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EFFECT OF MERCURIC CHLORIDE ON THE KINETICS OF CATIONIC AND SUBSTRATE ACTIVATION OF THE RAT BRAIN MICROSOMAL ATPase SYSTEM

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Abstract—Mercuric chloride ($HgCl_2$), a neurotoxic compound, inhibited the adenosine triphosphatase (ATPase) system in a concentration-dependent manner. Hydrolysis of ATP was linear with time with or without $HgCl_2$ in the reaction mixtures. Higher inhibition of (Na^+-K^+)ATPase activity by $HgCl_2$ was observed in alkaline (8.0 to 9.0) pH and at lower temperatures (17 to 32°). Activation energy values were increased slightly in the presence of $HgCl_2$. Activation of (Na^+-K^+)ATPase by ATP in the presence of $HgCl_2$ showed a decrease in V_{max} from 15.29 to 5.0 μmol of inorganic phosphate (P_i)/mg protein/hr with no change in K_m. Similarly, activation of K^+-stimulated *p*-nitrophenyl phosphatase (K^+-PNPPase) in the presence of $HgCl_2$ showed a decrease in V_{max} from 3.26 to 1.35 μmol of *p*-nitrophenol (PNP)/mg protein/hr with no change in K_m. K^+-activation kinetic studies indicated that $HgCl_2$ decreased V_{max} from 14.01 to 4.30 μmol P_i/mg protein/hr in the case of (Na^+-K^+)ATPase and from 3.45 to 2.40 μmol PNP/mg protein/hr in the case of K^+-PNPPase with no changes in K_m. Na^+-activation of (Na^+-K^+)ATPase in the presence of $HgCl_2$ showed a decrease in V_{max} from 11.06 to 3.23 μmol P_i/mg protein/hr and an increase in K_m from 1.06 to 2.08 mM. Preincubation of microsomes with sulfhydryl (SH) agents dithiothreitol, cysteine and glutathione protected $HgCl_2$-inhibition of (Na^+-K^+)ATPase. The data suggest that $HgCl_2$ inhibited (Na^+-K^+)ATPase by interfering with the dephosphorylation of the enzyme–phosphoryl complex.

A major target of mercuric compounds is the central nervous system [1, 2]. Numerous biochemical studies on mercury and other heavy metals have reported alterations in energy metabolism [3–5]. These compounds interfere with the cellular energy metabolism by inhibiting oligomycin-sensitive Mg^{2+}-ATPase (ATP synthesis) in mitochondria as well as (Na^+-K^+)ATPase (ATP hydrolysis) [5, 6]. Recently, increased attention has been focussed on elucidating the interaction of heavy metals with membrane-bound enzymes, particularly (Na^+-K^+)ATPase [3, 5]. Magnesium-dependent Na^+-K^+ stimulated ATPase is involved in several phases of the regulation of nerve cell activity including maintenance and re-establishment of the resting potential [7], transport of Na^+ and K^+ [8, 9], and uptake of neurotransmitters [10]. It is therefore of interest to study the inhibitory action of mercuric chloride ($HgCl_2$) on this enzyme system. It has been established that the reaction sequence of (Na^+-K^+) ATPase (E_1) involves a series of partial reactions observed *in vitro* including Na^+-dependent phosphorylation and its subsequent K^+-dependent dephosphorylation as shown below [11],

$$ATP + E_1 \xleftrightarrow{Mg^{2+},\,Na^+} E_1{\sim}P + ADP \quad (1)$$

$$E_1{\sim}P \xrightarrow{Mg^{2+}} E_2 - P \quad (2)$$

$$E_2 - P + K^+ \longleftrightarrow K - E_2 - P \quad (3)$$

$$K - E_2 - P + H_2O \longrightarrow K - E_2 + P_i \quad (4)$$

$$K - E_2 \longleftrightarrow K^+ + E_2 \quad (5)$$

$$E_2 \longleftrightarrow E_1 \quad (6)$$

K^+-stimulated *p*-nitrophenyl phosphatase (K^+-PNPPase) (E_2) represents the phosphatase moiety of the enzyme (equations 2 to 5) [12]. In the present study, we examined the *in vitro* effects of $HgCl_2$ on (Na^+-K^+)ATPase to help understand the mechanism of inhibition. Since the mercuric compounds are known inhibitors of enzymes containing active sulfhydryl (SH) groups such as (Na^+-K^+)ATPase, the protective effects by the representative thiol compounds dithiothreitol (DTT), cysteine and glutathione (GSH) against $HgCl_2$ inhibition of (Na^+-K^+)ATPase were also studied *in vitro*.

