J. Membrane Biol. 13, 263–282 (1973)

Harmaline: A Competitive Inhibitor of Na Ion in the $(Na^+ + K^+)$-ATPase System

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Received 5 February 1973

Summary. Experimental evidence is given that the hallucinogen harmaline (HME) behaves as an inhibitor of the $(Na^+ + K^+)$-ATPase system, specifically in the Na^+-dependent phosphorylation reaction. HME at 0.3 to 3 mM inhibited several membrane ATPase preparations such as those from human erythrocytes, rat brain and squid retinal axons. The same concentration blocked Na^+ outflow from squid giant axons. The behavior of several harmane derivatives such as harmine, harmalol and harmaline demonstrated that certain groups influenced the concentration for 50% inhibition of the ATPase system. The following evidence demonstrated that HME blocked the formation of the phosphorylated intermediate by competition with Na ions in the $(Na^+ + K^+)$-ATPase reaction in rat brain. (1) The HME effect on the overall $(Na^+ + K^+)$-ATPase reaction showed a fully competitive inhibition with respect to Na ion concentration. (2) The inhibition of the Na^+-stimulated phosphorylation by HME was fully competitive with respect to Na ions, with or without oligomycin present. (3) HME inhibited the effect of ADP on the phosphorylation reaction using ^{32}P–ATP. (4) HME did not accelerate the rate of membrane dephosphorylation by means of ^{32}P–ATP and cold ATP.

From the behavior of HME as a competitive inhibitor at Na ion sites of the $(Na^+ + K^+)$-ATPase reactions one may gain information about (a) The chemical nature of Na^+ sites which may be responsible for the selectivity of this cation, and (b) The sequence of Na^+ and ATP entrance into the Na^+-dependent phosphorylation reaction. The experimental evidence supports the hypothesis that the entrance of Na^+ into the enzyme system may precede the formation of the phosphorylated intermediate.

An interest in elucidating the nature of the molecular mechanism involved in the functioning of the sodium pump has led to the study of its behavior in isolated membrane fragments [1, 6] and in intact cells [13, 15]. From experiments with fragments of cell membranes with $(Na^+ + K^+)$-ATPase activity, several partial reactions have been described. The reaction sequence appears to involve a Na^+-stimulated phosphorylation of the enzyme followed by a K^+-dependent dephosphorylation [29].

Despite the wide range of blocking agents used to study the mechanism of the transport reaction, ouabain, oligomycin [11, 16, 17, 37] and N-ethylmaleimide (NEM) [10] still remain the most useful inhibitors of the later steps of the reaction. Considerable evidence indicates that ouabain inhibits ATPase by binding to the phosphorylated form of the enzyme but does not interfere with its formation [7, 34]. The Na^+-stimulated phosphorylation reaction is not inhibited by oligomycin nor NEM and it does not show inhibition by ouabain at low concentrations. However, the lack of a specific inhibitor of the earlier steps of the reaction has impeded the elucidation of the exact role of the phosphorylated enzyme in the ion translocation process.

The purpose of this study is to analyze in detail the inhibitory action of harmaline (HME) on the overall and partial ($Na^+ + K^+$)-ATPase reactions.

Harmaline is a psychotomimetic drug belonging to the lysergic acid diethylamide (LSD)-like class that produces disorders in thinking, feeling, behavior and perception (hallucination) [24, 33, 38]. This fluorescent molecule is a β-carboline (harmane), an indole compound, which can have substitutions in the rings such as seen in harmine, harmalol and methoxyharmalan [24].

The experiments reported here indicate that HME inhibits the stage involving the formation of the phosphorylated intermediate. Probably most important, the kinetics of this inhibition were fully competitive with respect to Na ions in the presence or absence of oligomycin.

Materials and Methods

Na-Outflow from Giant Axons

Finely dissected giant axons of the squid (*Dosidicus gigas*) with a mean diameter of 700 μ were microinjected with ^{22}Na (1×10^6 cpm/fiber) dissolved in 0.60 M KCl at pH 7.4, as described by Canessa-Fischer, Zambrano and Rojas [5]. A 25-μ Pt wire placed in the microinjection pipette allowed continuous intracellular recording of the action potential on a Tektronix 502 A oscilloscope. The axon was excited through external Pt electrodes at the frequency of 1/sec by means of a Grass S-5 stimulator.

The fibers were immersed in artificial seawater at 18 °C (420 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 50 mM $MgCl_2$, 5 mM Tris-Chloride, pH 7.4). The bathing solutions were changed at 3-min intervals and their radioactivity determined in a well-type gamma scintillation counter.

Cell Membrane Preparations

Squid retinal axon membranes were prepared from 40 g (wet weight) of nerve as described elsewhere [13]. The axons were rinsed in 0.25 M sucrose, 10 mM Tris-EDTA, at pH 7.4, and cut into very small pieces with scissors. The nerves were homogenized in 10 ml of 0.25 M sucrose, 5 mM Tris-EDTA, and 30 mM histidine, at pH 7.4 using a motor-driven glass-glass homogenizer in the presence of acid-washed glass beads (150- to

200-μ diameter). Sonication was performed at 20 kc output for 10 sec (Bronwill Biosonic B sonicator). The suspension was spun for 10 min at $1,500 \times g$ in a Sorvall refrigerated centrifuge, and the supernatant was spun twice at $10,000 \times g$ for 20 min. To obtain the final membrane fraction the last supernatant was centrifuged for 90 min at $105,000 \times g$ in a 30 rotor in a Spinco model L ultracentrifuge. The pellet was resuspended twice by gentle homogenization with a Teflon homogenizer in 0.25 M sucrose, 30 mM Tris-Chloride, 1 mM EDTA at pH 7.4, and centrifuged for 60 min at $105,000 \times g$. The final pellets were resuspended in 5 ml of 30 mM Tris-Chloride and 1 mM EDTA at pH 7.4. All steps were performed at 2 °C. The fraction sedimented at $100,000 \times g$ was lyophilized and stored at −20 °C under vacuum in sealed vials.

Retinal axon membranes were purified by NaI or KI treatment as described by Nakao, Tashima, Nagano and Nakao [28], suspending the pellet in a mixture of 2 M KI, 3.3 mM $MgCl_2$, 2.5 mM Tris-EDTA, 2.5 mM cysteine and 1.3 mM Tris-ATP at pH 8.0.

The suspension was mixed with magnetic stirrer and was allowed to stand at 0 °C for 30 min. It was then diluted with water to 0.8 M KI and centrifuged for 30 min at $100,000 \times g$. The resultant pellet was washed twice with 5 mM Tris EDTA (pH 7.4) and finally it was suspended in water (about 0.5 mg protein/ml/tube). Aliquots of this preparation were stored at −20 °C and used within 2 weeks.

The preparation of rat brain was carried out according to Nakao *et al.* [28], using KI instead of NaI as described for retinal axon membranes.

