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Sulfhydryl groups are essential for organic anion exchange in canine renal brush-border membranes

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The effect of several sulfhydryl-modifying reagents ($HgCl_2$, *p*-chloromercuribenzenesulfonic acid (PCMBS), *N*-ethylmaleimide) on the renal organic anion exchanger was studied. The transport of *p*-amino[^{3}H]hippurate, a prototypic organic anion, was examined employing brush-border membrane vesicles isolated from the outer cortex of canine kidneys. $HgCl_2$, PCMBS and *N*-ethylmaleimide inactivated *p*-aminohippurate transport with IC_{50} values of 38, 78 and 190 μM. The rate of *p*-aminohippurate inactivation by *N*-ethylmaleimide followed apparent pseudo-first-order reaction kinetics. A replot of the data gave a linear relationship between the apparent rate constants and the *N*-ethylmaleimide concentration with a slope of 0.8. The data are consistent with a simple bimolecular reaction mechanism and imply that one molecule of *N*-ethylmaleimide inactivates one essential sulfhydryl group per active transport unit. Substrate (1 mM *p*-aminohippurate) affected the rate of the *N*-ethylmaleimide (1.3 mM) inactivation: the $t_{1/2}$ values for inactivation in the presence and absence of *p*-aminohippurate were 7.4 and 3.7 min, respectively. The results demonstrate that there are essential sulfhydryl groups for organic anion transport in the brush-border membrane. Moreover, the ability of substrate to alter sulfhydryl reactivity suggests that the latter may play a dynamic role in the transport process.

Introduction

The secretion of the organic anion *p*-aminohippurate is localized to the proximal tubule of the mammalian kidney (for reviews see Refs. 1–3). Secretion involves transepithelial movement across both faces of the renal cell, and attempts to resolve the complexity of this transport process have been undertaken employing isolated plasma membrane vesicles. These studies have demonstrated that transport across both the basolateral and brush-border membrane is carrier-mediated [4]. *p*-Aminohippurate transport across the basolateral membrane is electrogenic and occurs via a Na^+-gradient-dependent anion-exchange mechanism [5], while the transport across the brush-border membrane occurs by an electroneutral Na^+-gradient-independent anion-exchange mechanism [4,6].

In the present study, the effect of sulfhydryl-modifying reagents on *p*-aminohippurate transport in the brush-border membrane was examined employing canine brush-border membrane vesicles. Previous studies with isolated rabbit and flounder tubules had shown that sulfhydryl modifying reagents decreased *p*-aminohippurate transport [7,8]. However, the relative contributions of the two membranes to the transport inactivation could not be determined. In the rat, it has been shown that

Abbreviations: PCMBS, *p*-chloromercuribenzenesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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p-aminohippurate transport in basolateral membrane vesicles [9,10] is sensitive to sulfhydryl modifying reagents, whereas *p*-aminohippurate transport in brush-border membrane vesicles is insensitive [11]. It remains to be established, however, whether this pattern holds for other species as well, i.e., sulfhydryl groups are essential for *p*-aminohippurate transport in the basolateral membrane but not in the brush-border membrane. For this reason and because of the paucity of data on the mechanism of organic anion transport in the brush-border membrane we undertook this study. We found that *p*-aminohippurate transport in the brush-border membrane of dog kidney is exquisitely sensitive to sulfhydryl-modifying reagents and the reactivity of these compounds is altered by the presence of substrate. Thus, the *p*-aminohippurate organic anion exchangers in the brush-border membrane of the dog and rat appear to be quite different from one another.

Methods

Membrane preparation

Brush-border membrane vesicles were isolated from canine kidney cortex using a cation-precipitation method [12]. The purified membranes (5.0–11.3 mg of protein per ml) were suspended in 10 mM Hepes/50 mM potassium gluconate/200 mM mannitol (pH 7.5) and stored at −70°C until use [13]. Protein concentration was assayed using bovine serum albumin as a standard [14].

