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TRANSPORT ATPase—THE DIFFERENT MODES OF INHIBITION OF THE ENZYME BY VARIOUS MERCURY COMPOUNDS

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Abstract—The effects of a number of mercurials on the partial reactions of Na^+,K^+-ATPase were studied. Results of these studies allow a classification of the mercurials into two groups: selective modifiers of the enzyme and unspecific inhibitors. Thimerosal, ethylmercury and methylmercury were shown to selectively inhibit Na^+,K^+-ATPase while not affecting K^+-NPPase, Na^+-ATPase, ADP–ATP exchange reaction and phosphoenzyme formation. In contrast, *p*-chloromercuribenzoic acid, *p*-chloromercuriphenylsulfonic acid, mersalyl and mercuric chloride inhibited Na^+,K^+-ATPase as well as several partial reactions of the enzyme in a parallel manner. The selective modification of Na^+,K^+-ATPase by compounds such as ethylmercury and methylmercury makes these agents useful probes of the reaction mechanism of the enzyme. Whether this mode of inhibition of the enzyme is related to the mechanism of toxicity of short-chain alkylmercury compounds remains to be determined.

Compounds of mercury are known to form covalent bonds with sulphur and are capable of inactivating many enzyme systems, particularly those requiring reduced sulfhydryl groups for activity [1]. The fact that mercurials inhibit Na^+,K^+-ATPase (ATP phosphohydrolase, EC 3.6.1.3) has been established through work concerning the effect of mercurial diuretics on the enzyme [2]. While these drugs have been shown to inhibit the enzyme *in vitro*, attempts to correlate the enzyme inhibition with diuretic action *in vivo* have not been uniformly successful [2].

Previous studies have focused on the mercurial inhibition of the Na^+ plus K^+-dependent activity and not on the various partial reactions of the enzyme. The enzyme catalyzes a variety of partial reactions including a Na^+-dependent ATPase, a Na^+-dependent ADP–ATP exchange, several reactions involving phosphorylated forms of the enzyme and hydrolyses of certain artificial substrates [3–5]. Our work concerning the reaction mechanism of this enzyme led to the discovery of an interesting compound with unique inhibitory effects on the enzyme. Selected concentrations of ethylmercurithiosalicylate (thimerosal) were found to inhibit Na^+,K^+-dependent ATPase activity, without inhibiting the partial reactions of the enzyme that have been studied to date [6, 7].

With the discovery of the above effects of thimerosal on the enzyme, the question arose as to whether other mercurial compounds have similar effects on the enzyme. The objective of this work was the comparison of the mechanism of the inhibitory effects of a variety of mercury compounds with that of thimerosal.

MATERIALS AND METHODS

Na^+, K^+-ATPase complex from guinea pig kidney was prepared by the method of Post and Sen [8]. The enzyme from dog kidney was made according to Lane *et al.* [9]. Hemoglobin-free membranes of human red cells were prepared by the method of Hoffman and Parker [10], and used for the determination of red cell Na^+,K^+-ATPase activity. The specific activities of all preparations were similar to those described in the original literature.

The various mercurial-treated enzymes were obtained by incubating the membranes (approximately 200 μg protein/ml) at 37° in a solution containing the designated mercurial and 50 mM Tris–HCl (pH 7.4). After 10 min, the membranes were collected by centrifugation at 35,000 *g* for 15 min and washed repeatedly with a solution containing 0.25 M sucrose, 30 mM histidine, and 1 mM EDTA (pH 6.8). The properties of the modified enzyme were always compared with those of the control enzyme that had been carried through the same procedure in the absence of mercurial.

The Na^+,K^+-ATPase activity of the enzyme was assayed at 37° in a medium containing 2 mM ATP, 3 mM $MgCl_2$, 1 mM EDTA, 100 mM NaCl, 25 mM KCl and 50 mM Tris–HCl (pH 7.4), in a total volume of 2.5 ml. To determine the $Na^+ + K^+$-independent portion of the activity, KCl and NaCl were omitted from the reaction mixture. The reaction was terminated after the appropriate time by the addition of 1.5 ml of 8% perchloric acid. A 3 ml aliquot was then assayed for orthophosphate according to the method of Fiske and Subbarow [11]. The ATP utilized in the assay was prepared by passage of the disodium salt of ATP (Sigma, St. Louis, MO) through a column of Dowex 50W-X8 (Sigma), and was readjusted to pH 7.4 with Tris base.

