bioscience Inc., or Aldrich.

**RESULTS**

**Time Course and Temperature Dependence of CH$_2$Hg-SG Uptake into cLPM Vesicles**—Although the CH$_2$Hg-SG complex is thermodynamically stable with an association constant of 1.28 × 10$^{14}$ M$^{-1}$/s$^{-1}$ (Simpson, 1961), this complex is kinetically labile, i.e. methylmercury can shuttle between competing sulphydryl groups, including those found on membrane vesicle proteins. To reduce this nonspecific binding, more clearly assess the specific transport of CH$_2$Hg-SG into the vesicles, DMPS was included in the stop solution (Fig. 1). DMPS is a hydrophilic chelating agent (molecular weight = 188) containing two sulphydryl groups which can compete for methylmercury bound to the outside of the vesicles.

Fig. 1A shows that CH$_2$Hg-SG (10 μM) uptake by cLPM vesicles increased as a function of time and was greatly diminished at 4°C. Note that in the absence of the chelator (Fig. 1B),
there was significant binding of $[^{203}\text{H}]$methylmercury to the vesicles, with an apparent uptake of nearly 600 pmol/mg of protein after 5 min of incubation at 4 °C (Fig. 1B), whereas in the presence of DMPS, this value dropped to about 50 pmol/mg of protein (Fig. 1A, filled symbols). Because the vesicle-associated $[^{203}\text{H}]$methylmercury at 4 °C is presumed to be due to nonspecific binding, DMPS was effective at diminishing this binding (note the different scales on the ordinate of Fig. 1. A and B), although it did not completely eliminate nonspecific binding (see below). All DMPS concentrations tested (250, 500, and 1000 μM) were effective at reducing binding (Fig. 1A). A DMPS concentration of 500 μM was added to the stop solution of all subsequent experiments.

Effect of Medium Osmolarity on CH$_3$Hg-SG Uptake in cLPM Vesicles—To further demonstrate that vesicle-associated $[^{203}\text{H}]$methylmercury was due to transport into an intravesicular space as opposed to nonspecific binding to the vesicles, the external medium osmolarity was increased by adding increasing concentrations of sucrose (0.25–0.85 M), resulting in a proportional decrease in the intravesicular volume. As illustrated in Fig. 2, as the osmolarity of the external medium increased, vesicle-associated $[^{203}\text{H}]$methylmercury decreased, indicating that CH$_3$Hg-SG was being transported into an osmotically active intravesicular space. Extrapolation of these data to an infinitely high medium osmolarity, or a negligible intravesicular volume, revealed that approximately 50% of the $[^{203}\text{H}]$methylmercury associated with the vesicles was due to membrane binding, even with DMPS in the stop solution (Fig. 2). This degree of binding is comparable to that seen with taurocholate (Meier et al., 1984b), but higher than with amino acids, such as alanine or glutamate (Ballatori et al., 1986; Meier et al., 1984); Simmons et al., 1992). To account for this nonspecific binding in subsequent experiments, CH$_3$Hg-SG uptake was routinely measured at 4 °C, and this value was subtracted from all measurements.

Time Course for CH$_3$Hg-SG Uptake in the Presence of Various Inwardly Directed Cation Gradients—The uptake of CH$_3$Hg-SG (10 μM) into cLPM vesicles was measured in the presence of inwardly directed 100 mM gradients of NaCl, KCl, and LiCl (Fig. 3). Replacing NaCl with either KCl or LiCl had no effect on CH$_3$Hg-SG uptake, indicating that CH$_3$Hg-SG transport is not dependent on a Na$^+$ gradient. CH$_3$Hg-SG uptake was also assessed at shorter time intervals (Fig. 3, inset) in order to select a time point that reflected initial rates of uptake. Because CH$_3$Hg-SG uptake was linear for up to 30 s, uptake for subsequent experiments was measured at 20 s.