MATERIALS AND METHODS

Male Sprague–Dawley rats weighing 175–200 g were obtained from Harlan Sprague–Dawley Inc. (Indianapolis, IN). All biochemicals used for the enzyme assays were obtained from the Sigma Chemical Co. (St. Louis, MO).

A stock solution of $HgCl_2$ was prepared by dissolving $HgCl_2$ in glass-distilled water. Ten microliters of the test solution was added to the reaction mixture to obtain the desired final concentration. To determine *in vitro* effects, the microsomes in the reaction mixture were preincubated with $HgCl_2$ for 5 min prior to the initiation of the reaction. The solutions of thiol compounds were freshly prepared by dissolving the compounds in distilled water and used (10 μL per reaction mixture) immediately to minimize oxidation of disulfides. The compounds were added

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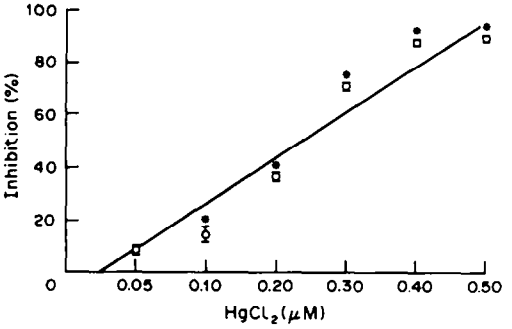

Fig. 1. Inhibition of rat brain microsomal (Na^+-K^+)ATPase activity by $HgCl_2$. Each value is the mean ± SE of four preparations, each assayed in triplicate. Key: (*) Significantly ($P < 0.05$) different from control (13.88 ± 0.24 μmol P_i formed/mg protein/hr).

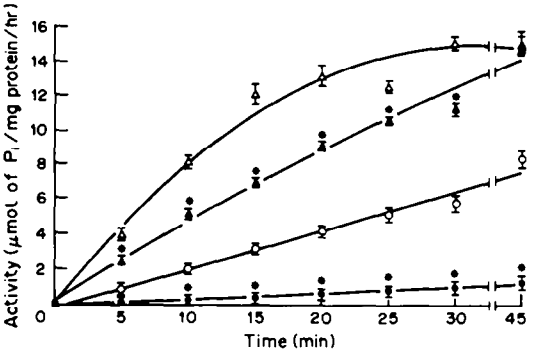

Fig. 3. Time course of the inhibition of rat brain microsomal (Na^+-K^+)ATPase activity by $HgCl_2$. Symbols: 15 μg protein [(○) control; (●) 2×10^{-7} M $HgCl_2$]; 50 μg protein [(△) control; (▲) 2×10^{-7} M $HgCl_2$]. Each value is the mean ± SE of four preparations, each assayed in triplicate. Key: (*) Significantly ($P < 0.05$) different from control.

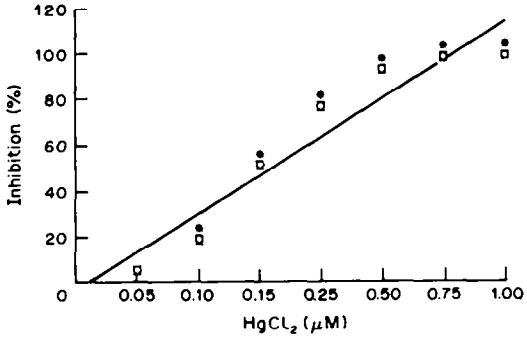

Fig. 2. Inhibition of rat brain microsomal K^+-PNPPase activity by $HgCl_2$. Each value is the mean ± SE of four preparations, each assayed in triplicate. Key: (*) Significantly ($P < 0.05$) different from control (2.83 ± 0.08 μmol PNP formed/mg protein/hr).

separately to the reaction mixtures prior to the addition of $HgCl_2$ (2.0×10^{-7} M), and then the samples were preincubated at 37° for 5 min before initiation of the reaction.

