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# Mercapturic Acids (*N*-Acetylcysteine *S*-Conjugates) as Endogenous Substrates for the Renal Organic Anion Transporter-1

JAMES M. POMBRIO, ADAM GIANGRECO, LIQIONG LI, MICHAEL F. WEMPE, M. W. ANDERS, DOUGLAS H. SWEET, JOHN B. PRITCHARD, and NAZZARENO BALLATORI

*Departments of Environmental Medicine (J.M.P., A.G., L.L., N.B.) and Pharmacology and Physiology (M.F.W., M.W.A.), University of Rochester School of Medicine and Dentistry, Rochester, New York; and Laboratory for Pharmacology and Chemistry, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina (D.H.S., J.B.P.)*

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## ABSTRACT

Mercapturic acids are *N*-acetyl-L-cysteine *S*-conjugates that are formed from a range of endogenous and exogenous chemicals. Although the kidney is a major site for elimination of mercapturic acids, the transport mechanisms involved have not been identified. The present study examined whether mercapturic acids are substrates for the renal basolateral organic anion transporter-1 (Oat1) from rat kidney. This carrier mediates uptake of organic anions from the bloodstream in exchange for intracellular $\alpha$-ketoglutarate. Uptake of [$^3$H]*p*-aminohippuric acid (PAH) in Oat1-expressing *Xenopus laevis* oocytes was strongly inhibited by *S*-(2,4-dinitrophenyl)-*N*-acetyl-L-cysteine (DNP-NAC) and by all other mercapturic acids tested, including the endogenous mercapturic acid *N*-acetyl-leukotriene E$_4$. Inhibition by the mercapturic acids was competitive, which is consistent with the hypothesis that these compounds are substrates for Oat1. This conclusion was supported by the direct demonstration of saturable [$^{35}$S]DNP-NAC uptake in Oat1-expressing oocytes. [$^{35}$S]DNP-NAC uptake was inhibited by PAH and other mercapturic acids and was stimulated in oocytes preloaded with glutarate. The apparent $K_m$ value for DNP-NAC uptake was only 2 $\mu$M, indicating that this mercapturic acid is a high affinity substrate for Oat1. Together, these data indicate that clearance of endogenous mercapturic acids is an important function of the renal organic anion transporter.

Many potentially toxic metabolites of both endogenous and exogenous origin are eliminated from the body after their conversion to the corresponding mercapturic acids [i.e., *N*-acetyl-L-cysteine *S*-conjugates (Boyland and Chasseaud, 1969; Chasseaud, 1979; Dekant et al., 1989; Stevens and Jones, 1989; Hinchman et al., 1991, 1998; Hinchman and Ballatori, 1994; Rebbeor et al., 1998; Wang and Ballatori, 1998)]. Mercapturic acid synthesis requires at least four enzymatic steps and three cell membrane transport events and is believed to require the interorgan shuttling of the metabolic intermediates (Stevens and Jones, 1989; Hinchman and Ballatori, 1994). The initial step in mercapturic acid synthesis is the conjugation of reactive compounds with glutathione, a process catalyzed by the intracellular glutathione *S*-transferases. The resulting glutathione *S*-conjugates are exported from the cell for subsequent metabolism by the ectoproteins γ-glutamyltranspeptidase and dipeptidases to form cysteine *S*-conjugates. Cysteine *S*-conjugates are trans-

ported back into the cell for acetylation by *N*-acetyltransferases to form mercapturic acids. The addition of the *N*-acetyl group converts zwitterionic cysteine *S*-conjugates to monovalent organic anions, thus increasing their water solubility and facilitating their export by organic anion transporters.

Mercapturic acids that are synthesized in the liver may be transported directly across the canalicular membrane into bile (Hinchman and Ballatori, 1994), whereas those synthesized in the kidney may be transported into urine, a major route for their elimination (Stevens and Jones, 1989). Mercapturic acids synthesized in other tissues enter the bloodstream and are eliminated by kidney and liver (Hinchman et al., 1998), although the relative contribution of these two organs, and possibly of other organs, remains to be established. Failure to remove mercapturic acids from either the blood or cells may result in toxicity and has been implicated as a causative factor in nephrotoxic disease (Dohn et al., 1985; Dekant et al., 1989; Stevens and Jones, 1989).

Although the renal proximal tubule is a major site of mercapturic acid excretion, the molecular mechanisms by which

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**ABBREVIATIONS:** Oat1, rat organic anion transporter-1; OAT1, human organic transporter-1; PAH, *p*-aminohippuric acid; LTE$_4$, leukotriene E$_4$; DNP-NAC, *S*-(2,4-dinitrophenyl)-*N*-acetyl-L-cysteine; NAC, *N*-acetyl-L-cysteine.

these compounds are removed from the circulation and excreted into renal tubular fluid is unknown. The present study tested the hypothesis that renal clearance of mercapturic acids is mediated in part by the kidney organic anion transporter-1 (Oat1; Lopez-Nieto et al., 1997; Sekine et al., 1997, 2000; Sweet et al., 1997; Wolff et al., 1997). This transporter is localized to the basolateral membrane of the S2 region of the renal proximal tubule (Tojo et al., 1999) and functions to take up a range of organic anions in exchange for intracellular α-ketoglutarate (Sweet and Pritchard, 1999). To date, Oat1 and Oat3 are the only organic anion uptake transporters that have been localized to the basolateral membrane of proximal tubules (Sweet et al., 1999; Tojo et al., 1999; Cha et al., 2001). The substrate selectivity of Oat1 is broad and includes *p*-aminohippurate (PAH), cyclic nucleotides, nucleoside phosphate analogs, nonsteroidal anti-inflammatory drugs, antibiotics, and other small organic anions (Lopez-Nieto et al., 1997; Sekine et al., 1997; Sweet et al., 1997; Wolf et al., 1997; Uwai et al., 1998; Apiwattanakul et al., 1999; Cihlar et al., 1999; Hosoyamada et al., 1999; Takeda et al., 1999; Inui et al., 2000). This selectivity suggests that Oat1 may play a central role in renal elimination of mercapturic acids. Our results demonstrate that Oat1 does transport mercapturic acids and that mercapturic acids may represent an important group of endogenous substrates for Oat1.

## Experimental Procedures

**Materials and Animals.** Mature *Xenopus laevis* frogs were purchased from Nasco (Fort Atkinson, WI). Animals were maintained under a constant light/dark cycle at a room temperature of 18°C. L-[$^{35}$S]Cysteine (1075 Ci/mmol) and [$^3$H]*p*-aminohippuric acid (PAH; 4.08 Ci/mmol) were purchased from PerkinElmer Life Science Products (Boston, MA). Iodoethane, iodopropane, iodobutane, 2,4-dinitrofluorobenzene, allyl iodide, 3-bromopropanol, 1-bromo-3-phenylpropane, acetyl bromide, pentafluorophenol, 1-bromododecane, and trichloroethylene were purchased from Aldrich Chemical (Milwaukee, WI). L-Cysteine hydrochloride, *N*-acetyl-L-cysteine, *N*-acetyl-LTE$_4$, *p*-aminohippuric acid, and *S*-benzyl-L-cysteine were purchased from Sigma Chemical Co. (St. Louis, MO). *N*-Acetyl-*S*-benzyl-L-cysteine was purchased from Schweizerhall, Inc. (Plainfield, NJ), and *N*-Acetyl-L-norleucine was obtained from Indofine Chemical Co., Inc. (Somerville, NJ).

