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EFFECTS OF ATP AND MAGNESIUM IONS ON THE FLUORESCENCE OF HARMALA ALKALOIDS. RESTRICTIONS FOR THE USE OF HARMALA ALKALOIDS AS FLUORESCENT PROBES FOR $(Na^+ + K^+)$-ATPase

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SUMMARY

1. Harmine and harmaline were investigated as potentially useful fluorescent inhibitors of $(Na^+ + K^+)$activated ATPase.

2. From spectroscopic measurements both compounds were shown to form 1 : 1 complexes with ATP, the dissociation constants being 0.65 mM and 1.83 mM for harmine and harmaline respectively. Addition of Mg^{2+} and enzyme further affected these equilibria.

3. Although it was possible to demonstrate a competitive effect of harmine at the sodium-loading site of the enzyme, other inhibitory effects, including inhibitions of ouabain binding and the ouabain-insensitive ATPase were found.

4. It was concluded that the harmala alkaloids can inhibit $(Na^+ + K^+)$-activated ATPase in a complex way involving both Na- and ATP-binding sites. This severely limits their usefulness as spectroscopic probes.

INTRODUCTION

The hallucinogenic drugs harmine and harmaline are members of the linear tricyclic group of harmala alkaloids which induce disturbances in behaviour and perception [1, 2]. Recently these compounds have been shown to inhibit the $(Na^+ + K^+)$-ATPase systems of squid retinal axon, rat brain and human erythrocyte membranes, where their mechanism of action is reported to be competitive inhibition at the Na^+-activation site of the system [3, 24]. A similar effect of harmaline as a competitive inhibitor has been reported for Na-dependent amino acid transport in kidney and intestine [4], Na : Ca systems in skeletal muscle* and Na transport in frog skin and crayfish gill [5].

These interesting psychomimetic compounds are spectroscopically active with strong blue fluorescence between 420 and 485 nm. This combination of properties makes these agents potentially useful site-specific fluorescent probes to investigate the

* Lea, T. J., Ashley, C. C. and Ellory, J. C. (1976) submitted for publication.

molecular mechanism of Na-dependent membrane transport processes, and $(Na^+ + K^+)$-ATPase activity in particular.

We have therefore examined the properties of harmine and harmaline both in free solution and when bound to membranes containing $(Na^+ + K^+)$-ATPase activity. Our results suggest that the interaction of these drugs with membrane ATPase systems and their various ligands, is much more complex than has been reported [3, 4, 24] making the interpretation of the fluorescence data more difficult and limiting the usefulness of these agents as membrane probes.

MATERIALS AND METHODS

Membranes rich in $(Na^+ + K^+)$-ATPase activity were prepared from sheep and pig kidney cortex and outer medulla by the procedure recently described by Charnock and Bashford [6]. A membrane preparation of $(Na^+ + K^+)$-ATPase was obtained from crab leg nerve (*Cancer pagurus*) following 30-s disruption in the cold in a Willems Ultra-Turrax mincer (Model TP 18/2 Manufactured by Janke and Kunkel KG) in a medium of 0.25 M sucrose/30 mM histidine/20 mM Tris base/2 mM Na_2 EDTA, adjusted to pH 7.6 by the addition of 1 M HCl. The crude mince was centrifuged at $1500 \times g$ for 10 min to sediment the coarse debris, followed by centrifugation at $12\,000 \times g$ for 30 min to obtain a post-mitochondrial fraction. The supernatant was then centrifuged at $38\,000 \times g$ for 120 min to yield a microsomal fraction containing $(Na^+ + K^+)$-ATPase activity. The kidney enzyme had a specific activity of 1–6 μmol phosphate released/mg protein per h, and was 65–90 % ouabain inhibitable. The crab material gave activities from 2–7 μmol phosphate released/mg protein per h and was 40–70 % ouabain-sensitive Human red cell membranes were obtained by the method of Fortes et al. [7], whose procedure was also used for all determinations of enzyme activity. Protein was determined by the method of Lowry et al. [8], and $(Na^+ + K^+)$-ATPase activity was calculated as the difference in activity in the presence and absence of 0.1 mM ouabain (kidney) or 1 mM (crab), following incubation for 15–30 min in 100 mM NaCl/10 mM KCl/1 mM Na_3ATP/0.5 mM $MgCl_2$/15 mM Tris · HCl pH 7.6 at 37 °C.