Assay of Na^+-ATPase activity was conducted at a low ATP concentration (1 μM) utilizing γ-^{32}P-labeled nucleotide obtained from New England Nuclear (Boston, MA). All media for these assays contained 3 mM $MgCl_2$, 1 mM EDTA, 100 mM NaCl and 50 mM Tris–HCl (pH 7.4). The Na^+-independent portion of the activity was determined by omitting NaCl from the reaction mixture. The released P_i was converted to phosphomolybdate, extracted into isobutanol and counted [12].

The standard assay mixture for *p*-nitrophenylphosphatase (NPPase) contained the following:

50 mM Tris–HCl (pH 7.4), 3 mM $MgCl_2$, 1 mM EDTA, 2 mM Tris-*p*-nitrophenylphosphate (NPP), 25 mM KCl and appropriate amounts of enzyme, in a total volume of 2.5 ml. The reaction was terminated after the appropriate time interval by the addition of 1.5 ml of 3 N NaOH, iced for 5 min and then centrifuged. Optical densities were then read at 410 nm on a Spectronic 20, Bausch and Lomb. The Tris-*p*-nitrophenylphosphate was prepared by passing the sodium salt of *p*-nitrophenylphosphate (Sigma) through a column of Dowex 50W-X8, and was readjusted to pH 7.4 with Tris base.

The ADP–ATP exchange rate was determined as described previously [7] and the determination of ^{32}P-labeled phosphoenzyme was carried out as described in an earlier publication [13].

The mercurials used in modification studies of the Na^+,K^+-ATPase were: ethylmercurithiosalicylate(thimerosal), mersalyl acid, *p*-chloromercuribenzoic acid (PCMB) and *p*-chloromercuriphenylsulfonic acid (PCMPS)(Sigma); ethylmercuric chloride and methylmercuric chloride, (ICN Pharmaceuticals, Plainview, NY); and mercuric chloride (Fischer Chemicals, Pittsburgh, PA).

RESULTS

Effects of thimerosal on Na^+, K^+-*ATPase and* K^+-*NPPase of various membrane preparations.* Figure 1 compares the effects of varying concentrations of thimerosal on Na^+,K^+-ATPase and K^+-NPPase activities in membrane preparations from dog kidney and erythrocyte ghosts. It is evident from the data that, with increasing concentrations of thimerosal, Na^+,K^+-dependent ATPase activity is more sensitive to the inhibitory effects of thimerosal. For the dog kidney enzyme, Fig. 1A, a 10^{-3} M concentration of thimerosal produced 93 per cent inhibition of Na^+,K^+-ATPase activity; in contrast, K^+-NPPase activity was not affected significantly. In order to obtain 50 per cent inhibition of the Na^+,K^+-ATPase, approximately a 10^{-4} M concentration of thimerosal was required.

Comparing the results above to those obtained from human erythrocyte ghosts, (Fig. 1B), the same general trend was seen; thimerosal selectively inhibited Na^+,K^+-ATPase. At a 10^{-3} M concentration of thimerosal, 100 per cent of the red cell Na^+,K^+-ATPase was inhibited and a 50 per cent inhibition level of the enzyme activity occurred at approximately 5×10^{-5} M. Throughout the concentration range of thimerosal tested in Fig. 1, K^+-NPPase was not substantially inhibited. However, we have demonstrated in other experiments that if sufficiently high thimerosal concentrations are used, inhibition of the K^+-NPPase can also be attained.