Effect of ATP on CH$_3$Hg-SG and Taurocholate Uptake in cLPM Vesicles—The transport of CH$_3$Hg-SG (10 μM) into cLPM vesicles was studied by adding 100 mM potassium gluconate to the external medium in the presence and absence of valinomycin (Table I). These conditions allow potassium to rapidly diffuse into valinomycin-treated vesicles generating a transient inside-positive potential. As shown in Table I, addition of valinomycin significantly stimulated the uptake of CH$_3$Hg-SG. Under identical experimental conditions, uptake of [H]GSH was also enhanced by valinomycin, suggesting that the membrane potential could provide the driving force for the transport of these compounds across the canalicular membrane into bile.

Concentration Dependence of Initial Rates of CH$_3$Hg-SG Uptake in cLPM Vesicles—Initial rates (20 s) of CH$_3$Hg-SG uptake into cLPM vesicles were examined as a function of increasing substrate concentration (5 μM–5 mM). Fig. 5A presents the uptake of CH$_3$Hg-SG at concentrations of 5–300 μM, whereas Fig. 5B shows uptakes at higher concentrations. Net uptake of CH$_3$Hg-SG was obtained by subtracting uptake values at 4 °C from those at 25 °C (dashed lines). CH$_3$Hg-SG uptake followed Michaelis-Menten kinetics providing evidence for a carrier-mediated transport system. An Eadie-Hofstee plot of all the data (Fig. 5, inset) revealed the presence of at least two saturable components: a high affinity system with an apparent $K_m$ of 12

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2 N. Ballatori and W. J. Dutczak, unpublished observations.
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### FIG. 4. Effect of ATP on CH$_3$Hg-SG and taurocholate uptake in cLPM vesicles.**

Canalicular membrane vesicles were resuspended in a solution containing 190 mM sucrose, 100 mM KCl, 10 mM Hepes/Tris, pH 7.5, and 0.25 mM acivicin. Vesicles were pretreated with valinomycin (10 g/mg protein), and uptake of 10 mM CH$_3$Hg-SG and 1 mM (H)TAUROCHOLATE was measured at 37 °C in an incubation solution containing 190 mM sucrose, 100 mM KCl, 10 mM Hepes/Tris, pH 7.5, 10 mM MgCl$_2$, and with and without 5 mM ATP. The stop solution contained 10 mM Hepes/Tris, pH 7.5, 0.2 mM CaCl$_2$, 10 mM MgCl$_2$, 100 mM KCl, and 500 g DMPS. Data are means ± S.E. of three experiments, performed in triplicate. *a* significantly different from control (p < 0.05) as analyzed by Student’s t test.

### FIG. 5. Concentration dependence of initial rates of CH$_3$Hg-SG uptake in cLPM vesicles.**

Vesicles containing 250 mM sucrose, 10 mM Hepes/Tris, pH 7.5, 20 mM KCl, and 0.25 mM acivicin were incubated at either 25 °C or 4 °C in the same media supplemented with CH$_3$Hg-SG (5 µM to 5 mM) along with a 20% molar excess of GSH, and uptake was measured after 20 s. Vesicles were pretreated with valinomycin (10 g/mg protein). DMPS, 500 g, was added to the stop solution. Values are means ± S.E. of three experiments, each performed in triplicate.

### TABLE I: Substrate specificity of CH$_3$Hg-SG uptake in cLPM vesicles

Canalicular membrane vesicles containing 250 mM sucrose, 10 mM Hepes/Tris, pH 7.5, 20 mM KCl, and 0.25 mM acivicin were incubated at 25 °C and 4 °C in the same media containing 10 µM CH$_3$Hg-SG and 40 µM GSH. Vesicles were pretreated with valinomycin (10 g/mg protein), and transport was measured at 20 s. Values were calculated by subtracting 4 °C uptake from uptake at 25 °C. DMPS, 500 g, was added to the stop solution. The concentration of inhibitors is given below. Uptake in the absence of added inhibitor was assigned a relative value of 100%. BCH, 2-amino-bicyclo[2.2.1]heptane-2-carboxylic acid; DNP-SG, S-dinitrophenyl glutathione. Data are means ± S.E. of three to eight experiments, each performed in triplicate.