Preparation of microsomal fractions. The whole rat brain was removed after decapitation and homogenized in 9 vol. of ice-cold 0.32 M sucrose solution (pH 7.5) containing 10 mM imidazole and 1 mM ethylenediaminetetraacetic acid (homogenizing medium). Microsomes were prepared according to the procedures described by Koch [13]. The microsomal pellets obtained from the 100,000 *g* centrifugation were resuspended and diluted in the ice-cold sucrose solution, quick frozen in liquid nitrogen, and stored at −85° until used.

Determination of (Na^+-K^+) *ATPase activity.* The microsomal (Na^+-K^+)ATPase activity was measured using the end point phosphate analysis. A 1-mL reaction mixture contained: 5 mM ATP, 5 mM Mg^{2+}, 100 mM Na^+, 20 mM K^+, 135 mM imidazole/HCl buffer (pH 7.5), and 35–40 μg of enzyme protein. The reaction rate was proportional to the amount of protein used in this study. The total cationic ligand-stimulated ATPase activity was measured with Na^+, K^+, and Mg^{2+} present in the reaction mixture. The Mg^{2+}-ATPase was measured in the presence of 1 mM ouabain, a specific inhibitor of (Na^+-K^+)ATPase. The (Na^+-K^+)-activated component of ATPase was determined as the difference between total ATPase and Mg^{2+}-ATPase. Inorganic phosphate (P_i) was determined by the method of Lowry and Lopez [14] as modified by Phillips and Hayes [15]. Protein was determined by the method of Lowry *et al.* [16] using bovine serum albumin as a standard. Enzyme activity is expressed as micromoles of P_i formed per milligram of protein per hour.

K^+-PNPPase activity. K^+-PNPPase activity in the brain microsomal preparation was measured using methods described by Ahmed and Judah [17] and Albers and Koval [18]. Hydrolysis of the substrate *p*-nitrophenyl phosphate (PNPP) was analyzed in the presence of 5 mM Mg^{2+}, 10 mM K^+, 5 mM PNPP, 100 mM Tris/HCl buffer (pH 7.4) and 40–50 μg of microsomal protein at 37° in a final volume of 1.0 mL. Incubation time was 20 min, after which trichloroacetic acid [at a final concentration of 5% (w/v)] was added to stop the reaction. The reaction mixture was diluted with 1.0 M Tris (pH 10.4), and the optical density was determined at 400 nm against a blank. K^+-PNPPase was measured as the activity in the presence of Mg^{2+} and K^+ minus the activity in the presence of Mg^{2+}. The K^+-activated PNPPase activity is expressed as micromoles of *p*-nitrophenol (PNP) per milligram of protein per hour.

Kinetic analysis. All kinetic analyses were performed as per the methods described by Ahmed *et al.* [19] and Phillips *et al.* [20]. Activation energy (ΔE) values were calculated as described by Dixon and Webb [21].

Expression of results. Each point on the graphs indicates the mean ± SE of at least three to four different microsomal preparations, and each preparation was assayed three times. Double-reciprocal plots of kinetic data were constructed according to the method of Lineweaver and Burk [22] using a computer program.* Data were subjected to

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Table 1. Effect of pH on $HgCl_2$ (2×10^{-7} M) inhibition of rat brain microsomal (Na^+-K^+)ATPase activity

pH	(Na^+-K^+)ATPase (μmol P_i formed/mg protein/hr)		% Inhibition
	Control	$HgCl_2$	
6.0	6.70 ± 0.22	2.09 ± 0.46*	68.8
6.5	10.49 ± 0.90	3.02 ± 0.30*	71.2
7.0	12.41 ± 0.26	4.11 ± 0.38*	66.9
7.5	14.53 ± 0.14	5.85 ± 0.95*	59.7
8.0	13.63 ± 0.83	3.04 ± 0.41*	77.7
8.5	12.66 ± 0.49	2.11 ± 0.32*	83.3
9.0	9.83 ± 0.32	2.71 ± 0.22*	72.4

Each value is the mean ± SE of three independent studies, each assayed in triplicate.
* Significantly ($P < 0.05$) different from control.