[$^{35}$S]DNP-NAC was synthesized by a modification of the method of Saxena and Henderson (1996). L-[$^{35}$S]Cysteine in 10 mM dithiothreitol (255 μl) was mixed with 2.5 μl of freshly prepared 10 mM L-cysteine and lyophilized in a 5-ml conical vial containing a stir vane. Dimethylformamide (96 μl) and 5 μl of 0.66 M pentafluorophenoxyacetic acid were added, sealed, and stirred at room temperature (4 h). While stirring, 20 μl of water, 10 μl of 1.43 M KHCO$_3$, and 100 μl of 50 mM 2,4-dinitrofluorobenzene in ethanol were added, and the mixture was stirred for 2 h. The product was purified by high-performance liquid chromatography [Varian model 5000 with a Varichrom adjustable wavelength spectrophotometric detector (Varian Assoc. Inc., Sunnyvale CA)] and an HP 3394A integrator (Hewlett-Packard Co., Palo Alto, CA). The column [Bakerbond NP Octadecyl (C$_{18}$) column, 4.5 × 250 mm, 5 μm (J. T. Baker Research Products, Phillipsburg, NJ)] was eluted with a mobile phase of acetonitrile/water (25:75 with 0.1% H$_3$PO$_4$, v/v) and a flow rate of 1.0 ml/min. [$^{35}$S]DNP-NAC, which cochromatographed with an authentic sample of DNP-NAC, was detected at 365 nm and quantified by the external standard method using the area under the peak (15.9% yield).

*S*-Ethyl-*N*-acetyl-L-cysteine, *S*-propyl-*N*-acetyl-L-cysteine, *S*-butyl-*N*-acetyl-L-cysteine, *S*-allyl-*N*-acetyl-L-cysteine, *S*-3-phenylpro-pyl-*N*-acetyl-L-cysteine, *S*-3-hydroxypropyl-*N*-acetyl-L-cysteine, and *S*-dodecyl-*N*-acetyl-L-cysteine were synthesized as described by Grenby and Young (1960) and purified as described by Uttamsingh et al. (1998). Pentafluorophenyl acetate was prepared as described by Kisfaludy et al. (1979), and DNP-NAC as described by Hinchman et al. (1991).

**NMR Analysis.** The $^1$H NMR were recorded at 300 MHz on a General Electric QE-300 spectrometer (DMSO-d$_6$ or D$_2$O). The chemical shifts are reported in ppm and the splitting patterns are denoted as: s, singlet; d, doublet; t, triplet; q, quartet; p, pentet; hex, hextet; hep, heptet; m, multiplet; and br, broad: *N*-acetyl-*S*-2,4-dinitrophenyl-L-cysteine (DNP-NAC), 1.81 (s, 3H), 3.33–3.44 (m, 2H), 3.59–3.65 (m, 1H), 4.49–4.56 (m, 1H), 7.91–7.94 (d, 1H, J = 9 Hz), 8.42–8.51 (m, 2H), and 8.84–8.85 (d, 1H); *p*-aminohippuric acid (PAH), (D$_2$O) 3.68 (s, 2H), 4.60 (2, 4H), 6.58–6.61 (d, 2H, J = 9Hz), and 7.39–7.42 (d, 2H, J = 9Hz); *N*-acetyl-L-cysteine (NAC), 1.86 (s, 3H), 2.39–2.42 (m, 1H), 2.64–2.89 (m, 2H), 4.35–4.37 (m, 1H), and 8.15–8.18 (d, 1H); *N*-Acetyl-L-norleucine, 0.81–0.86 (t, 3H), 1.23–1.33 (br, 4H), 1.51–1.68 (m, 2H), 1.82 (s, 3H), 3.4 (br, 1H), 4.08–4.15 (m, 1H), and 8.06–8.08 (d, 1H); *N*-acetyl-*S*-ethyl-L-cysteine, 1.12–1.17 (t, 3H), 1.84 (s, 3H), 2.47–2.49 (q, 2H), 2.70–2.83 (m, 2H), 3.4 (br, 1H), 4.34–4.36 (m, 1H), and 8.21–8.23 (d, 1H); *N*-acetyl-*S*-allyl-L-cysteine, 1.84 (s, 3H), 2.59–2.82 (m, 2H), 3.12–3.15 (d, 2H), 3.4 (br, 1H), 4.33–4.37 (m, 1H), 5.05–5.12 (m, 2H), 5.67–5.73 (m, 1H), and 8.22–8.24 (d, 1H); *N*-acetyl-*S*-propyl-L-cysteine, 0.87–0.91 (t, 3H), 1.46–1.53 (hex, 2H), 1.83 (s, 3H), 2.44–2.49 (q, 2H), 2.64–2.85 (m, 2H), 3.4 (br, 1H), 4.32–4.36 (m, 1H), and 8.19–8.22 (d, 1H); *N*-acetyl-*S*-butyl-L-cysteine, 0.82–0.87 (t, 3H), 1.28–1.38 (hex, 2H), 1.41–1.51 (hex, 2H), 1.83 (s, 3H), 2.47–2.52 (t, 2H), 2.64–2.87 (mm, 2H), 3.4 (br, 1H), 4.31–4.35 (m, 1H), and 8.27–8.29 (d, 1H); *N*-acetyl-*S*-dichlorovinyl-L-cysteine, 1.84 (s, 3H), 3.08–3.15 (m, 1H), 3.34–3.40 (m, 1H), 4.33–4.37 (m, 1H), 6.99 (s, 1H), and 8.29–8.32 (d, 1H); *N*-acetyl-*S*-Cl-triF-ethyl-L-cysteine, 1.85 (s, 3H), 3.10–3.34 (m, 3H), 4.40–4.50 (m, 1H), 7.09–7.11 & 7.24–7.28 (mixture of diastereomers, 1H), and 8.40–8.42 (d, 1H); *N*-acetyl-*S*-benzyl-L-cysteine, 1.85 (s, 3H), 2.58–2.78 (m, 2H), 3.38 (br, 1H), 3.73 (s, 2H), 4.36–4.44 (m, 1H), 7.29 (brs, 5H), 8.24–8.27 (d, 1H); *N*-acetyl-*S*-BrCl-diF-ethyl-L-cysteine, 1.85 (s, 3H), 3.08–3.35 (m, 3H), 4.39–4.44 (m, 1H), 7.01–7.03 (t, 1H), and 8.38–8.40 (d, 1H); *N*-acetyl-*S*-diBr-diF-ethyl-L-cysteine, 1.84 (s, 3H), 3.07–3.32 (m, 3H), 4.38–4.45 (m, 1H), 6.83–6.96 (t, 1H), and 8.37–8.40 (d, 1H); *N*-acetyl-*S*-3-phenylpropyl-L-cysteine, 1.86 (s, 3H), 2.04–2.09 (t, 2H), 2.47–2.86 (m, 5H), 3.44–3.49 (t, 2H), 4.31–4.37 (m, 1H), 7.14–7.29 (m, 5H), and 8.09–8.11 (d, 1H); *N*-acetyl-*S*-pentaCl-butadiene-L-cysteine, 1.85 (s, 3H), 2.05 (s, 1H), 3.24–3.31 (m, 1H), 3.47–3.53 (m, 1H), 4.41–4.46 (m, 1H), and 8.36–8.39 (d, 1H); $^{13}$C NMR: (DMSO-d$_6$) 22.74, 35.05, 52.23, 118.44, 124.96, 125.768, 134.42, 169.86, and 171.62; *N*-acetyl-*S*-dodecyl-L-cysteine, 0.80–0.84 (t, 3H), 1.10–1.46 (br, 21H), 1.82–1.88 (m, 4H), 2.66–2.87 (m, 2H), 3.24 (br, 1H), 4.26–4.43 (m, 1H), 8.13–8.16 (d, 1H).

