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EFFECT OF SUBSTRATES AND PRODUCTS ON BEEF BRAIN MICROSOMAL ($Na^+ + K^+ + Mg^{2+}$)-ATPase ACTIVITY

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SUMMARY

A microsomal ($Na^+ + K^+ + Mg^{2+}$)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) preparation from beef brain grey matter is inhibited by inorganic phosphate. Decreasing the ATP concentration leads to an increase in the ($Na^+ + K^+ + Mg^{2+}$)-ATPase activity. This activity passes through a definite maximum and then falls as the ATP concentration is lowered further. Preincubation of the enzyme preparation at 37° for up to 2 h does not lead to marked inactivation.

INTRODUCTION

We wish to report some studies on the effects of substrates and products on the rate of ATP hydrolysis by a microsomal ATPase (ATP phosphohydrolase, EC 3.6.1.3) preparation, fractionated according to the method of SCHWARTZ, BACHELARD AND MCILWAIN[1] from beef brain grey matter, which is maximally activated in the presence of Mg^{2+} by the monovalent ions Na^+ and K^+ together. The essential chemical features of the reaction catalyzed by the enzyme system are:

$$ATP^{4-} + H_2O = ADP^{3-} + HPO_3^{2-} + H^+ \quad (1)$$

SKOU[2] discovered such an ATPase activity in crab nerve homogenates. He has recenty outlined in a review[3] many of the general features of these enzyme systems and the properties they have in common with the ($Na^+ + K^+$)-coupled cation pump mechanism of active transport. POST *et al.*[4] and DUNHAM AND GLYNN[5] have demonstrated that the enzyme system is located within the membrane of human red blood cells and BONTING *et al.*[6,7] have demonstrated its wide distribution in a variety of mammalian tissues.

EXPERIMENTAL PROCEDURES

Materials

All chemicals were of reagent grade or specially purified from British Drug

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Houses or Sigma Chemical Company. The water used was deionized and distilled from $KMnO_4$. Tris–ATP and NaADP from Sigma were used. The NaADP solutions were neutralized with NaOH.

Enzyme preparations

Microsomal fractions showing ATPase activity were obtained from beef brain grey matter essentially according to the procedures of SCHWARTZ, BACHELARD AND MCILWAIN[1] and RODNIGHT, HEMS AND LAVIN[8]. The homogenizing and storage medium was usually 0.32 M sucrose–0.5 mM EDTA (tetraTris), (pH 7.4) (KOH or Tris, 5°). The preparations were stored at −20° as 5–10-ml aliquots in sealed plastic tubes at a concentration of 1–15 mg protein per ml. To improve the specific activity and increase the percentage of $(Na^+ + K^+ + Mg^{2+})$-ATPase activity in the total ATPase activity some of the microsomal preparations were extracted according to the sodium iodide procedure of NAGANO *et al.*[9].

Enzyme assay

Enzyme activity was tested in two assay media. One contained 100 mM NaCl, 25 mM KCl, 0.5 mM tetraTris–EDTA, 30 mM Tris–HCl (pH 7.4, 37°) and the other 25 mM KCl, 0.5 mM tetraTris–EDTA, 130 mM Tris–HCl (pH 7.4, 37°). The final protein concentration in the assay media was routinely about 100 μg protein per ml of assay medium. Any additional reagents which were tested for effects on activity were contained in these media. Reactions were usually started by the addition of ATP (to give a concentration of 3 mM unless otherwise indicated), preceded by 5 min thermal equilibration at 37°, and followed by 10 min reaction at 37°. Reactions were stopped by quickly transferring the glass test tubes to crushed ice and adding 1.5 ml of ice-cold 10% trichloroacetic acid to the 1.0 ml assay test to give a final trichloroacetic acid concentration of 6% (w/v). The resultant deproteinized suspensions were filtered on ice through Whatman No. 1 filter paper, and 1-ml aliquots were taken from the filtrate for phosphate determinations. These samples were kept until tested on ice or at −20°.

In order to correct for the non-enzymatic hydrolysis of ATP, assay controls were run for each experimental data point with both the Na^+-containing and the Na^+-free assay medium. This was done by incubating ATP-containing media of both types for 10 min at 37°, followed by the addition of trichloroacetic acid and then the enzyme preparation at 0°. The ATPase activity was corrected for the non-enzymatic hydrolysis of ATP by subtracting the P_i released in the appropriate control assay medium from the apparent enzymatic hydrolysis values in the corresponding test assay medium. The ATPase specific activity was calculated in μmoles P_i released per h per mg protein. By taking the difference between the specific activity values obtained in the Na^+-containing and the Na^+-free media the values reported as the $(Na^+ + K^+ + Mg^{2+})$-ATPase activity were obtained.