These observations with dog kidney and erythrocyte membrane preparations substantiate further the results of earlier studies dealing with the interaction of thimerosal and guinea pig kidney enzyme. When the guinea pig kidney enzyme had been incubated with thimerosal, at a concentration of approximately 10^{-4} M, a modified enzyme with intact K^+-NPPase but almost completely inhibited Na^+,K^+-ATPase activity was obtained. This modified enzyme could also catalyze the ADP–ATP exchange reaction and initiate phosphoenzyme formation from ATP [6, 7].

Comparison of the effects of other mercurials on the enzyme with those of thimerosal. With the discovery of the effects of thimerosal on the enzyme, the question arose as to whether other mercurial compounds have similar effects. Therefore, the interactions with the enzyme of mercuric chloride, *p*-chloromercuribenzoic acid (PCMB), *p*-chloromercuriphenylsulfonic acid (PCMPS), mersalyl, ethylmercury and methylmercury were studied. Of the tested compounds, ethylmercury and methylmercury proved to have effects similar to those of thimerosal. Data for ethylmercury are presented below. Figure 2, the effects of varying concentrations of ethylmercury on Na^+,K^+-ATPase, K^+-NPPase and Na^+-ATPase, shows that selective

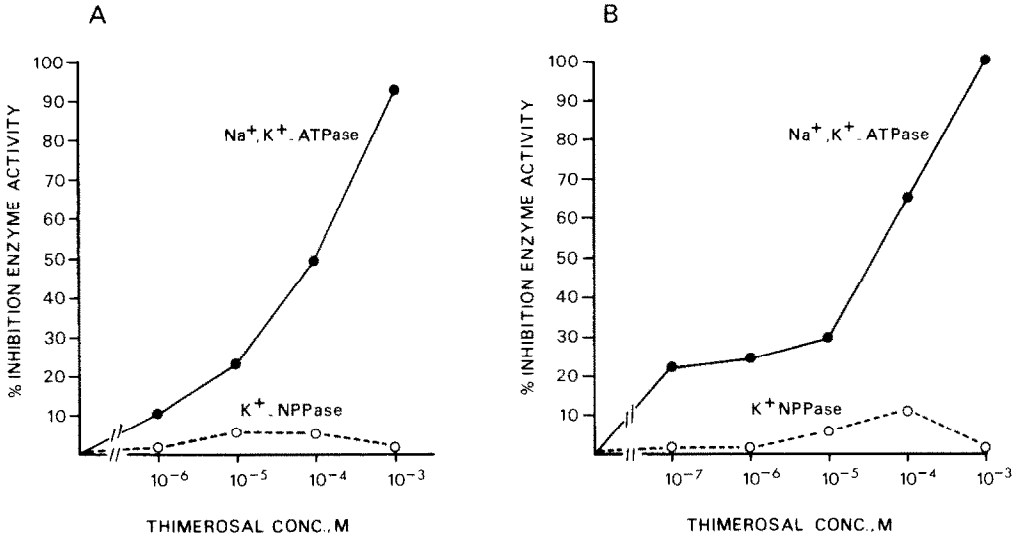

Fig. 1. Effects of varying concentrations of thimerosal on Na^+, K^+-dependent ATPase and K^+-NPPase activities of the dog kidney (A) and human erythrocyte ghost (B) membrane preparations. Conditions were as described under Materials and Methods.

