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Inhibition of Renal Na^+-Pi Cotransporter by Mercuric Chloride: Role of Sulfhydryl Groups

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Abstract We studied the role of sulfhydryl groups in Na^+-Pi cotransport across the renal brush border membrane (BBM), using $HgCl_2$, an agent which penetrates membranes freely. $HgCl_2$ inhibited the initial Na^+-dependent ^{32}Pi transport in a dose-dependent manner (IC_{50} = 54 μM). Na^+-independent transport was not affected. The inhibitory effect persisted under Na^+ equilibrium–exchange conditions. Additionally, $HgCl_2$ had no effect on the diffusional uptake of ^{22}Na up to 1 min incubation. Exposure to $HgCl_2$ had no effect on vesicle integrity as determined by osmotic shrinking experiments. BBM vesicle (BBMV) volume, determined by D-glucose equilibrium uptake, was not affected at low $HgCl_2$ concentrations, but decreased at higher concentrations (> 100 μM). Vesicle volumes, determined by flow cytometry, were not changed after exposure to $HgCl_2$. Kinetic studies showed a reduction in the apparent Vmax for Pi transport from 1.40 ± 0.13 to 0.75 ± 0.19 nmoles/mg protein/5 sec, without a significant change in the apparent Km. In protection studies, dithiothreitol (DTT) completely protected against inhibition, but Pi, phosphonoformic acid (PFA), and Na^+ gave no protection. The data suggest that sulfhydryl groups are essential for the function of Na^+-Pi cotransporter of renal BBM.

Key words: phosphate transport, sulfhydryl, mercuric chloride, brush border membrane

Inorganic phosphate (Pi) is transported across the renal proximal tubular brush border membrane (BBM) via an active Na^+-dependent carrier-mediated transport mechanism [1,2]. Phosphate regulatory factors (hormonal, metabolic, and dietary) exert their effects through this Na^+-dependent transport [1,2]. It is postulated that Pi transport is effected through a Na^+-Pi cotransporter, an integral protein which spans the membrane with binding sites for Pi and Na^+. The putative Na^+-Pi cotransporter has not been isolated and the molecular mechanisms of Pi transport remain largely unknown. Attempts at isolating the Na^+-Pi cotransporter, using direct isolation techniques, have so far been unsuccessful. This is due in part to the very low concentration of the transporter in the membrane [3] as well as the unavailability of suitable probes. Recently, Dousa et al. described the use of phosphonocarboxylic acids as specific inhibitors of Na^+-Pi cotransport in renal and intestinal BBM vesicles (BBMV) [4,5]. Unfortunately, these inhibitors are reversible and not suitable as affinity labels for the isolation of Na^+-Pi cotransporter. Furthermore, they may react with other Na^+-dependent anion transporters [6]. Other derivatives of phosphonocarboxylic acids, particularly halogenated forms, may prove more useful for isolation and eventual purification of the Na^+-Pi cotransporter [7].

The use of indirect methods, aimed at identifying various essential groups associated with the cotransporter, can provide useful information about its structure. Modification of an essential group will result in the inhibition of transport. Additionally, if an essential group is at or near the substrate binding site(s) on the cotransporter, the transport may be protected from inhibition by the substrate or its analogues. Studies of the intestinal Na^+/D-glucose cotransporter have identified several essential groups, including: disulfide bonds [8,9], sulfhydryl [10,11], lysine [12], tyrosine [13,14], and amino

Abbreviations used: Pi: inorganic phosphate; BBM: brush border membrane; BBMV: brush border membrane vesicle; MTH: 300 mM mannitol, Tris HEPES buffer; DTT: dithiothreitol; PFA: phosphonoformic acid (foscarnet); Na_o^+: extravesicular Na^+; Na_i^+: intravesicular sodium; K_o^+: extravesicular K^+; K_i^+: intravesicular K^+; PCMBS: p-chloromercuriphenylsulfonate.

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groups [15,16]. Studies of L-proline transport have also demonstrated the presence of lysine and tyrosine groups [17] at the transporter (imino carrier) site. Modification of arginine [18,19] and tyrosine [20] residues as well as sulfhydryl groups [20] has resulted in the inhibition of Pi transport in renal BBMV.

Preliminary studies, using fluorescent derivatives of phenylglyoxal (an arginine modifying agent), have identified a 130 KDa polypeptide as the putative Na^+-Pi cotransporter of intestinal BBM [21]. Similarly, Debiec and Lorenc have described a 155 KDa protein with affinity binding sites for both Pi and Na^+ [22]. Using the amino acid–modifying agent, N-acetyl-imidazole, Wuarin et al. [23] have identified four proteins of 31, 53, 104, and 176 KDa as possible candidates for Na^+-Pi cotransporter in OK cells, an established renal epithelial cell line.

To determine whether sulfhydryl groups are involved in the function of the Na^+-Pi cotransporter, we tested the effect of $HgCl_2$, a specific sulfhydryl-modifying agent, on Pi transport across rat renal cortical BBMV. The results show that sulfhydryl groups are essential for the function of renal Na^+-Pi cotransporter, but are not involved in Pi or Na^+ binding.