Erythrocyte membranes were prepared from stored human blood by gradual osmotic lysis [40].

ATPase Assays

For squid nerve membrane, the assay for overall ATPase activity was carried out at 37 °C in a final volume of 2 ml at pH 7.4 containing 200 mM NaCl, 40 mM KCl, 4 mM $MgCl_2$, 2 mM Tris-ATP, and 100 mM Tris-Cl. For kinetic studies, initial rates of inorganic phosphate release were used.

Samples of 40 to 60 μg of membrane proteins were preincubated for 10 min, the reaction was started with ATP addition, incubated 10 to 20 min and stopped by addition of 0.1 ml of 100% trichloroacetic acid (TCA). Since hallucinogenic agents interfered with the phosphate (Pi) analysis, two extractions with 2.5 ml of isobutanol were performed to remove the drug from the aqueous solutions. One-milliliter aliquots of the aqueous phase were used for colorimetric Pi determination as described elsewhere [4, 9].

For rat brain membranes similar conditions were used. The reaction was carried out in a final volume of 2 ml of ionic medium containing 100 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$ and 250 μg of membrane protein. The assay for erythrocyte membranes differed by the use of twice the concentration of KCl and 350 μg of membrane protein in the same volume. To calculate $(Na^+ + K^+)$-ATPase activity, the Mg^{++} ATPase activity assayed in the absence of NaCl and KCl was subtracted from the total ATPase activity assayed in the presence of Na^+ and K^+.

Proteins were determined according to Lowry, Rosebrough, Farr and Randall [27].

A Pi calibration curve was carried out in the presence of HME to determine the linearity and reproducibility in triplicate.

Preparation of ^{32}P-ATP

The γ-phosphate labeling of ATP was prepared by the procedure described by Post and Sen [30], but the purification of ATP from other components was carried out as described by Glynn and Chappell [20].

The eluate from Dowex-1 column containing the terminally labeled free ^{32}P-ATP was neutralized with a 2-M Tris solution and stored at −20 °C in several aliquots. Each

aliquot was thawed just once. The yield of the preparation was 90%. The ^{32}P-ATP was analyzed by its optical density at 260 nm, by the determination of total phosphorus, and by 7-min hydrolyzed phosphate.

^{32}P-Labeling of a ($Na^+ + K^+$)-ATPase Intermediate

All the glassware for these experiments was rinsed in water, twice distilled in glass. The assays were carried out in triplicate at 0 °C in a final volume of 1 ml containing 100 mM Tris-Cl at pH 7.6, 50 μM $MgCl_2$, 0.2 μM Tris – ^{32}P-ATP, (100,000 to 200,000 cpm), NaCl varied as indicated and 100 to 150 μg of membrane protein. The reaction was started by the addition of ^{32}P-ATP, incubated for 5 to 120 sec and then stopped by the addition of 1 ml of 10% TCA. The membranes were filtered through a 0.1-μ Millipore filter and washed three times with a cold solution containing 10 mM HCl, 0.1 mM ATP and 0.1 mM Na_2HPO_4. The filters were placed in scintillation vials containing 5 ml of H_2O. Cerenkov counting for ^{32}P was performed in a Mark I scintillation counter.

For experiments studying the kinetics of phosphorylation the reagents were added with Schwarz and Hamilton automatic pipettes. In experiments with oligomycin, this compound was dissolved in ethanol and added to the incubation medium and incorporated before the addition of the enzyme. In all experiments with HME, fresh aqueous solutions were prepared.

Reagents

Harmaline, harmalol and harmine as the chlorhydrate form were obtained from Regis Co., Chicago, Illinois or Sigma Chemical Co., St. Louis, Missouri. ATP, ADP, oligomycin, ouabain, Tris 121, EDTA and the enzymes required for ^{32}P-ATP synthesis were from Sigma Chemical Co., St. Louis. Dowex 50–100 mesh and Dowex 1 were from Biorad Lab., Rockville Center, N.Y. Cysteine from Mann Research, New York. Isobutanol from Merck, Darmstadt. Carrier-free ^{32}P and ^{22}Na (10 mC/mg) were from New England Nuclear, Boston, Massachusetts.

Results

Effect of HME on the Na^+ Outflow from Squid Giant Axons

Eight squid fibers, ranging between 700 and 850 μ in diameter, were microinjected with ^{22}Na to measure sodium outflow into artificial seawater. HME in artificial seawater (ASW) produced a striking fall in sodium extrusion and in intracellular action potential (IAP) (Fig. 1 *a*, *b*). Fig. 1 *a* shows that 0.7 mM HME-ASW produced a marked inhibition of sodium outflow; 30 min later, after the addition of harmaline, the outward movement of Na^+ was only 50% of the initial flow. The action potential, however, falls faster, since 90% inhibition was observed 12 min after the hallucinogen was added. If HME-ASW is replaced by ASW, Na^+ extrusion starts to recover prior to IAP, reaching 70% of the initial rate 50 min later. The effect of 0.3 mM HME was reversed 30 min after removal from seawater (data not shown).

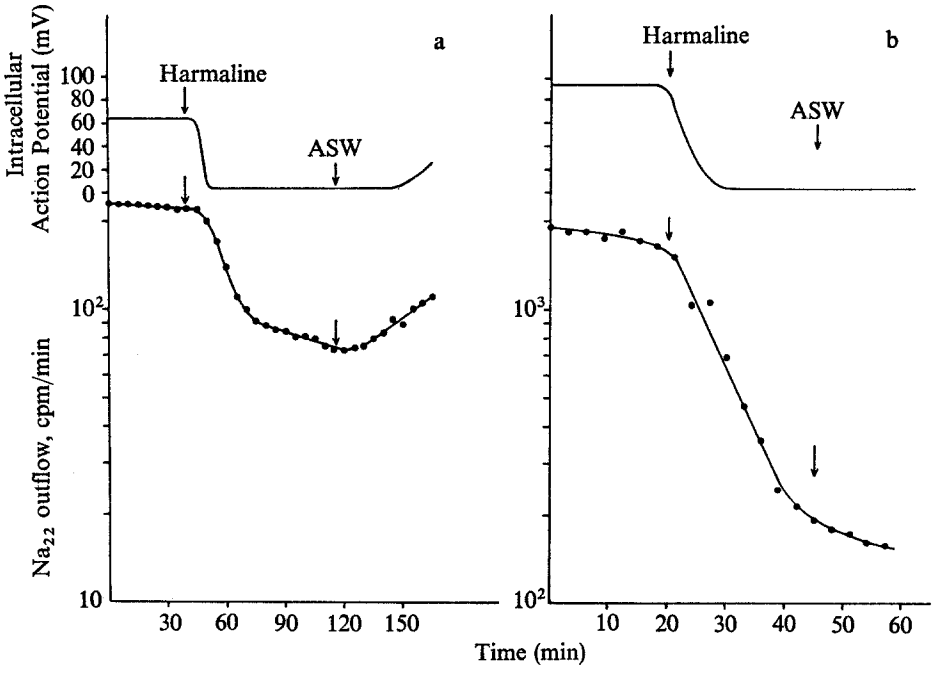


Fig. 1. The effect of harmaline on Na outflow from squid giant axons. The bathing solution was artificial seawater (ASW) maintained at 18 °C. (*a*) Axon diameter was 690 μ and area 0.354 cm^2. The outflow was measured first with ASW for 40 min; at the time indicated by the first arrow, the external solution was changed to seawater containing 0.7 mM harmaline, adjusted to pH 7.4. At the time indicated by the second arrow, the external solution was replaced by ASW. (*b*) Axon diameter 720 μ; area 0.360 cm^2. As above, but with 2.8 mM harmaline