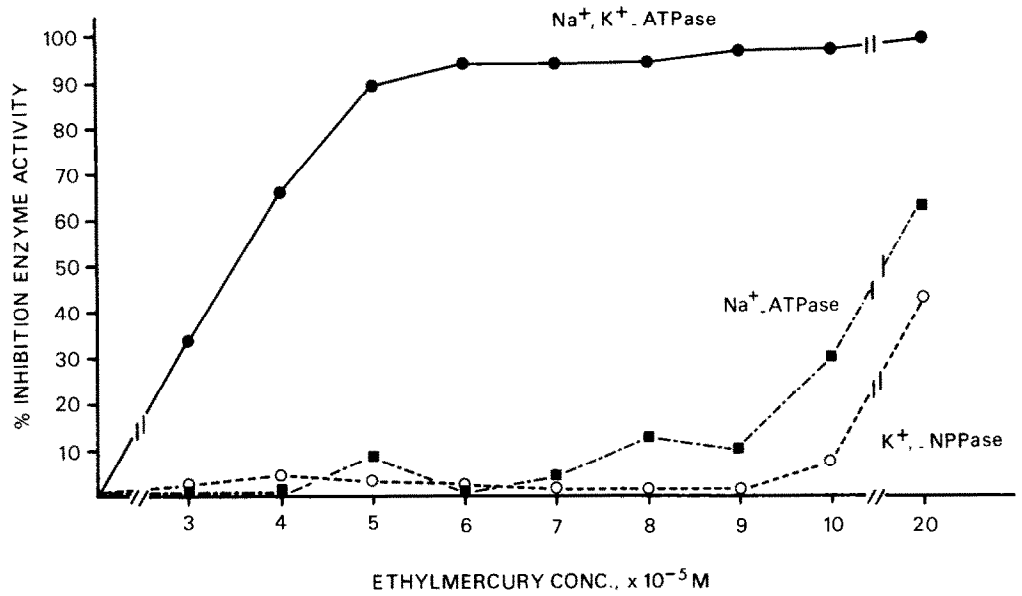

Fig. 2. Effects of varying concentrations of ethylmercury chloride on Na^+, K^+-dependent ATPase, Na^+-ATPase and K^+-NPPase activities of the dog kidney membrane preparation. Conditions were as described under Materials and Methods.

inhibition of Na^+,K^+-ATPase activity without the inhibition of the two partial reactions can be achieved with this compound. As is evident from the data, half-maximal inhibition of Na^+,K^+-ATPase is obtained at 3.5×10^{-5} M ethylmercury, indicating that ethylmercury is a more potent inhibitor of the enzyme than thimerosal.

To determine the effects of ethylmercury on two other partial reactions, namely ADP–ATP exchange and labeling of the enzyme by [^{32}P]-ATP, the same enzyme that was treated with 8×10^{-5} M ethylmercury for the experiments of Fig. 2 was utilized. As the data of Table 1 show, the Na^+-dependent ADP–ATP exchange activity of this preparation is the same as that of control enzyme. Since this exchange activity is thought to represent the reversal of the initial phosphorylation of the enzyme by ATP, the data suggests that ethylmercury, at a concentration that completely inhibits Na^+,K^+-ATPase activity, does not affect the Na^+-dependent phosphorylation of the enzyme by ATP. That this is indeed the case is proved directly by the experiments of Fig. 3. These data show the time dependence of phosphorylation by [^{32}P]-ATP for control and ethylmercury-treated enzyme. For both enzymes, incubation with Na^+ produces an increased level of phosphorylation which reaches a maximum at approximately 10 sec, and then falls to a steady state level. This level is fairly constant throughout the 20-sec period although some spontaneous discharge is evident with the treated enzyme. In the presence of Na^+ plus K^+, both enzymes show a lower level of phosphorylation which is maintained at a fairly constant level. The data of Fig. 3 also show the effect of K^+ on the dephosphorylation of the enzyme. The decrease in the level of ^{32}P-incorporation that is obtained upon addition of EDTA represents the spontaneous discharge of the phosphoenzyme. The simultaneous addition of EDTA and K^+ increases the rate of discharge. As is evident from the data, this stimulatory effect of K^+ on the breakdown of the phosphoenzyme is observed in both the control and the ethylmercury-treated enzymes. The combined data of Figs. 2 and 3 and Table 1 clearly show that it is possible to react the enzyme with an appropriate dose of ethylmercury to obtain a preparation in which Na^+,K^+-ATPase is inhibited completely without the inhibition of all tested partial reactions of the enzyme.

Table 1. Comparison of ADP–ATP exchange rates of control and ethylmercury-treated enzymes*

Enzyme	ADP–ATP exchange rate (nmoles [^{14}C]ATP/mg/min)		
	Mg^{2+}	$Mg^{2+} + Na^+$	Na^+ dependent
Control	12.0	53.6	41.6
Ethylmercury	12.3	53.2	40.9

* The dog kidney enzyme preparation was treated with 8×10^{-5} M ethylmercury chloride. The reaction mixture for control and treated enzymes contained 0.1 mM $MgCl_2$ with and without 150 mM NaCl. Other conditions and procedures were as described in Materials and Methods.