**Computational Methods.** Chemical structures were drawn with CS ChemDraw Ultra and copied into CS Chem3D Pro (version 5.0; Cambridge Soft Corporation; Cambridge, MA). Molecular mechanics energy minimizations were performed with a root-mean-square gradient of 0.001. Molecular geometries were generated in the Gaussian Z-matrix style with the CS MOPAC PRO application. For each molecule, an Austin-model 1 (AM1) semiempirical calculation and an ab initio (3–21G basis set) calculation was performed with the Gaussian 98 software package (Gaussian, Inc., Carnegie, PA). The plausibility of the structures was confirmed with CS ChemDraw Pro, and both the theoretical gas-phase dipole (Debye units) and COSMO van der Waals surface (Å$^2$) were calculated with the CS MOPAC PRO application. Water, with a dielectric constant of 78.4 at 25°C, was used as the solvent.

**Synthesis of Capped cRNA.** Rat Oat1 cDNA was prepared as previously described (Sweet et al., 1997; Li et al., 1998). Capped complementary RNA (cRNA) was transcribed in vitro with T7 RNA polymerase (Ambion, Austin, TX), the cRNA was precipitated with lithium chloride, and resuspended in RNase-free water for oocyte

injection. When electrophoresed on an RNA gel, this cRNA gave a single band corresponding to the predicted size for Oat1 message.

***X. laevis* Oocytes Preparation and Microinjection.** Isolation of *X. laevis* oocytes was performed as described by Goldin (1992) and previously employed in our laboratory (Ballatori et al., 1996; Li et al., 1998). Frogs were anesthetized by immersion for 15 min in ice-cold water containing 0.3% tricaine (Sigma). Oocytes were removed from the ovary and washed with $Ca^{2+}$-free OR-2 solution (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES-Tris, pH 7.5) and incubated at room temperature with gentle shaking for 90 min in OR-2 solution supplemented with 2 mg/ml of collagenase (type IA, Sigma). Oocytes were transferred to fresh collagenase solution after the first 45 min of incubation. Collagenase was removed by extensive washing in OR-2 solution at room temperature. Stage V and VI defolliculated oocytes were selected and incubated at 18°C in modified Barth's solution [88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, and 20 mM HEPES-Tris, pH 7.5], supplemented with gentamycin (50 μg/ml). After 3 to 4 h of incubation, oocytes were injected with 50 nl of Oat1 cRNA (5 ng/oocyte). Control oocytes were injected with a corresponding volume of sterile $H_2O$. Injected oocytes were cultured at 18°C with a daily change of modified Barth's medium containing gentamycin. Healthy oocytes with a clean, brown animal half and a distinct equator line were selected for experiments.

**PAH and DNP-NAC Uptake into Oocytes.** Uptake studies were performed 3 days after injection of cRNA. From 6 to 8 oocytes were incubated at 25°C for 1 h in 100 μl of modified Barth's solution in the presence of [³⁵S]DNP-NAC or [³H]PAH (Ballatori et al., 1996; Li et al., 1998). Adding 2.5 ml of ice-cold modified Barth's solution stopped the uptake, and the oocytes were washed three times each with 2.5 ml of ice-cold modified Barth's solution. Two oocytes were placed in a polypropylene scintillation vial with 0.2 ml of 10% SDS, and radioactivity was measured in a Packard model 4530 scintillation spectrometer after addition of 5 ml of Opti-Fluor (Packard Instruments, Downers Grove, IL).

**Efflux Studies.** To preload oocytes with [³H]PAH, they were incubated with 10 μM [³H]PAH for 2 h (1 μCi/ml). After this incubation, the oocytes were washed rapidly three times in modified Barth's solution, and efflux was measured over the next 60 min in modified Barth's solution containing various substrates or inhibitors. To preload with 2 mM glutarate, oocytes were microinjected with 50 nl of a 22 mM stock of glutarate dissolved in water, or water for control. After injection, the oocytes were incubated at room temperature for 20 to 30 min, washed three times with modified Barth's solution, and uptake of either 20 μM [³H]PAH or 1 μM [³⁵S]DNP-NAC was measured for 1 h at 25°C.

**Statistical Analysis.** A bivariate nonlinear logistic regression model was used to analyze the relationships between [³⁵S]DNP-NAC uptake, dipole moment, and van der Waals surface. The dependent variable was [³⁵S]DNP-NAC uptake and the two independent variables were dipole moment and van der Waals surface. As is customary for these models, the independent variables were log-transformed for analysis. This model is similar to the standard logistic model used in radioimmunoassay. The model also included a parameter that determined the maximum level of the dependent variable, whereas the minimum possible level was assumed to be zero. In addition, because the variance did not seem to be constant, a power of the mean model was used for the variance (Finney, 1978). The value of the power parameter was estimated as an additional parameter of the model, using the method of maximum likelihood. Initial values were chosen by analysis of the transformed data.

## Results

**Mercapturic Acids Are *cis*-Inhibitors of Oat1-Mediated [³H]PAH Transport.** To test the possibility that mercapturic acids are transported by Oat1, initial studies exam-

ined the effects of DNP-NAC on the uptake of [³H]PAH in Oat1-expressing *X. laevis* oocytes. DNP-NAC was an effective inhibitor of 20 μM [³H]PAH uptake (Fig. 1). Strong inhibition (63%) was observed at a DNP-NAC concentration of 5 μM, and inhibition was essentially complete at 100 μM. In contrast, *N*-acetyl-L-cysteine (NAC) itself was a relatively weak inhibitor of Oat1-mediated PAH transport, requiring millimolar concentrations to elicit significant effects (Fig. 1), indicating that Oat1 prefers the complete mercapturic acid for interaction with the carrier.

Consistent with this result, other mercapturic acids were also strong inhibitors of Oat1-mediated [³H]PAH transport (Fig. 2). The extent of inhibition was generally correlated with molecular size, hydrophobicity, and negative charge on the mercapturic acids. For example, 1 mM *S*-ethyl-NAC produced only 28% inhibition, whereas 1 mM *S*-propyl-NAC (92%) and *S*-butyl-NAC (95%) produced progressively more inhibition, and *S*-dodecyl-NAC inhibited [³H]PAH uptake by 83% at a concentration of only 500 μM (Fig. 2). Of particular importance, the endogenous mercapturic acid *N*-acetyl-$LTE_4$ inhibited 20 μM PAH uptake by 38 ± 3% at a concentration of 5 μM (±S.E., $n = 3$).

The inhibition of PAH uptake by DNP-NAC, *N*-acetyl-$LTE_4$, *S*-ethyl-NAC, and *S*-benzyl-NAC, was competitive in nature (Fig. 3). The apparent $K_m$ value for PAH uptake in Oat1-expressing oocytes was 11 ± 5 μM, and the calculated $K_i$ values for these four mercapturic acids were 1.9 ± 0.2 μM, 9 ± 8 μM, 1.1 ± 0.5 mM, and 0.4 ± 0.4 mM, respectively (Fig. 3). Our $K_m$ value for PAH transport agrees with previously published results (Lopez-Nieto et al., 1997; Sekine et al., 1997; Sweet et al., 1997; Wolf et al., 1997), whereas the low $K_i$ values for DNP-NAC and *N*-acetyl-$LTE_4$ suggests that they may be high-affinity substrates for Oat1.

