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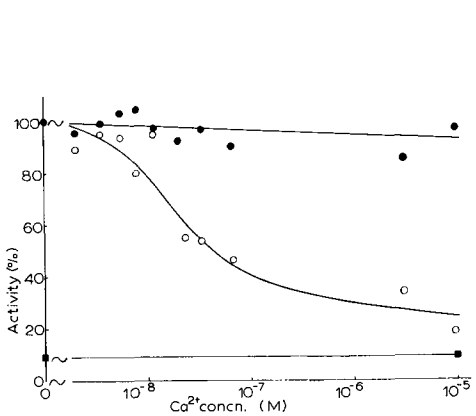
Ca^{2+}-dependent inhibition of (Na^+ + K^+)-dependent ATPase by hydroxylamine

Our present knowledge about the action of the (Na^+ + K^+)-dependent ATPase (ATP phosphohydrolase, EC 3.6.1.3) of cell membranes is as follows: An intermediate[1] is phosphorylated from the terminal phosphate of ATP in the presence of Na^+ and Mg^{2+}. The phosphate is attached to the enzyme in an acylphosphate bond forming an acylphosphoprotein[2]. Its dephosphorylation is accelerated by a K^+-dependent acylphosphatase (acyl phosphate phosphohydrolase, EC 3.6.1.7)[3]. SCHONER, KRAMER AND SEUBERT[4] failed to get an inhibition of the (Na^+ + K^+)-dependent ATPase when they incubated it in the presence of H_2NOH, concluding that an acyl phosphate cannot be an intermediate in the hydrolysis of ATP to ADP and P_i. CHIGNELL AND TITUS[5] also failed to obtain an inhibition by H_2NOH. The results presented here show that H_2NOH inhibits the (Na^+ + K^+)-dependent ATPase when Ca^{2+} is present in low concentrations.

The (Na^+ + K^+)-dependent ATPase prepared from rabbit kidney cortex[6] was



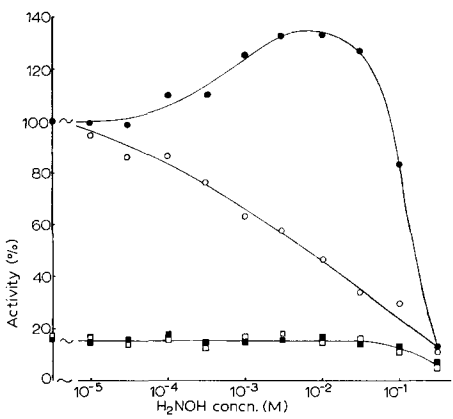


Fig. 1. Dependence of H_2NOH inhibition of (Na^+ + K^+)-dependent ATPase on Ca^{2+} concentration. The concentration of free Ca^{2+} was calculated from the ratio Ca^{2+}: ethylene glycol-bis-(aminoethyl)-tetraacetic acid in the incubation medium[7]. ●, 150 mM NaCl, 30 mM KCl; ○, 150 mM NaCl, 30 mM KCl, 20 mM H_2NOH; ■, 0.3 mM ouabain, 20 mM H_2NOH.

Fig. 2. Inhibition of (Na^+ + K^+)-dependent ATPase by H_2NOH. ●, 150 mM NaCl, 30 mM KCl; ○, 150 mM NaCl, 30 mM KCl, 0.06 mM $CaCl_2$; ■, 0.3 mM ouabain; □, 0.3 mM ouabain, 0.06 mM $CaCl_2$.

incubated at 37° with 6 mM $MgCl_2$, 4 mM ATP, 20 mM Tris–*N*-tris(hydroxymethyl)-methyl-2-aminoethane sulfonic acid, pH 7.8, and 0.05 mM ethyleneglycol-bis(aminoethyl)-tetraacetic acid. To this was added either 150 mM NaCl and 30 mM KCl or 0.3 mM ouabain. In all experiments $H_2NOH \cdot HCl$ was neutralized with Tris to pH 7.8. Under these conditions H_2NOH did not inhibit the (Na^+ + K^+)-dependent ATPase. However, when the concentration of free Ca^{2+} gradually increased, H_2NOH began to inhibit the enzyme (Fig. 1). Half-maximal inhibition at 20 mM H_2NOH was ob-

tained with a Ca^{2+} concentration of $3 \cdot 10^{-8}$ M. Ca^{2+} itself had only a slight inhibitory effect at the highest concentration shown when H_2NOH was missing. Without H_2NOH, half maximal inhibition was obtained with about 0.5 to 1 mM Ca^{2+}. Mg^{2+}, Ba^{2+} and Sr^{2+} could not substitute for Ca^{2+}.