Changes in the fluorescence intensity of harmine and harmaline both in solution and after binding to $(Na^+ + K^+)$-ATPase-containing membranes were determined at room temperature using a Hitachi-Perkin Elmer MPF-2A spectrofluorometer. The excitation and emission spectra of both agents were not affected by the catalytic amounts of enzyme protein that were employed in this study.

The binding of [^{3}H]ouabain to human erythrocyte membranes and the determination of *p*-nitrophenylphosphatase activity of sheep kidney $(Na^+ + K^+)$-ATPase-containing preparations were determined by methods described previously [7].

Both harmine and harmaline were obtained from The Sigma Chemical Co. St. Louis, Mo. and were purified by recrystallisation from ethanol.

RESULTS AND DISCUSSION

Harmine fluorescence

The fluorescence excitation and emission spectra of harmine were recorded at room temperature in 10 mM Tris buffer pH 7.4. The excitation spectrum showed a

TABLE I

EFFECT OF ATP ADDITION ON HARMINE FLUORESCENCE

Fluorescence intensity measured at 320 nm excitation and 420 nm emission. Values given have been corrected for ATP addition and volume dilution. At 320 nm the absorbance of 5 μM harmine was 0.086 in the absence of ATP.

ATP (mM)	Corrected fluorescence intensity (%)	% Quenching
Control	78.7	nil
0.025	78.0	1.0
0.049	77.1	2.1
0.074	76.1	3.3
0.098	75.5	4.1
0.147	74.2	5.7
0.195	73.2	7.0
0.243	72.1	8.4
0.290	71.3	9.4
0.336	70.5	10.4
0.383	69.3	12.0
0.429	68.7	12.8
0.474*	68.2	13.4

* The addition of 0.45 mM $MgCl_2$ to this system enhanced the fluorescence intensity (E) by 3.5 %.

maximum of 324 nm and a shoulder at 357 nm. The emission spectrum gave a single maximum at 413 nm. These values were taken from uncorrected machine spectra. None of the peaks were shifted by the addition of the catalytic amounts of enzyme protein used in this study (less than 10 μg protein/ml final concentration).

The fluorescence emission of harmine was quenched 10–20 % by the addition of ATP in normal substrate concentrations (< 2.5 mM). When 350 nm exciting light was employed there was a measurable contribution to the fluorescence emission from the ATP solution. This troublesome artefact was markedly reduced by shifting to the lower 324 nm excitation peak and in all subsequent experiments excitation was with 320 nm light and the emission was measured at 420 nm.

Table I gives the degree of fluorescence quenching measured in a 5 μM solution of harmine upon the serial addition of ATP. In this experiment the degree of quenching reaches a maximum of 13.4 %. The quenching was partially reversed (3.5 %) by the addition of equimolar $Mg\,Cl_2$ to ATP.

The ATP-induced quenching of harmine fluorescence (5 μM) was measured at a number of different concentrations of $Mg\,Cl_2$. Harmine fluorescence was reduced in the presence of 0.05 mM $Mg\,Cl_2$ and abolished in the presence of 0.3 mM $Mg\,Cl_2$. These results obtained with harmine in free solution suggest that a complex interaction between the enzyme inhibitor, the enzyme substrate and the enzyme ligand is possible as well as the interaction of the drug with the Na^+ activation site which has been reported [3, 4, 24].

The mechanism of the fluorescence quenching can be either "static" or "dynamic" [9, 10]. Dynamic quenching is a diffusion controlled process described by the classical Stern-Volmer relation [9–11].

