DISCUSSION

The administration of AITC, thiourea and thiouracil reduced blood coagulation time by 30–40 per cent confirming previous results [4]. It appears that goitrogenic compounds in general are powerful agents for shortening the blood coagulation time. When thyroxine was administered with the antithyroid compounds, this effect on blood coagulation time is largely reversed.

The total phospholipid concentration of plasma was raised by 30–35 per cent in the groups treated with AITC, thiourea and thiouracil. Analysis of the individual phospholipid showed that the greatest increase was in the phosphatidyl ethanolamine fraction (55–60%). As blood platelets contain a considerable amount of phospholipids which are essential for the coagulation process [5], this increase in the concentration of plasma phospholipids may accelerate the coagulation process. In the thyroxine treated animals no such change occurred and the effects of antithyroid compounds were greatly reduced by simultaneous administration of thyroxine.

These results might explain why blood coagulation in rats is accelerated by administration of antithyroid compounds. It was shown previously [13] that the effect of feeding different fats on blood coagulation was related to changes in the phospholipid content of the plasma.

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Institute of Nutrition,
University of Dacca,
Dacca, Bangladesh

RAFIDA IDRIS
K. AHMAD

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Harmaline inhibits the $(Na^+ + K^+)$-dependent ATPase by affecting both Na^+ and K^+ activation

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The judicious use of inhibitors is frequently of enormous assistance in efforts to understand enzymatic processes. For the $(Na^+ + K^+)$-dependent ATPase, which is the enzymatic representation of the sodium/potassium pump of the cell membrane [1], further assistance in such efforts is greatly needed, in light of the overall complexity of the reaction mechanism [2]. This is particularly evident in terms of defining the cation sites that activate the enzyme and that are involved in the actual translocation process, since conflicting formulations describing such sites await resolution [3]. Currently, there are available several agents that modify affinities of the cation sites, including (1) phlorizin [4] and (2) deuterium oxide [5], both of which increase the apparent affinity for K^+ but decrease it for Na^+, and (3) dimethylsulfoxide [6–8], which increases the apparent affinity for K^+ of the associated K^+-dependent phosphatase reaction, but also acts as a "competitive" activator toward some substrates of the reaction and, in addition, is an uncompetitive inhibitor toward Na^+. Beyond these, a reagent that affected only activation by one cation would be particularly desirable. Canessa *et al.* [9] recently reported inhibition of this ATPase by harmaline (3,4-dihydro-7-methoxy-1-methyl-9-pyrid [3,4-bis]indole) that was competitive toward Na^+ but not toward K^+, an observation deserving further exploration.

The enzyme preparation used here was obtained from rat brain microsomes after treatment with deoxycholate and NaI, as described previously [10]. To determine ATPase activity, the enzyme preparation (0·5 mg/ml final concentration) was first incubated for 10 min at 37° in 30 mM histidine·HCl–Tris (pH 7·8) in the absence or presence of harmaline (obtained from Sigma Chemical Co.); the assay incubation was then initiated by adding 4 vol. of media to bring the final concentrations, under control conditions, to 30 mM histidine·HCl–Tris (pH 7·8), 3 mM Tris–ATP, 3 mM $MgCl_2$, 90 mM NaCl, and 10 mM KCl (and, where harmaline was originally present, maintaining its concentration). ATPase activity was measured in terms of inorganic phosphate production [10]. Although the maximal harmaline concentration present in these analyses, 60 μM, did not affect the phosphate method of Lowry and Lopez [11] measurably, all determinations were rou-

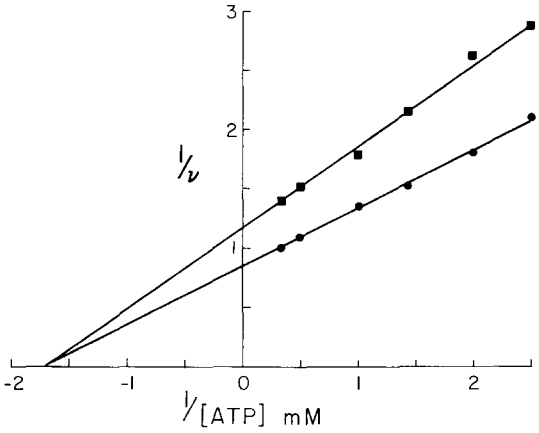

Fig. 1. Effect of harmaline on substrate kinetics. The enzyme preparation was first equilibrated for 10 min at 37° in the absence or presence of 0·4 mM harmaline; the $(Na^+ + K^+)$-dependent ATPase activity was then measured, as described in the text, in the standard medium but with the concentrations of ATP indicated (the $MgCl_2$ concentration was kept constant at 3 mM). Harmaline, when present in the equilibration medium, was maintained in the assay incubation at the same concentration. Data are presented in Lineweaver–Burk form from experiments without (●) or with (■) harmaline.

