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Diethyl stilbestrol—Mode of inhibition of the ($Na^+ + K^+$)-dependent adenosine triphosphatase*

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RECENT experiments with an enzyme preparation from rat brain[1] demonstrated that phlorizin and phloretin inhibited the ($Na^+ + K^+$)-dependent adenosine triphosphatase (ATPase) but stimulated the K^+-dependent phosphatase activity, although the latter appears to represent the terminal hydrolytic process of the ATPase.[2] Kinetic analysis showed that for both enzymatic activities phlorizin increased the apparent affinity for K^+ while decreasing it for Na^+, an effect that can explain the disparate stimulation/inhibition and that also could be relevant to considerations of the mode of inhibition by these compounds of Na^+-dependent sugar transport.[1] In the erythrocyte, sugar transport is inhibited not only by these compounds but also by diethyl stilbestrol (DES) in an apparently competitive fashion.[3] This study was undertaken to compare the effects of DES with those of phlorizin and phloretin on the enzymatic activities: the data show that DES is a potent inhibitor, decreasing the apparent affinity for both Na^+ and K^+. A recent report[4] described inhibition of ATPase activity in rat uteri after systemic administration of estradiol after a lag period of several hours; reports on the effects of DES on the enzyme *in vitro* are not available.

The enzyme preparation was obtained from rat brain microsomes after treatment with deoxycholate and NaI as previously described, and was largely free of monovalent cation-independent activity.[1, 2, 5] ($Na^+ + K^+$)-dependent ATPase activity was measured in terms of inorganic phosphate liberation as previously described;[5] the standard medium contained 50 mM Tris-HCl (pH 7·8), 3 mM ATP (Tris salt), 3 mM $MgCl_2$, 90 mM NaCl, 10 mM KCl, and 70 mM ethanol (0·4%). Incubation was for 5–10 min at 30°. K^+-dependent phosphatase activity was measured with *p*-nitrophenylphosphate (NPP) in terms of *p*-nitrophenol liberation as previously described;[2] the standard medium contained 50 mM Tris-HCl (pH 7·8), 3mM NPP (Tris salt), 3 mM $MgCl_2$, 10 mM KCl, and 70 mM ethanol. Incubation was for 10–20 min at 30°. Ethanol was included as the solvent for DES and other agents tested; this concentration of ethanol inhibited the ATPase activity 3 ± 1 per cent and the phosphatase activity 7 ± 2 per cent (± S.E.M.) compared to media without it. Enzyme velocities are presented relative to that in the standard medium defined as 1·0. Experimental data are the averages of five or more experiments performed in duplicate, and straight lines in the Hill plots were drawn by the method of least squares.

TABLE 1. INHIBITION OF K^+-DEPENDENT PHOSPHATASE ACTIVITY*

Reagents	Effect (Per cent of control activity) With 0·1 mM reagent	With 0·01 mM reagent
DES	14	71
Hexestrol	25	76
Estradiol	71	86
Estriol	85	94
Estrone	92	97
Phenolphthalein	20	64
Ouabain	53	74
Phloretin	174	171

* K^+-dependent phosphatase was measured after incubations in media containing 50 mM Tris-HCl (pH 7·8), 3 mM NPP, 3 mM $MgCl_2$, 1 mM KCl, and 70 mM ethanol. The effect of adding the reagents at the concentration indicated is presented as a percentage of the control activity in their absence.

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K^+-dependent phosphatase activity averaged, with fresh enzyme preparations, 0·05 μmoles NPP hydrolyzed/mg protein/min, and was quite sensitive to concentrations of DES in the range 0·01–0·10 mM (Table 1; Fig. 1). The relative inhibition varied with the ionic composition of the media (Fig. 1), with a K_i of about 0·015 mM. Of a series of other estrogenic compounds major inhibition was apparent at these concentrations only with hexestrol (Table 1; Fig. 1); the concentration for 50 per cent