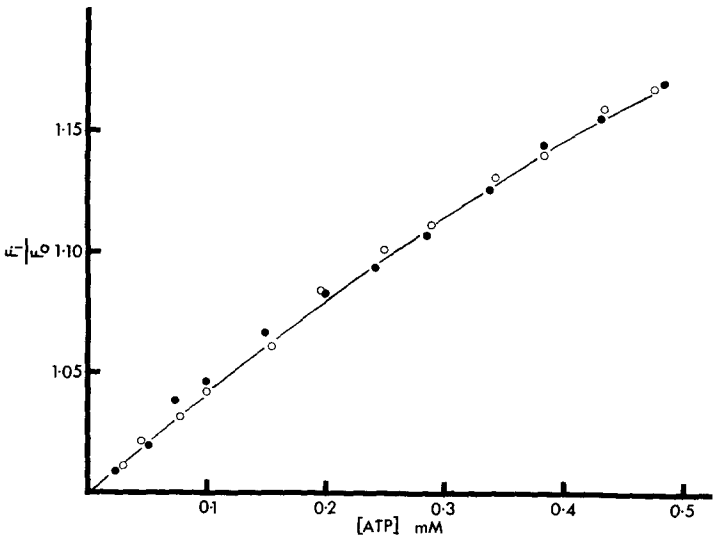

Fig. 1. Stern and Volmer plot for the quenching of harmine fluorescence by ATP. F_1/F_0 is the ratio of the fluorescence intensity of free harmine with the intensity in the presence of ATP. Harmine concentration 5 μM in 10 mM Tris pH 7.4 at 25 °C. ATP was added serially as the sodium salt adjusted to pH 7.5 with NaOH. Intensities were all corrected for changes in cuvette volume. ○–○, control in the absence of $MgCl_2$; ●–●, in the presence of crab nerve ($Na^+ + K^+$)-ATPase. The curve approaches a limiting value for F_1/F_0 of 1.52.

$F_0/F_1 = 1/1+K[Q]$ where F_0 is the observed fluorescence in the presence of quencher, F_1 the initial fluorescence in the absence of quencher and K the quenching constant. Quenching of this type is characterised by linear plots of F_1/F_0 versus $[Q]$ of slope K. In the experiments reported here such plots are non-linear and the value of F_1/F_0 tends to a maximum at high concentration of ATP (Fig. 1). This implies that quenching occurs by a static mechanism possibly by the formation of a ground state complex with reduced fluorescence. Support for this type of mechanism is afforded by the observation that the optical density at 320 nm of a 5 μM harmine solution was reduced by the addition of ATP. Under such circumstances the dissociation constant for the harmine/ATP interaction can be calculated from the fluorescence data alone.

The fluorescence enhancement can be defined as $E = F_0/F_1$ where F_0 is the observed fluorescence and F_1 the initial fluorescence in the absence of interacting ligand. The limiting enhancement (E_b) is the value of E when all the fluorophore is complexed, and C is the fraction of fluorophore complexed. Now

$$F_0/F_1 = E = 1-C+C \cdot E_b$$
$$C-E_b \cdot C = 1-E$$
$$C = \frac{1-E}{1-E_b}$$

A double reciprocal plot of $1/1-E$ versus $1/ATP$ will yield a value for $1/1-E_b$ at the ordinate intercept. This value can then be used to calculate C, the fraction of fluorophore complexed, at all points on the titration curve. The dissociation constant (K_D)

TABLE II

DISSOCIATION CONSTANT (K_D) OF HARMINE/ATP MEASURED UNDER A VARIETY OF EXPERIMENTAL CONDITIONS

n = number of binding sites per mol of harmine.

Experiment	Conditions	K_D	n
a	Control	0.64±.05	1.09±.06
b	Control	0.69±.05	0.98±.05
c	+Pig $(Na^+ + K^+)$-ATPase	0.79±.10	1.03±.10
d	+Crab $(Na^+ + K^+)$-ATPase	0.75±.13	0.72±.09

for the ATP/harmine interaction and a value for n, the number of binding sites per mol can then be obtained from a graphical solution of the Scatchard relationship [12].

The results of duplicate experiments yielded values for K_D (ATP/harmine) of 0.64 mM±0.05 and 0.69 mM±0.05 with values for n of 1.09±0.06 and 0.98±0.05 respectively. Clearly there is a 1 : 1 harmine/ATP complex formed under these conditions with a dissociation constant near 0.7 mM.