### TABLE II: Substrate specificity of CH$_3$Hg-SG uptake in cLPM vesicles

Canalicular membrane vesicles containing 250 mM sucrose, 10 mM Hepes/Tris, pH 7.5, 20 mM KCl, and 0.25 mM acivicin were incubated at 25 °C and 4 °C in the same media containing 10 µM CH$_3$Hg-SG and 40 µM GSH. Vesicles were pretreated with valinomycin (10 g/mg protein), and transport was measured at 20 s. Values were calculated by subtracting 4 °C uptake from uptake at 25 °C. DMPS, 500 g, was added to the stop solution. The concentration of inhibitors is given below. Uptake in the absence of added inhibitor was assigned a relative value of 100%. BCH, 2-amino-bicyclo[2.2.1]heptane-2-carboxylic acid; DNP-SG, S-dinitrophenyl glutathione. Data are means ± S.E. of three to eight experiments, each performed in triplicate.

### CH$_3$Hg-SG and previously characterized transport systems located on the canalicular membrane, we tested the inhibitory effects of substrates for some of these transporters on the high affinity component of CH$_3$Hg-SG uptake in cLPM vesicles. Table II shows that uptake of 10 µM CH$_3$Hg-SG was strongly inhibited by GSH. The low affinity component of CH$_3$Hg-SG uptake was also inhibited by GSH (data not shown). In addition, a number of glutathione conjugates and the GSH analog ophthalmic acid also decreased CH$_3$Hg-SG uptake (Table II). In contrast, GSSG, bile acids, amino acids, and P-glycoprotein inhibitors had no effect.

**Kinetics of GSH Inhibition of CH$_3$Hg-SG Uptake in cLPM Vesicles**—Because GSH strongly inhibited CH$_3$Hg-SG uptake into cLPM vesicles (Table II), the kinetics of this inhibition were examined. CH$_3$Hg-SG uptake (5–100 µM) was assessed in the absence and presence of 500 µM GSH. Fig. 6 is an Eadie-Hofstee plot of the data. As illustrated in this figure, the inhibition of CH$_3$Hg-SG uptake by GSH (K = 83 µM) led to an increase in the apparent K for CH$_3$Hg-SG transport. These results are consistent with a competitive type of inhibition, providing further evidence that CH$_3$Hg-SG and GSH share common carriers.

**Trans-Stimulation of CH$_3$Hg-SG Uptake in cLPM Vesicles**—Trans-stimulation experiments (Table III) provided additional evidence that CH$_3$Hg-SG and GSH are transported by common transport systems. When vesicles were preloaded with 1 mM CH$_3$Hg-SG or GSH, a significantly greater uptake of
bound to GSH (Omata et al. 1978). Methylmercury is then transported across the canalicular membrane into bile by a process that is dependent on intracellular GSH (Ballatori and Clarkson, 1985b); however, the transport mechanism involved has also not been identified. Because biliary excretion is a major route for the elimination of methylmercury, transport of this metal across the canalicular membrane into bile may be the limiting step in its detoxification.

In the present study, purified rat liver canalicular plasma membrane vesicles were used to identify and characterize a carrier-mediated transport system for the GSH complex of methylmercury (CH₃Hg-SG) that may be responsible for biliary excretion of this toxin. The data demonstrate that CH₃Hg-SG is not a substrate for the ATP-dependent canalicular GSSG or glutathione S-conjugate carriers, but appears to be transported into bile by carriers that also transport GSH. Transport of CH₃Hg-SG was independent of Na⁺ and ATP, but was electrogenic, indicating that the membrane potential could provide some of the force for CH₃Hg-SG excretion into bile. Furthermore, GSH competitively inhibited and trans-stimulated uptake of this organometal complex, providing strong evidence for shared transport mechanisms.