**DNP-NAC Is a Substrate for Oat1.** To directly confirm that mercapturic acids are substrates for Oat1, we synthesized [³⁵S]DNP-NAC and measured its uptake in Oat1-ex-
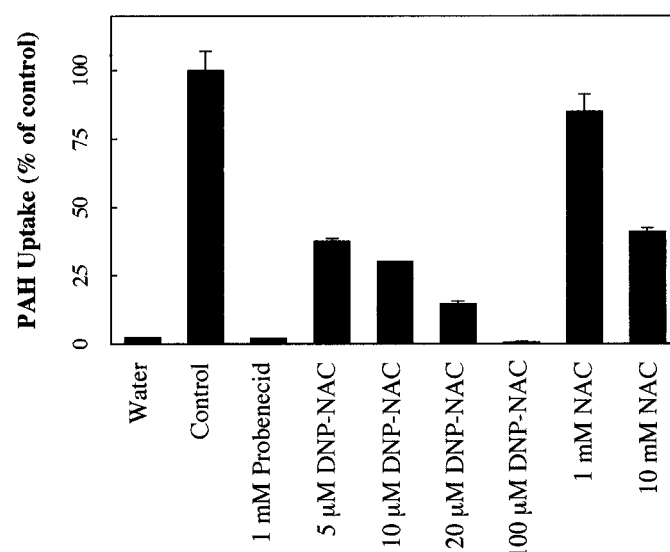


**Fig. 1.** 2,4-Dinitrophenyl *N*-acetylcysteine (DNP-NAC) *cis*-inhibits uptake of [³H]PAH in *Xenopus laevis* oocytes expressing rat Oat1. Oocytes were injected with 5 ng of Oat1 cRNA or with 50 nl of water (water injected) and cultured for 3 days at 18°C. Uptake of 20 μM [³H]PAH was measured at 25°C for 1 h in the absence or presence of inhibitors in cRNA-injected oocytes. Values are shown as means ± S.E., $n = 3$ separate experiments, each performed in triplicate.

pressing oocytes. There was little transport of [35S]DNP-NAC in control (water-injected) oocytes, whereas there was significant accumulation of this compound in Oat1-expressing oocytes (Fig. 4A). Uptake of 1 $\mu$M [35S]DNP-NAC was completely inhibited by 1 mM probenecid (Fig. 4A), a known inhibitor of Oat1 (Sekine et al., 1997; Sweet et al., 1997; Uwai et al., 1998). Kinetic analysis of [35S]DNP-NAC uptake revealed that this mercapturic acid is a high-affinity substrate for Oat1, with an apparent $K_m$ value of $2 \pm 1$ $\mu$M (Fig. 4B).

[35S]DNP-NAC uptake was *cis*-inhibited by PAH and by all mercapturic acids tested (Fig. 5). In general, the effects of the mercapturic acids on [35S]DNP-NAC uptake were similar to their effects on [3H]PAH uptake (Figs. 5 and 2, respectively). Analysis of the relationship between [35S]DNP-NAC uptake and either dipole moment or van der Waals surface (as an indication of solvated molecular size) revealed that both of these parameters are important determinants of inhibitory potential (Fig. 6 and Table 1). The extent of inhibition seemed to increase as either dipole moment (Fig. 6A) or van der Waals surface (Fig. 6B) was increased. A bivariate, nonlinear logistic regression model was used to analyze the relationships between [35S]DNP-NAC uptake, dipole moment, and van der Waals surface. The dependent variable was [35S]DNP-NAC uptake and the two independent variables
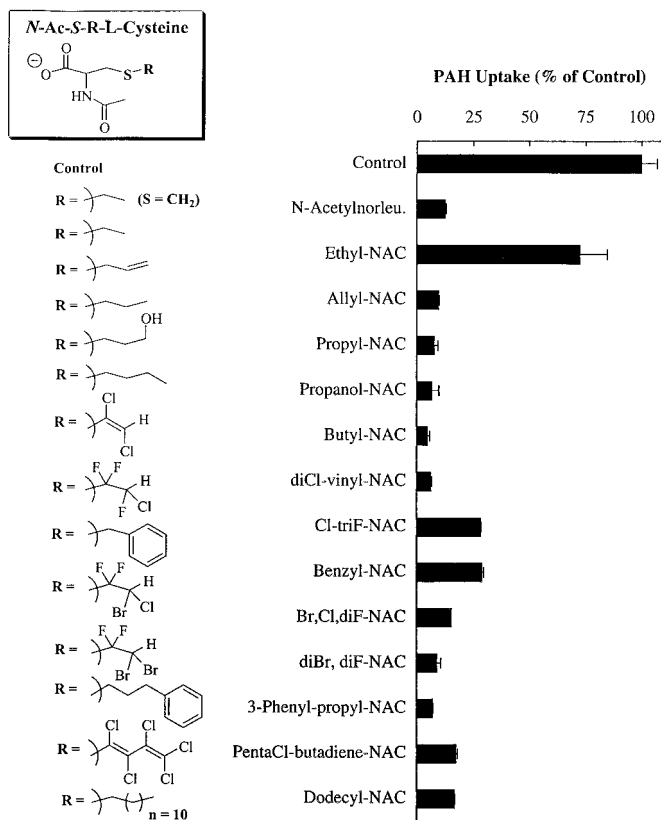
**PAH Uptake (% of Control)**

Control
N-Acetylnorleu.
Ethyl-NAC
Allyl-NAC
Propyl-NAC
Propanol-NAC
Butyl-NAC
diCl-vinyl-NAC
Cl-triF-NAC
Benzyl-NAC
Br,Cl,diF-NAC
diBr, diF-NAC
3-Phenyl-propyl-NAC
PentaCl-butadiene-NAC
Dodecyl-NAC

*N-Ac-S-R-L-Cysteine*

**Fig. 2.** Mercapturic acids inhibit [3H]PAH uptake in rat Oat1-expressing *X. laevis* oocytes. Uptake of 20 $\mu$M [3H]PAH was measured for 1 h at 25°C in oocytes injected with 5 ng of Oat1 cRNA in the absence or presence of various inhibitors. All inhibitors were present at a concentration of 1 mM with the exception of *S*-butyl-NAC, *S*-Br,Cl,diF-NAC, *S*-diBr,diF-NAC, *S*-pentachlorobutadiene-NAC, and *S*-dodecyl-NAC, which were present at 0.5 mM. Chemical structures of the compounds are provided. Values represent the mean of cRNA-injected minus water-injected oocytes $\pm$ S.E. ($n = 6–8$ samples from two independent experiments). See Table 1 for names of acids.