When harmine was bound to ATP in the absence of Mg^{2+} but in the presence of either pig or crab membranes containing $(Na^+ + K^+)$-ATPase there was no significant difference in K_D from the controls although there was apparently some reduction in the value of n for the crab preparation (Table II). However in the presence of Mg^{2+} the interaction becomes complex yielding more than one value for K_D and n (Fig. 2). Such a result could arise if there were more than one site for harmine interaction under these conditions; apparently the presence of enzyme is incidental to this result.

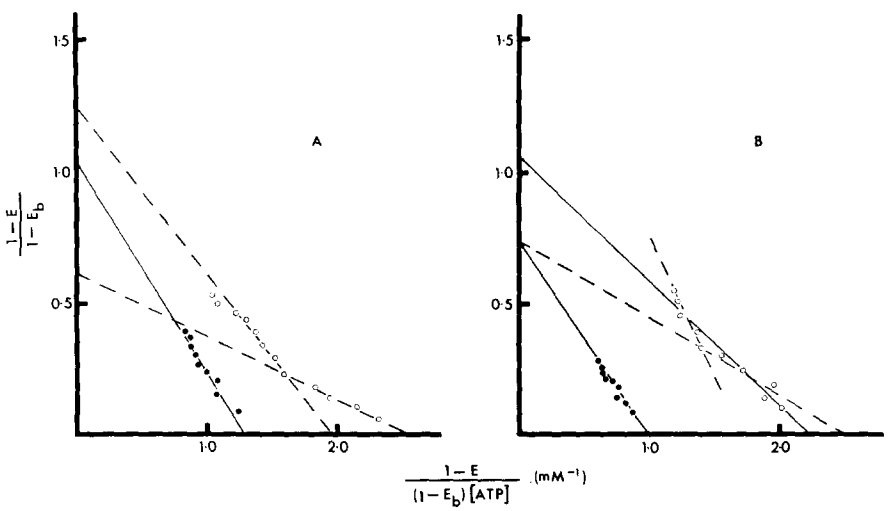

Fig. 2. The effect of Mg^{2+} on the ATP-induced quenching of harmine fluorescence in the presence of $(Na^+ + K^+)$-ATPase-containing membranes. Excitation and emission wavelengths as in Fig. 1. Harmine was 5 μM. 10 mM ATP added as the Tris salt adjusted to pH 7.5 with 0.01 M HCl. Part A: Pig kidney $(Na^+ + K^+)$-ATPase, ●–● control, no Mg^{2+} added; ○–○, 10 mM $MgCl_2$. Part B: Crab nerve $(Na^+ + K^+)$-ATPase, ●–● control, no Mg^{2+} added; ○–○, 10 mM $MgCl_2$.

TABLE III

EFFECT OF ATP ON HARMALINE FLUORESCENCE

Fluorescence intensity measured at 380 nm excitation and 480 nm emission. Values given here have been corrected for ATP addition and volume dilution.

ATP (mM)	Corrected fluorescence intensity (%)	% Quenching
Control	83.4	nil
0.62	76.7	8.0
1.25	73.1	12.4
1.87	70.9	15.0
2.50	68.7	17.6
3.12	67.2	19.4
3.75	66.2	20.6
4.37	65.0	22.1
5.00*	64.2	23.0

* The addition of 5 mM $MgCl_2$ to this system enhanced the fluorescence intensity (E) by 11.2 %.

Similar experiments with 1 μM harmaline, the more saturated analogue of harmine also indicate a marked quenching of fluorescence on the addition of ATP, which could again be partially reversed by the subsequent addition of Mg^{2+} (Table III).

Calculation of the harmaline/ATP dissociation constant in the absence of membrane enzyme gave a value of $K_D = 1.83$ mM with $n = 0.97$. In the presence of

