Temperature-Dependence of Activation and Inhibition of Rat-Brain Adenosine Triphosphatase Activated by Sodium and Potassium Ions

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1. The adenosine-triphosphatase activity of rat-brain microsomes was measured between 0° and 37°. The stimulatory effect of Na⁺ plus K⁺ on the Mg²⁺-dependent adenosine-triphosphatase activity decreased sharply with decreasing temperature and became negligible at 0°. An Arrhenius plot drawn from the experimental data showed two discontinuities: one at about 6° and the other at about 20°. 2. The increment in activity induced by Na⁺ plus K⁺ was more sensitive to oligomycin at lower than at higher temperatures, but the opposite was observed for ouabain. The action of oligomycin showed a biphasic character, since below a certain concentration it caused slight activation of Na⁺-plus-K⁺-activated adenosine triphosphatase. 3. Where oligomycin increased the activity of the enzyme, it also enhanced the accumulation of an acid-precipitable phosphorylated compound formed through the transfer of the γ -phosphate group of [³²P]ATP to the enzyme system. Stimulatory concentrations of oligomycin did not interfere with K⁺-mediated dephosphorylation of the intermediate, though high concentrations of oligomycin counteracted the effect of K⁺. 4. The temperature profile of K⁺-stimulated microsomal phosphatase qualitatively resembled that of microsomal adenosine triphosphatase.

The retardation of K⁺-linked Na⁺ transport by low temperatures has been observed by various investigators (Whittam & Davies, 1953; Page, Goerke & Storm, 1964; Willis, 1964). Impairment of energy supply could be one reason for the temperature effect, or the enzyme system that is involved in the transport of Na⁺ and K⁺ across biological membranes could be directly affected by low temperatures. Skou (1965) and Ahmed & Judah (1965a) have shown that (NaK)-ATPase,* present in membrane fragments and supposedly closely connected to the transport of alkali-metal ions, possesses an unusually high temperature coefficient.

The object of the present work was a more detailed study of the effect of temperature on (NaK)-ATPase. The experiments described below confirm and supplement previous reports on non-activation of the enzyme by Na⁺ plus K⁺ at low temperatures. It is also shown that the temperature-dependent changes in enzyme activity are

accompanied by changes in its sensitivity to inhibitors.

MATERIALS

Reagents. The reagents used were obtained from the following sources: [³²P]phosphate from The Radiochemical Centre, Amersham, Bucks.; ATP, *p*-nitrophenyl phosphate (disodium salt), ouabain, oligomycin and glyceraldehyde 3-phosphate dehydrogenase from Sigma Chemical Co., St Louis, Mo., U.S.A.; 3-phosphoglycerate kinase from C. F. Boehringer und Soehne G.m.b.H., Mannheim, W. Germany. All other chemicals used were obtained from commercial sources and were of analytical grade.

Animals. Male albino rats of a local strain were used.

METHODS

Enzyme preparation. For the preparation of rat-brain membrane ATPase, the procedure of Skou (1962) was slightly modified to avoid contamination with oligomycin-sensitive mitochondrial ATPase. Whole rat brain was homogenized with 10 parts (v/w) of 0.25 M-sucrose containing EDTA (1 mM) and tris buffer (50 mM) adjusted to pH 7.4 with HCl. The homogenate was centrifuged at 10000g for 20 min. at 0°, sedimented material was discarded and the centrifugation was repeated twice more. The final supernatant was then centrifuged at 30000g for 30 min.

* Abbreviations: ATPase, adenosine triphosphatase; in accordance with the suggestion by Wheeler & Whittam (1964), the activity observed in the presence of Mg²⁺ alone is designated (Mg)-ATPase and that measured in the presence of Mg²⁺, Na⁺ and K⁺ as (NaK)-ATPase.

and the precipitate suspended in ice-cold 0.25M-sucrose containing sodium deoxycholate (0.2%, w/v) and EDTA (5mM). Subsequent steps were as described by Skou (1962).

Preparation of [32 P]ATP labelled in the γ -phosphate group. The procedure described by Glynn & Chappell (1964) was followed. The specific activity of the preparation was in the range 4×10^6 – 20×10^6 counts/min./ μ mole. The labelled ATP was obtained in the form of its tris salt and stored in the frozen state.

