that nerve stimulation causes the release of nucleotides from the membrane. Furthermore, Caldwell and Keynes[14] have proposed that ATP provides phosphate bond energy for sodium extrusion in giant axons. Nachmansohn[15] also mentions a requirement for phosphate bond energy in or near the axonal membrane in order that acetylcholine may be resynthesized for its role in permeability changes of the axonal membrane during activity. Secondly, Holton[16] reported that ATP was liberated from sensory nerve endings following antidromic stimulation. However, this seems to be an unlikely explanation of the effects observed in the gut since a preganglionic efferent innervation of the non-adrenergic inhibitory neurones has been described.[3, 17, 18] Thirdly, ATP is known to have a binding function in the catecholamine containing vesicles of adrenergic neurones in the adrenal medulla.[19] However, Stjärne[20] has been unable to find evidence of ATP release from spleen following stimulation of adrenergic nerves (even though it is now well established that it is released with catecholamines from cells in the adrenal medulla[19]), but the possibility that ATP may have a function in the storage and/or release of other transmitter substances cannot be discounted, despite a lack of supporting evidence.

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Sodium- and potassium-activated ATPase of beef brain—Effects of some tranquilizers

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THE ROLE of sodium- and potassium-activated ATPase (Na^+K ATPase, EC 3.6.1.4) upon active cation translocation across the cellular membrane is well established.[1] A number of central nervous system depressants have been shown to inhibit this enzyme activity.[2–10] Tranquilizing actions of

chlorpromazine and its analogs have been correlated with their inhibitory actions upon brain Na^+K ATPase activity.[3] On the other hand, Festoff and Appel[11] reported that diphenylhydantoin stimulated Na^+K ATPase activity of brain synaptosomal fractions when the ratio of Na:K was above 25:1 and was inhibitory at lower ratios. The present study will report effects of some tranquilizers upon partially purified Na^+K ATPase of beef brain. Three chemically unrelated agents were studied: a piperazine derivative, hydroxyzine; a butyrophenone derivative, haloperidol; and a benzodiazepine derivative, diazepam.

The enzyme was prepared from the microsomal fraction of beef brain homogenate according to the methods of Nakao *et al.*[12] and Squires,[9] with the modification of Israel and Salazar.[6] Fresh beef brain cortices were collected with a spatula and were homogenized with 10 vol. of 0·25 M sucrose, 0·003 M EDTA, 0·01 M tris–HCl and 0·1% deoxycholate at pH 7·0 with a Waring blender. The homogenate was centrifuged at 10,000 *g* for 10 min. The supernatant fluid was adjusted to pH 5·0 with 1 M sodium acetate–acetic acid buffer, pH 4·5. After 15 min it was centrifuged at 12,000 *g* for 35 min. The precipitate was suspended with one-tenth of the original volume of 0·25 M sucrose, 0·001 M EDTA, 0·01 M tris–HCl, pH 7·4, and the pH was readjusted to 7·4 with solid tris and was centrifuged at 60,000 *g* for 1 hr. The precipitate was suspended with 0·25 M sucrose, 0·001 M tris–HCl, pH 7·4. To this, a mixture of 6 M NaI, 0·005 M EDTA, and 0·05 M histidine, pH 8·0, was added in 15 min to give a final NaI concentration of 2 M. After standing another 15 min, an equivalent amount of water was added to this suspension and centrifuged at 60,000 *g* for 1 hr. The precipitate was suspended with 0·25 M sucrose, 0·01 M tris–HCl, pH 7·4, and the pH was readjusted to 7·4 with HCl. The preparation was washed three times by repeating centrifugation at 60,000 *g* for 1 hr and suspending the precipitate in 0·25 M sucrose and 0·01 M tris–HCl, pH 7·4. The final sediment was suspended with the same buffer to give a protein concentration of 4–5 mg/ml, divided into small quantities and kept at −20°.

The enzyme activity was measured by incubating 0·05 ml of enzyme with substrate in a total volume of 2·15 ml at 38° for 30 min. The enzyme preparation was diluted from the frozen stock to appropriate concentrations to give linear inorganic phosphate liberation in a 30-min incubation. The standard reaction media consisted of 3 mM ATP, 6 mM $MgCl_2$, 30 mM tris–HCl, pH 7·4, with appropriate amount of NaCl and KCl. The reaction was terminated by the addition of 0·1 ml of 80% (w/v) trichloroacetic acid. The liberated inorganic phosphate was determined by the molybdovanadate method reported elsewhere.[13] Protein was determined by the biuret method as described by Weichselbaum.[14] Because Tris gave the positive biuret reaction, the standard solution was prepared by dissolving bovine serum fraction V in 0·01 M tris.