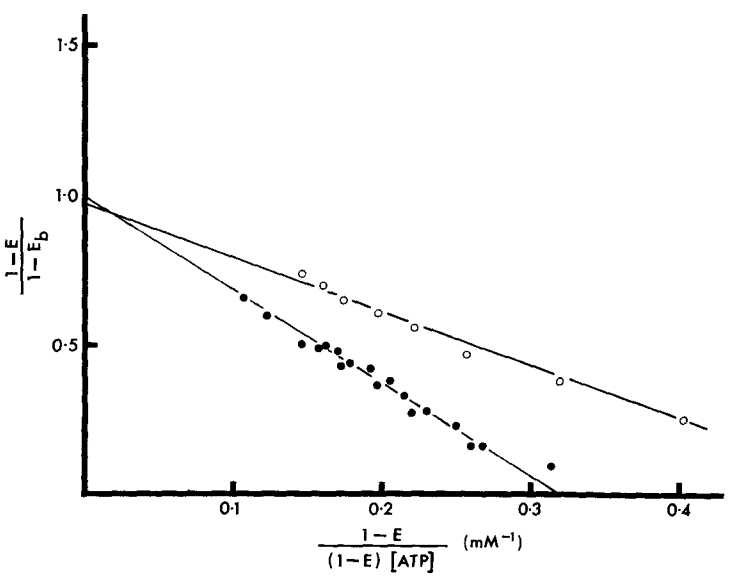

Fig. 3. The effect of ($Na^+ + K^+$)-ATPase membranes on the ATP-induced quenching of harmaline fluorescence. Excitation wavelength 380 nm; emission wavelength 480 nm. Harmaline concentration 1 μM; ATP added as the Tris salt adjusted to pH 7.5 with 0.01 M HCl. ●–●, in the presence of sheep kidney ($Na^+ + K^+$)-ATPase containing membranes; ○–○, control, no enzyme present.

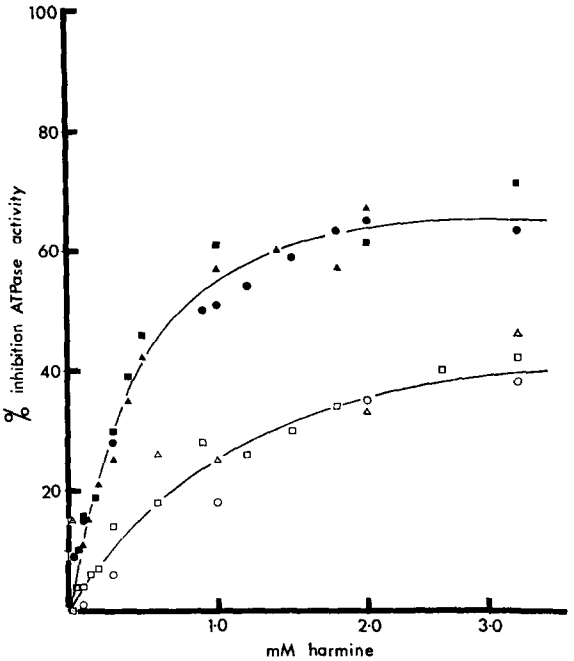

Fig. 4. Concentration dependence of the harmine inhibition of the ATPase activity of sheep kidney membranes. Assay system contained 100 mM Na^+/10 mM K^+/1 mM disodium ATP/0.5 mM $MgCl_2$. 0.1 mM ouabain added where required. Ouabain-sensitive ($Na^+ + K^+$)-ATPase (solid symbols); ouabain-insensitive Mg-ATPase (open symbols). Data are the mean of duplicate assays on three different membrane preparations.

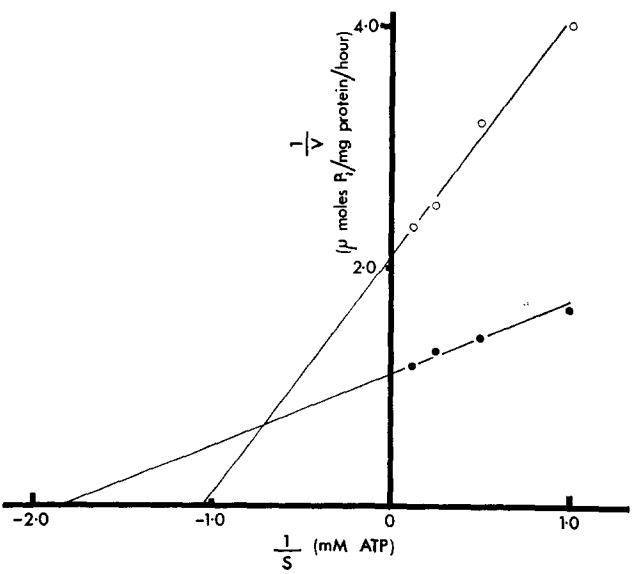

Fig. 5. The effect of harmine on the reaction velocity of sheep kidney ($Na^+ + K^+$)-ATPase. A double reciprocal plot of $1/v$ vs $1/s$. ●–●, control, no harmine added. ○–○, 3 mM harmine. The calculated apparent K_m for ATP in the absence of harmine was 0.54 mM. K_I for harmine was 0.94 mM. Data are the mean of duplicate determinations on a single enzyme preparation.