TABLE 1
Summary of computational analysis for van der Waals surface, dipole moment, atomic charge, and electrostatic potential for the various mercapturic acids and other inhibitors of Oat1-mediated transport, and comparison with their ability to inhibit 1 $\mu$M [35S]DNP-NAC transport at a concentration of 1 mM.

| Compound | Mopac AM1 | | [35S]DNP-NAC Uptake | Gaussian AM1 | | Gaussian 3-21G | | |
| | van der Waals COSMO | Dipole | | Atomic Charge[a] | Electrostatic Potential | Dipole | Atomic Charge[a] | Electrostatic Potential |
| | Å² | Debye | % control | | S | Debye | | S |
| p-Aminohippuric acid | 97.88 | 16.880 | 7 | | | 12.218 | | |
| N-Acetyl-L-cysteine | 84.06 | 7.492 | 58 | 0.021512 | -4.249561 | 4.946 | -0.557607 | -59.191508 |
| N-Acetyl-L-norleucine | 95.38 | 10.886 | 33 | | | 8.741 | | |
| N-Acetyl-S-ethyl-L-cysteine | 96.48 | 9.047 | 51 | 0.042494 | -4.234000 | 5.896 | 0.153826 | -59.079785 |
| N-Acetyl-S-allyl-L-cysteine | 103.90 | 9.884 | 13 | 0.057778 | -4.217712 | 6.300 | 0.173386 | -59.072646 |
| N-Acetyl-S-propyl-L-cysteine | 104.96 | 14.279 | 29 | 0.056792 | -4.225401 | 5.100 | 0.156153 | -59.078778 |
| N-Acetyl-S-propanol-L-cysteine | 109.84 | 14.310 | 32 | 0.063822 | -4.227486 | 8.326 | 0.170609 | -59.071887 |
| N-Acetyl-S-butyl-L-cysteine | 112.48 | 16.188 | 4 | 0.057749 | -4.224219 | 4.558 | 0.155599 | -59.076946 |
| N-Acetyl-S-dichlorovinyl-L-cysteine | 116.98 | 16.305 | 24 | 0.232308 | -4.112969 | 8.100 | 0.416882 | -59.026438 |
| N-Acetyl-S-Cl-triF-ethyl-L-cysteine | 113.13 | 9.995 | 17 | 0.126033 | -4.144872 | 6.062 | 0.304376 | -59.027901 |
| N-Acetyl-S-benzyl-L-cysteine | 117.90 | 14.773 | 30 | 0.044221 | -4.208984 | 9.670 | 0.162123 | -59.068327 |
| N-Acetyl-S-BrCl-diF-ethyl-L-cysteine | 123.77 | 11.829 | 6 | 0.120330 | -4.148114 | 7.217 | 0.308328 | -59.028978 |
| N-Acetyl-S-diBr-diF-ethyl-L-cysteine | 127.02 | 12.843 | 0 | 0.119333 | -4.156045 | 16.618 | 0.424838 | -59.030739 |
| N-Acetyl-S-3-phenylpropyl-L-cysteine | 137.73 | 17.212 | 9 | 0.049869 | -4.216265 | 6.085 | 0.168957 | -59.070011 |
| N-Acetyl-S-pentaCl-butadiene-L-cysteine | 151.81 | 19.213 | 3 | 0.213526 | -4.090848 | 17.249 | 0.563808 | -59.010127 |
| N-Acetyl-S-dodecyl-L-cysteine | 168.76 | 26.542 | 1 | 0.023932 | -4.234851 | 24.173 | 0.137728 | -59.075049 |
| N-Acetyl-S-2,4-DNP-L-cysteine | 137.02 | 12.942 | 1 | 0.223480 | -4.107553 | 8.386 | 0.398940 | -59.024311 |

[a] Atomic charges with hydrogens summed into heavy atoms.

were dipole moment and van der Waals surface. A significant association was found with both predictors: the regression coefficients (SD) were −58.51 (15.99) for der Waals surface and 18.27 (6.71) for dipole moment. The parameter for the maximum level of [$^{35}$S]DNP-NAC uptake was 29.86 (6.24). When the effects of these two parameters were examined simultaneously, an interesting relationship was noted: for compounds with a relatively low dipole moment (~8 Debye), inhibitory potential reached its maximum level as the van der Waals surface increased from only about 95 to 105 Å$^2$. In contrast, for compounds with a relatively high dipole (~20–25 Debye), a much larger increase in molecular size was needed to elicit maximal inhibition (from about 130–150 Å$^2$). Thus, for this group of mercapturic acids, the relatively hydrophobic compounds are the best inhibitors of Oat1-mediated DNP-NAC transport.

To further test whether DNP-NAC and other mercapturic acids are substrates for Oat1 and to examine the mechanism of transport, additional studies measured efflux of [$^3$H]PAH from preloaded oocytes into either buffer alone or buffer containing various mercapturic acids (Fig. 7). [$^3$H]PAH efflux from control oocytes was a first-order process, with about 30 to 35% of the compound released over 60 min (Fig. 7A). The addition of 1 mM unlabeled PAH to the medium significantly stimulated [$^3$H]PAH efflux in Oat-1 expressing oocytes, indi-

cating *trans*-stimulation, whereas 1 mM probenecid almost completely blocked [$^3$H]PAH efflux (Fig. 7A). Probenecid also partially blocked the efflux that was stimulated by extracellular PAH (Fig. 7B), supporting previous data demonstrating that probenecid binds strongly to the carrier, but is very poorly transported. When oocytes were exposed to 0.1 mM DNP-NAC, there was a stimulation of [$^3$H]PAH efflux, whereas a concentration of 1 mM DNP-NAC inhibited efflux, although the latter difference was not statistically significant (Fig. 7B). Benzyl-NAC also inhibited [$^3$H]PAH efflux, whereas NAC stimulated efflux, and the other mercapturic acids had no effect (Fig. 7B). The inhibition of [$^3$H]PAH efflux by high concentrations of benzyl-NAC and DNP-NAC indicates that these relatively hydrophobic compounds are either entering the oocytes in sufficient quantities to inhibit transport from an intracellular site or are acting like probenecid, which binds to the transporter and markedly decreases its turnover rate. Unfortunately, the present data do not allow us to distinguish between these possibilities.

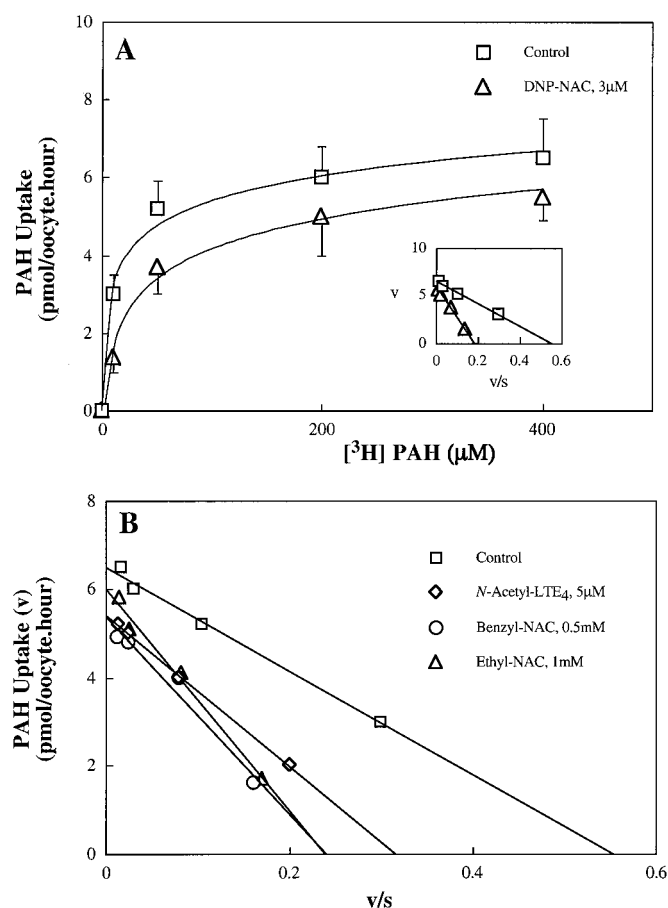Because rat Oat1 functions as an exchanger with intracel-

**Fig. 3.** Kinetic characteristics of [$^3$H]PAH transport in Oat1-expressing oocytes. Uptake of increasing concentrations of [$^3$H]PAH was measured for 1 h at 25°C in oocytes injected with 5 ng of rat Oat1 cRNA, in the absence (□) or presence (△) of either 3 μM DNP-NAC (A); or with 5 μM *N*-acetyl-LTE$_4$ (◇), 0.5 mM *S*-benzyl-NAC (○), or 1 mM *S*-ethyl-NAC (△; panel B). Values are means ± S.D., *n* = 3–5 separate experiments.