Assay of ATPase activity. The standard reaction mixture consisted of 50 mM-tris buffer adjusted to pH 7.4 with HCl; 0.5 mM-MgCl₂, [32 P]ATP (50mM; specific activity 1×10^5 – 5×10^5 counts/min./ μ mole), and the enzyme preparation in an amount equivalent to 0.4mg. of protein/ml. Where indicated, NaCl (100mM) and KCl (20mM) were also added. The total volume was 0.5 ml. The reaction was started by adding ATP to the mixture equilibrated for 10min. at the required temperature. At 24° the time of incubation was 1 min.; at other temperatures it was chosen so as to liberate approximately the same amount of P_i as at 24° in 1 min. Approx. 50% of the ATP was hydrolysed. The progress of the reaction was found to be nearly linear up to 50% hydrolysis of the ATP. The reaction was stopped with 0.05 ml. of 50% (w/v) trichloroacetic acid. After centrifugation, 0.2 ml. of the supernatant was removed and the liberated P_i separated from nucleotides by extraction with 2-methylpropan-1-ol-benzene (Nielsen & Lehninger, 1955; Avron, 1960). The organic phase (1 ml.) was taken for the radioactivity determination. Controls, in which trichloroacetic acid was added before ATP to the pre-equilibrated reaction mixture, yielded corrections less than 5% for the counts contributed by [32 P]phosphate present in the [32 P]ATP preparation and liberated non-enzymically. The pH of the reaction mixture, which was 7.4 at room temperature, varied between 7.2 and 7.9 in the range of temperatures investigated. Since, however, the corresponding changes in the activity of (Mg)-ATPase and (NaK)-ATPase were less than 5%, no correction was applied.

ATPase activity was expressed as m μ moles of P_i liberated/mg. of protein.

Labelling of an acid-precipitable fraction by [32 P]ATP. The procedure of Post, Sen & Rosenthal (1965) was used, with slight modifications. The reaction mixture consisted of tris buffer (50mM) adjusted to pH 7.4 with HCl, [32 P]ATP (0.4mM; specific activity 5×10^5 counts/min./ μ mole), MgCl₂ (0.4mM) and the ATPase preparation in an amount equivalent to 0.8mg. of protein/ml. Where indicated, NaCl (25mM) and KCl (5mM) were added. The total volume was 1 ml. The reaction was started by adding ATP to the mixture, equilibrated at 24° for 19min., and terminated after 15sec. by adding 5 ml. of ice-cold 0.5N-HClO₄ under vigorous stirring. The mixture was centrifuged immediately at 6000 g for 10min. at 0°. To remove any absorbed radioactive material from the precipitate it was washed four times by centrifugation (6000 g for 10min. at 0°) with 5 ml. of ice-cold 0.1N-HClO₄ that contained unlabelled P_i (1mM) and ATP (0.6mM). The washed precipitate was finally resuspended in 1.0 ml. of 0.1N-NaOH containing Na₂CO₃ (2%, w/v) and boiled for 15min. After cooling a sample was taken for radioactivity determination.

Determination of phosphatase activity. The procedure followed in determining the phosphatase activity of the membrane particles in which the ATPase activity was

located was essentially that described by Ahmed & Judah (1964), with slight modifications. The mixture consisted of tris buffer (50mM) adjusted to pH 7.4 with HCl, MgCl₂ (5mM), *p*-nitrophenyl phosphate (5mM) and the preparation in an amount equivalent to 0.4mg. of protein/ml. Where indicated, KCl (10mM) was also added. The total volume was 1.0 ml. The reaction was started by adding the *p*-nitrophenyl phosphate to the mixture, which was pre-equilibrated for 10min. at the required temperature. The time of incubation was 5min. at 23°, and at other temperatures the time was chosen so as to yield approximately the same amount of free phenol as at 23° in 5min. The reaction was terminated by adding 0.1 ml. of 50% (w/v) trichloroacetic acid. After centrifugation (6000g for 5min. at 0°), 0.5 ml. of the supernatant was diluted to a final volume of 3 ml. with 1.0M-tris solution, pH 10.4. The E_{400} of the yellow solution was then read on the Zeiss PMQ II spectrophotometer (1 cm. path length) against a blank of 1.0M-tris solution. Readings were corrected for free phenol present by running controls in which the trichloroacetic acid was added to the mixture before the substrate.