Fig. 1*b* shows that a higher dose of HME (2.8×10^{-3} M) started to block the sodium outflow and action potential in 1 min. 86% inhibition was reached 30 min later. Upon removal of HME, both processes still remained blocked during the following 60 min. It was also found that HME action did not modify the membrane resting potential. Studies carried out by Rojas, Herrera, Delpiano and Riobó [32] in frog sciatic and rat saphenous nerves have shown that 1.4×10^{-3} M harmaline markedly decreased the magnitude of the action potential prior to a rise in the stimulation threshold and slowing of conduction velocity. They also found that HME was blocking specifically the inward Na^+ current. We were unable to perform further experiments on the effect of HME on giant axons due to the scarcity of the squid *Dosidicus gigas* in the Chilean coast after March, 1970.

We have also found that similar concentrations of HME were inhibitory to the short-circuit current across the frog skin.

Table 1. Effect of harmaline and ouabain on the $(Na^+ + K^+)$-ATPase activity of several membrane preparations[a]

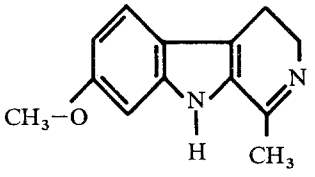
Membrane preparations	$(Na^+ + K^+)$-ATPase specific activity (μmole/mg prot/hr)	% Inhibition		
		Ouabain 0.1 M	Harmaline 0.5 mM	5.25 mM
(a) Squid retinal axon	160 ± 16.5 (15)[b]	100 ± 0.0	10	92 ± 2.2
(b) Rat brain ATPase	39 ± 2.7 (12)	68 ± 7.3	30	88 ± 5.4
(c) Human erythrocyte membranes	2.5 ± 0.3 (3)	70 ± 4.2	50	85 ± 4.1

[a] Plasma membranes were prepared from squid retinal axons [13] and purified according to Nakao *et al.* [28]; the rat brain membranes were purified by using KI on the Nakao procedure; erythrocyte membranes were prepared by gradual osmotic lysis [40]. Assay conditions were (mM): ATP 2, Tris-Cl 100.

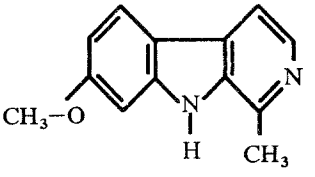
(a) Mg^{2+} 4, Na^+ 200, K^+ 40; pH 7.4 and 8.5 for 0.5 and 5.25 mM HME, respectively.
(b) Mg^{2+} 4, Na^+ 100, K^+ 20, pH 7.4.
(c) Mg^{2+} 4, Na^+ 100, K^+ 40, pH 7.4.

To calculate the $(Na^+ + K^+)$-ATPase activity, the Mg^{++}-ATPase activity assayed in the absence of NaCl and KCl, was subtracted from the total ATPase activity, assayed in the presence of Na and K ions.

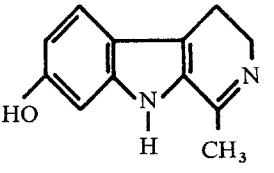
[b] Mean values ± SD. ( ) = Number of membrane preparations.



Harmaline



Harmine



Harmalol

Fig. 2. The structure of harmala alkaloids. These psychoactive compounds are hallucinogens derived from a β-carboline skeleton which is a linear tricyclic structure related both to indole and to pyridine. They are the active compounds of several species of Banisteriopsis used by the natives of South America to prepare the infusion ayahuasca yagé [33]. Harmaline: 4.9 dihydro, 7 methoxy, 1-methyl, 3 H-pyrido [3,4-b] indole hydrochloride. Harmine: 7 methoxy-1-methyl-9 H-pyrido [3,4-b] indole hydrochloride. Harmalol: 4.9 dihydro, 1 methyl, 3 H-pyrido [3,4-b] indol-7-ol hydrochloride

Effect of HME on Several Membrane ATPase Preparations

Table 1 shows that HME blocked the enzyme activity significantly in three types of membrane preparations. A 90% decrease in the ATPase activity was reached at a rather high concentration of HME (5 mM), in the presence of 100 to 200 mM Na^+, the optimal concentration for each membrane. It can be seen that 0.5 mM HME was more inhibitory of erythrocyte membranes whose enzyme had the lowest specific activity; HME concentrations of the same order of magnitude blocked sodium outflow from squid giant axons (Fig. 1) and inhibited the $(Na^+ + K^+)$-ATPase activity of isolated axonal membranes.

Effect of Harmaline-Analogues on the $(Na^+ + K^+)$-ATPase Activity

Fig. 2 shows the structure of several β-carboline derivatives. The effect of these hallucinogens on the ATPase system from squid retinal axon membranes was studied. In Fig. 3, the effect of harmaline, harmine and harmalol on ATPase from the retinal axon membranes is shown. It is clear that all