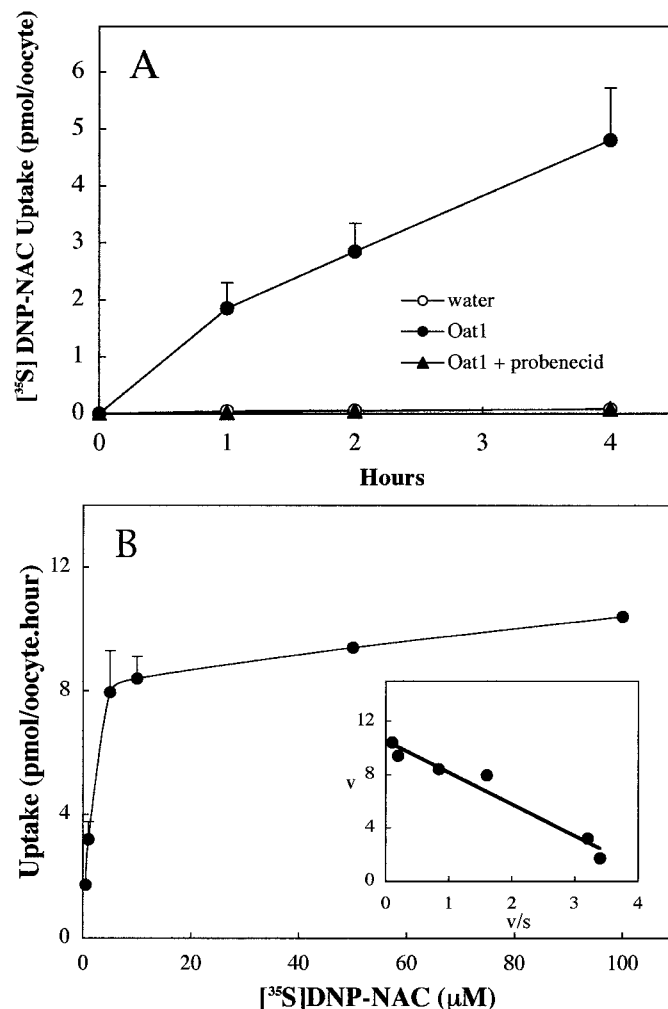**Fig. 4.** DNP-NAC is a substrate for rat Oat1. A, uptake of 1 μM [$^{35}$S]DNP-NAC was measured at 25°C over a 4-h interval in Oat-injected oocytes (5 ng cRNA, ●), water-injected oocytes (○), or Oat1-injected oocytes in the presence of 1 mM probenecid (▲). B, uptake of [$^{35}$S]DNP-NAC was measured for 1 h at 25°C. Data points represent Oat1-injected oocytes (16 ng cRNA) minus water-injected oocytes. Eadie-Hofstee plot (inset) reveals an apparent Km of 2 ± 1 μM. Values shown are means ± S.E., *n* = 2 to 3 independent experiments of six samples each.

lular dicarboxylates (α-ketoglutarate), uptake of [35S]DNP-NAC in Oat1-expressing oocytes should be stimulated when oocytes are preloaded with a high concentration of a dicarboxylate. To test this hypothesis, uptake of both [3H]PAH and [35S]DNP-NAC was measured in control oocytes and oocytes preloaded with 3 mM glutarate. Uptake of both compounds was significantly stimulated by glutarate (Fig. 8), consistent with uptake on Oat1 by exchange with intracellular dicarboxylates.

## Discussion

Mercapturic acids constitute a large and important group of cellular metabolites that are eliminated in urine. They are formed from many endogenous and exogenous compounds (Boyland and Chasseaud, 1969; Chasseaud, 1979; Dekant et al., 1989; Stevens and Jones, 1989; Ballatori, 1994; Wang and Ballatori, 1998). The exogenous compounds include drugs, natural toxins, and environmental pollutants, and the endogenous compounds include leukotrienes, prostaglandins, hepoxilin, nitric oxide, hydroxyalkenals, ascorbic acid, dihydroxyphenylalanine, dopamine, and maleic acid (Wang and Ballatori, 1998). When their excretion is compromised, nephrotoxic disease is observed (Dohn et al., 1985; Dekant et al., 1989; Stevens and Jones, 1989). Although it has long been known that mercapturic acids are excreted in urine, the molecular mechanisms have not been identified. The data presented above indicate that their urinary excretion is mediated by Oat1, and thus, that this transporter may play an important protective role in the elimination of these compounds.

The known properties of Oat1 support such a role in the handling of mercapturic acids; however, Oat1 may not be the only transporter involved. Recent studies demonstrate that human OAT3 is also present on the basolateral membrane of renal proximal tubules and that this transporter displays overlapping substrate specificity with human OAT1 or rat Oat1 (Cha et al., 2001). Thus, it is likely that mercapturic acids are also substrates for human OAT3, although this has not yet been examined.

Oat1 was cloned from rat, mouse, and flounder in 1997 and was shown to mediate basolateral uptake of organic anions in exchange for intracellular α-ketoglutarate (Lopez-Nieto et al., 1997; Sekine et al., 1997; Sweet et al., 1997; Wolf et al., 1997). This is the uphill, rate-limiting step in the net secretion of organic anions by the proximal tubule (Pritchard and Miller, 1993). Thus, Oat1 is a critical transporter in the renal elimination of organic anions. Its tissue-specific and subcellular localization, its mode of energy coupling, and its substrate selectivity all fit with this functional role (Lopez-Nieto
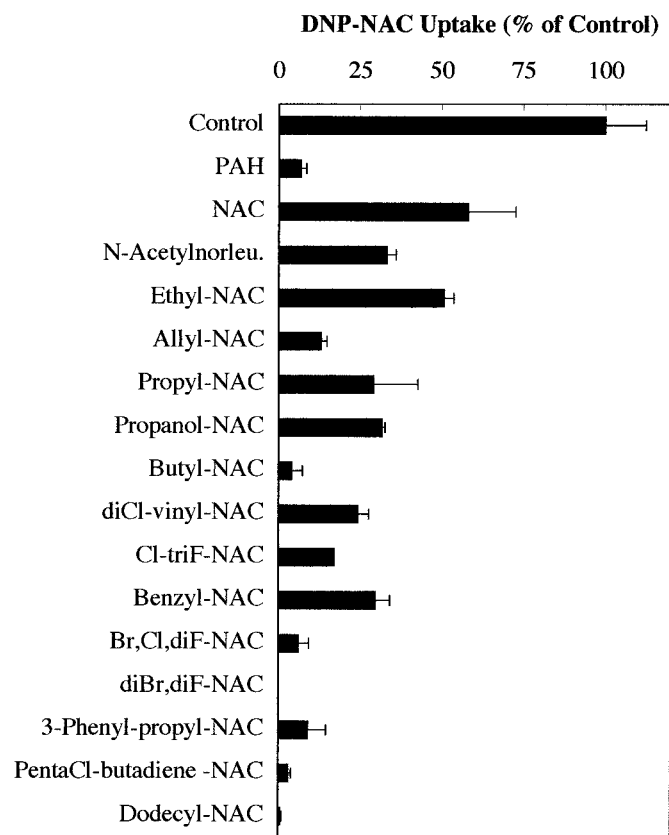