Experiments similar to those of Figs. 2 and 3 and Table 1 were done with methylmercury. The results were qualitatively the same. The only difference between ethylmercury and methylmercury was the slightly lower potency of the latter. Half-maximal inhibition of Na^+,K^+-ATPase activity was obtained at 4.7×10^{-5} M methylmercury.

The reactions of the remainder of the tested mercury compounds with the enzyme led to a different pattern of inhibition of the enzyme. This is exemplified by the data of Fig. 4. Here the effects of varying concentrations of mersalyl on Na^+,K^+-ATPase, K^+-NPPase and Na^+-ATPase activities are shown. Comparison

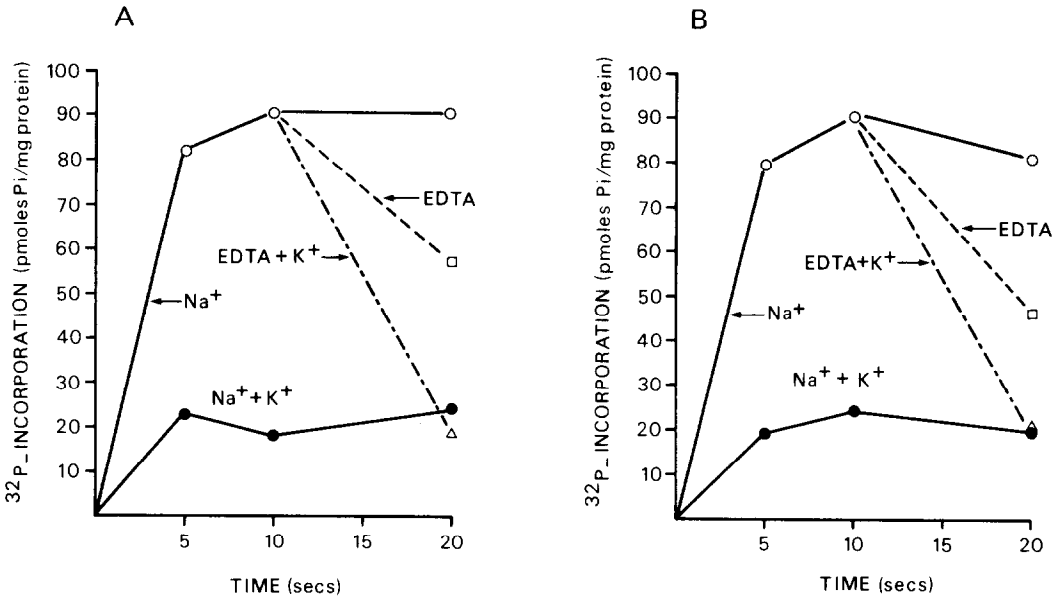

Fig. 3. Effects of Na^+ and K^+ on phosphoenzyme formation and breakdown in control (A) and ethylmercury-treated (B) enzymes. The studies were done on the guinea pig kidney enzyme in the presence of (1) $Na^+ + K^+$, (2) Na^+ alone, (3) Na^+ with the addition of EDTA after 10 sec and (4) Na^+ with the addition of EDTA and K^+ after 10 sec. The final concentrations of the indicated reagents were: Na^+, 100 mM; K^+, 20 mM; and EDTA, 15 mM. Final concentrations of other reagents were: Tris-HCl, 30 mM (pH 7.4); $MgCl_2$, 3 mM; and [^{32}P]-ATP, 50 μM. Other conditions and procedures were the same as described in Materials and Methods.

of these data with those of Fig. 2 show clearly the difference between the effects of ethylmercury and mersalyl on the enzyme. Although at any concentration of mersalyl some differences in the extent of inhibitions of the three tested activities are observed (Fig. 4), the difference in the sensitivities of the three activities to mersalyl are not as great as those to ethylmercury (Fig. 2). Thus, with mersalyl it is not possible to obtain complete inhibition of Na^+,K^+-ATPase activity without affecting the partial reactions. The dose–response curves for other tested mercury compounds were similar to those presented in Fig. 4