Transport measurements

p-Aminohippurate influx experiments. Transport of *p*-aminohippurate into brush-border membrane vesicles was assayed employing a pH gradient and a rapid filtration technique [5]. Brush-border membrane vesicles were allowed to equilibrate at 25°C for 30 min. The influx was initiated by diluting the brush-border membrane vesicles 10-fold with the reaction solution (10 mM Hepes/50 mM potassium gluconate/200 mM mannitol (pH 6.5)) containing 50 μM *p*-amino[^{3}H]hippurate. The specifically mediated transport was examined by looking at 50 μM *p*-aminohippurate uptakes in the presence of 0.50 mM probenecid, a competitive inhibitor [4]. The reaction was terminated by adding 3 ml of ice-cold buffer to the reaction vessel at times specified in the figures. The quench buffer employed was identical to the reaction solution buffer. The reaction vessel was rinsed twice with 3 ml ice-cold buffer and the contents poured onto prewetted 0.3 μ Millipore filters where the solution was removed under vacuum (500 Torr). The filters were rinsed with an additional 3 ml of ice-cold buffer and transferred to liquid scintillation vials. 10 ml of scintillation fluid (Filtron X, National Diagnostics, Somerville, NJ) were added which completely solubilized the membranes and filters to yield a homogeneous solution. The amount of radioactivity taken up by the vesicles was determined by standard liquid scintillation techniques using an external standard to correct for quench. Corrections for the amount of radioactivity bound to the filters were determined by running filter controls (reaction solution without brush-border membrane vesicles) concurrently with each experiment.

Transport measurements in presence of various reagents. Stock solutions of $HgCl_2$, *N*-ethylmaleimide and PCMBS were prepared fresh for each experiment. After the appropriate preincubation, the transport assays were performed as outlined above. Control transport was performed in the absence of the reagent employing an equivalent amount of the buffer. The concentrations of all reagents employed were normalized per mg of protein in the reaction vessel.

The data are presented as means ± S.E. unless otherwise noted. Linear regression and the Student's *t*-test were employed where appropriate [15].

Materials

p-Amino[^{3}H]hippurate (431 mCi/mmol) was obtained from Amersham (IL). Mercuric chloride was purchased from Mallinckrodt Chemical Works (NY) and L-(+)-cysteine hydrochloride was purchased from Eastman Kodak (Rochester, NY). The other chemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO): unlabeled *p*-aminohippurate, probenecid, *N*-ethylmaleimide and PCMBS.

Results

p-Aminohippurate crosses the brush-border membrane via an anion exchanger [6,16]. The

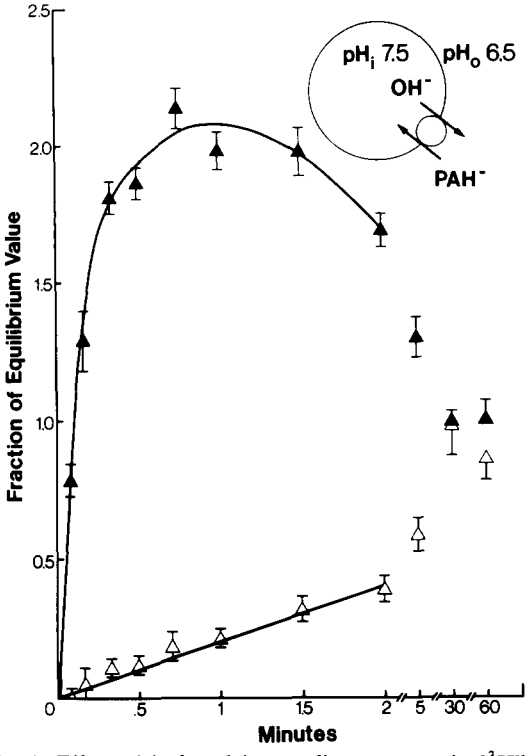

Fig. 1. Effect of hydroxyl ion gradient on *p*-amino[^{3}H]hippurate uptake into brush-border membrane vesicles. Brush-border membrane vesicles that had been prepared in 10 mM Hepes/50 mM potassium gluconate/200 mM mannitol (pH 7.5); (10 μl) were allowed to equilibrate at 25°C for 15 min. Influx was initiated by adding 90 μl of 50 μM *p*-amino[^{3}H]hippurate (prepared in 10 mM Hepes/50 mM potassium gluconate/200 mM mannitol (pH 6.5)) and examined in the presence (△) or absence (▲) of 0.50 mM probenecid. The data are presented as fractions of the equilibrium value (30 min time point). Each point represents the mean ± S.E. of a single membrane preparation performed in quadruplicate (6.0 mg protein/ml).