When assayed with 150 mM Na^+ and 20 mM K^+, the specific activity of the preparation was 685 nmoles inorganic phosphate liberated per minute per milligram of protein. In the absence of Na^+ and K^+, the specific activity was 10. At 20 mM K^+, Na^+ activated the preparation half maximally at 18 mM. At 150 mM Na^+, K^+ activated the preparation half maximally at 1·7 mM. The enzyme was inhibited by $CaCl_2$; a half maximal inhibition was observed at 1·5 mM Ca^{2+} when assayed with 150 mM Na^+ and 20 mM K^+.

The effects of hydroxyzine, haloperidol and diazepam upon the enzyme preparation were studied in a standard media with several cationic concentrations: 150 mM Na^+ and 20 mM K^+, 150 mM Na^+ and 2 mM K^+, 18 mM Na^+ and 20 mM K^+, and 15 mM Na^+ and 5 mM K^+. The last combination was included to simulate condition *in vivo* of nervous tissue where intracellular Na^+ is about 15 mM and extracellular K^+ may be 2 to 5 mM. The media described by Festoff and Appel[11] were also used. In all cationic concentrations, stimulation of the enzyme activity was not observed at the concentrations of the tranquilizers between 10^{-3} and 10^{-12} M. They all showed inhibition when their concentrations exceeded 10^{-5} M. The tranquilizers did not reverse the inhibitory action of Ca^{2+} upon the enzyme activity when studied with standard media with 150 mM Na^+, 20 mM K^+ and 1·5 mM Ca^{2+}. Hydroxyzine, haloperidol and diazepam at 0·5 mM inhibited the preparation 30, 29 and 15 per cent of the original activity.

At 15 mM Na^+ and 5 mM K^+, hydroxyzine, haloperidol and diazepam inhibited the preparation 50 per cent at 0·15, 0·25 and 0·60 mM respectively (Fig. 1). When the cationic concentrations were 150 mM Na^+ and 20 mM K^+, hydroxyzine, haloperidol and diazepam at 0·5 mM inhibited the preparation 35, 33 and 16 per cent respectively. When the concentration of K^+ was reduced, the inhibition became more evident. At concentrations of 150 mM Na^+ and 2 mM K^+, they inhibited the preparation 71, 70 and 51 per cent respectively. Kinetic studies with Lineweaver–Burk plots showed noncompetitive inhibition against ATP. When Lineweaver–Burk plots were constructed between the reciprocals of reaction velocity and K^+ concentrations, a competitive inhibition was demonstrated (Fig. 2). Apparent inhibitor constants were estimated by constructing Dixon plots upon 2 mM and 10 mM K^+ concentrations and found to be: hydroxyzine, 0·1 mM; haloperidol, 0·1 mM, and diazepam, 0·2 mM. These concentrations were in excess of clinical doses by a factor of about 100 when judged from the dose for preanesthetic sedation.

The reversibility of the inhibition was studied by incubating 0·1 ml of the enzyme preparation, containing about 0·4 mg of enzyme protein, with 0·5 mM of hydroxyzine, haloperidol or diazepam

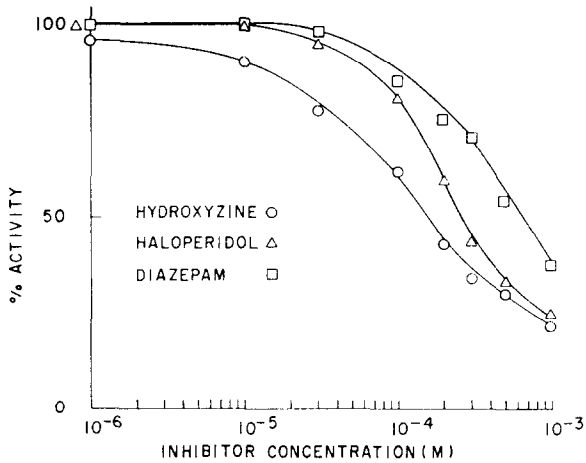

FIG. 1. Effects of hydroxyzine, haloperidol and diazepam upon Na^+K ATPase preparation of beef brain. Enzyme activity expressed as per cent of control value is plotted against logarithmic scale of inhibitor concentrations. The reaction media contained 15 mM NaCl, 5 mM KCl, 3 mM ATP and 6 mM $MgCl_2$.