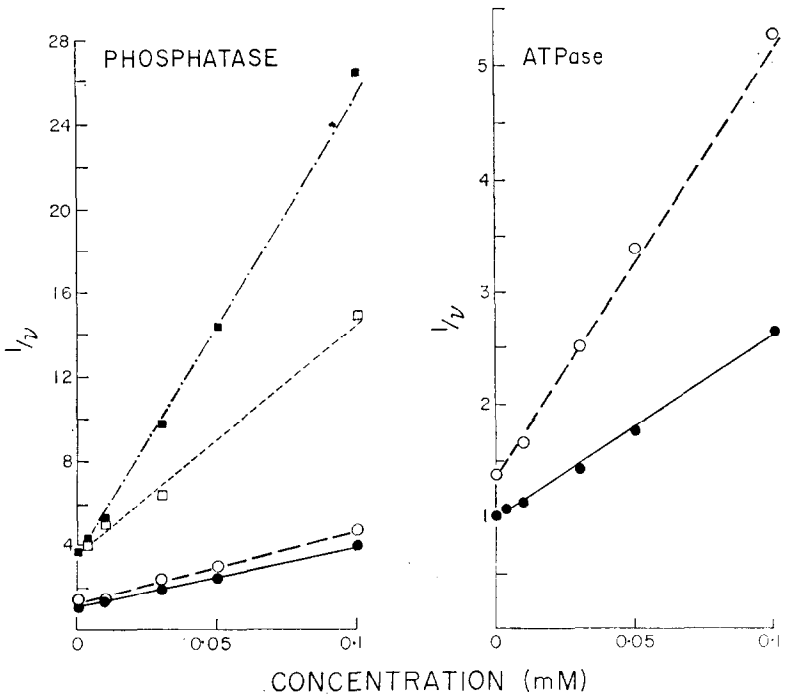

FIG. 1. Effects of DES on K^+-dependent phosphatase and (Na^+ + K^+)-dependent ATPase activity. In the left hand panel the effect of various concentrations of DES and hexestrol on phosphatase activity is shown for various cation complements in the form of a Dixon plot. Phosphatase activity was measured after incubations in media containing 50 mM Tris-HCl (pH 7·8), 3 mM NPP, 3 mM $MgCl_2$, 70 mM ethanol, and 10 mM KCl with DES (● ——— ●), 10 mM KCl plus 10 mM NaCl with DES (○ - - - - - - ○), 1 mM KCl with DES (■ - · - · - ■), and 1 mM KCl with hexestrol (□ – – – □). In the right hand panel analogous experiments with the ATPase are presented as a Dixon plot. ATPase activity was measured after incubation in media containing 50 mM Tris-HCl (pH 7·8), 3 mM ATP, 3 mM $MgCl_2$, 70 mM ethanol, and 90 mM NaCl plus 10 mM KCl with DES (● ——— ●), and 10 mM NaCl plus 10 mM KCl with DES (○ - - - - - - ○).

inhibition was 0·017 mM with DES and 0·035 mM with hexestrol in the presence of 1 mM KCl (Fig. 1). For comparison, inhibition by phenolphthalein and ouabain and stimulation by phloretin are shown for these conditions (Table 1).

DES altered the apparent affinity of the phosphatase for K^+; at a concentration of 0·03 mM it increased the $K_{0.5}$ (the concentration for half-maximal activity) from 2·2 to 4·0 mM (Fig. 2) while

having no effect on the cooperative response to K^+, as indicated by the slope of the Hill plot, n. However, DES also decreased V_{max} by 30 per cent.

($Na^+ + K^+$)-dependent ATPase activity averaged, with fresh enzyme preparations, 0·6 μmoles, P_i liberated/mg protein/min, and was similarly sensitive to DES (Fig. 1). The K_i for the ATPase was also about 0·015 mM (Fig. 1).

DES altered the apparent affinity of the ATPase for both Na^+ and K^+ (Fig. 3). At a concentration of 0·03 mM, it increased the $K_{0.5}$ for K^+ from 0·84 to 1·1 mM and the $K_{0.5}$ for Na^+ from 4·6 to 8·5 mM. DES had no effect on the cooperative response to the cations, but decreased V_{max} by 30 per cent.

DES was a potent inhibitor of both the phosphatase and ATPase activities, and this inhibition was associated with a decrease in the apparent affinity for the activating cations and a decrease in V_{max}. By contrast, phlorizin[1] increased the apparent affinity for K^+ while decreasing it for Na^+, and had little effect on V_{max}.