sheep kidney enzyme the harmaline/ATP dissociation constant was $K_D = 3.1$ mM with $n = 0.99$ (Fig. 3). Thus, although there is still only one binding site per mol the interaction of harmaline with ATP is weaker than that previously found for harmine and ATP.

It is important to note that this decrease in dissociation constant is in agreement with the decrease in inhibitor potency for these agents reported by Canessa et al. [3].

As all these results strongly suggest a direct interaction of both harmine and harmaline with ATP as a potential cause of the inhibition of $(Na^+ + K^+)$-ATPase, or for that matter any other membrane ATPase, a further examination of the mechanism of inhibition of harmine seemed warranted.

A plot of the effect of increasing harmine concentration versus the degree of inhibition of both ouabain-sensitive $(Na^+ + K^+)$-ATPase and ouabain-insensitive Mg^{2+}-ATPase is given in Fig. 4. It is clear that both membrane ATPase systems are inhibited by harmine which in these experiments was able to produce only 65 % inhibition of $(Na^+ + K^+)$-ATPase and 40 % inhibition of Mg^{2+}-ATPase activity at 3 mM drug concentration.

When the effect of harmine on $(Na^+ + K^+)$-ATPase was examined at variable concentrations of ATP, it can be seen from the double reciprocal plots shown in Fig 5 that the intercept is neither on the x nor on the y axis, consistent with some form of mixed inhibition [13].

As Canessa et al. [3] have reported competitive inhibition with Na^+ and harmaline on $(Na^+ + K^+)$-ATPase we therefore re-examined the effect of Na^+ at

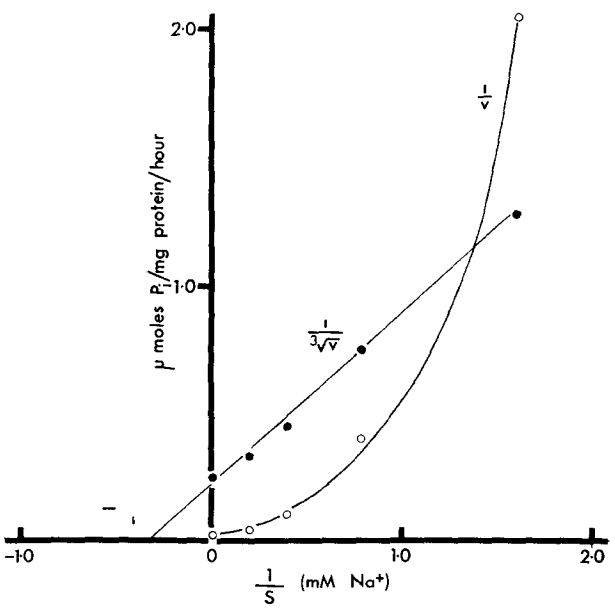

Fig. 6. Comparison of a cubic double reciprocal plot ($1/3\sqrt{v}$ vs. $1/s$) and a double reciprocal plot ($1/v$ vs. $1/s$) of the effect of $[Na^+]$ on the reaction velocity of sheep kidney $(Na^+ + K^+)$-ATPase. Assay system was described under Methods and in Fig. 4. Data are the mean of triplicate determinations on a single enzyme preparation.