Carrier-mediated transport systems functioning to translocate a variety of substrates across the canalicular membrane into bile have been identified and partially characterized, including carriers for GSH, GSSG, glutathione conjugates, organic anions, and bile acids. Transport of GSH in rat cLPM vesicles is reported to be electrogenic (Inoue et al., 1983) and independent of either a Na⁺ gradient (Inoue et al., 1983) or ATP (Fernandez-Cchea et al., 1992). Although both Fernandez-Cchea et al. (1992) and Inoue et al. (1983) found GSH transport to consist of a single saturable component, there are discrepancies in the reported values for the kinetic parameters. Inoue et al. (1983) have identified a high affinity transport system for GSH with an apparent $K_m$ of 0.33 mM and a $V_{max}$ of 1.47 nmol·mg of protein$^{-1}$·20 s$^{-1}$. Fernandez-Cchea et al. (1992) only detected a low affinity component with an apparent $K_m$ of 16 mM and a $V_{max}$ of 6.7 nmol·mg of protein$^{-1}$·15 s$^{-1}$. In contrast to these reports, recent studies of the transport properties of GSH in rat cLPM vesicles indicate both high (0.24 mM) and low ($K_m = 17$ mM) affinity components for GSH transport in rat cLPM vesicles. The high affinity component of GSH transport is inhibited by glutathione S-conjugates and other γ-glutamyl compounds, indicating that it might function to transport these compounds into bile.

An ATP-dependent transport system for organic anions including some glutathione S-conjugates, sometimes referred to as the multispecific organic anion transporter, has also been detected within the canalicular membrane of rat hepatocytes (Kobayashi et al., 1990; Akerboom et al., 1991). This transport system has been studied using S-dinitrophenyl glutathione (DNP-SG) as a model substrate. ATP-dependent DNP-SG uptake into rat cLPM vesicles is saturable and consists of a single component. The reported $K_m$ values for DNP-SG transport range from 4 μM (Kobayashi et al., 1990) to 71 μM (Akerboom et al., 1991). This transport system has a relatively broad substrate specificity, with DNP-SG uptake being inhibited by structurally diverse compounds such as GSSG (Kobayashi et al., 1990), taurocholate (Akerboom et al., 1991), and cysteinyl leukotrienes (Ishikawa et al., 1990), but not by GSH (Akerboom et al., 1991; Kobayashi et al., 1990) or the low molecular weight glutathione S-conjugate, S-methyl glutathione (Kobayashi et al., 1990). Interestingly, studies by Kobayashi et al. (1990) suggest that as the carbon skeleton of an S-substituted glutathione adduct increases, the conjugate becomes a better substrate for the ATP-dependent glutathione conjugate transporter, with S-buty1, S-pentyl, and S-nonyl glutathione inhibiting DNP-SG

![Graph](https://via.placeholder.com/150)

**Table III**

<table>
<thead>
<tr>
<th>Test compound</th>
<th>$n$</th>
<th>CH₃Hg-SG uptake</th>
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<tbody>
<tr>
<td></td>
<td></td>
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</tr>
<tr>
<td>$\text{CH}_3\text{Hg-SG}$</td>
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</tr>
<tr>
<td>GSH</td>
<td>4</td>
<td>127.8 ± 7.2$^a$</td>
</tr>
<tr>
<td>$S$-Methyl glutathione</td>
<td>3</td>
<td>114.0 ± 8.1</td>
</tr>
<tr>
<td>$S$-Ethyl glutathione</td>
<td>3</td>
<td>108.0 ± 6.7</td>
</tr>
<tr>
<td>DNP-SG</td>
<td>4</td>
<td>97.8 ± 2.6</td>
</tr>
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$^a$ Significantly different from control ($p < 0.05$), using paired t test.

$\text{CH}_3\text{Hg-SG}$ was observed when vesicles were not preloaded. $S$-Methyl and $S$-ethyl glutathione increased uptake by 14 and 8%, respectively; however, these differences were not statistically significant (Table III). An acceleration in $\text{CH}_3\text{Hg-SG}$ uptake was not observed when vesicles were preloaded with DNP-SG.