Table 2. Effect of temperature on $HgCl_2$ (2×10^{-7} M) inhibition of rat brain microsomal (Na^+-K^+)ATPase activity

Temperature (°C)	(Na^+-K^+)ATPase (μmol P_i formed/mg protein/hr)		ΔE (Cal/mole × 10^3)	
	Control	$HgCl_2$	Control	$HgCl_2$
17	1.49 ± 0.11	0.46 ± 0.04* (−69.1)		
22	3.33 ± 0.26	0.83 ± 0.07* (−75.1)		
27	6.14 ± 0.29	1.93 ± 0.13* (−68.6)		
32	9.11 ± 0.43	2.60 ± 0.23* (−71.5)		
37	14.49 ± 0.23	6.13 ± 0.60* (−59.7)		
17–27			55.69 ± 1.47	57.32 ± 2.29 (+2.93)
27–37			36.67 ± 2.27	48.85 ± 1.62* (+33.2)

Each value is the mean ± SE of three independent studies each assayed in triplicate. The values in parentheses are percent changes over control.
* Significantly ($P < 0.05$) different from control.

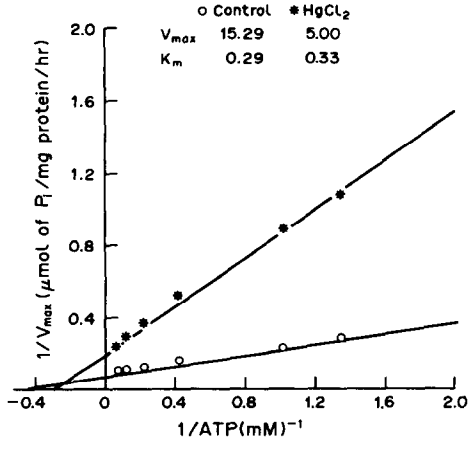

Fig. 4. Effect of $HgCl_2$ (2×10^{-7} M) on ATP-activation kinetics of rat brain microsomal (Na^+-K^+)ATPase. Each value is the mean of three different preparations, each assayed in triplicate.

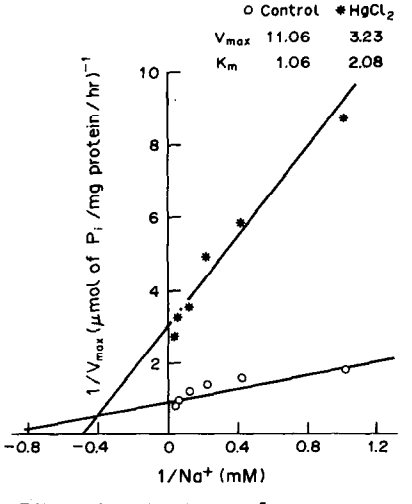

Fig. 5. Effect of $HgCl_2$ (2×10^{-7} M) on Na^+-activation kinetics of rat brain microsomal (Na^+-K^+)ATPase. Each value is the mean of three different preparations, each assayed in triplicate.