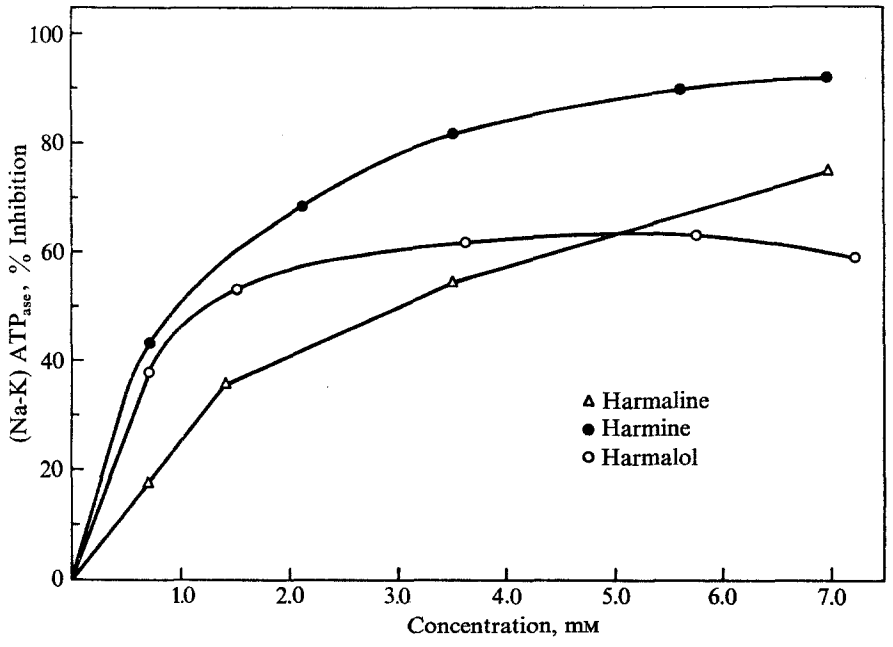


Fig. 3. The inhibition by harmaline-analogues of the $(Na^+ + K^+)$-ATPase from retinal axon membranes of the squid. The enzymic activity was assayed in a final volume of 2 ml of the following composition (mM): Na^+ 200, K^+ 40, Mg^{++} 4, ATP 2, Tris-Cl 100, pH 7.4 and 50 μg of membrane protein. The 3 β-carboline were extracted as indicated in Materials and Methods before performing phosphate analysis. The $(Na^+ + K^+)$-ATPase specific activity of the membrane preparation was 110 μM/mg protein/hr

three compounds showed a striking inhibition of the enzyme system when assayed at pH 7.4 and 200 mM Na^+. The concentrations for 50% inhibition were 3 mM for HME, 1.2 mM for harmalol and 1.0 mM for harmine. Harmine displayed higher inhibition than the other two drugs, since it inhibited by 90% at 4 mM and 200 mM Na^+. It was also found that at pH 8.5, HME significantly increased its ability to block $(Na^+ + K^+)$-ATPase activity, since 92% inhibition was obtained at 5.25 mM (Table 1) whereas the inhibition by harmalol remained unchanged. The effect of harmine could not be studied at a pH higher than 7.4 because of its low solubility in water. A comparison of the inhibitory effect of these derivatives deserves a more complete investigation. The effect of other indole-like molecules was also studied. Preliminary experiments showed that the concentration for 50% inhibition for the diethylamide of lysergic acid was 0.5 mM. The maximal inhibition obtained with mescaline was 26% at 2 mM. Between 1 and 5 mM 5-hydroxy-tryptamine did not inhibit $(Na^+ + K^+)$-ATPase activity.

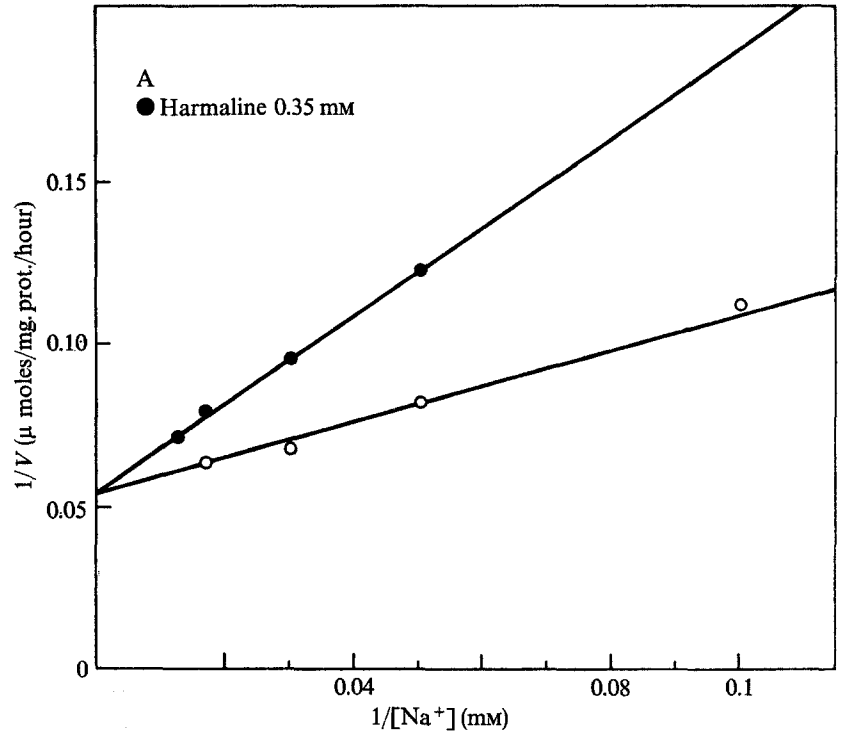
Kinetics of HME Inhibition of the $(Na^+ + K^+)$-ATPase Reaction

The rather high HME concentration needed for ATPase inhibition led us to investigate a possible competition of HME with some of the reaction substrates. The effect of 1 mM HME on the reaction velocity was studied as a function of the ATP concentration (0 to 20 mM) and at a constant concentration of Na^+ (100 mM) and K^+ (20 mM). The experiments indicate that HME inhibition was not a function of the ATP concentrations.

With the concentration of Na^+ at 100 mM and that of K^+ varying between 0 and 30 mM, the inhibition by 1 mM HME was constant ($\pm 10\%$). However, with the concentration of K^+ constant and that of Na^+ varying, inhibition of ATPase activity was inversely proportional to Na^+ concentration.

When the squid axon ATPase was assayed at 20 mM KCl and NaCl was varied from 1 to 20 mM, HME behaved as a competitive inhibitor with respect to Na ions (Fig. 4*A*). 0.8 mM HME increased the $K_{0.5}$ for Na ions of the rat brain enzyme from 9.1 to 15.3 (Fig. 4*B*).

Fig. 4. Lineweaver-Burke plot of the relation of Na^+-concentration to $(Na^+ + K^+)$-ATPase activity. (*A*) The membrane used for enzymic assays were from squid retinal axons. The ionic medium was (mM): Mg^{++} 4, K^+ 40, ATP 2, Tris-Cl 100, pH 7.4. ○, Without harmaline; ●, With 0.35 mM harmaline. (*B*) The membranes used were from rat brain purified with KI [3]. The ionic medium was (mM): Mg^{++} 5, K^+ 20, ATP 2, Tris-Cl 100, pH 7.4. ○, Without harmaline; ●, 0.8 mM harmaline was added to the incubation medium


A
● Harmaline 0.35 mM
0.15
0.10
0.05
0
1/V (μ moles/mg. prot./hour)
0.04
0.08
0.1
1/[Na+] (mM)