MATERIALS AND METHODS

Preparation of BBMV

Sprague-Dawley rats weighing 250–300 g were used in all experiments. BBMV were prepared by a divalent cation precipitation method [5,24]. After bilateral nephrectomy under Na^+ pentobarbital anesthesia (50 mg/kg B.W./IP), the cortices were dissected and suspended in a buffer containing 50 mM mannitol, 2 mM Tris/HEPES (pH 7.5). After homogenization with a glass-teflon homogenizer and further dilution in the same buffer, the tissue was again homogenized with a Polytron homogenizer. To this homogenate, $CaCl_2$ was added to a final concentration of 10 mM, and periodically agitated for 20 min. After centrifugation at 2,000*g* for 10 min, the supernatant was centrifuged at 35,000*g* for 30 min. The pellet was resuspended in a buffer containing 300 mM mannitol, 5 mM Tris/HEPES (pH 7.5) (MTH buffer), and homogenized using a tight-fitting Dounce homogenizer and recentrifuged at 35,000*g* for 20 min. The final pellet was resuspended in MTH buffer and used for transport studies.

In most experiments BBMV were exposed to $HgCl_2$ for an appropriate time (generally 5 min at 20°C), then washed in 15 ml of ice-cold MTH buffer and centrifuged at 35,000*g* for 20 min. The pellet was resuspended in MTH and used for transport measurements. The stocks of $HgCl_2$ were protected from light and used within 2 weeks. The protein concentration of the final BBMV suspension was always adjusted to ~7 mg/ml. Other experimental conditions are described in legends to figures.

Transport Measurements

Transport measurements were carried out at 20°C on freshly prepared BBMV, using a rapid filtration method as previously described [4,5,32]. The stop solution contained 135 mM NaCl, 10 mM $NaHAsO_4$, 5 mM Tris/HEPES (pH 7.5). Preliminary experiments showed that treatment of BBMV with $HgCl_2$ does not interfere with the inhibitory effect of arsenate in the stop solution. The percent inhibition of ^{32}Pi transport by 1 mM arsenate was $-46.1 \pm 2.6\%$ in BBMV exposed to $HgCl_2$, compared to $-54.4 \pm 2.1\%$ in control BBMV ($n = 3$). These differences were not statistically significant.

Media for transport contained, in final concentrations: 100 mM NaCl or KCl, 100 mM mannitol, 5 mM Tris/HEPES (pH 7.5), and either 0.1 mM ^{32}Pi or 0.05 mM D-[^{3}H] glucose. The uptake in the presence of KCl was less than 3% of the total uptake in the presence of NaCl, and was not routinely measured. In experiments where ^{22}Na diffusional uptake was measured, the uptake medium contained 100 mM $^{22}NaCl$, 100 mM mannitol, 5 mM Tris/HEPES (pH 7.5) and the stop solution contained 150 mM KCl, 10 mM Tris/HEPES (pH 7.5). The radioactivity retained after washing the filters with incubation media (blanks) was subtracted from sample counts. For ^{22}Na, the blanks were $29 \pm 4\%$ and $10 \pm 1\%$ of sample counts at 15 sec and 90 min respectively ($n = 3$). For other solutes, the blanks were $<10\%$ of sample counts. All measurements were carried out in quadruplicate and the results expressed relative to BBMV protein concentration determined by the Lowry method [25]. Each experiment was repeated at least twice on separate BBMV preparations.

Flow Cytometry

For measurement of BBMV dimensions by flow cytometry, following exposure to $HgCl_2$ and centrifugation, BBMV were suspended in MTH buffer at a final protein concentration of 0.5–1.0 mg/ml. Cross-sectional areas and volumes were

determined by linear regression from mean forward light scatter measurements, using beads with diameters ranging from 0.48 to 1.33 μm as standards. For each determination, 6,000 to 10,000 vesicles were counted. The following formulae were used for calculations: cross-sectional area = $\pi D^3/4$; volume = $\pi D^3/6$, where D is the cross-sectional diameter.

Enzymatic Assays

The BBM enzymes routinely measured included: alkaline phosphatase, gamma-glutamyltransferase, maltase, and leucine aminopeptidase. These were measured by colorimetric methods previously described [26,27,28]. Compared to the crude homogenate, BBM-associated enzymes were enriched 11 to 14 times. Na^+-K^+ ATPase was measured by a modification of the method of Kinsolving et al. [29]. The activity of this enzyme was enriched 0.80 ± 0.12 times (n = 8). Succinate dehydrogenase (a mitochondrial enzyme) was determined by the method of Pennington [30]. Its activity was markedly reduced (0.04 ± 0.01 times, n = 13).

The results are expressed as mean ± SE of several experiments or of replicate samples. Data comparisons were made by Student's t test for group or paired comparisons. Where appropriate, one-way ANOVA was used for multiple comparisons. Values of $P \geq 0.05$ were considered nonsignificant.