transport of *p*-aminohippurate is symmetrical, so that one can study either influx or efflux in brush-border membrane vesicles [4], representing reabsorption and secretion, respectively. For convenience, *p*-aminohippurate influx was followed in the experiments to be described. The effect of a hydroxyl ion gradient *trans* to the *p*-aminohippurate was examined and the results from a representative experiment are shown (Fig. 1). (The *trans* compartment is defined as that toward which the radioactive species is moving.) The presence of a pH gradient (inside alkaline) produced an overshoot of *p*-amino[^{3}H]hippurate uptake which surpassed the equilibrium value by a factor of 2. Equilibrium was achieved within 30 min and yielded an intravesicular volume of 3.6 ± 0.4 μl per mg of protein; a value consistent with those obtained by others for intestinal and renal epithelia [17,18]. Quantitative variations are seen among different membrane preparations in that the extent of the overshoot can vary as well as the time point of the apex. A 10-fold excess of a competitive inhibitor, probenecid [4], prevented the overshoot and the uptake increased linearly with time, consistent with a nonmediated process (i.e., one which follows Fick's law of diffusion). At the 15 s time point, more than 90% of the total *p*-aminohippurate uptake was blocked by probenecid (Fig. 1). The difference between the total and the probenecid-resistant uptake is taken as the specifically mediated transport and was calculated in each experiment from the 15 s uptake. The specifically mediated transport in controls employing pH gradients was found to be 524 ± 52 (S.E., $n = 30$) pmol per min per mg protein (nine different membrane preparations were employed).

The effect of various sulfhydryl-modifying reagents on the specifically mediated transport of *p*-aminohippurate in the brush border membrane was examined. $HgCl_2$, PCMBS and *N*-ethylmaleimide inactivated the specifically mediated anion-driven *p*-aminohippurate transport (Fig. 2). They differed, however, in the extent of the inactivation they produced. The mercurials $HgCl_2$ and PCMBS inactivated greater than 96% of the transport, whereas *N*-ethylmaleimide inactivated only 77 ± 8% of the transport. The IC_{50} values for the inactivation of *p*-aminohippurate transport are shown in Table I and increased in the following order: $HgCl_2$ < PCMBS < *N*-ethylmaleimide. The data are presented in two ways, i.e., by molarity and by nmol per mg protein. The latter is convenient for comparing results from various laboratories which employ different concentrations of protein in their final reaction solution. The effect was not due to the disruption of vesicles, since the equilibrium values in the presence of the reagents were not different from that of the controls (3.6 ± 0.4 μl per mg of protein). The values for intravesicular volumes calculated from equilibrium points for brush-border membrane vesicles treated with the highest concentrations of *N*-ethylmaleimide