P_i was determined by the method of DRYER, TAMMES AND ROUTH[10] at 750 mμ in a Beckman DB spectrophotometer using KH_2PO_4 in a 6% trichloroacetic acid solution of assay medium as a standard. Protein was determined by the method of LOWRY *et al.*[11] using crystalline bovine serum albumin as a standard.

RESULTS AND DISCUSSION

The microsomal ATPase preparation used in this investigation was activated by Na^+ and Mg^{2+} in the usual way. Maximal stimulation was observed with 50 mM Na^+ and 5 mM Mg^{2+}. The concentrations required for half maximal stimulation of the ($Na^+ + K^+ + Mg^{2+}$)-ATPase were 10 mM and 1.25 mM for Na^+ and Mg^{2+}, respectively.

Fig. 1 demonstrates a decrease in activity at high ATP concentrations similar

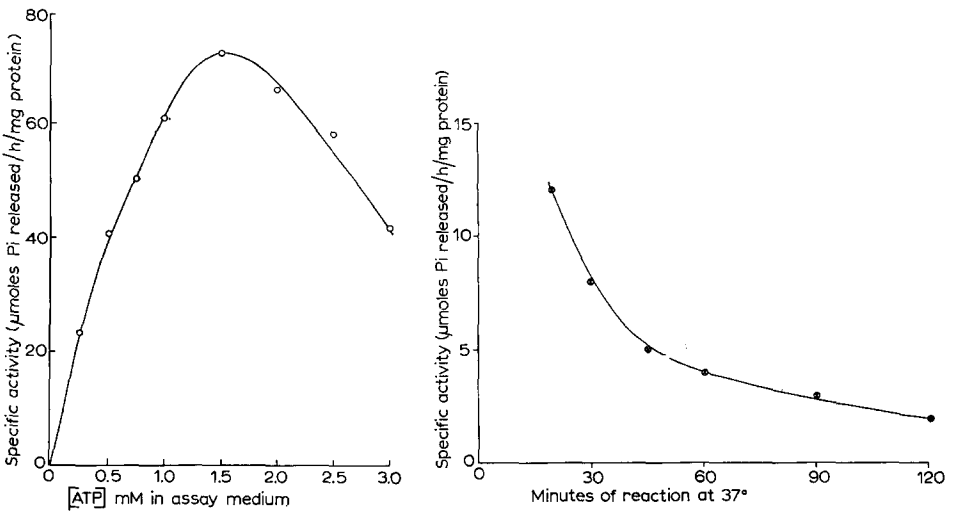


Fig. 1. Effect of ATP concentration on ($Na^+ + K^+ + Mg^{2+}$)-ATPase activity. NaI-extracted preparation. Mg^{2+} concentration, 5 mM.

Fig. 2. Relationship between reaction time and ($Na^+ + K^+ + Mg^{2+}$)-ATPase activity.

to that observed by SKOU[12] with a crab nerve preparation and DUNHAM AND GLYNN[5] with a red blood cell preparation. Our results for both NaI-extracted and unextracted systems suggest an optimal Mg^{2+}/ATP ratio between about 2–3 for the beef brain ($Na^+ + K^+ + Mg^{2+}$)-ATPase activity. Thus it would appear that our results agree with the 2:1 Mg^{2+}/ATP ratio SKOU[12] reported necessary for maximal activity, since the 0.5 mM EDTA present in our media will bind an equimolar concentration of Mg^{2+}, leaving 1.5–2.5 Mg^{2+} per ATP.

Fig. 2 shows that after 10–20 min of reaction the specific activity dropped sharply as the reaction was allowed to progress. During the first 10–20 min of reaction the specific activity was constant. However, after about 45 min of reaction the specific activity was decreased by almost 60%.

To investigate the possibility that denaturation of the enzyme preparation at 37° might be significant, the enzyme was incubated for varying lengths of time in the absence of ATP. ATP was than added to start the reaction. As shown by Fig. 3, the activity appears to increase for about the first 40 min preincubation at 37°. Very little inactivation occurs even after nearly 2 h preincubation at 37°. These changes upon thermal incubation at 37° might result from changes in the microsomal membrane structural organization thereby facilitating substrate accessibility to the enzyme.