**Fig. 5.** *cis*-Inhibition of [35S]DNP-NAC uptake in Oat1-expressing *X. laevis* oocytes. Uptake of 1 μM [35S]DNP-NAC was measured for 1 h at 25°C in the absence (control) or presence of PAH or mercapturic acids. All inhibitors were used at a concentration of 1 mM. Values are shown as means ± S.E., *n* = 3.
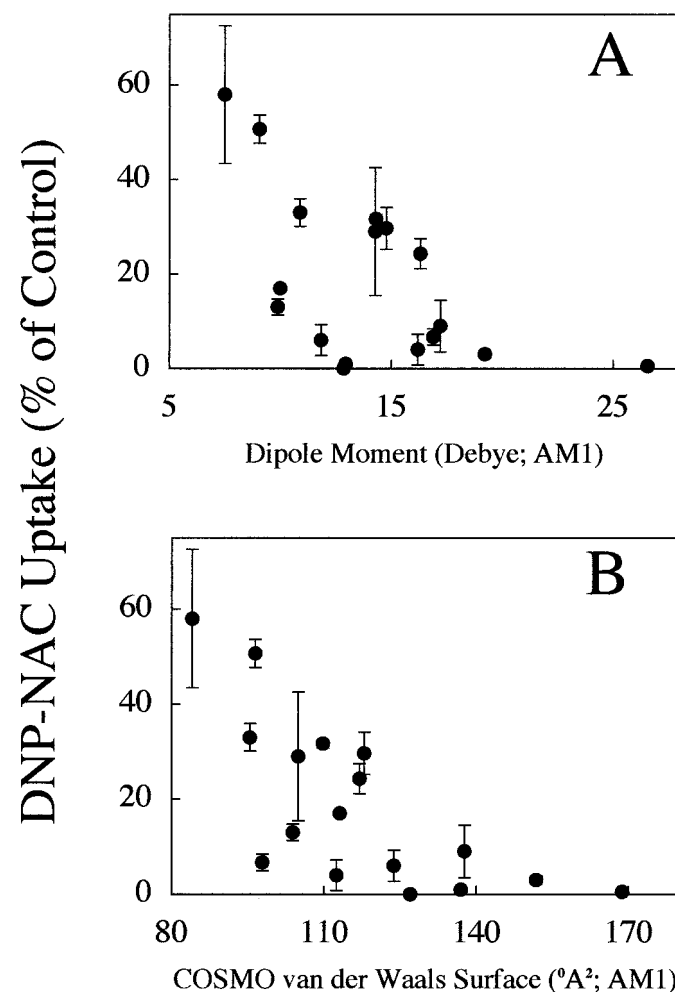


**Fig. 6.** Relation between calculated relative dipole moments and van der Waals surface and rates of [35S]DNP-NAC uptake. AM1 semiempirical calculations were performed on the inhibitors used in Fig. 5 (data are provided in Table 1). A trend was observed between the inhibition of [35S]DNP-NAC uptake and both dipole moment (A) and van der Waals surface (B). Values are shown as means ± S.E., *n* = 3.

et al., 1997; Sekine et al., 1997; Sweet et al., 1997; Wolf et al., 1997). Oat1 is highly expressed in the kidney and is localized to the basolateral membranes of the S2 segment of the renal proximal tubule (Sweet et al., 1999; Tojo et al., 1999), the site of mercapturic acid secretion within the nephron (Stevens and Jones, 1989). Thus, Oat1 seems to be a major basolateral membrane transporter that can mediate the accumulative transport of a multitude of organic anions from the peritubular fluid. Because the $\alpha$-ketoglutarate electrochemical gradient is large, this mode of energy coupling provides a powerful driving force for uptake of solutes into renal proximal tubular cells. Because of this large driving force, organic

anion secretion is very effective, often mediating complete clearance of substrates from renal plasma in a single pass through the kidney (Pritchard and Miller, 1993). Oat1 is also multispecific; i.e., it mediates uptake of a variety of structurally unrelated extracellular organic molecules, including nonsteroidal anti-inflammatory drugs, $\beta$-lactam antibiotics, and many other small anionic or neutral molecules (Burckhardt and Wolff, 2000). Together, these properties argue strongly that the organic anion system, more specifically Oat1, should be capable of transporting and secreting mercapturic acids.

Our results demonstrate that Oat1-mediated PAH uptake is effectively inhibited by mercapturic acids (Figs. 1 and 2). Furthermore, PAH transport was competitively inhibited by several of these compounds, including $S$-ethyl-NAC, $S$-benzyl-NAC, DNP-NAC, and $N$-acetyl-LTE$_4$ (Fig. 3). Apparent $K_i$ values for two of these mercapturic acids, DNP-NAC and $N$-acetyl-LTE$_4$, were 10 $\mu$M or less (Fig. 3). Because the $K_m$ value for the classical organic anion substrate, PAH, was 11 $\mu$M in our system, these mercapturic acids seem to be high affinity substrates for Oat1. Direct kinetic measurements using [$^{35}$S]DNP-NAC yielded an apparent $K_m$ value of 2 $\pm$ 1 $\mu$M (Fig. 3B), confirming its high affinity for rat Oat1. It is important to note that this $K_m$ value for DNP-NAC is lower (i.e., higher affinity) than most of the other substrates that have been tested with Oat1 (Burckhardt and Wolff, 2000), indicating that mercapturic acids may be the preferred substrates for this transporter. Once taken up into renal proximal tubular cells, mercapturic acids may then be secreted into tubular fluid for excretion in urine; however, the trans-
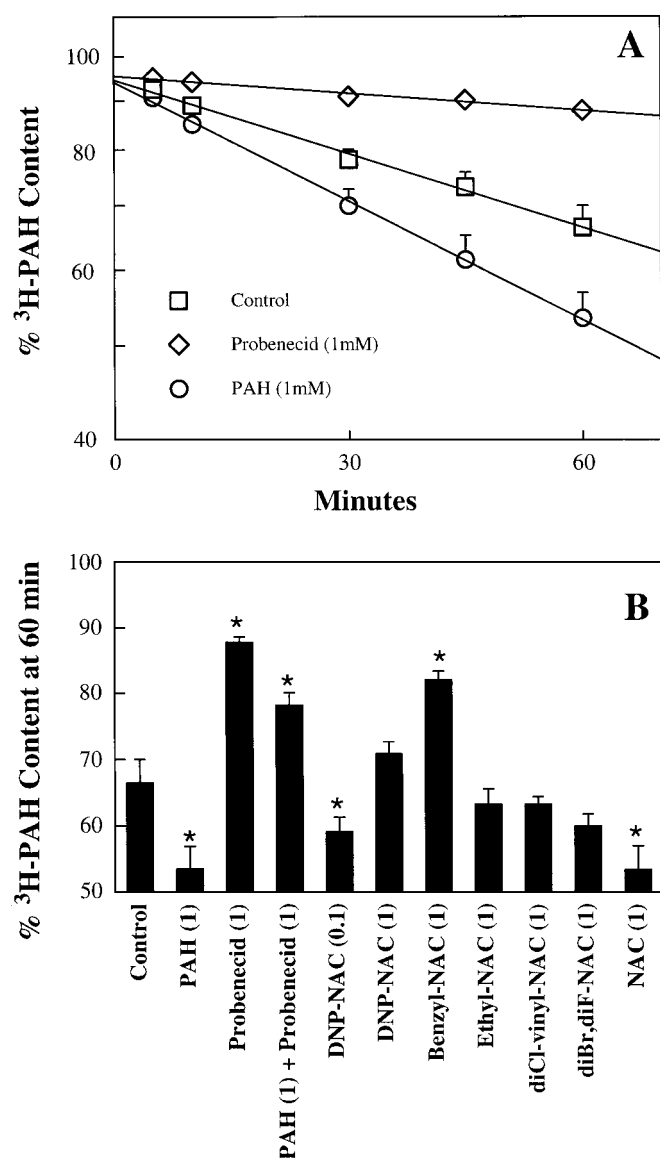