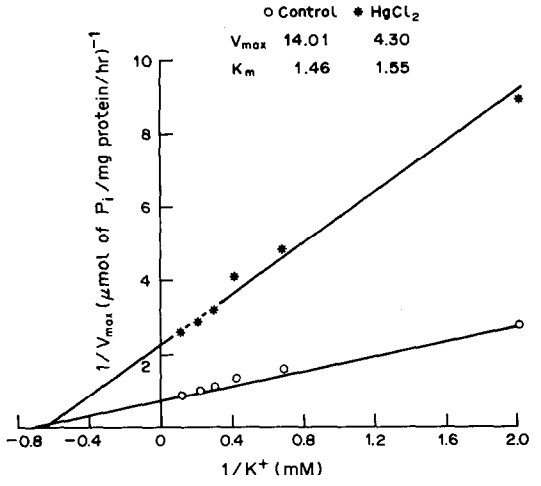

Fig. 6. Effect of $HgCl_2$ (2×10^{-7} M) on K^+-activation kinetics of rat brain microsomal (Na^+-K^+)ATPase. Each value is the mean of three different preparations, each assayed in triplicate.

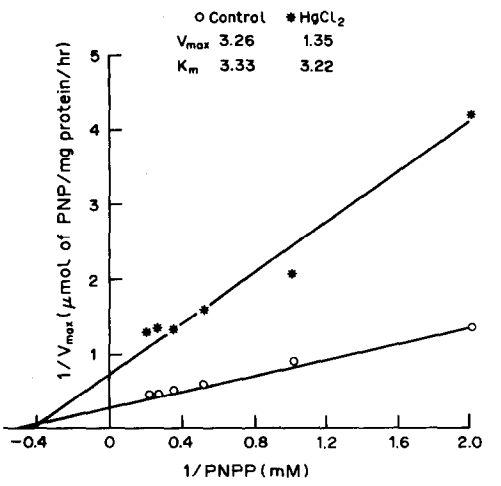

Fig. 7. Effect of $HgCl_2$ (2×10^{-7} M) on PNPP-activation kinetics of rat brain K^+-PNPPase. Each value is the mean of three different preparations, each assayed in triplicate.

regression analysis, and the regression lines were plotted for the best straight-line fit. Data were also analyzed by Student's *t*-test to determine the differences between control and experimental treatments; a value of $P < 0.05$ was considered significant.

RESULTS

Inhibition of the microsomal ATPase system by $HgCl_2$. (Na^+-K^+)ATPase was inhibited significantly by $HgCl_2$ in a concentration-dependent manner with an estimated IC_{50} of 2.35×10^{-7} M (Fig. 1). As shown in Fig. 2, K^+-PNPPase was also inhibited by $HgCl_2$ with an IC_{50} of 2.7×10^{-7} M.

Time–course study of inhibition of ATPase by $HgCl_2$. Inhibition of (Na^+-K^+)ATPase by $HgCl_2$ was dependent on the enzyme concentration and independent of the incubation time. At 15 or 50 μg of microsomal protein, a linear rate of ATP hydrolysis was observed for 15–20 min without $HgCl_2$, whereas similar linearity was observed throughout a 45-min incubation with $HgCl_2$ (Fig. 3).

Effect of pH on inhibition of ATPase by $HgCl_2$. The pH of individual incubation mixtures was varied from 6.0 to 9.0 in imidazole/HCl buffer. Inhibition was higher in alkaline pH (8.0 to 9.0), suggesting that $HgCl_2$ inhibition of (Na^+-K^+)ATPase was pH dependent (Table 1).

Effect of temperature on $HgCl_2$ *inhibition of ATPase*. Temperature of individual reaction mixtures was varied from 17 to 37°. $HgCl_2$ markedly inhibited (Na^+-K^+)ATPase activity at all temperatures studied. However, it was observed that inhibition was greater at lower temperatures (17–32°) than at 37° (Table 2), suggesting that $HgCl_2$ inhibition of (Na^+-K^+)ATPase was temperature dependent. An increase in ΔE values for (Na^+-K^+)ATPase by $HgCl_2$ at 27–37° (Table 2) suggests that the enzyme was catalytically less efficient in the presence of $HgCl_2$.