fixed concentrations of substrate (Mg^{2+}-ATP) and inhibitor (harmine). Since (Na^+-K^+)-activated ATPase is an asymmetric membrane-bound multisite enzyme, with both external and internal affinities for Na^+ and K^+, kinetic experiments are difficult to interpret unequivocally. Several authors have analysed the Na^+/enzyme interaction in terms of multisite kinetics [14–17]. It now seems generally accepted, however, that results can be well described in terms of three internal Na-loading sites with an affinity for both Na (0.19 mM) and K (9 mM) [18]. Making the cubic reciprocal plot for ouabain-sensitive ATPase activity versus reciprocal Na concentration (Fig. 6) yields a good straight line (in contrast to the simple reciprocal plot which is markedly parabolic). Using this approach harmine can be shown to be behaving as a competitive inhibitor for Na, in confirmation of the results of Canessa et al. [3] (Fig. 7). From five inhibition experiments, the mean value for the x-intercept, representing the apparent K_m for Na but containing a term for K inhibition at the Na site, was 3.26$\pm$ 0.11 mM for each of three sites. From inhibitor experiments at harmine concentrations from 0.15–1 mM (ATP remaining constant at 2.6 mM), the derived apparent K_i for harmine was 0.28$\pm$0.03 mM. Although these values do not represent true kinetic constants they enable us to say that harmine is an effective competitive inhibitor at the Na-loading site.

In order to examine the specificity of harmine inhibition further we examined several of the partial reactions of ($Na^+ + K^+$)-ATPase which are believed to separate to some extent the requirements for various ligands [19].

For example, we found that 1 mM harmine was without significant effect upon the K-dependent *p*-nitrophenylphosphatase activity of the kidney enzyme preparation, while at relatively low concentrations it inhibited the binding of [^{3}H]ouabain to

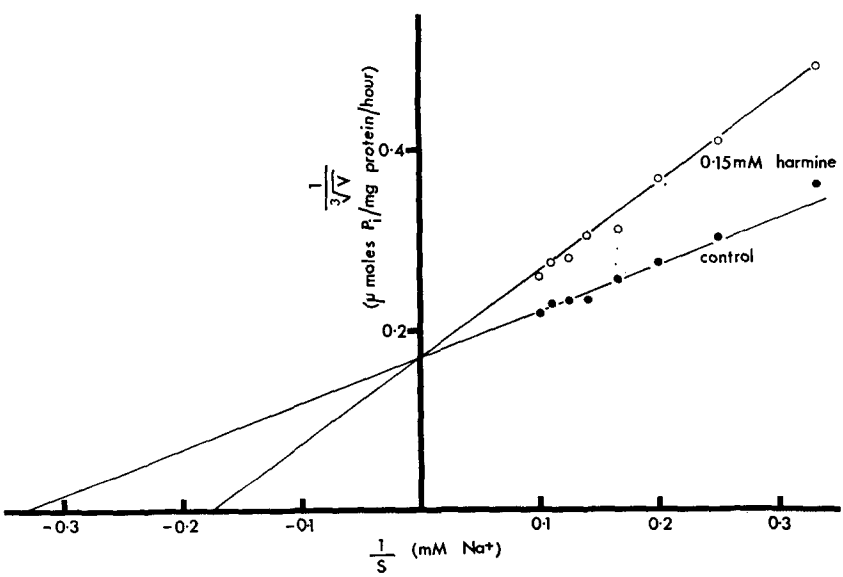

Fig. 7. Cubic reciprocal plot of the effect of [Na^+] on the reaction velocity of pig kidney ($Na^+ + K^+$)-ATPase in the presence and absence of harmine. Assay system and results as described under Methods and in Fig. 4 except that [Mg ATP] was 2.6 mM. ●–●, control no harmine added. ○–○, 0.15 mM harmine.