**Fig. 7.** Efflux of [$^3$H]PAH in medium containing putative substrates and inhibitors of Oat1. Oocytes were preloaded with [$^3$H]PAH by incubating with 10 $\mu$M [$^3$H]PAH for 2 h (1 $\mu$Ci/ml) at 25°C. Oocytes were then washed to remove extracellular radioactivity and efflux was measured over the next 60 min in modified Barth's solution containing various substrates or inhibitors. A, time course of efflux in modified Barth's solution (control; □) or in the same solution containing either 1 mM PAH (○) or 1 mM probenecid (◇). B, the percentage of [$^3$H]PAH remaining in oocytes after 60 min of incubation with various substrates or inhibitors. The concentrations of these compounds are provided in parentheses (mM). Values are shown as means $\pm$ S.E., $n = 4$.
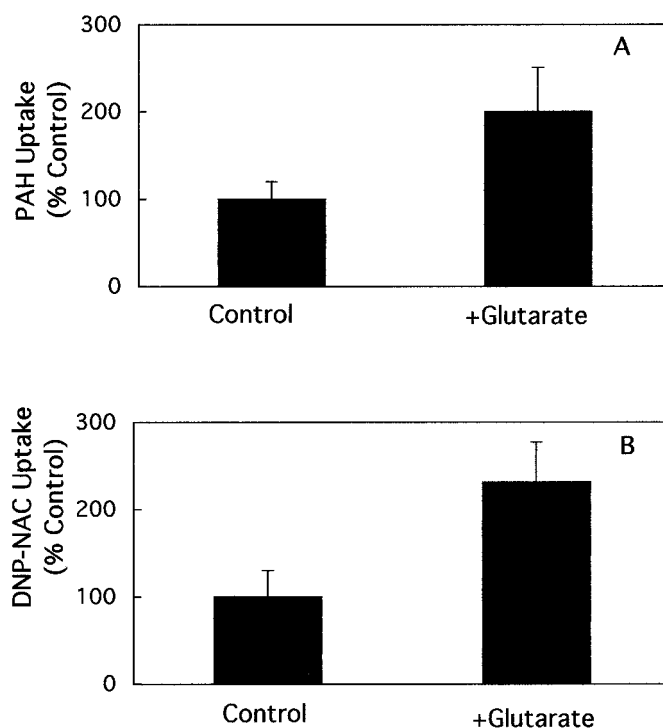


**Fig. 8.** Stimulation of PAH and DNP-NAC uptake in oocytes preloaded with glutarate. To preload with 2 mM glutarate, oocytes were microinjected with 50 nl of a 22 mM stock of glutarate in water, or water for control. After injection, the oocytes were incubated at room temperature for 20 to 30 min, washed three times with modified Barth's solution, and uptake of either 20 $\mu$M [$^3$H]PAH or 1 $\mu$M [$^{35}$S]DNP-NAC measured for 1 h at 25°C. Values are shown as means $\pm$ S.E., $n = 3$.

porter responsible for export across the apical membrane of the cell into the tubular fluid is unknown.

Our results also support the previous finding that Oat1 has a broad substrate selectivity and demonstrate that molecular size, mass/charge ratio, and dipole moment are important factors in substrate recognition (Fig. 6 and Table 1; Ullrich, 1997). Quantum mechanical calculations were used in the preliminary characterization of substrate specificity for some mercapturic acids, and trends were noted between inhibitory potential and molecular size, mass/charge ratio, and dipole moment (Fig. 6 and Table 1). All of the mercapturic acids used in this study have a fundamental formal charge of $-1$. The anion is mildly delocalized through the two resonance contributors, from the carboxylic acid, and not readily stabilized by field effects. If one makes the assumption that the formal charge is in fact $-1$, then there is an absolute mass/charge or COSMO/charge relationship for this system, just as was reported recently for the for voltage-gated potassium channels exposed to quaternary ammonium ions (Wempe, 2001). These trends indicate that molecular size and hydrophobicity are important determinants of inhibitory potency for this group of anions. From an experimental standpoint, this means that one should use a radiolabeled probe of comparable molecular mass. For example, [$^3$H]PAH should only be used to compare small mercapturic acid or other inhibitors of relatively low molecular mass (COSMO 80–110 Å$^2$), whereas a larger substrate, such as [$^{35}$S]DNP-NAC, may be a better substrate for analysis of inhibitors with a larger COSMO range (80–180 Å$^2$).

Quantitative analysis of these molecular and chemical relationships may be useful for other classes of substrates and may help in the design of newer, more potent therapeutic drugs. This information, when combined with data on transporter localization and substrate selectivity, would allow drugs to be targeted more accurately to their desired sites of action, with greater efficacy, and perhaps with more predictable pharmacokinetic/toxicokinetic profiles.

Finally, the demonstration that mercapturic acids are substrates for Oat1 raises an interesting question as to its possible role in development. In the adult, Oat1 is predominantly expressed in the kidney (Sekine et al., 1997; Sweet et al., 1997). However, during early fetal development Oat1 is highly expressed in brain before its expression in kidney (Nakajima et al., 2000; Pavlova et al., 2000). It is conceivable that Oat1 may play a role in brain transport of endogenous mercapturic acids such as leukotrienes and prostaglandins in the developing fetus, whereas in adults it functions as a broad-specificity renal transporter for removing organic anions from the bloodstream.

In summary, mercapturic acids were shown to be excellent substrates for Oat1. In the adult animal, these compounds probably form an important class of endogenous substrates for excretory transport via Oat1. In the developing fetus there may be additional roles for Oat1, including perhaps the transport of prostaglandins and leukotrienes.

## Acknowledgments

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**Address correspondence to:** Ned Ballatori, Ph. D., Department of Environmental Medicine, Box EHSC, University of Rochester School of Medicine and Dentistry, 575 Elmwood Avenue, Rochester, NY 14642. E-mail: ned_ballatori@urmc.rochester.edu

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