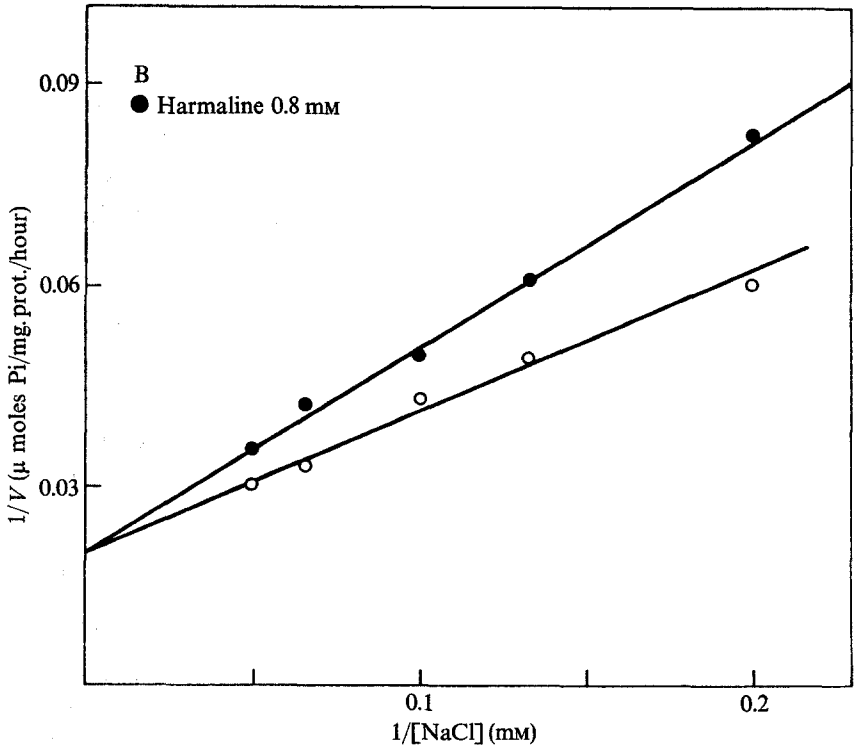

B
● Harmaline 0.8 mM
0.09
0.06
0.03
1/V (μ moles Pi/mg. prot./hour)
0.1
0.2
1/[NaCl] (mM)

Fig. 4A and B

HME Inhibition of the Na^+-Dependent Phosphorylation of the ATPase Reaction

^{32}P incorporated from ^{32}P-ATP into rat brain ATPase preparations were studied at 0 °C and pH 7.6 using a KI-purified enzyme. The labeling of the enzyme was stimulated specifically by sodium ions according to findings of Post, Sen and Rosenthal [31] and Fahn, Koval and Albers [12]. In our hands, the KI preparation showed a phosphorylation process with saturation kinetics. The NaCl concentration for half-maximum activity was 2 mM. The presence of KCl decreased the amount of ^{32}P incorporated. Preincubation with 0.1 mM ouabain did not inhibit the incorporation of radioactivity into the membrane at 0 °C. The Na^+-independent phosphorylation was found to be negligible when the glassware and reagents were carefully washed with double distilled water. This permitted reproducible results when studying HME inhibition of the phosphorylation reaction of $(Na^+ + K^+)$-ATPase at low NaCl concentrations.

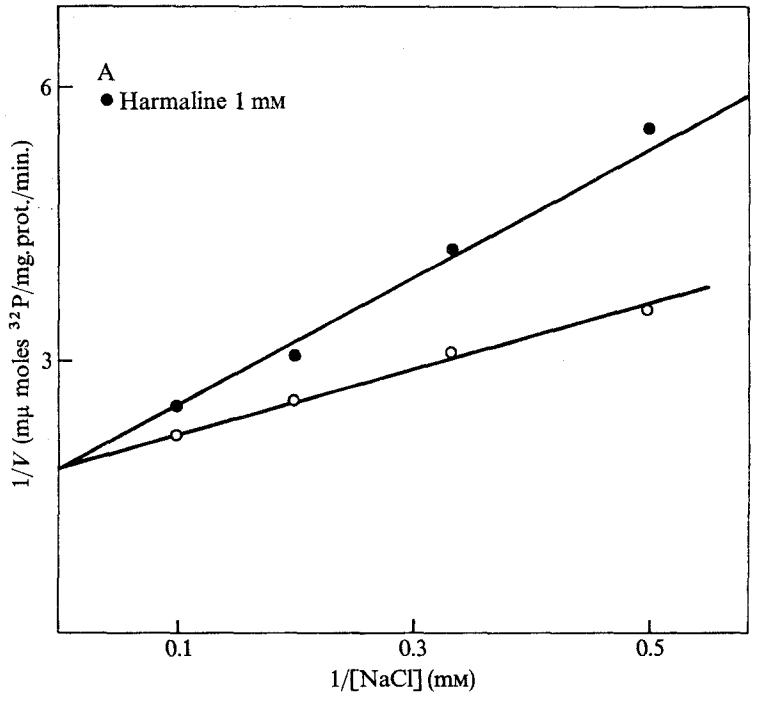
Table 2 shows the effect of several HME concentrations on the phosphorylation reaction of $(Na^+ + K^+)$-ATPase. It can be seen that the percentage of inhibition increased when the NaCl concentration decreased. HME, at 4 mM, completely blocked the enzyme at 1 mM NaCl but only inhibited it by 42% at 50 mM NaCl.

Fig. 5*A* shows a Lineweaver-Burke analysis of phosphate labeling of the enzyme as a function of NaCl concentration. In these experiments initial rates of phosphorylation were measured at 10 sec with labeled ATP. It can be seen that 1 mM HME showed a competitive type of kinetics with respect to sodium ions. The apparent $K_{0.5}$ for Na^+ increased from 2.12 to 5.35 mM with 1 mM harmaline.

It can also be observed that a 100% inhibition was obtained at 1 mM Na^+ (Table 2). According to Dixon and Webb [8] this would indicate that we are dealing with a "fully competitive type" (Type I*a*) inhibitor in which HME combines with Na^+ sites.