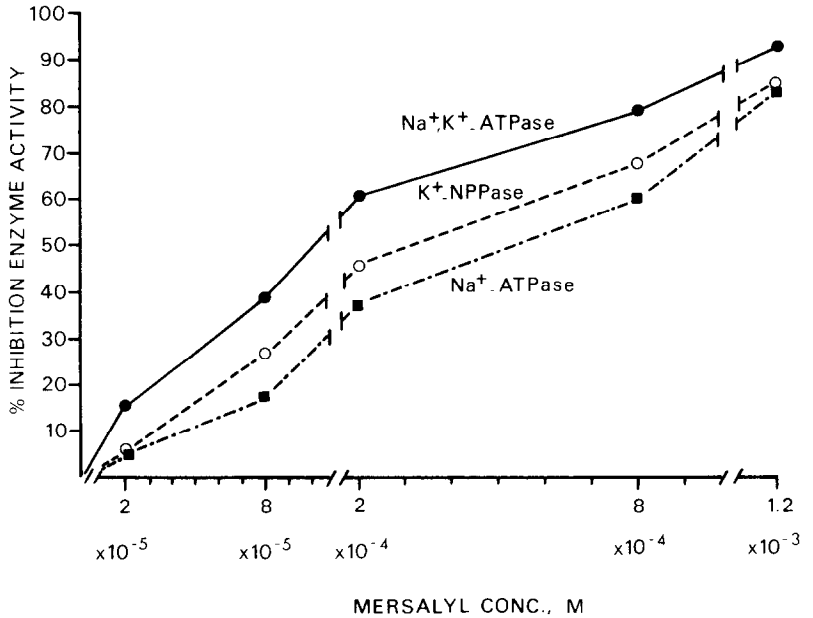

Fig. 4. Effects of varying concentrations of mersalyl on Na^+, K^+-dependent ATPase, Na^+-ATPase and K^+-NPPase activities of the dog kidney membrane preparation. Conditions were as described in Materials and Methods.

Table 2. Comparison of I_{50} values (molar) of various mercurials for three enzymatic activities of the dog kidney enzyme

Mercurial	Na^+,K^+-ATPase	Na^+-ATPase	K^+-NPPase
Mercuric chloride	0.83×10^{-4}	0.70×10^{-4}	1.0×10^{-4}
Mersalyl	1.0×10^{-4}	5.5×10^{-4}	3.0×10^{-4}
PCMB	1.8×10^{-4}	1.9×10^{-4}	2.4×10^{-4}
PCMPS	2.8×10^{-4}	3.5×10^{-4}	6.7×10^{-4}
Thimerosal	1.0×10^{-4}	2.5×10^{-3}	5.0×10^{-3}
Ethylmercury	3.5×10^{-5}	2.0×10^{-4}	2.5×10^{-4}
Methylmercury	4.7×10^{-5}	3.3×10^{-4}	4.1×10^{-4}

for mersalyl. The I_{50} of each compound (concentration resulting in half-maximal inhibition) for each of the three tested activities is presented in Table 2. For comparison, values for thimerosal, ethylmercury and methylmercury are also included.

DISCUSSION

The present study demonstrates that both ethylmercury and methylmercury produce a unique inhibitory pattern on Na^+,K^+-ATPase. At specified concentrations, these compounds selectively inhibit the Na^+,K^+-dependent ATPase activity while leaving the K^+-NPPase activity and Na^+-ATPase activity intact. In addition, all other partial reactions of the enzyme remain uninhibited including the ADP–ATP exchange reaction and phosphoenzyme formation from ATP.

These effects of ethylmercury and methylmercury are identical to those observed previously with thimerosal in the guinea pig kidney [6, 7]. The effects of thimerosal are also seen in the highly purified enzyme preparation from dog kidney and in the relatively impure enzyme preparation from human erythrocyte ghosts.