RESULTS

Mercuric chloride, which penetrates biological membranes freely and covalently binds to proteins, was used to assess the relationship of sulfhydryl groups with the Na^+-Pi cotransporter of the BBM. In preliminary studies $HgCl_2$ proved to be the most potent among several sulfhydryl reagents tested. These reagents included p-chloromercuribenzoate (PCMB), p-chloromercuriphenylsulfonate (PCMBS), N-ethylmaleimide (NEM), and 5,5′-dithio-bis-(2-nitrobenzoate) (DTNB). The inhibitory effect on ^{32}Pi transport was not reversible by extensive (3×) washing, consistent with covalent modification by $HgCl_2$.

Effect of $HgCl_2$ on Linearity of ^{32}Pi Transport

Under our experimental conditions, Na^+ gradient–dependent ^{32}Pi transport was linear at least up to 15 sec incubation. Exposure of BBMV to $HgCl_2$ resulted in a significant inhibition of ^{32}Pi transport at all incubation times studied. After exposure of BBMV to $HgCl_2$, ^{32}Pi transport remained linear at least up to 20 sec (Fig. 1). Fifteen-second transport was, therefore, used for most subsequent experiments.

Time-Dependency of Inhibition

To determine the dependency of inhibition on incubation time, BBMV were preincubated with $HgCl_2$ (50 μM) or MTH buffer (controls) for varying time periods ranging from 1 to 60 min, washed and repelleted, and ^{32}Pi transport was determined. Inhibition of the initial Na^+-dependent Pi transport increased with preincubation time with $HgCl_2$, up to 5 min. Beyond 5 min, there was no further inhibition (Fig. 2). There was no change in ^{32}Pi transport after preincubation with MTH buffer (controls) for up to 60 min. In subsequent experiments, 5 min incubation times were used.

Dose-Response Studies

$HgCl_2$ inhibited the initial Na^+ gradient–dependent ^{32}Pi transport in a dose-dependent manner, with a half maximal inhibitory concentration (IC_{50}) calculated from Dixon plots, of approximately 54 μM (Fig. 3). The inhibitory concentration was dependent upon the protein concentration of the BBM at the time of exposure to $HgCl_2$. Increasing membrane protein concentration resulted in a decrease in the inhibitory effect of $HgCl_2$ on ^{32}Pi transport (Table I). To eliminate the variability between different experiments, BBMV protein concentration was always adjusted to ~7 mg/ml (chosen arbitrarily) prior to incubation with $HgCl_2$.

Effect on Time Course of ^{32}Pi Transport

In the presence of 100 mM Na^+ gradient, ^{32}Pi transport exhibited a rapid increase with time (Fig. 4), reaching a maximum at about 1 min (overshoot phenomenon), followed by a gradual efflux of ^{32}Pi from the vesicles, reaching an equilibrium (measured at 120 min). Uptake in the presence of a K^+-gradient was markedly reduced and represented less than 2% of that in the presence of Na^+. It increased gradually, reaching an equilibrium without overshoot. When ^{32}Pi transport in BBMV exposed to $HgCl_2$ was compared to controls, the initial Na^+-dependent ^{32}Pi transport was markedly reduced, with a significant reduction in the height of the overshoot. The percent inhibition was 41.8 ± 7.2% at 15 sec, 43.6 ± 6.0% at 1 min ($P < 0.05$),

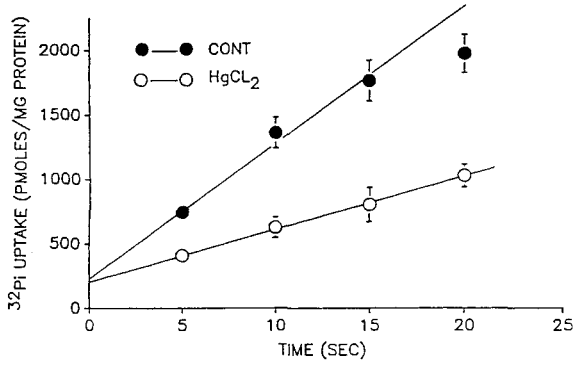

Fig. 1. Linearity of ^{32}Pi transport after exposure to $HgCl_2$. BBMV were incubated with $HgCl_2$ (50 μM) or MTH (control) for 5 min at 20°C, then washed and centrifuged at 35,000g for 20 min. ^{32}Pi transport was measured at times indicated, in the presence of 100 mM Na^+ gradient. Results are mean ± SE of three separate experiments. r = 0.98 for controls and 0.97 for $HgCl_2$.

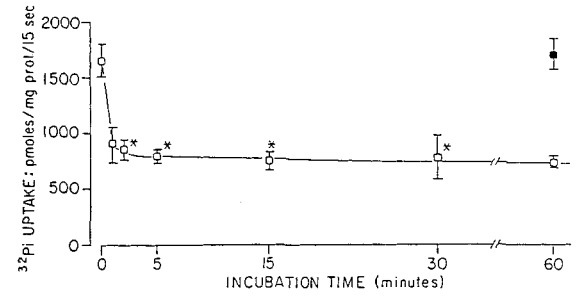

Fig. 2. Time-dependency of $HgCl_2$ effect on ^{32}Pi transport. BBMV were incubated with $HgCl_2$ (50 μM) or MTH (control), at 20°C, for various times shown, then washed and centrifuged. ^{32}Pi transport was measured at 15 sec in the presence of 100 mM Na^+ gradient. Control (-■-), $HgCl_2$ (-□-). *P < 0.05, paired t test (n = 4).