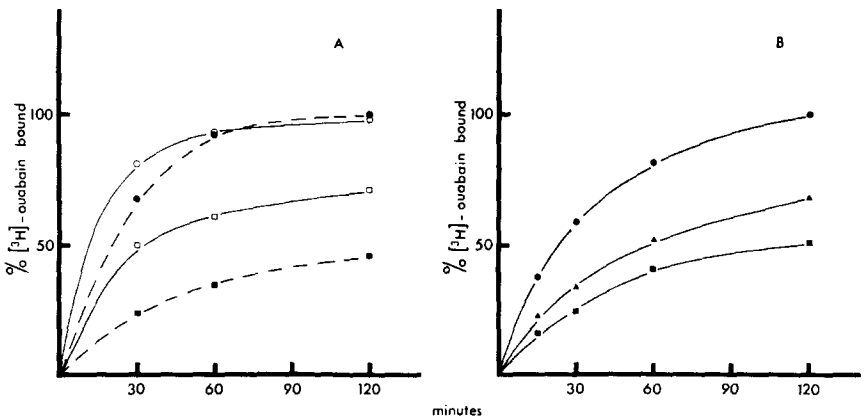

Fig. 8. The effect of harmine on the rate of [^{3}H]ouabain binding to human red cell ghosts. Experimental conditions: 8 nM [^{3}H]ouabain, 100 mM NaCl, 3 mM $MgCl_2$. 10 mM Tris (pH 7.5 at 37 °C), 3 mM Na_3 ATP or 5 mM phosphate where required. Part A: ○–○, ATP, Na^+ and Mg^{2+}; □–□, ATP, Na^+ and Mg^{2+}, plus 1 mM harmine; ●–●, Mg^{2+} and P_i; ■–■, Mg^{2+} and P_i plus 1 mM harmine. Part B: ●–●, Mg^{2+} and P_i control no harmine added; ▲–▲, Mg^{2+} and P_i plus 0.3 mM harmine; ■–■, Mg^{2+} and P_i plus 0.6 mM harmine.

human red cell membranes whether the binding of [^{3}H]ouabain was supported by either ATP and Na^+, or by Mg^{2+} and P_i [20–23]. The results of the [^{3}H]ouabain binding experiments are given in Fig. 8. Thus it is clear that reactions of $(Na^+ + K^+)$-ATPase which superficially do not involve either Na^+ or ATP can also be inhibited by harmine. In other experiments directed towards the specificity of harmine inhibition we re-examined the effect of harmine upon the ouabain-insensitive Mg^{2+}-dependent ATPase of our membrane enzyme preparations, which in the experiments reported

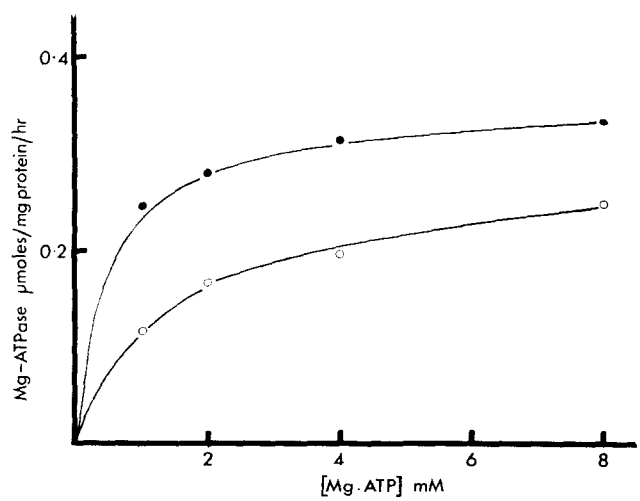

Fig. 9. Effect of harmine on Mg ATPase activity of sheep kidney membranes. Assay conditions as described under Methods and in Fig. 4. 0.1 mM ouabain present in all tubes. ●–●, control, no harmine added; ○–○, 3 mM harmine.

above had already shown considerable inhibition in the presence of 3 mM harmine (cf. Fig. 4).

By varying the concentration of Mg-ATP we could demonstrate that the level of inhibition of Mg-ATPase activity was decreased with increasing substrate concentration (Fig. 9). In view of our earlier findings of the effect of Mg^{2+} on harmine/ATP fluorescence quenching it seems likely that the Mg-ATP interaction is stronger than the harmine/ATP interaction. However, both reactions are probably important in determining the degree of inhibition of these systems. In general it would seem that the very complex interactions of harmine with both the substrate for these reactions (Mg-ATP) and as a competitive inhibitor with the activating ligand (Na^+) would suggest that the inhibitory action of these agents is not confined to competition with Na^+ at the sites of Na^+ activation [3, 4, 24]. This lack of specificity will greatly limit the usefulness of these agents as fluorescent probes in an examination of the molecular mechanism of membrane transport reactions.

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