It is not possible to compare these values with those obtained for the overall ATPase reaction since the temperatures and ATP concentration are

Fig. 5. Lineweaver-Burke plot of the Na^+-dependent phosphorylation of rat brain membranes. The ^{32}P-labeling of the membranes was assayed as described in Table 2 and Materials and Methods. (*A*) 1 mM harmaline was added to the incubation medium before the reaction was started by the addition of ATP. The reaction mixture contained 120 μg of membrane proteins, 197,000 cpm of ^{32}P-ATP, 0.2 μM Tris-ATP and an incubation time of 10 sec was used in the reaction. (*B*) 2 mM harmaline plus 60 μg/ml of oligomycin were present in the incubation medium before the reaction was started by the addition of 200,000 cpm of ^{32}P-ATP; other conditions were similar to Fig. 5*A*


A
● Harmaline 1 mM
6
3
1/V (mμ moles 32P/mg.prot./min.)
0.1
0.3
0.5
1/[NaCl] (mM)

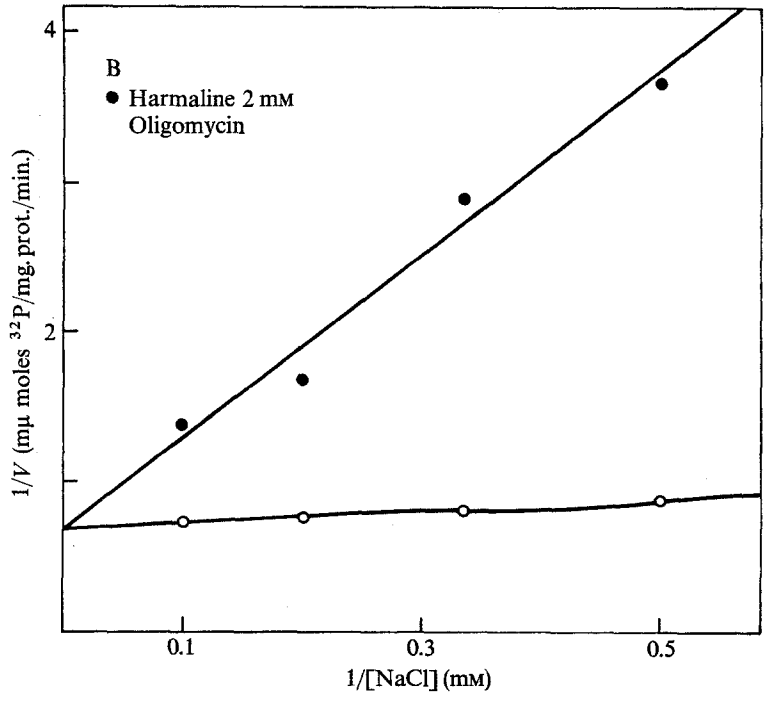

B
● Harmaline 2 mM
Oligomycin
4
2
1/V (mμ moles 32P/mg.prot./min.)
0.1
0.3
0.5
1/[NaCl] (mM)

Fig. 5A and B

Table 2. Effect of harmaline concentration on the Na^+-dependent phosphorylation of the rat brain ATPase system

NaCl concentration (mM)	^{32}P-incorporated (cpm) Harmaline (mM)				
	0.0	0.4	1.0	2.0	4.0
1	4,060	3,710	2,620	1,500	0
10	8,820	9,675	8,570	5,870	2,580
50	11,073	11,753	11,160	8,380	5,020

The content or concentration of the 1 ml final volume of the reaction mixture was: 136 μg of rat brain membrane protein, 0.05 mM Mg^{++}, 0.2 μM ATP, 2×10^5 cpm of ATP^{32}, 100 mM Tris-Cl at pH 7.6. The reaction was started with ATP. Incubation time: 60 sec.

1,420 cpm incorporated in the presence of Mg^{++} were subtracted from each value. The reaction was stopped by 1 ml of 10% TCA, followed by filtration through millipore filters and washing three times with 0.01 N HCl, 100 μM ATP-Tris, 100 μM K_2HPO_4. The filters were counted in 5 ml of water by use of the Cerenkov effect.

different for both types of reactions. Moreover, the presence of K^+ in the ATPase assays might change the affinity of the membrane enzyme for Na^+.

The conventional formulation of the ATPase reaction sequence proposes a conformational change of the phosphorylated enzyme E_1-P to E_2-P that is sensitive to Mg^{2+} concentration [12]; this appears to be the point of action of oligomycin when assayed for the overall reaction. It was important to investigate if HME in the presence of oligomycin was still able to block the Na^+-activated phosphorylation competitively with respect to sodium.

Fig. 5*B* shows a Lineweaver-Burke analysis of the phosphorylated intermediate formation as a function of NaCl concentration in the presence of oligomycin and harmaline. HME, at 2 mM, displayed again clearly competitive kinetics with respect to sodium ions. It can be seen that the $K_{0.5}$ for Na^+, in the presence of oligomycin, is reduced from 2.12 to 0.46 mM as reported previously by Fahn, Hurley, Koval and Albers [10]. HME increased the $K_{0.5}$ for Na^+ from 0.46 to 6.6 mM. These data also indicate that the HME site of action must be restricted to the initial steps of the ATPase reaction under these experimental conditions.

Experiments with ^{32}P-ATP have shown two different kinds of labeling, one that decreases when all the ATP is hydrolyzed and another that does not [38], called by J. C. Skou, the "unstable" and "stable" labeling, respectively. At low ATP concentrations an increase in Na^+ concentration increases the "unstable" labeling.

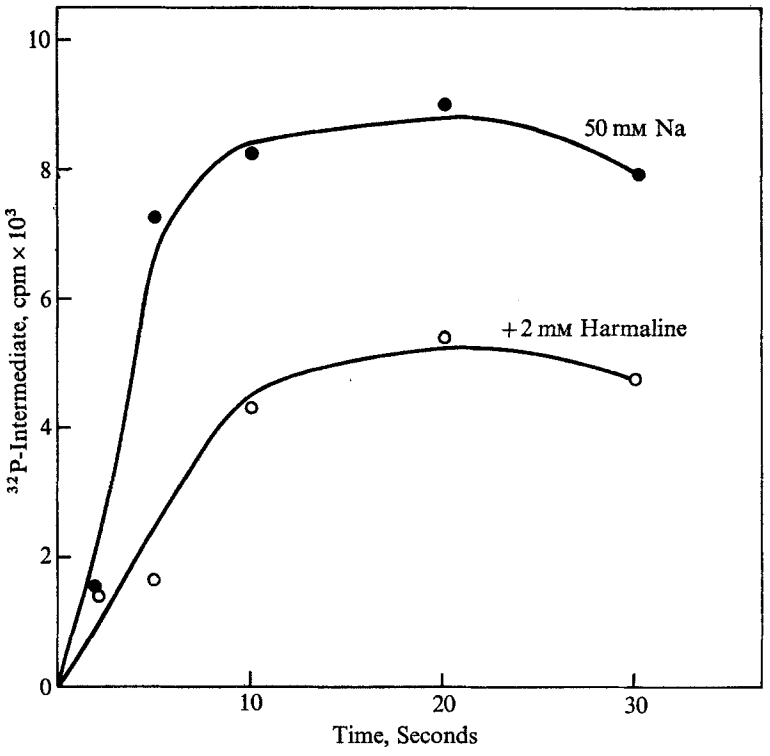


Fig. 6. The effect of harmaline on the kinetics of the Na^+-dependent phosphorylation from rat brain preparation. The ^{32}P-labeling of the membrane ATPase was assayed as described in Table 2 and in Materials and Methods. The incubation medium contained 50 mM NaCl, 114 μg membrane protein, 100,000 cpm ^{32}P-ATP, 0.36 μM Tris-ATP, μM $MgCl_2$ and 100 mM Tris-Cl at pH 7.6. The reaction was started by addition of ATP. •, The time course of Na^+-dependent phosphorylation; ○, 2 mM harmaline were added to the incubation medium before the reaction was started by the addition of ^{32}P-ATP. The time of ATP addition was zero time

Fig. 6 shows the kinetics of the phosphorylation reaction activated by 50 mM NaCl and the effect of 2 mM HME. It can be seen that a plateau of labeling was reached between 10 to 15 sec after addition of ATP. When the reaction was started in the presence of 2 mM HME, an 88% inhibition of enzyme labeling can be observed as early as 5 sec. Later, the inhibition of the ^{32}P-ATP incorporation decreased slowly to 54% at 10 sec, and 43% at 20 and 30 sec. This would indicate that HME is inhibiting the formation of the phosphorylated intermediate.