TABLE I. Dependency of ^{32}Pi Transport Inhibition on BBMV Protein Concentration*

Protein concentration (mg/ml)	^{32}Pi transport (pmoles/mg/15 sec)	% inhibition
18.6	1202 ± 97	23
10.1	975 ± 14	51
5.1	362 ± 4	77
2.5	55 ± 3	96
0.8	31 ± 3	98

*BBMV were suspended in MTH buffer at various protein concentrations and incubated with $HgCl_2$ (50 μM) for 5 min at 20°C. After washing, ^{32}Pi transport was measured at 15 sec in the presence of 100 mM Na^+ gradient. Results shown are of a representative experiment performed in quadruplicate and repeated twice. Control uptake was 1560 ± 60 pmoles/mg/15 sec.

and 27.8 ± 2.9% at 120 min (P = 0.25) (n = 3). Na^+-independent ^{32}Pi transport (Na^+ replaced by K^+) was not affected by $HgCl_2$ at any time point.

Effect on Na^+ Conductance

Since the inhibitory effect of $HgCl_2$ on ^{32}Pi transport might be secondary to dissipation of the Na^+ gradient ($Na_o^+ > Na_i^+$) imposed at the beginning of the transport measurements, Na^+-dependent ^{32}Pi transport was determined under Na^+ equilibrium–exchange conditions ($Na_o^+ = Na_i^+$), thus eliminating the Na^+ gradient (Fig. 5). Under these conditions, Na^+-dependent ^{32}Pi transport did not show an overshoot. The inhibitory effect of $HgCl_2$ persisted under Na^+ equilibrium–exchange conditions (−24 ± 6% at 15 sec

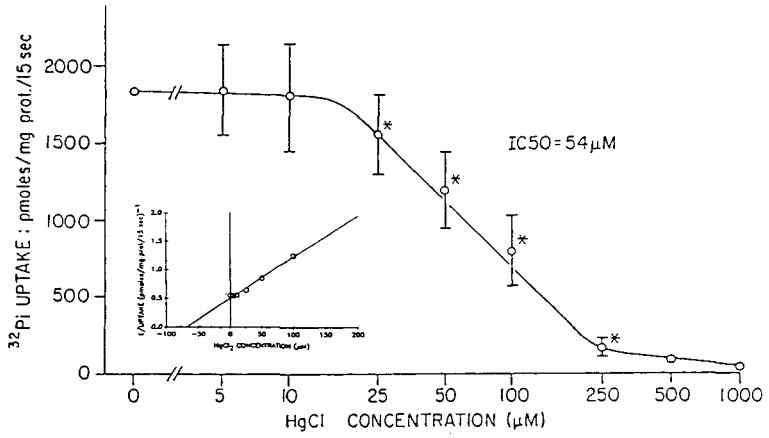

Fig. 3. Dose-dependency of inhibition of ^{32}Pi transport by $HgCl_2$. BBMV were exposed to different concentrations of $HgCl_2$ for 5 min, at 20°C, then washed and centrifuged. ^{32}Pi transport was measured at 15 sec in the presence of 100 mM Na^+ gradient. Results are shown as a semi-logarithmic plot. Insert: Dixon plot of the same data. *P < 0.05, one way ANOVA and paired t test (n = 4).

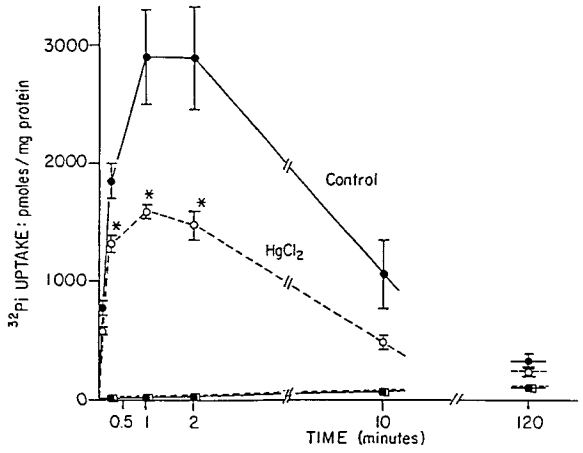

Fig. 4. Effect of $HgCl_2$ on the time course of ^{32}Pi transport under Na^+ gradient conditions. BBMV were incubated with $HgCl_2$ (50 μM) for 5 min at 20°C, then washed and centrifuged. ^{32}Pi transport was measured at various incubation times given, in the presence of either 100 mM Na^+ (-●-, -○-) or K^+ (-■-, -□-) gradient. (n = 3). *$P < 0.05$, group t test.