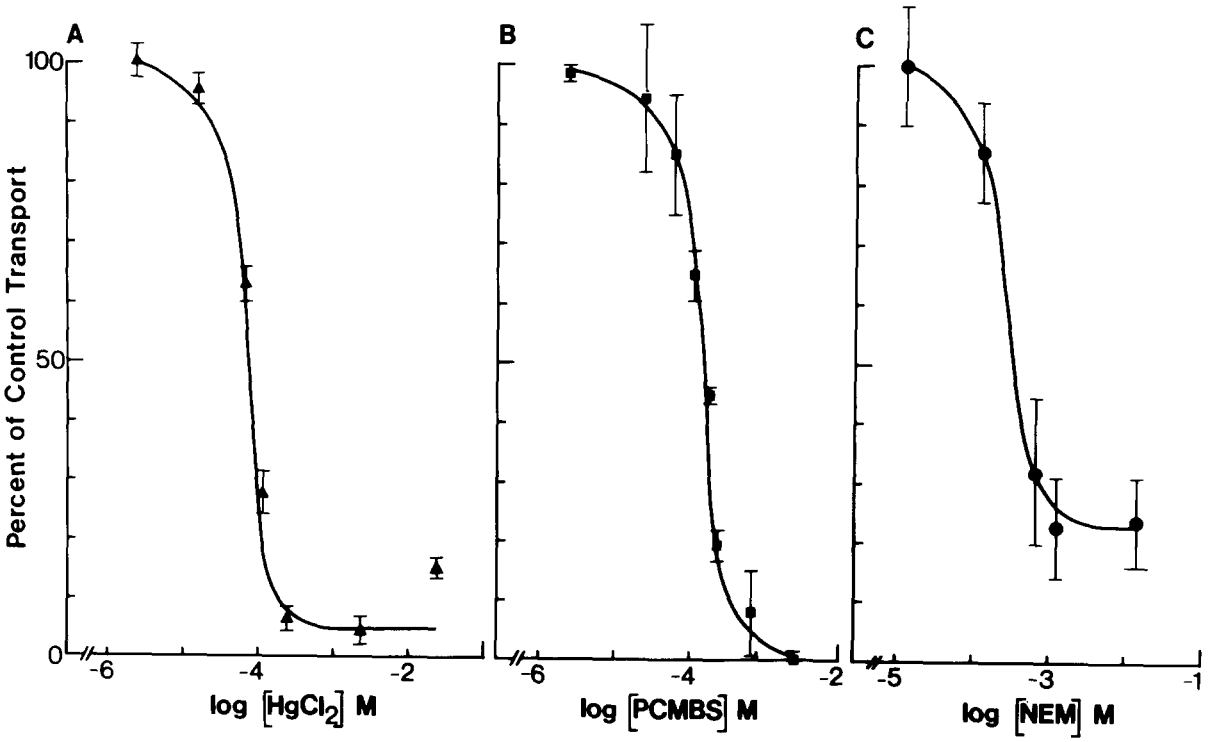

Fig. 2. $HgCl_2$, PCMBS and *N*-ethylmaleimide inactivation of *p*-amino[^{3}H]hippurate transport. Brush-border membrane vesicles prepared in 10 mM Hepes/50 mM potassium gluconate/200 mM mannitol (pH 7.5) were preequilibrated with $HgCl_2$ (▲, panel A), PCMBS (■, panel B) or *N*-ethylmaleimide (●, panel C) for 30 min. The influx was started by adding 50 μM *p*-amino[^{3}H]hippurate (90 μl) prepared in 10 mM Hepes/50 mM potassium gluconate/200 mM mannitol (pH 6.5) to brush-border membrane vesicles (10 μl) for 15 s. This was done in the presence and absence of 0.50 mM probenecid to determine the specifically mediated transport (see Methods). Data are presented as percentages of control transport. Each symbol gives the mean ± S.E. for 2–4 experiments, each conducted in quadruplicate. 1–4 different membrane preparations were employed. The protein concentration in the reaction vessel was 5 mg/ml.

and $HgCl_2$ employed (see Fig. 2) were 3.2 ± 0.1 ($P < 0.05$) and 4.4 ± 0.2 μl per mg of protein ($P < 0.02$), respectively. Concentrations of PCMBS at the IC_{50} value produced intravesicular volumes similar to the control (3.4 ± 0.2 μl per mg of protein, $P < 0.05$). However, higher concentrations of PCMBS produced smaller intravesicular volumes. This is believed to be a result of increased nonspecific membrane permeability. The observations are consistent with the ability of organic mercurials to increase membrane permeability to various ions (for review see Ref. 19).

TABLE I

INHIBITORY CONSTANTS FOR SULFHYDRYL-MODIFYING REAGENTS

The IC_{50} values from the inactivation of *p*-aminohippurate transport by various sulfhydryl-modifying reagents is presented. The data represent means ± S.E. of four experiments each conducted in quadruplicate employing three different membrane preparations.

Reagent	IC_{50}	
	μM	nmol/mg protein
N-ethylmaleimide	190 ± 40	44 ± 9
PCMBS	76 ± 16	17 ± 4
$HgCl_2$	38 ± 12	9 ± 3

It is conceivable that the observed inhibition was due to an alteration in membrane permeability to OH^-, thereby affecting the driving force, rather than an alteration in the transporter itself. This possibility was tested by examining the effect of PCMBS and *N*-ethylmaleimide on *p*-aminohippurate transport under nongradient-stimulated conditions (i.e., where pH inside = pH outside). Brush-border membrane vesicles were treated with

either 0.5 mM PCMBS or 0.5 mM *N*-ethylmaleimide for 30 min, washed, and assayed for *p*-aminohippurate transport in a medium at same pH value (7.5). The specifically mediated transport was calculated and compared to control brush-border membrane vesicles not exposed to these reagents but otherwise treated in an identical manner. The *p*-aminohippurate transport capacity in the controls was 74.9 ± 20.5 pmol/min per mg protein (mean ± S.E. of three experiments each conducted in quadruplicate). *p*-Aminohippurate