Effect of $HgCl_2$ *on ATP substrate activation kinetics*. When ATP concentration was varied from 0.075 to 2.0 mM with all other conditions constant (Fig. 4), the V_{max} of ATP-stimulated (Na^+-K^+)ATPase activity fell from 15.29 to 5.0 μmol P_i/mg protein/hr without significant changes in the apparent K_m (0.29 to 0.33 mM) in the presence of 2.0×10^{-7} M $HgCl_2$. These results indicate that the effect of $HgCl_2$ on (Na^+-K^+)ATPase was independent of substrate ATP at low-affinity binding sites, suggesting noncompetitive inhibition.

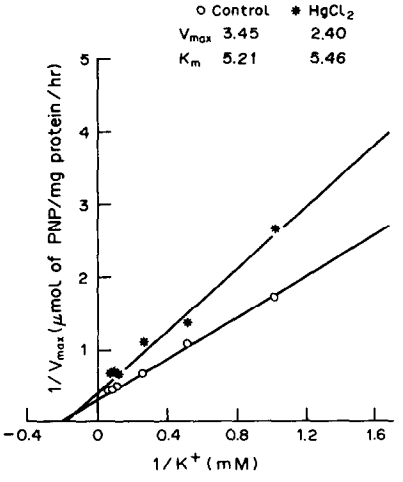

Fig. 8. Effect of $HgCl_2$ (2×10^{-7} M) on K^+-activation kinetics of rat brain microsomal K^+-PNPPase. Each value is the mean of three different preparations, each assayed in triplicate.

Effect of $HgCl_2$ *on cationic activation kinetics*. At optimal Na^+ concentration (100 mM) K^+ was varied from 0.5 to 10 mM, while at optimal K^+ concentration (20 mM) Na^+ was varied from 1.0 to 50.0 mM with all other conditions constant. Double-reciprocal plots for Na^+ activation showed a mixed type of inhibition of (Na^+-K^+)ATPase by $HgCl_2$.

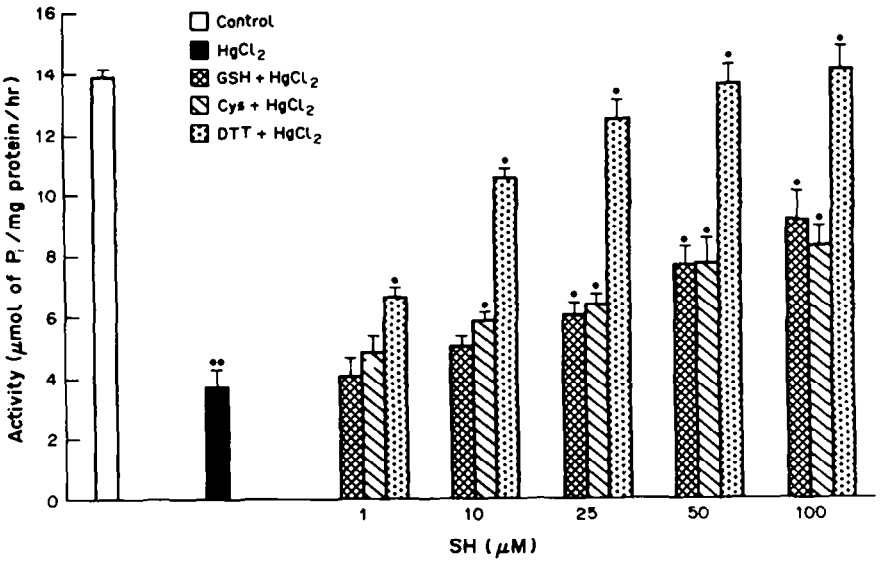

Fig. 9. Protective effects of DTT, cysteine (Cys) and GSH against $HgCl_2$ (2×10^{-7} M) inhibition of (Na^+-K^+)ATPase in rat brain microsomes. Each value is the mean ± SE of four preparations, each assayed in triplicate. Key: (*) Significantly ($P < 0.05$) different from $HgCl_2$; and (**) significantly ($P < 0.05$) different from control.

The apparent V_{max} decreased from 11.06 to 3.23 μmol P_i formed/mg protein/hr and K_m increased from 1.06 to 2.08 mM (Fig. 5). Inhibition of K^+-activated (Na^+-K^+)ATPase by $HgCl_2$ (Fig. 6) was noncompetitive. In the presence of $HgCl_2$, the apparent V_{max} was decreased from 14.01 to 4.3 μmol P_i/mg protein/hr without change in the apparent K_m.