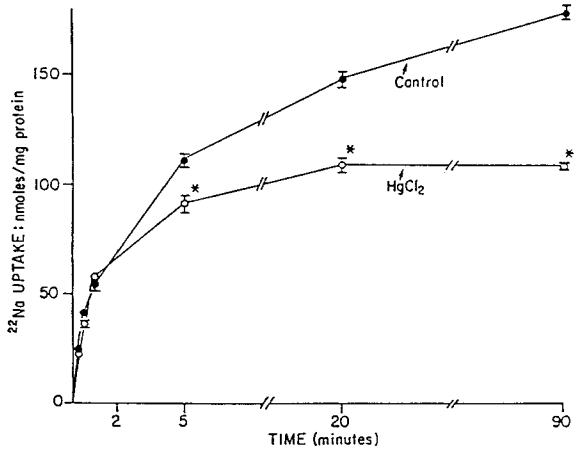

Fig. 6. Effect of $HgCl_2$ on ^{22}Na diffusional uptake. BBMV were incubated with 100 μM $HgCl_2$ for 5 min at 20°C, then washed in MTH buffer and centrifuged. ^{22}Na uptake was determined at various incubation times, in a medium containing 100 mM $^{22}NaCl$, 100 mM mannitol, 5 mM Tris/HEPES (pH 7.5). Results are mean ± SE of a representative experiment performed in quadruplicate and repeated twice. *$P < 0.05$.

and −23 ± 3% at 1 min), indicating that it is independent of a possible effect on Na^+ conductance. When ^{32}Pi transport was determined under K^+ equilibrated conditions ($K_o^+ = K_i^+$), the initial uptake was approximately 40% of that in the presence of Na^+. Furthermore, ^{32}Pi transport under K^+ equilibrium conditions was similar in BBMV exposed to $HgCl_2$ compared to control BBMV.

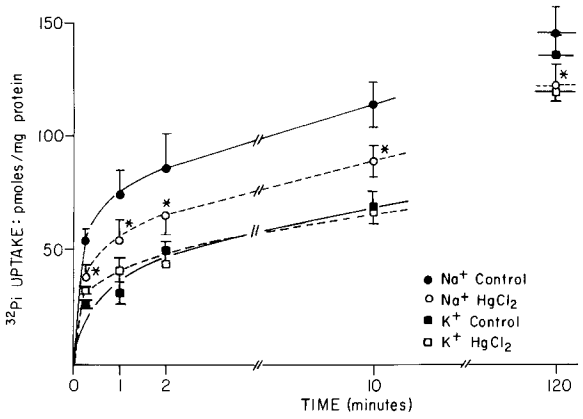

Fig. 5. Effect of $HgCl_2$ on the time course of ^{32}Pi transport under equilibrium-exchange conditions. BBMV were suspended at 20°C for 60 min in media containing 200 mM mannitol, 5 mM Tris/HEPES (pH 7.5) and 50 mM NaCl (-●-, -○-) or KCl (-■-, -□-), then washed and resuspended in the same media. BBMV were then incubated with 50 μM $HgCl_2$ for 5 min at 20°C, then washed and resuspended in same media. ^{32}Pi transport was measured at various incubation times indicated. Uptake media were same as preloading media, but contained, in addition, 0.1 mM $K_2H^{32}PO_4$ (n = 2–6). *$P <$ 0.05.

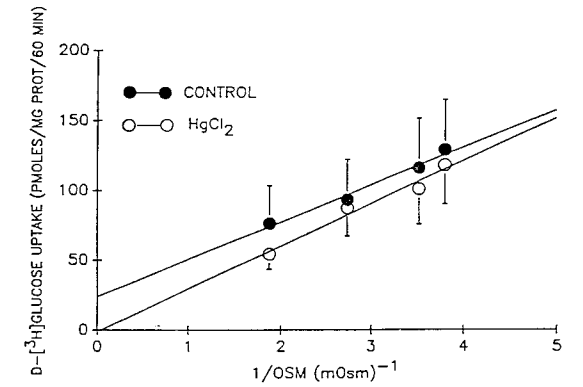

Fig. 7. Effect of $HgCl_2$ on vesicle integrity. BBMV were prepared in a medium containing 200 mM mannitol, 5 mM Tris/HEPES (pH 7.5). After exposure to 50 μM $HgCl_2$, for 5 min at 20°C, BBMV were washed twice and repelleted in the same medium. Twenty microliters of BBMV (150 μg protein) were added to 80 μl of transport media with varying osmolarities obtained by the addition of sucrose. Equilibrium uptake of D-[^{3}H]-glucose was determined after 60 min incubation. Results are mean ± SE of four separate experiments. (-●-) control, (-○-) $HgCl_2$. r = 0.98 for control and 0.95 for $HgCl_2$.

In additional experiments, the effect of $HgCl_2$ on Na^+ conductance was directly tested by measuring the time-course of ^{22}Na diffusional uptake in BBMV exposed to $HgCl_2$. The results of a representative experiment are shown in Figure 6. $HgCl_2$ had no effect on ^{22}Na uptake up to 1 min incubation.