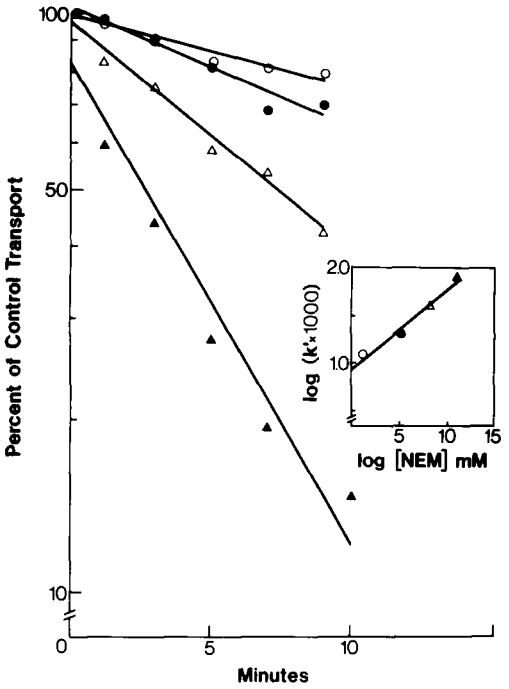

Fig. 3. Time course of *N*-ethylmaleimide inactivation of *p*-amino[^{3}H]hippurate transport into brush-border membrane vesicles. Brush-border membrane vesicles prepared in 10 mM Hepes/50 mM potassium gluconate/200 mM mannitol (pH 7.5) were first equilibrated for 30 min at 25°C. Various concentrations of *N*-ethylmaleimide were added to the brush-border membrane vesicles (○, 130 μM; ●, 325 μM; △, 650 μM; ▲, 1.3 mM). At the appropriate time interval an aliquot of brush-border membrane vesicles was removed (10 μl). 15 s influx was measured by adding 50 μM *p*-amino[^{3}H]hippurate prepared in 10 mM Hepes/50 mM potassium gluconate/200 mM mannitol (pH 6.5). This was done in the presence and absence of 0.50 mM probenecid. Data are expressed as percents of control transport. Each symbol represents the mean for 1–3 different membrane preparations performed in quadruplicate. Inset: Double-logarithmic plot of observed pseudo-first-order rate constants of inactivation, k', min^{-1} (calculated from the linear regressions) vs. *N*-ethylmaleimide concentration. For convenience in evaluating the data, the inactivator concentration was multiplied by 10. The slope of the line is 0.8 ($r = 0.9889$). The final protein concentration in the reaction vessel was 5 mg/ml.