When HME was added at different times after the phosphorylation reaction was initiated by ATP, the percentage of inhibition at initial time was also a function of the Na^+ concentration.

Effect of HME on the Dephosphorylation of ATPase

Albers, Koval and Siegel [2], postulated that the E-P phosphorylated compound first formed from ATP, which they called E_1-P, is converted to another form, E_2-P, by a reaction which requires Mg^{2+} and which is blocked by NEM or oligomycin. The two forms of the phosphorylated enzyme can be distinguished by their sensitivity to breakdown in the presence of ADP or K^+ [29]. E_1-P should be split only when ADP is added and E_2-P should be split only when K^+ is added.

These tests were applied to the brain enzyme using oligomycin to block the K^+-sensitive breakdown reaction (Table 3). The effect of ADP on the Na^+-dependent phosphorylation was studied in a reaction medium containing oligomycin. When 5 μM ADP was present initially in the incubation medium, the ^{32}P-ATP incorporated into the enzyme was reduced by 41% as compared with the control. When 4 mM HME was present, only 22% of the ^{32}P-ATP was incorporated. These experiments indicate that HME inhibits the effect of ADP on the E_1-P intermediate.

It is quite possible that HME inhibits the backward phosphorylation reaction; thus, the hallucinogen may be competing again with sodium ions at an early step of the transport ATPase reaction.

Experiments were carried out using ^{14}C-ATP as the labeled substrate under similar incubation conditions. A few counts were incorporated into the membrane but incorporation was not stimulated by sodium ions and was not inhibited by HME.

Further evidence that HME does not inhibit the formation of the E_2-P intermediate comes from experiments designed as described by Post, Kume, Tobin, Orcutt and Sen [29]. The nerve membrane preparation was allowed to incorporate ^{32}P from ^{32}P-ATP in the presence of sodium ions, and then,

Table 3. Effect of ADP on the Na^+-dependent phosphorylation of $(Na^+ + K^+)$-ATPase reaction in rat brain membranes

Incubation media	Control (cpm)	Added ADP (cpm)	(% reduction)
Oligomycin	3,330	1,960	41
HME + oligomycin	2,000	1,560	22

The content or concentration of the 1 ml final volume of the reaction mixture was: 50 mM NaCl, 100 mM Tris-Cl at pH 7.6, 0.05 mM Mg^{+2}, 5 μM ADP, 60 μg oligomycin; 4 mM harmaline, 0.2 μM ATP and 100 μg of membrane protein from rat brain. The reaction was started by the addition of ATP. Incubation time: 15 sec. Temperature: 5 °C. The reaction was stopped as indicated in Table 2. Nonspecific phosphatase activity was not detectable at this temperature.

Table 4. Effect of KCl and harmaline on the rate of breakdown of the Na^+-dependent phosphorylation

5-sec ^{32}P-ATP phosphorylation	Control	HME 2 mM	KCl 10 mM	10 mM KCl + 2 mM HME
0 time after cold ATP-Mg	10,050			
2 sec after cold ATP-Mg	7,300			
5 sec after cold ATP-Mg	4,270	5,003	2,290	1,870

The content or concentration of the 1 ml final volume of the reaction mixture was: 0.05 mM $MgCl_2$, 50 mM NaCl, 100 mM Tris-Cl at pH 7.6, 120 μg membrane protein; 1×10^6 cpm of ^{32}P-ATP, 0.2 μM ATP. Temperature 4 °C, volume 1 ml; after 5 sec labeling, 100 μM cold ATP was added. The reaction was stopped as indicated in Table 2. HME, KCl, KCl plus HME were added 3 sec after the chase of cold ATP.

at a time corresponding to zero time, a large amount of unlabeled ATP was added. Since any further incorporation would have been due mainly to unlabeled phosphorus, the subsequent loss of radioactivity gives a measure of the rate of breakdown of the intermediate.

Table 4 shows the rate of dephosphorylation of the native enzyme; it can be seen that it is rapid enough to suggest that a trace of K^+ may have been carried along with the enzymes after the treatment with concentrated KI. However, the behavior of the ATPase reaction showed that the dialyzed and nondialyzed membrane displayed the same $K_{0.5}$ for K ions in the presence of 100 mM Na^+.

It can be seen (Table 4) that potassium ions greatly accelerated the breakdown of the phosphorylated intermediate. The addition of HME caused only a slight decrease on the *E*-P decay as compared with the control.

Under the conditions used in these experiments, the addition of KCl and HME did not modify significantly the rate of breakdown of the phosphorylated intermediate caused by K ions alone.

The fact that KCl is still able to dephosphorylate the *E*-P intermediate in the presence of HME argues against a competition of the β-carboline with oligomycin. It should be noted, however, that we have not tested the effect of HME at lower K^+ concentrations.

Discussion

The data given in this paper indicate that harmaline behaves as an inhibitor of the $(Na^+ + K^+)$-ATPase system at the Na^+-stimulated phosphorylation step of the reaction. It should be noted that the HME concentrations used in this work are at least one order of magnitude greater than those estimated to be required for its psychotomimetic action [24].

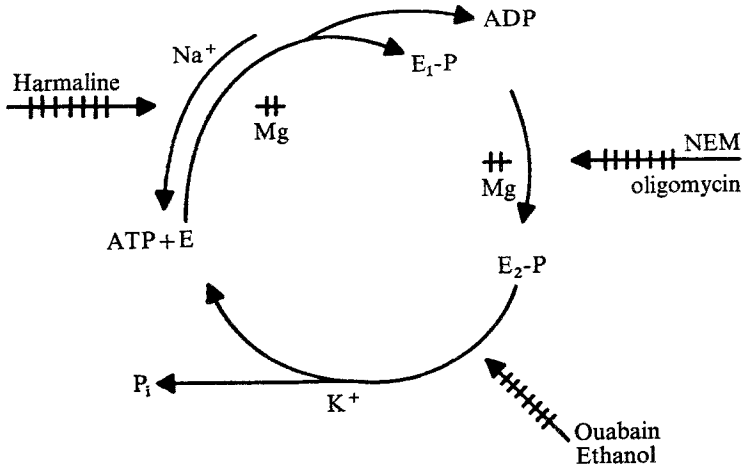


Fig. 7. Scheme to explain the site of action of HME on the partial reaction of the Na pump. The scheme is modified from Siegel and Albers [35] and Post *et al.* [29]. The site of inhibition of harmaline, ouabain, oligomycin, NEM and ethanol [25] are shown by the arrows

A study of the partial reactions of the ATPase showed that the formation of the phosphorylated intermediate from ATP was the stage blocked by HME (Fig. 7). This statement is based on the following experimental findings:

(1) HME inhibited the $(Na^+ + K^+)$-ATPase and the Na^+-dependent phosphorylation reactions at similar concentrations, fully competitively with respect to Na ions.