TABLE II. Effect of $HgCl_2$ on BBMV Dimensions*

	Control	50 μM	250 μM	500 μM
Diameter (μm)	0.704 ± 0.07	0.690 ± 0.09	0.643 ± 0.05	0.619 ± 0.03
Volume (μm^3)	0.458 ± 0.06	0.470 ± 0.01	0.437 ± 0.130	0.335 ± 0.05

*BBMV were preincubated with different concentrations of $HgCl_2$ for 5 min, followed by washing and centrifugation at 35,000*g* for 20 min. Final pellets were resuspended in MTH buffer and used for flow cytometry. Results are mean ± SE of three experiments. Mean diameter and volume were not significantly different from controls.

Effect of $HgCl_2$ on Vesicle Integrity

Following exposure to $HgCl_2$, BBMV were washed twice and equilibrium uptake of D-[^{3}H]-glucose was determined after 60 min incubation in media with varying osmolarities obtained with the addition of sucrose. When the data were plotted as glucose uptake versus the reciprocal of medium osmolarity, a linear relationship was obtained both in control BBMV and in BBMV exposed to $HgCl_2$ (Fig. 7). The results suggest that these BBMV behave as sealed, osmotically active vesicles. The vesicle integrity is, therefore, not affected by prior treatment with $HgCl_2$.

Effect of $HgCl_2$ on Vesicle Volume

Since the uptake of solutes by BBMV is directly proportional to their volume, it can be surmised that the reduction of Na^+-dependent ^{32}Pi transport may be secondary to a reduction of the vesicle volume by $HgCl_2$. To test this possibility, BBMV volume was determined by flow cytometry. Following exposure to $HgCl_2$, BBMV were washed and repelleted, then resuspended in MTH at a final concentration of 1 mg/ml protein. The mean BBMV volume and cross-sectional diameter were not significantly different between control BBMV and BBMV exposed to 50 to 500 μM $HgCl_2$ (Table II).

Another commonly used method for evaluation of vesicle volume change is the measurement of the equilibrium uptake of D-[^{3}H]-glucose. Using this method, BBMV volume was not significantly affected by concentrations of $HgCl_2$ as high as 100 μM. Above 100 μM, there was a significant reduction in BBMV volume (Fig. 8). The concentration of $HgCl_2$ which produced 50% reduction of vesicle volume was 240 μM, significantly higher than the IC_{50} for ^{32}Pi transport inhibition. Since $HgCl_2$ can affect D-glucose transport [9], it is, therefore, possible that complete equilibrium could not be achieved with our experimental conditions, explaining the discrepancy between D-glucose equilibrium uptake and flow cytometry.

Effect on Stoichiometry of Na^+-Pi Cotransport

Stoichiometry was determined in BBMV exposed to $HgCl_2$, by the measurement of Na^+-dependent ^{32}Pi transport in the presence of Na^+ concentrations ranging from 10 to 150 mM. The mean Hill coefficients were 2.0 ± 0.22 and 1.86 ± 0.14 for control and $HgCl_2$-treated vesicles, consistent with a stoichiometry of 2 Na^+:1 Pi under both conditions.

Kinetic Experiments

Kinetic experiments were conducted after exposure of BBMV to 100 μM $HgCl_2$ (Fig. 9). Na^+-dependent ^{32}Pi transport was measured at 5 sec, in media with increasing ^{32}Pi concentrations. Analysis of the data by double-reciprocal plots showed a reduction in the apparent Vmax of ^{32}Pi transport from 1.40 ± 0.13 to 0.75 ± 0.19 nmoles/mg protein/5 sec ($P < 0.05$), with no significant change in the apparent Km (77 ± 10 μM vs. 107 ± 19 μM for control and $HgCl_2$ respectively).

Protection Studies

To determine the location of the essential SH groups within the Na^+-Pi cotransporter, we tested the ability of substrates (Na^+, Pi) and a specific inhibitor of ^{32}Pi transport, namely phosphonoformic acid (PFA), to protect against inhibition by $HgCl_2$. BBMV were first incubated with the tested compounds, added at 20- to 1,000-fold molar excess compared to $HgCl_2$, followed by the addition of 50 μM $HgCl_2$ (Table III). As expected, the sulfhydryl reducing agent, dithiothreitol (DTT), completely protected against the inhibitory effect of $HgCl_2$ on ^{32}Pi transport. However, neither Pi nor PFA afforded any protection. The results were not different in the presence or absence of 100 mM NaCl at the time of incubation. Similarly, Na^+ alone did not have any protective effect on transport inhibition. Identical results were obtained when various substrates were added following

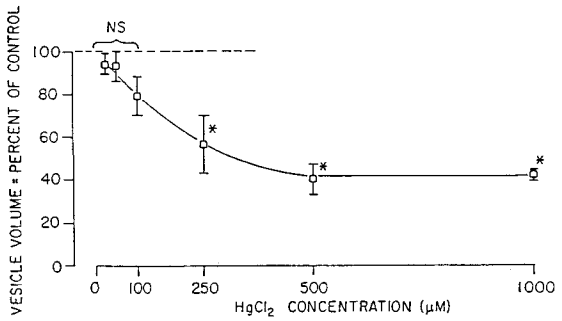

Fig. 8. Effect of $HgCl_2$ on vesicle volume. BBMV were incubated with $HgCl_2$ at various concentrations for 5 min at 20°C. After washing and centrifugation, equilibrium uptake of D-[^{3}H]-glucose was measured after 60 min incubation in the presence of 100 mM Na^+ gradient. Results are expressed as percent of control. Control uptake: 67 ± 6 pmoles/mg protein/60 min (n = 4). *$P < 0.05$; NS: not significant.