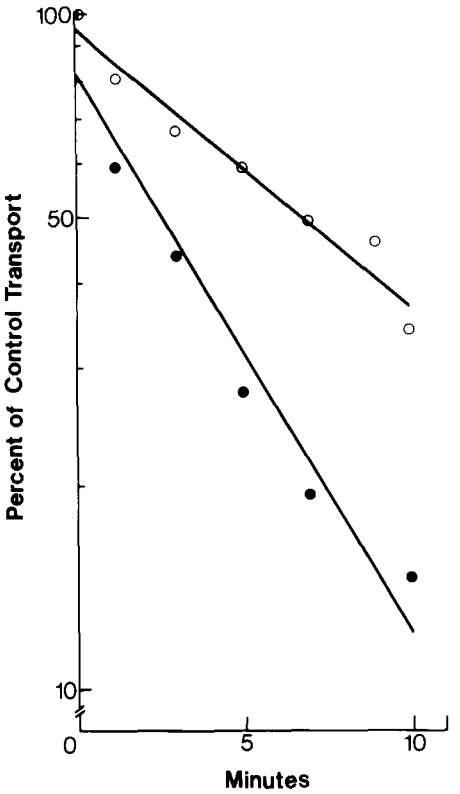

Fig. 4. Effect of substrate on the rate of *N*-ethylmaleimide inactivation of *p*-amino[^{3}H]hippurate transport into brush-border membrane vesicles. Brush-border membrane vesicles prepared in 10 mM Hepes/50 mM potassium gluconate/200 mM mannitol (pH 7.5), were first preequilibrated with 1.25 mM *p*-aminohippurate (○), or buffer (●), for 30 min and then with 1.3 mM *N*-ethylmaleimide. At the appropriate time interval an aliquot of brush-border membrane vesicles was removed (10 μl) and 15 s influx was measured by adding 50 μM *p*-amino[^{3}H]hippurate (90 μl) prepared in 10 mM Hepes/50 mM potassium gluconate/200 mM mannitol (pH 6.5). This was done in the presence and absence of 0.50 mM probenecid in order to determine the specifically mediated transport. Data are presented as percentage of control transport. Each symbol represents the mean ± S.E. for three different membrane preparations performed in quadruplicate. From the linear regression the $t_{1/2}$ values of the inactivation were calculated. In the absence of substrate $t_{1/2} = 3.7$ min ($r = -0.9778$) and in the presence of 1.25 mM *p*-aminohippurate $t_{1/2} = 7.4$ min ($r = -0.9802$). The final protein concentration in the reaction vessel was 5 mg/ml.

transport was less than 1 pmol/min per mg protein in the PCMBS-treated membranes and was 15.6 ± 9.4 pmol/min per mg protein in the *N*-ethylmaleimide-treated brush-border membrane vesicles.

Brush-border membrane vesicles inactivated with PCMBS (IC_{50} concentration) were treated with an exogenous thiol, L-cysteine (20 mM) to restore transport. The excess cysteine will cause the equilibrium to shift from the PCMBS-protein sulfhydryl complex to a PCMBS-cysteine complex, thereby resulting in recovery of the protein sulfhydryl group. This procedure restored 20% of the transport. The inability to totally restore transport may reflect irreversible denaturation of the protein (for reviews see Refs. 19, 20).

The rate of *p*-aminohippurate inactivation by *N*-ethylmaleimide was examined and was consistent with an apparent pseudo-first-order-reaction mechanism (Fig. 3). A double-logarithmic plot of the apparent rate constants vs. the inactivator concentration gave a straight line with a slope of 0.8 (Fig. 3, inset). The data support a simple bimolecular reaction mechanism and imply that in the rate-limiting step, one molecule of *N*-ethylmaleimide inactivates one sulfhydryl group per active transport unit. The effect of substrate (*p*-aminohippurate) on the rate of the *N*-ethylmaleimide inactivation of anion driven *p*-aminohippurate transport was examined (Fig. 4). *p*-Aminohippurate altered the reactivity of sulfhydryls to *N*-ethylmaleimide. The $t_{1/2}$ values for the inactivation in the presence and absence of substrate were 7.4 and 3.7 min, respectively.

The disulfide-reducing agent, dithiothreitol, did not affect transport at concentrations up to 25 mM. This observation makes it unlikely that *p*-aminohippurate–hydroxyl exchange occurs via a disulfide–sulfhydryl exchange mechanism.

Discussion

The results demonstrate that sulfhydryl groups are essential for *p*-aminohippurate transport in the canine brush-border membrane. The finding that *p*-aminohippurate alters their reactivity suggests that they are present at or near the active site. However, one cannot preclude the possibility that substrate binding, rather, affects the reactivity of a sulfhydryl group at another site on the transporter. At this time, we cannot discriminate between neighboring or distal sulfhydryls. Since these reagents used are highly specific for sulfhydryl groups, in all likelihood the results reflect sulfhydryl modification [19–21]. Among the mercurials, the organic compounds such as PCMBS are believed to be the most specific sulfhydryl modifiers [22]. *N*-Ethylmaleimide, although it has a preference for sulfhydryl groups, can interact with the ε-amino of lysine although at a rate of two orders of magnitude slower [21]. Therefore, we think the data are most consistent with sulfhydryl modification. We can exclude the possibility that the findings were due to changes in the permeability of the membrane to OH^- because: first, *p*-aminohippurate transport under nongradient-stimulated conditions was decreased to a similar extent to that found under gradient-stimulated conditions; and second, in the presence of substrate (*p*-aminohippurate) the rate of inactivation by *N*-ethylmaleimide was less and the 'protected' brush-border membrane vesicles were capable of pH-driven transport.