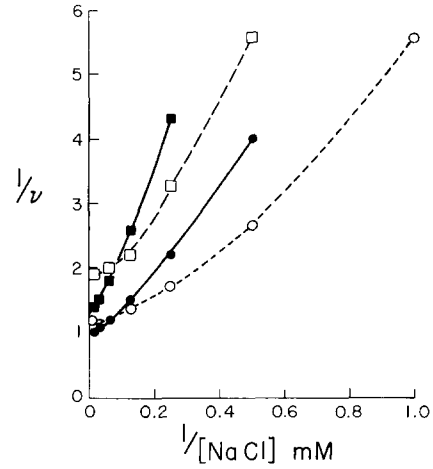

Fig. 2. Effect of harmaline on activation by Na^+. Experiments were performed and the data are presented as in Fig. 1, except that the assay incubations were performed with the concentrations of NaCl shown: in the presence of 10 mM KCl after equilibration in the absence (●) or presence (■) of 0·4 mM harmaline, and in the presence of 2 mM KCl after similar equilibration in the absence (○) or presence (□) of harmaline. With 10 mM KCl, harmaline increased the $K_{0.5}$ for Na^+ from 5·0 to 7·9 mM; with 2 mM KCl harmaline increased the $K_{0.5}$ from 2·5 to 3·1 mM.

tinely adjusted to contain this level of harmaline. Velocities of the $(Na^+ + K^+)$-dependent ATPase activity [10] are expressed relative to that in the standard medium defined as 1·0. Phosphatase activity was determined analogously in terms of umbelliferone production from umbelliferone phosphate; the enzyme was equilibrated for 10 min in the absence or presence of harmaline, and the assay incubation then initiated by adding 4 vol. of media to bring the final concentrations, under control conditions, to 30 mM histidine·HCl–Tris (pH 7·8), 1 mM Tris–umbelliferone phosphate, 3 mM $MgCl_2$, and 20 mM KCl. After termination of the incubation, umbelliferone was measured fluorometrically, as described previously [12]; all assays were routinely adjusted, as above, to contain the same amount of harmaline. Velocities of the K^+-dependent phosphatase activity [13] are expressed relative to that in the standard medium defined as 1·0. Data presented are averages of four or more determinations performed in duplicate.

Harmaline, at a concentration of 0·4 mM, inhibited the $(Na^+ + K^+)$-dependent ATPase activity in the standard medium by 30 per cent. The extent of this inhibition was constant during the incubation period, provided that the enzyme was first equilibrated with the drug. The inhibition was reversible, and simple washing (dilution of the incubation medium by a 20-fold excess of cold 0·25 M sucrose, centrifugation, and resuspension of the sedimented enzyme preparation for assay) restored activity essentially to control values.

Harmaline was a typical non-competitive inhibitor toward the substrate, ATP (Fig. 1). By contrast, harmaline decreased the apparent affinity of the enzyme for Na^+ (Fig. 2): the concentration for half-maximal activation, $K_{0.5}$, was increased from 5·0 to 7·9 mM in the presence of 10 mM KCl. However, harmaline was not here a simple competitive inhibitor, for the extent of inhibition did not approach zero as the concentration of Na^+ was increased (Fig. 2): the apparent V_{max} was also decreased by harmaline. This is at variance with the report of Canessa *et al.* [9] in which V_{max} was unchanged; the discrepancy may relate to the fact that, in the earlier study, concentrations of NaCl greater than 20 mM were not examined with the mammalian enzyme, thus requiring a greater extrapolation to V_{max} in the Lineweaver–Burk plot.