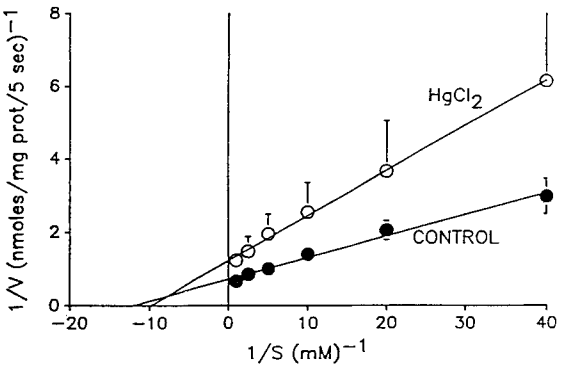

Fig. 9. Kinetics of ^{32}Pi transport inhibition by $HgCl_2$. BBMV were incubated with 100 μM $HgCl_2$ for 5 min at 20°C, repelleted and used for transport measurements. ^{32}Pi transport was measured at 5 sec, in the presence of 100 mM Na^+ gradient, using Pi concentrations ranging from 0.025 to 1.0 mM. Results are mean of three experiments. r = 0.996 for control and 0.998 for $HgCl_2$.

the addition of $HgCl_2$ (displacement or reversibility studies; results not shown).

DISCUSSION

Sulfhydryl groups are structural components of many proteins, including enzymes and cotransporters. These groups have been shown to be essential for the function of Na^+/D-glucose cotransporter of intestinal BBM, as assessed by studies of D-glucose transport and phlorizin binding [8,9].

To determine if sulfhydryl groups play a role in the function of Na^+-Pi cotransporter of renal BBM, we performed covalent modifications of these groups using $HgCl_2$, a potent and specific sulfhydryl-modifying agent. Among several sulfhydryl reagents studied, $HgCl_2$ proved to be the most potent (IC_{50} = 54 μM). As it had been previously reported for intestinal BBMV [9,22], the inhibitory potency was directly proportional to the protein concentration of the BBMV at the time of exposure to $HgCl_2$. This suggests that a large number of reactive sites are present on the BBM, thus reducing the concentration of the available inhibitor. The IC_{50} value, as calculated, may therefore overestimate the inhibitory potency of $HgCl_2$.

TABLE III. Protection of $HgCl_2$-Induced Inhibition of ^{32}Pi Transport*

1st addition	2nd addition	^{32}Pi transport	% change
1. MTH	MTH	1646 ± 87 (11)	—
2. MTH	$HgCl_2$	934 ± 128[a] (11)	−43.2
3. DTT	$HgCl_2$	1921 ± 59[NS] (8)	+16.7
4. K_2HPO_4	$HgCl_2$	881 ± 137[a] (7)	−46.4
5. PFA	$HgCl_2$	854 ± 125[a] (11)	−48.1
6. NaCl	$HgCl_2$	991 ± 139[a] (7)	−39.8

*BBMV were first incubated (10 min, 20°C) with either MTH buffer (control, No. 1), or MTH containing 1 mM DTT or 50 mM NaCl. For Pi and PFA protection, the incubation medium contained 100 mM NaCl and 1 mM Pi or 10 mM PFA. BBMV were then incubated with 50 μM $HgCl_2$ for 5 min at 20°C followed by three washing and centrifugation cycles (35,000*g* for 20 min) with MTH buffer. *32*Pi transport was measured at 15 sec, in the presence of 100 mM Na^+ gradient. Numbers in parentheses refer to the number of experiments. [a]$P < 0.05$ v. control. NS, not significant.

Will and Hopfer [31] demonstrated that the inhibition of transport of neutral amino acids by PCMBS in intestinal BBMV is due to an increase in Na^+ conductance of the membrane. The uptakes of L-valine and D-glucose were not affected by PCMBS when transport was measured under equilibrium conditions. Additionally ^{22}Na uptake measured in the same membranes was increased after PCMBS treatment. Our results differ from those of Will and Hopfer in several points: We studied Pi transport in *renal,* rather than intestinal, BBMV. The inhibitory effect, although reduced, persisted under Na^+-equilibrium conditions. Furthermore, direct measurements of ^{22}Na uptake showed that, in BBMV treated with $HgCl_2$, the uptake was unchanged during the initial time period (<1 min) when ^{32}Pi transport was measured. Taken together, we conclude that sulfhydryl reagents inhibit renal BBMV Pi transport through a direct interaction with the Na^+-Pi cotransporter, and not by an increase in BBMV Na^+ conductance. Will and Hopfer [31] also found that