Other mercurials tested did not show the selective inhibition seen with thimerosal, ethylmercury and methylmercury. With these compounds, at any tested concentration, inhibitions of the Na^+,K^+-ATPase and the partial reactions were obtained. This dichotomy in the response of the enzyme to the mercurials justifies a classification of the mercurials tested into two main types: (1) selective modifiers, whose interaction with the enzyme leads to the inhibition of Na^+,K^+-dependent ATPase without any apparent inhibitory effects on the various partial reactions of the enzyme (in this group are thimerosal, ethylmercury and methylmercury); and (2) unspecific inhibitors, those mercurials that at any inhibitory concentration affect the Na^+,K^+-dependent ATPase activity and the tested partial reactions. This group includes *p*-chloromercuribenzoic acid, *p*-chloromercuriphenylsulfonic acid, mersalyl and mercuric chloride.

The selective modifier, thimerosal, has been shown to have potential in studying the mechanistic relationships of the ATPase complex. The characteristics of this unique modification have been integrated into the generalized reaction sequence of the enzyme and interpreted as further evidence for the existence of two nucleotide binding sites on the enzyme [6, 7]. The studies presented here show that ethylmercury and methylmercury, like thimerosal, may be used as probes of the reaction mechanism of Na^+,K^+-ATPase.

The finding that various mercurials interact with Na^+,K^+-ATPase *in vitro* by different mechanisms raises some interesting questions concerning the mechanism of the *in vivo* effects of the mercurials. For example, since it is known that symptoms and consequences of methylmercury poisoning are in some respects different from those of poisoning by inorganic mercury compounds [14], one may wonder whether the variations in the toxic manifestation could be due to the different mechanisms of enzyme inhibition by these compounds at the molecular level. Although inhibition of brain Na^+,K^+-ATPase in experimental methylmercury poisoning has been demonstrated [15], it is not known whether the mode of inhibition of the enzyme under those conditions resembles that described in this paper. Further studies on the nature of Na^+,K^+-ATPase inhibition in experimental poisoning by mercury compounds may be of value in the understanding of the mechanism of toxicity of these environmental hazards.

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REFERENCES

1. J. L. Webb, *Enzyme and Metabolic Inhibitors*, Vol. 2, p. 719. Academic Press, New York (1966).
2. B. R. Nechay, *Ann. N.Y. Acad. Sci.* **242**, 501 (1974).
3. I. M. Glynn and S. J. D. Karlish, *A. Rev. Physiol.* **37**, 13 (1975).
4. L. E. Hokin and J. L. Dahl, in *Metabolic Pathways* (Ed. L. E. Hokin), Vol. 6, p. 267. Academic Press, New York (1972).
5. A. Schwartz, G. E. Lindenmayer and J. C. Allen, *Pharmac. Rev.* **27**, 3 (1975).
6. G. R. Henderson and A. Askari, *Biochem biophys. Res. Commun.* **69**, 499 (1976).
7. G. R. Henderson and A. Askari, *Archs Biochem. Biophys.* **182**, 221 (1977).
8. R. L. Post and A. K. Sen, in *Methods in Enzymology* (Eds. R. W. Estabrook and M. E. Pullman), Vol. 10, p. 762. Academic Press, New York (1967).
9. L. K. Lane, J. H. Copenhaver, Jr., G. E. Lindenmayer and A. Schwartz, *J. biol. Chem.* **218**, 7197 (1973).
10. J. F. Hoffman and J. C. Parker, *J. gen. Physiol.* **50**, 893 (1967).
11. C. H. Fiske and Y. Subbarow, *J. biol. Chem.* **66**, 375 (1925).
12. A. H. Neufeld and H. M. Levy, *J. biol. Chem.* **244**, 6493 (1969).
13. A. Askari and S. N. Rao, *Biochem. biophys. Res. Commun.* **49**, 1323 (1972).
14. T. W. Clarkson, *CRC Crit. Rev. Toxic.* **1**, 203 (1972).
15. M. Su and G. T. Okita, *Fedr. Proc.* **34**, 810 (1975).