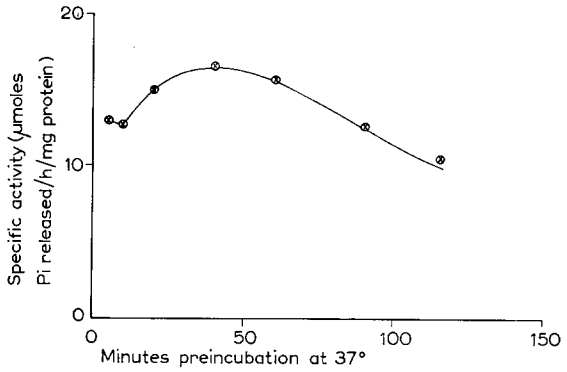
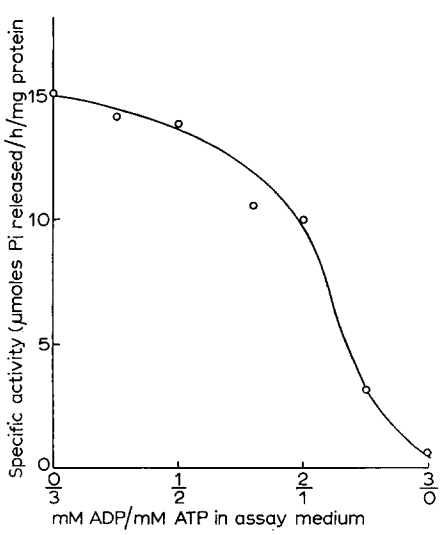


Fig. 3. Effect of preincubation time on ($Na^+ + K^+ + Mg^{2+}$)-ATPase activity.

Possible inhibition of the ATPase activity by the protons released during the hydrolysis reaction (Eqn. 1) will depend on their ability to effect a pH change in the assay medium. Therefore, the buffering capacity of the assay media was tested by adding per ml 3 μmoles of H^+ as HCl. There was no significant change to 0.01 pH units with the Na^+-free assay medium. As it was estimated that after 120 min less than 1 μmole of an initial 3 μmoles ATP per ml would be hydrolyzed in the experimental procedure, pH effects were not examined further.

Fig. 4 shows that the variation of the ADP/ATP ratio at a constant (ADP + ATP) concentration of 3 mM does not affect the specific activity markedly until relatively high (2:1) ADP/ATP ratios are reached. In view of this ratio not being obtained during the marked decrease in specific activity (Fig. 3) another source of inhibition was sought.



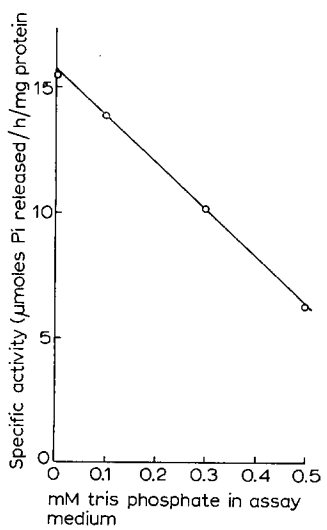


Fig. 4. Effect of ADP/ATP ratio at constant adenosine phosphate concentration on ($Na^+ + K^+ + Mg^{2+}$)-ATPase activity. (ADP + ATP) concentration, 3 mM.

Fig. 5. Inhibition of ($Na^+ + K^+ + Mg^{2+}$)-ATPase activity by P_i.

The results shown in Fig. 5 strongly suggest a likely product inhibitor to be the P_i generated by the reaction. As RODNIGHT, HEMS AND LAVIN[8] reported under different experimental conditions that P_i does not appear to inhibit a $(Na^+ + K^+ + Mg^{2+})$-ATPase from the same source, we examined our controls for linearity in the experimental range studied. We found that the experimental controls containing varying amounts of P_i were linear within experimental error in both the assay media containing Na^+ and those without Na^+.

The main conclusion of this study is that inorganic phosphate markedly inhibits the activity of a $(Na^+ + K^+ + Mg^{2+})$-ATPase preparation from beef brain microsomes. We did not find such an affect for ADP at a constant (ADP + ATP) concentration of 3 mM until relatively high levels of ADP/ATP ratios were reached. Product inhibition, particularly by ADP but also by P_i has been reported by BENDALL[13] for rabbit cardiac-muscle ATPase.

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