Differences in the extent of the inhibition produced by the various sulfhydryl reagents were observed. The inorganic mercurial $HgCl_2$ was more reactive than the organic mercurial PCMBS which in turn was more reactive than the sulfhydryl-alkylating reagent *N*-ethylmaleimide. The higher potency of $HgCl_2$ may be due to its smaller size and to its capability to penetrate and to react with sulfhydryl groups inaccessible to larger molecules such as PCMBS and *N*-ethylmaleimide. Besides being more potent, $HgCl_2$ and PCMBS totally inactivated the specifically mediated *p*-aminohippurate transport, while *N*-ethylmaleimide did not (Fig. 2). There are several possible explanations for *N*-ethylmaleimide-resistant transport. First, the sulfhydryl group(s) may be inaccessible to *N*-ethylmaleimide and second, modifications of some sulfhydryl groups may render others less reactive or completely unreactive to the reagent. It is plausible that there are many sulfhydryls present on the carrier and the binding of several *N*-ethylmaleimide molecules to both endofacial and exofacial sites may be necessary to inactivate the transport. Unexpectedly, we observed that concentrations of $HgCl_2$ greater than 2.5 mM in-

activated transport less than that maximally seen at lower concentrations. Perhaps the anomalous behavior of $HgCl_2$ can be explained by some of the reasons given above for *N*-ethylmaleimide. Another possibility is that the effective concentration is decreased due to complex formation [20].

Our findings with dog brush-border membrane vesicles are quite different from those of Inui et al. [11] who found that *p*-aminohippurate transport in rat brush-border membrane vesicles was unaffected by sulfhydryl-modifying reagents. Several other differences between the two species have been noted. For example, we reported that the cephalosporins share a common transport system with *p*-aminohippurate in the dog brush-border membrane [23]. On the other hand, Inui et al. [11] states that in the rat brush-border membrane, cephalosporin and *p*-aminohippurate transport systems are separate. It is a well-documented phenomenon that various species handle this class of chemical compounds, i.e., organic anions, differently [1,3]. The results presented here raise the interesting possibility that the organic anion exchangers present in the brush-border membrane of the various species may in fact be very different proteins, suggesting that a molecular basis underlies the functional differences.

Others have shown that *p*-aminohippurate transport in the rat basolateral membrane is sensitive to sulfhydryl modification [9,10]. Unfortunately, the effect of substrate on sulfhydryl reactivity was not examined in these studies, precluding comparison with our results. Also, they were unable to achieve complete inactivation, in contrast to our findings with the brush-border membrane. Additionally, Tse et al. [9] found that the basolateral membrane transporter was most sensitive to PCMBS, intermediate to $HgCl_2$ and least to *N*-ethylmaleimide. This is consistent with an interpretation that the transporters in the two membranes are different. Interestingly, they found an *N*-ethylmaleimide-resistant fraction of *p*-aminohippurate transport and that some high concentrations of $HgCl_2$ were not as effective at inhibiting transport as were the somewhat lower concentrations.

Many membrane-transport systems are known to be sensitive to sulfhydryl-group modification [19,22]. The question is what role, if any, do these sulfhydryl groups have in the transport process. It is difficult to give a definitive interpretation of these modification studies, since a bulky group has been added to the transporter, and how that might affect the transport mechanism is unclear at this time. Some have postulated that disulfide–sulfhydryl exchange mechanisms are involved in transport systems [24]. In fact, we have raised the possibility that organic cations are transported in canine brush-border membrane vesicles by a disulfide–sulfhydryl exchange [18]. The basis for our hypothesis is that organic cation transport is inhibited by both disulfide- and sulfhydryl-modifying reagents, and that the inactivation is prevented by the presence of substrate. On the other hand, we were unable to uncover any effect of reductants on *p*-aminohippurate transport in dog brush-border membrane vesicles. Based upon this information, it appears unlikely that disulfide–sulfhydryl exchange plays a part in *p*-aminohippurate transport in brush-border membrane vesicles.

In conclusion, sulfhydryl modifying reagents inactivate *p*-aminohippurate transport in the brush-border membrane and the substrate (*p*-aminohippurate) protects against the inactivation, suggesting that thiols play a dynamic role in the transport process. Our findings also suggest that there may be a molecular basis underlying the known differences in the renal handling of organic anions by different species. Presumably, the active site of these transport proteins are highly conserved; however, our data illustrate that the overall primary structures may be quite different.

Acknowledgements

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