Since the ATPase system displays a competitive inhibition of Na ion activation by high concentrations of K^+ and a competitive inhibition of K ion activation by high concentrations of Na^+, a kinetic analysis of the HME inhibition at various concentrations of Na and K ions might give us useful information with regard to the nature of Na and K sites.

(2) HME also inhibited the Na^+-stimulated phosphorylation competitively with respect to Na ions when the formation of E_2-P from E_1-P was blocked by oligomycin.

(3) HME inhibited the effect of ADP on the phosphorylation reaction in the presence of oligomycin. When the phosphorylation reaction was started with an ADP-ATP ratio of 25, a 40% decrease of the phosphorylation reaction was observed. This effect of ADP was inhibited by the addition of HME.

Recent findings have shown that in the absence of Mg^{2+}, ADP displaces ATP from its binding sites [23]; so it is quite possible that the ADP present initially in the incubation medium may have prevented ATP from binding to the active center by simple competition before phosphorylation.

Even though the design of the ADP experiment may not distinguish between inhibition of the forward reaction and acceleration of the backward reaction, this interpretation is also supported by experiments in which phosphorylation by ^{32}P-ATP was interrupted with a chase of cold ATP. HME did not accelerate the rate of enzyme dephosphorylation studied by means of additions of cold ATP.

The physical and chemical properties of HME may help to elucidate the geometry of Na^+ sites in the ATPase system, and the sequence of Na^+ and ATP entrance into the early partial reactions of the Na^+ pump.

Even though the kinetic data shows a clear-cut competitive inhibition of harmaline with sodium for sodium-loading sites of the pump, it is possible that HME stabilizes a conformation of the enzyme which does not bind Na^+; thus, HME would produce its competitive effect indirectly and not by displacing Na^+ from its binding sites.

It would be important to investigate whether the charged or uncharged species is involved in mechanism of action of the HME.

The concentration of positively charged harmaline ions must be high for the chlorhydrate salt in aqueous solution (at pH 7.4), so that one would have to postulate that the affinity of the enzyme for the harmaline ion might be similar to the affinity for the sodium ion.

Since the HME molecule has a high partition coefficient in isobutanol/H_2O, it is also possible that the uncharged species of the inhibitor may combine to sites in a hydrophobic environment of the membrane adjacent to polar negative sites responsible for the selectivity for Na^+ ions. The increase of HME inhibition produced by an increase in pH suggests that the uncharged HME is acting on the $(Na^+ + K^+)$-ATPase system.

Accordingly, two molecular interpretations can be made regarding the geometry of Na^+ sites: (*i*) an uncharged Na^+ carrier is formed having some ionic groups useful for infiltration of the binding sites; (*ii*) a charged Na^+ site is formed neighboring a hydrophobic environment and in the vicinity of the phosphorylation site.

Knowing that HME behaves as a competitive inhibitor of Na ions a question of utmost interest from a mechanistic point of view is the step in the reaction sequence of the $(Na^+ + K^+)$-ATPase at which Na ions might interact.

The process of Na activation of the enzyme phosphorylation can be represented in the following steps:

$$E + \mathrm{ATP} \overset{\mathrm{I}}{\rightleftharpoons} E - \mathrm{ATP} \overset{\mathrm{II}}{\rightleftharpoons} E - \mathrm{P} + \mathrm{ADP}$$

which involves the binding of ATP and the formation of an *E*-P compound; on this scheme Mg ions are omitted since according to Hegyvary and Post [23], ATP combines with the enzymes without Mg^{++}, but other kinetic evidence suggests that Mg-ATP is the only true substrate [37].

In this sequence, it is possible for Na ions to interact at two different steps. Step I: Na ions should react first with enzyme favoring the binding of ATP. Step II: Na ions should react with *E*-ATP complex favoring the formation of the phosphorylated intermediate with the release of ADP.

As demonstrated by Hegyvary and Post [23], Na ions showed no significant change of ATP binding, in the absence of Mg ions. This result is in accordance with the lack of effect of HME on ^{14}C-ATP labeling in the presence of Mg and Na ions. Our experiments show that HME blocked the formation of phosphorylated intermediate competitively with respect to Na ions. The entrance of Na ions at Step II would be supported by these findings as well as by the experiments showing that HME did not increase the rate of dephosphorylation but blocked the ADP inhibition of the phosphorylation reaction.

Thus, a more likely site of action for Na ions will be the interaction with the enzyme system prior to the phosphorylation reaction.

The proposed order of events in this enzymic catalysis reaction may imply that Na ions might be involved in the orientation of enzyme groups into a configuration favoring the phosphorylation reaction. The binding with Na ions would facilitate bond changing reactions and confer selectivity to the Na^+-dependent phosphorylation step.

In this scheme, the nature of the transport must involve a step subsequent to the transphosphorylation reaction. This is also in accordance with evidences recently discussed by Glynn and Hoffman [21] and Glynn, Hoffman and Lew [22]. ADP may act to reverse a reaction of which it is a product, being necessary for Na^+-Na^+ exchange. A reversible transphosphorylation associated with the inward and outward movement of Na^+ explains also why the shuttling of the cation requires ADP as well as ATP. Since oligomycin inhibited the Na^+-Na^+ exchange (transport reaction) [17], but stimulated the ATP-ADP exchange [3], Glynn *et al.* proposed that Na^+ exchange involved steps beyond the formation of the phosphorylated intermediate [22].

We think that to approach the nature of the Na^+ transport site, investigators may take advantage of the HME inhibition on the Na-dependent phosphorylation. For instance, harmaline fluorescence properties may allow spectroscopy on critical membrane components.

The excellent technical assistance of Mrs. Gricelda Bravo and the secretarial work of Mrs. Ildina Cerda is gratefully acknowledged. Thanks are due to Dr. Siegmund Fischer for his continuous support and to Dr. R. L. Post for discussing our manuscript. This research was supported by the "Comisión de Investigación Científica, Universidad de Chile", Project No. 82–70.

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