intravesicular volume (as measured by D-glucose equilibrium uptake) was decreased after treatment of BBMV with high concentrations of PCMBS (> 0.5 mM). However, the mean vesicle volume calculated by electron microscopy decreased much less than the volume measured by D-glucose equilibrium uptake. Furthermore, this effect was completely reversed by DTT, indicating that changes in vesicle shape rather than true volume were present. Our results on the effect of $HgCl_2$ on vesicle volume are in agreement with those of Will and Hopfer. $HgCl_2$ reduced vesicle volume, as determined by D-glucose equilibrium uptake, at high concentrations (> 100 μM). However, significant inhibition of Pi transport was observed at concentrations which did not produce a significant change in volume. It is therefore unlikely that Pi transport inhibition by mercurials is solely due to a reduction of vesicle volume. To determine the possible contribution of decreased vesicle volume on the IC_{50} of $HgCl_2$, the dose-response data from Figure 3 were recalculated after correcting the uptake at each $HgCl_2$ concentration for the corresponding vesicle volume derived from data in Figure 8. The corrected IC_{50} was not significantly higher than that obtained without correcting for volume changes (67.2 ± 4.1 μM vs. 53.8 ± 5.4 μM). It should be pointed out, however, that measurements of vesicle volume by flow cytometry clearly demonstrated a lack of effect of $HgCl_2$ on vesicle volume, even at high concentrations (Table II). This also indicates that D-glucose equilibrium uptake may not be a reliable measure of vesicle volume after exposure to $HgCl_2$.

The osmotic shrinking experiments performed after exposure of BBMV to $HgCl_2$ demonstrate the presence of osmotically active, sealed vesicles, suggesting that vesicle integrity is maintained.

The effect of $HgCl_2$ may not be totally specific for sulfhydryl groups. Besides sulfhydryl groups, $HgCl_2$ can, in principle, react with other groups on the proteins, including carboxyl and amino groups. However, the affinity of $HgCl_2$ for sulfhydryl groups is much higher [13]. Pratt and Pedersen [20] have shown that covalent modification of amino, carboxyl, or histidyl groups results in an inhibition of Na^+-dependent Pi transport in renal BBMV. However, high concentrations (5–10 mM) of these reagents were necessary for inhibition. Furthermore, the fact that the inhibitory effect of $HgCl_2$ on Pi transport was totally protected or reversed by DTT suggests that other groups were not involved in the action of $HgCl_2$.

The nature of the inhibitory effect of $HgCl_2$ on Pi transport is speculative. Covalent modification of sulfhydryl groups within the Na^+-Pi cotransporters is likely, since the inhibition of ^{32}Pi transport persisted after extensive washing of the BBMV. The reduction of the apparent Vmax for Pi is also compatible with covalent modification, although other possibilities cannot be ruled out. A reduction in the number of functional Na^+-Pi cotransporters, presumably through conformational changes, or a reduced turnover rate of cotransporters can also be envisaged. It should be pointed out that these changes are not mutually exclusive and may occur concomitantly.

The fact that exposure to $HgCl_2$ did not result in a change in the stoichiometry of Na^+-Pi cotransport suggests that sulfhydryl groups are not located at or near the Na^+ binding sites on the cotransporter. It is also possible that $HgCl_2$ interacted with sulfhydryl groups at the Na^+ sites without producing conformational changes in the carrier or affecting the Na^+ gradient across the BBM.

The results of protection studies are compatible with the presence of essential sulfhydryl groups on the Na^+-Pi cotransporter, since DTT, which reduces S-S to SH, completely protected against inhibition of Pi transport by $HgCl_2$. The protection by DTT, added prior to the addition of $HgCl_2$, is also compatible with increased availability of reactive sulfhydryl groups. This is less likely in view of identical results obtained when DTT was added to BBMV, following preincubation with $HgCl_2$. The latter studies indicate that $HgCl_2$ may have been displaced from essential sulfhydryl groups on the Na^+-Pi cotransporter, resulting in the reversal of its inhibitory effect on Pi transport. The lack of protection by an excess of unlabeled Pi or PFA in the presence of Na^+ indicates that the reactive sulfhydryl groups are not involved in substrate binding. Similarly, Na^+ alone was not protective, indicating that sulfhydryl groups are also not involved in Na^+ binding. It is also possible that $HgCl_2$ produced conformational changes in the cotransporters, rendering these sites inaccessible to their respective substrates.

Since the highly permeable $HgCl_2$ has access to all the sulfhydryl groups within the BBM, the results of the present experiments do not allow us to determine the location of essential sulfhy-

dryl groups of the Na^+-Pi cotransporter in relation to the plane of the membrane. Recent studies by Pratt and Pedersen [20] suggest the presence of essential tyrosine and sulfhydryl groups on the cytoplasmic side of the BBM. Our recent studies aimed at characterizing the location of sulfhydryl groups of the renal Na^+-Pi cotransporter are also consistent with the presence of these groups on the intravesicular (cytoplasmic) side of the membrane [32].

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