Effect of $HgCl_2$ *on substrate (PNPP) and* K^+*-activated kinetics of PNPPase.* When PNPP concentration was varied from 0.5 to 5.0 mM at optimal K^+ concentration (20 mM) with all other assay conditions constant, the apparent V_{max} fell from 3.26 to 1.35 μmol PNP/mg protein/hr without change in the apparent K_m (3.33 to 3.22 mM) in the presence of $HgCl_2$ (2.0×10^{-7} M) (Fig. 7). By varying the K^+ concentration (1.0 to 20.0 mM) with other assay conditions constant, the apparent V_{max} was altered from 3.45 to 2.4 μmol PNP/mg protein/hr by $HgCl_2$ but no change was observed in the apparent K_m, confirming that the inhibition was noncompetitive with respect to the K^+-induced hydrolysis reaction (Fig. 8).

Alteration of $HgCl_2$ *inhibition by SH reagents.* The SH reagent alone had no effect on (Na^+-K^+)ATPase (Fig. 9). However, these compounds reduced the inhibitory effects of $HgCl_2$ on microsomal (Na^+-K^+)ATPase in a concentration-dependent manner. AT 100 μM, DTT restored specific activity completely to normal, whereas at 100 μM cysteine and 100 μM GSH the recovery was 60 and 66% respectively.

DISCUSSION

The results of the present study indicate that $HgCl_2$ is a potent inhibitor of brain microsomal (Na^+-K^+) stimulated ATPase and K^+-activated PNPPase. Similar observations on the inhibition of rat brain ATPase by heavy metals, chlorinated hydrocarbons and organotin compounds were reported earlier [5, 23, 24].

$HgCl_2$ inhibited microsomal (Na^+-K^+)ATPase and K^+-PNPPase noncompetitively with respect to substrate and cation-activation, indicating that $HgCl_2$ does interfere with ion transport across cell membranes [24]. These studies also suggested that $HgCl_2$ interacts with (Na^+-K^+)ATPase and K^+-PNPPase at sites not associated with substrate or cation (K^+) binding.

The kinetic effects observed in the present study may be due to induced conformational changes in the enzyme complex resulting from binding of $HgCl_2$ at critical SH moieties. Protection of the enzyme from inhibitory effects of $HgCl_2$ was achieved by preincubating the enzyme with SH reagents. $HgCl_2$ may be binding to microsomal membrane at SH sites which play a major role in the enzyme reaction [25]. Klonne and Johnson [26] reported inhibition of microsomal (Na^+-K^+)ATPase in mouse kidney by $HgCl_2$ and its protection by DTT. Protection by SH reagents of brain ATPase inhibited by heavy metals [26] and organotin compounds [24] was reported earlier. The higher protection by DTT compared to cysteine and GSH may be due to greater redox potential of DTT. It has been reported that DTT is a superior reducing agent [27] and can act *in vitro* to restore enzyme activity lost by oxidation of SH groups [28]. The present data suggest that $HgCl_2$ may have a property similar to that of SH-blocking reagents [25].

(Na^+-K^+)ATPase activity has been shown to be a biochemical manifestation of the Na^+ pump [8]. $HgCl_2$ increases the intracellular Na^+ concentration either by inhibiting (Na^+-K^+)ATPase or by increasing the permeability of the plasma membrane to Na^+

[29, 30]. The present data suggest that the inhibition of (Na^+-K^+)ATPase in rat brain microsomes may be due to its interference in the formation of $E_1 \sim P$ (Equation 1) and subsequent K^+-dependent dephosphorylation (Equation 4) by binding at or near the Na^+- and K^+-activation sites. Inhibition of K^+-PNPPase by $HgCl_2$ further supports this contention. The observed effects of $HgCl_2$ on this important enzyme indicate marked alterations in the Na^+ pump.

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