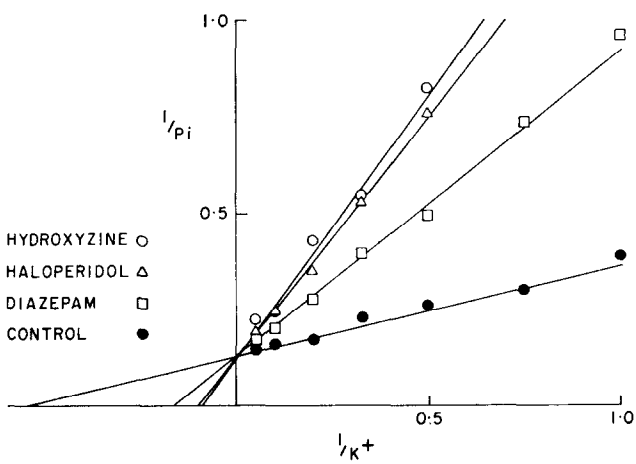

FIG. 2. Lineweaver Burk plots of inhibitions of Na^+K ATPase preparation by hydroxyzine, haloperidol and diazepam. Ordinate: Reciprocal of reaction velocity expressed as 10^{-7} M inorganic phosphate released per milligram of enzyme protein per minute. Abscissa: Reciprocal of potassium ion concentrations in 10^{-3} M. The reaction media contained 150 mM NaCl, 3 mM ATP and 6 mM $MgCl_2$. The concentrations of inhibitors were 0·5 mM.

in a 1-ml medium with 150 mM Na^+, 20 mM K^+ and 30 mM tris–HCl, pH 7·4 for 30 min at 38°. At the end of the incubation, the mixture was diluted to about 13 ml with iced 0·25 M sucrose and 30 mM tris–HCl, pH 7·4, and then centrifuged at 65,000 *g* for 1 hr. The sediment was washed twice by suspending the sediment with 13 ml of the sucrose Tris–HCl mixture and centrifuging it at 65,000 *g* for 1 hr. The washed protein was taken up with 5 ml of standard substrate mixture which contained 150 mM Na^+ and 20 mM K^+ and incubated for 30 min at 38°. Controls were treated identically without the inhibitors. All inhibitions were found to be reversible under this condition.

The dependency of the inhibition to pH was studied with 150 mM Na^+ and 2 mM K^+. When hydroxyzine (pK_{a1} = 2·05, pK_{a2} = 6·75) was studied at pH 7·75, the inhibition was about 41 per cent at 0·1 mM. At pH 6·75 and 5·75 the inhibitions were 21 and 10 per cent respectively. Haloperidol (pK_a = 8·2) 0·1 mM inhibited the enzyme activity 70, 37 and 14 per cent at pH 9·2, 8·2 and 7·2

respectively. These results appear to indicate that their free base and not protonated forms is preferred for the binding to the enzyme preparation. Diazepam ($pK_a = 3{\cdot}5$) did not show appreciable pH dependency in its effect upon Na^+K ATPase activity and inhibited the preparation about 22 per cent. Since pK_a value is low, this compound would have existed predominently in its base form throughout the pH range where this study was performed.

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Studies on enzymatic hydrolysis of aziridines—I. The conversion of 2β,3β-imino-5α-cholestane into 2β-amino-3α-hydroxy-5α-cholestane by liver microsomes

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RECENT studies on the role of liver microsomes have shown the presence of a new type of enzyme, named epoxide hydrolase[1] or epoxide hydrase,[2] which catalyzes the hydrolysis of a variety of epoxides of olefins[3–7] and arenes[8–11] to glycols. Activity of this microsomal enzyme is so potent that except in a few cases[2,3,12] epoxides formed during the oxidation of olefins by the NADPH-dependent mixed function oxygenase (epoxidase), also located in microsomes, are immediately hydrolyzed to make recognition of their existence difficult. In view of detoxication, existence of highly active epoxide hydrolase, relative to epoxidase, appears to be important to the animal body, for most epoxides are well known to be more or less toxic. It is of interest that 2,3-epoxycholestanes are stereoselectively hydrolyzed by the hydrolase to yield a single glycol with the same absolute configuration as that of the product obtained by their acid-catalyzed hydrolysis and also that the enzyme reaction is strongly inhibited with 2β, 3β-imino-5α-cholestane **1**, suggesting that the active center of the hydrolase has a dissociating hydrogen ($Enz^- H^+$) by which the enzyme interacts with the oxiran oxygen of the