**DISCUSSION**

Methylmercury is a ubiquitous environmental contaminant capable of producing severe neurologic dysfunction (Inskeep and Piotrowski, 1985). Ingestion is the main route of exposure, and greater than 90% of this organometal is absorbed and delivered to the liver via the portal circulation (Aberg et al., 1969). Neither the mechanism by which methylmercury traverses the sinusoidal membrane nor the process by which this metal is translocated across the hepatocyte to the canalicular membrane has yet been identified. However, it is well established that within the cells a large fraction of the methylmercury is loaded with DNP-SG.
uptake by 16, 28, and 81%, respectively.

The present findings demonstrate that CH$_2$Hg-SG is not a substrate for the ATP-dependent canicular organic anion transport systems, but that it shares common transport mechanisms with GSH. As previously reported for GSH transport$^2$ (Inoue et al., 1983; Fernandez-Checa et al., 1992, 1993), CH$_2$Hg-SG transport into cLPM vesicles was not dependent on a Na$^+$ gradient (Fig. 3) or ATP (Fig. 4) and was not affected by GSSG (Table II). CH$_2$Hg-SG uptake into cLPM vesicles was competitively inhibited by GSH (Fig. 6). In addition, transport of both CH$_2$Hg-SG and GSH is stimulated by a valinomycin-induced K$^+$ diffusion potential, indicating that transport of both solutes in cLPM vesicles is electrogenic, and that the membrane potential could supply the driving force for CH$_2$Hg-SG and GSH transport across the canalicular membrane into bile.

Inhibition studies demonstrate that CH$_2$Hg-SG transport is not affected by bile acids, amino acids, or P-glycoprotein substrates, indicating that these systems are not involved in CH$_2$Hg-SG transport (Table II). Inhibition studies also indicate that the chemical structure of the GSH adduct is a factor in defining the substrate specificity of the carrier, with S-methyl, S-ethyl, S-butyl, S-hexyl, S-ctyyl, and S-dinitrophenyl glutathione inhibiting CH$_2$Hg-SG transport, whereas the homoconjugate GSSG had no effect (Table II). Trans-stimulation experiments revealed that preloading vesicles with either CH$_2$Hg-SG or GSH accelerated CH$_2$Hg-SG uptake into vesicles (Table III). Not only do these results argue for transport of GSH and CH$_2$Hg-SG by common carriers, they also indicate that the carriers function bidirectionally.

CH$_2$Hg-SG transport was saturable, with at least two kinetic components: a high affinity component with an apparent $K_m$ of 12 ± 2 μM and a $V_{max}$ = 0.23 ± 0.02 nmol·mg$^{-1}$·20 s$^{-1}$ as well as a low affinity component with a $K_m$ of 1.47 ± 0.22 μM and a $V_{max}$ of 1.23 ± 0.14 nmol·mg$^{-1}$·20 s$^{-1}$. As indicated earlier, studies from this laboratory have detected both high and low affinity transport systems for GSH in rat cLPM vesicles.$^2$ Because the concentration of CH$_2$Hg-SG within the hepatocyte is in the micromolar range and the $K_m$ for the high affinity component of CH$_2$Hg-SG transport is 12 μM, this component is probably of physiologic relevance and may function to transport CH$_2$Hg-SG and perhaps other metal complexes into bile. Furthermore, because the high affinity components for CH$_2$Hg-SG (Table II) and GSH$^2$ transport are inhibited by various glutathione S-conjugates, this high affinity system may also function to transport these compounds into bile by an ATP-independent, electrogenic mechanism. In contrast, the low affinity GSH system may function as a high capacity transporter capable of pumping large amounts of GSH across the canalicul membrane into bile.

In summary, the results indicate that CH$_2$Hg-SG is transported across the canalicul membrane into bile by carriers that also transport GSH. These transport systems may function to efficiently remove methylmercury from the liver. Because GSH transporters are present in all mammalian cells, they may provide a common mechanism for the removal of univalent metals that form 1:1 GSH complexes. In contrast, divalent metals such as inorganic mercury, zinc, copper, lead, and cadmium are believed to be secreted into bile as multivalent GSH complexes, e.g. GS-Hg-SG (Ballatori 1991), thus it is more likely that these complexes are transported on carriers for GSSG or glutathione S-conjugates. Additional studies are needed to resolve these questions.

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REFERENCES