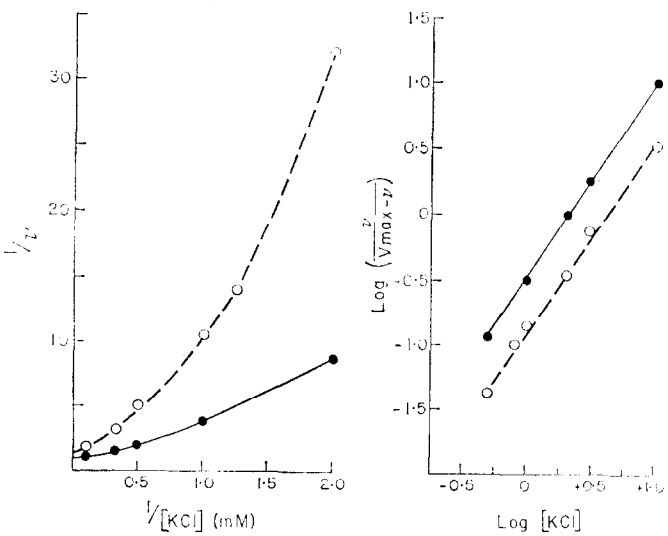

FIG. 2. Effects of DES on the response of the K^+-dependent phosphatase to KCl. Phosphatase activity was measured after incubations in the standard media but with the concentration of KCl indicated, in the absence (● ——— ●), or presence (○ - - - - - ○) of 0·03 mM DES. In the left hand panel the data are presented in the form of a Lineweaver–Burk plot, and in the right hand panel in the form of a Hill plot. Kinetic parameters in the absence and presence of DES are respectively: $K_{0.5}$:2·2 and 4·0 mM; n: 1·49 and 1·44; V_{max}: 1·1 and 0·75 relative units.

The cation-dependent ATPase has been suggested as a possible paradigm for cation-dependent sugar transport.[1] In this vein the inhibition of sugar transport by DES could reflect its reduction in the apparent affinity of the ATPase for Na^+, an action shared with phlorizin. Furthermore, such an effect of DES could explain its competitive inhibition of sugar transport. Several studies[3, 6, 7] have shown that not only phlorizin, which contains a glucosidic group, but also the aglucone phloretin and DES are apparent competitive inhibitors (vs glucose) of glucose transport. In the intestine the effect of Na^+ on glucose transport is to increase the apparent affinity for glucose[8] (i.e. Na^+ is a "competitive" activator). Hence a substance, such as DES, that could reduce the affinity of the transport apparatus for Na^+ would then appear to be a competitive inhibitor (vs. glucose) of transport.

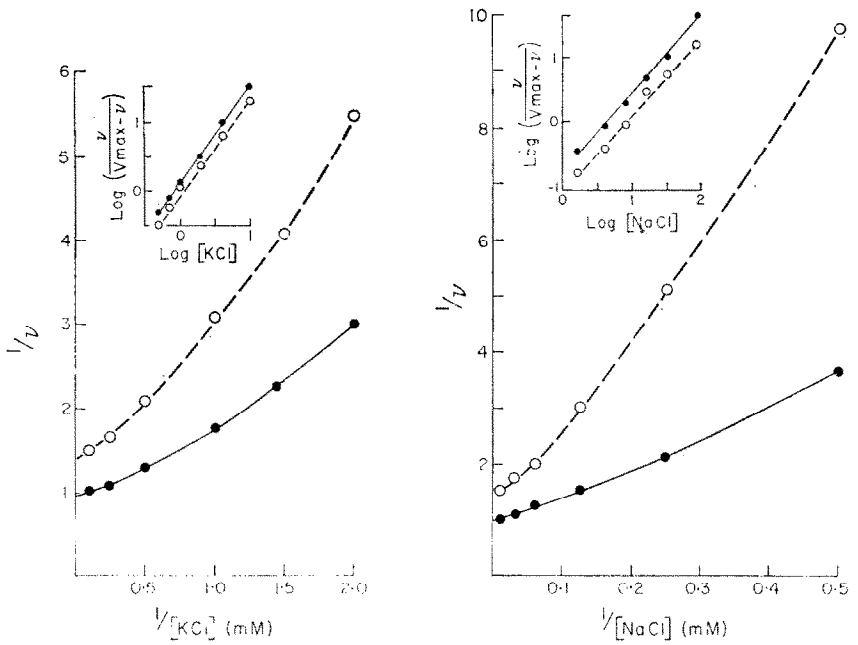

FIG. 3. Effects of DES on the response of the $(Na^+ + K^+)$-dependent ATPase to KCl and to NaCl. In the left hand panel the response of the enzyme to varying concentrations of KCl is shown: ATPase activity was measured after incubations in the standard media (with 90 mM NaCl) but with the concentration of KCl indicated, in the absence (● ——— ●) or presence (○ - - - ○) of 0·03 mM DES. The data are presented as a Lineweaver–Burk plot and, in the inset, as a Hill plot. Kinetic parameters in the absence and presence of DES are respectively: $K_{0.5}$: 0·84 and 1·1 mM; n: 1·43 and 1·36; V_{max}: 1·03 and 0·69 relative units. In the right hand panel the results of analogous experiments with differing concentrations of NaCl (with 10 mM KCl) are presented. Kinetic parameters in the absence and presence of DES are respectively: $K_{0.5}$: 4·6 and 8·5 mM; n: 1·18 and 1·19; V_{max}: 1·03 and 0·70 relative units.

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