Fig. 2 shows the inhibition of the $(Na^+ + K^+)$-dependent ATPase with different H_2NOH concentrations, with and without Ca^{2+}. Half maximal inhibition was obtained with about 3 mM H_2NOH in the presence of 0.01 mM free Ca^{2+}. When Ca^{2+} was omitted, the $(Na^+ + K^+)$-dependent ATPase activity increased between 0.1 and 30 mM H_2NOH. A similar increase was observed by SCHONER, KRAMER AND SEUBERT[4]. At higher concentrations, H_2NOH inhibition without Ca^{2+} was probably due to ionic strength inhibition by the Tris–HCl present[3]. H_2NOH had no effect on the ouabain insensitive hydrolysis of ATP by the enzyme, and it could not substitute for K^+.

Table I shows the effect of preincubation of the enzyme with H_2NOH. Inhibition was observed only when the enzyme was preincubated with H_2NOH in the presence of Ca^{2+} and ATP. None of the other samples showed any significant inhibition.

TABLE I

Activity of $(Na^+ + K^+)$-dependent ATPase pretreated with H_2NOH. The preincubation medium contained 150 mM NaCl, 30 mM KCl, 6 mM $MgCl_2$, 20 mM Tris–*N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid , pH 7.8, and either 1 mM ethyleneglycol-bis(aminoethyl)-tetraacetic acid or 0.01 mM $CaCl_2$ with or without 4 mM ATP. Preincubation time was 20 min at 23°. After preincubation, the enzymes were centrifuged at 30 000 × *g* and washed twice with 3 mM imidazole·HCl, pH 7.1, containing 15 mM NaCl and 1 mM EDTA. The final precipitate was tested for $(Na^+ + K^+)$-dependent hydrolysis of ATP[6]. Each value given is the mean value ± S.E. from 5 experiments.

Preincubation		*μmoles P_i per mg protein per h ± S.E.*
Control		69 ± 1.6
20 mM H_2NOH		
1 mM ethyleneglycol-bis(aminoethyl)-tetraacetic acid,	4 mM ATP	71 ± 1.1
	0 mM ATP	67 ± 0.9
0.01 mM $CaCl_2$	4 mM ATP	25 ± 2.7
	0 mM ATP	61 ± 4.8

The results indicate that H_2NOH in the presence of ATP interferes with the active site of the enzyme by forming a stable hydroxamate of the intermediate. This is additional proof that the acylphosphoprotein[2] found after incubation of the $(Na^+ + K^+)$-dependent ATPase with [γ-^{32}P]ATP in the presence of Na^+ is an intermediate in the $(Na^+ + K^+)$-dependent hydrolysis of ATP by the enzyme[1]. The results also show that Ca^{2+} in low concentration plays a yet unknown role in the activity of the $(Na^+ + K^+)$-dependent ATPase, possibly by regulating the access of substrate or inhibitor to the enzyme. The increase in activity with H_2NOH in the absence of Ca^{2+} is not yet understood.

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A kinetic study on the inactivation of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase by bromopyruvate

Three distinct isoenzymes of 7-phospho-2-oxo-3-deoxy-D-*arabino*-heptonate D-erythrose-4-phosphate-lyase (pyruvate phosphorylating), EC 4.1.2.15 (referred to hereafter as 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthase), which catalyze the first step of the biosynthesis of the aromatic amino acids, have been detected in *Escherichia coli* K 12 (ref. 1). The formation of DAHP from erythrose 4-phosphate and phosphoenolpyruvate (PEP) by isoenzyme 1a is inhibited allosterically by phenylalanine[2]. The mechanism of action of the phenylalanine-sensitive DAHP synthase is "ping-pong" and the first substrate of the enzyme is PEP (ref. 3). MELOCHE[4] observed that bromopyruvate inactivates the 6-phospho-2-keto-3-deoxy-D-gluconate D-glyceraldehyde-3-phosphate-lyase (EC 4.1.2.14) and pyruvate protects the enzyme against the inactivating effect of bromopyruvate. We have found a similar inactivating effect of bromopyruvate on the phenylalanine-sensitive DAHP synthase and the present paper describes the kinetics of inactivation of the enzyme by bromopyruvate and the kinetic analysis of the protection of the enzyme by PEP.

The growth conditions of the wild-type *E. coli* K 12 and the purification of the enzyme were described previously[3]; bromopyruvate was prepared chemically[5].

As Fig. 1a shows, bromopyruvate inactivates the enzyme and the rate constant k_1 of the pseudo-first-order reaction may be expressed as

$$k_1 = \frac{\log E_0 - \log E_t}{t} 2.3 \qquad (1)$$

where E_0 and E_t are the amounts of active enzyme at zero time and time t respectively.

Abbreviations: DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate; PEP, phosphoenolpyruvate.