The cause of the decrease in apparent V_{max} for Na^+ in Fig. 2 becomes apparent when the effect of harmaline on the activation of the enzyme by K^+ is examined. Harmaline decreased the apparent affinity for K^+ as well (Fig. 3): the $K_{0.5}$ for K^+ was increased at both levels of NaCl. As would be expected for an agent that decreased the affinity for both activators, the apparent V_{max} at infinite concentration of one activator is depressed by harmaline when measured at a finite concentration of the other (Figs. 2 and 3). Canessa *et al.* [9] reported that inhibition by har-

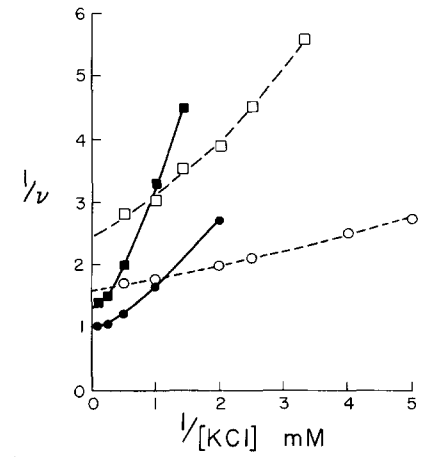

Fig. 3. Effect of harmaline on activation by K^+. Experiments were performed and the data are presented as in Fig. 2, except that the assay incubations were performed with the concentrations of KCl shown: in the presence of 90 mM NaCl after equilibration in the absence (●) or presence (■) of 0·4 mM harmaline, and in the presence of 5 mM NaCl after similar equilibration in the absence (○) or presence (□) of harmaline. With 90 mM NaCl, harmaline increased the $K_{0.5}$ for K^+ from 0·75 to 1·31 mM; with 5 mM NaCl, harmaline increased the $K_{0.5}$ from 0·16 to 0·34 mM.

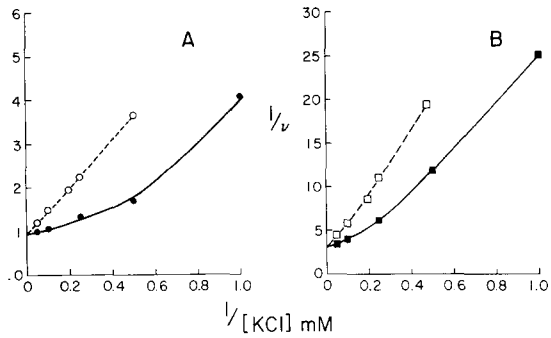

Fig. 4. Effect of harmaline on activation of the phosphatase reaction by K^+. Experiments were performed and the data are presented as in Fig. 3, except that the assay incubations measured K^+-dependent phosphatase activity, as described in the text, with the KCl concentrations shown. Assay incubations were performed in the absence of NaCl (●, ■) or presence of 10 mM NaCl (○, □) after equilibration in the absence (panel A) or presence (panel B) of 0·4 mM harmaline. In the absence of NaCl, harmaline increased the $K_{0.5}$ for K^+ from 2·0 to 4·3 mM; with 10 mM NaCl, harmaline increased the $K_{0.5}$ from 5·5 to 9·1 mM.

maline did not vary with K^+ concentration, but no data were presented.

$(Na^+ + K^+)$-dependent ATPase preparations exhibit K^+-dependent phosphatase activity that apparently reflects the terminal hydrolytic steps of the overall ATPase reaction [2,13]. In this reaction, Na^+ is not required, and, indeed, can be inhibitory [13]. Thus, examination of the effects of harmaline on this phosphatase activity permits effects on K^+-activation to be examined in the absence as well as the presence of Na^+. For these studies, umbelliferone phosphate was used as substrate [14], since liberation of the fluorescent product could be measured without interference from harmaline. Again, 0·4 mM harmaline inhibited the enzymatic activity, and again the apparent affinity for K^+ was decreased (Fig. 4). This increase in $K_{0.5}$ was also seen in the presence of Na^+, which itself increases the $K_{0.5}$ for K^+.

These studies indicate that harmaline inhibition of both the overall $(Na^+ + K^+)$-dependent ATPase and the related K^+-dependent phosphatase reaction is associated with decreases in the apparent affinities for K^+ as well as for Na^+. In this respect, harmaline resembles the more potent inhibitor, diethylstilbestrol [15], which inhibits these enzymatic activities and increases the $K_{0.5}$ for both Na^+ and K^+. Nevertheless, harmaline is of considerable potential value in studying cation activation, particularly if its absorption and fluorescent properties can be exploited.

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Department of Pharmacology,
State University of New York,
Upstate Medical Center,
Syracuse, N.Y. 13210, U.S.A.

JOSEPH D. ROBINSON

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