Centrifugation. Centrifugation was carried out in a Servall model RC-2 refrigerated centrifuge (rotor SS-34).

Radioactivity determination. The sample was diluted with Bray's solution (Bray, 1960) to a final volume of 10 ml. and counted in a Packard Tri-Carb model 3003 scintillation spectrometer.

Protein determination. The method of Lowry, Rosebrough, Farr & Randall (1951) was used.

RESULTS

Effect of temperature on the activity of (Mg)-ATPase and (NaK)-ATPase. The activity of brain membrane ATPase was measured at various temperatures and found to increase more rapidly with temperature in the presence of Mg²⁺ plus Na⁺ plus K⁺ than with Mg²⁺ alone. A typical experiment is presented in Fig. 1. The ratio of (NaK)-ATPase activity to (Mg)-ATPase activity increased from 1.05 at 0° to 4.90 at 37° (Fig. 1, inset). When the results are presented in the form of an Arrhenius plot, discontinuities in the slope (at 6° and 20° respectively) become apparent irrespective of the nature of the activating ions. Occasionally certain enzyme preparations showed a slight activation by Na⁺ plus K⁺ even at 0°, but this was, however, very small compared with the stimulation observed at 37° (cf. Table 1). Preparations that showed originally no activation at 0° could be transformed into the slightly activated form by incubating the particles in 50mM-tris buffer, pH 7.4, containing magnesium chloride (0.5mM) and subsequently cooling to 0°. The presence of Na⁺ plus K⁺ and of ATP was not required during the preincubation for this transformation to occur. Nevertheless, there is no obvious reason to assume that faulty temperature control during the preparation of the membrane ATPase caused the above described variations in activation at 0°.

Fig. 1. Effect of temperature on (Mg)-ATPase and (NaK)-ATPase. The reaction mixture contained tris buffer (50 mM) adjusted to pH 7.4 with HCl, [32 P]ATP (0.5 mM; specific activity 5×10^5 counts/min./ μ mole) and the enzyme preparation in an amount equivalent to 0.4 mg. of protein/ml.; other additions were: ○, MgCl_2 (0.5 mM); ●, MgCl_2 (0.5 mM), NaCl (100 mM) and KCl (20 mM). The total volume was 0.5 ml. The times of incubation, the method of assay and all other conditions were as described in the Methods section. ATPase activity is expressed as μ moles of P_i liberated/min./mg. of protein. The curve shown in the inset (●) represents a plot of the ratio of (NaK)-ATPase activity to (Mg)-ATPase activity against temperature. The lines in this Figure were drawn arbitrarily.

Inhibitory effect of ouabain on (NaK)-ATPase at different temperatures. According to Levi, Sharon & Koshland (1959), discontinuities in the Arrhenius plot signify changes in enzyme structure. It is conceivable that a change in the structure of the membrane ATPase will be manifested, not only in the extent of stimulation induced by Na^+ plus K^+ , but also in an altered sensitivity to inhibitors. The question was raised accordingly whether the action of ouabain, a specific inhibitor of (NaK)-ATPase (Dunham & Glynn, 1961), would be influenced by temperature. The results, summarized in Table 1, indicate that ouabain only exerts its effect on (NaK)-ATPase above 6° , and that its effectiveness as an inhibitor increases most significantly between 10 and 26° . Ahmed & Judah (1965b) have reported recently that the inhibitory effect of ouabain on rat-brain (NaK)-ATPase decreased steeply on reduction of the incubation temperature from 38° to 10° .

Effect of oligomycin on the activity of (NaK)-ATPase at different temperatures. Oligomycin is well known as a potent inhibitor of the 2,4-dinitrophenol-stimulated mitochondrial ATPase (Lardy, Johnson & McMurray, 1958; Jöbsis, 1963). More recently, it was found to inhibit at higher concentrations the membranal (NaK)-ATPase as well (Jöbsis & Vreman, 1963; Van Groningen & Slater, 1963). Jöbsis & Vreman (1963) reported that lower concentrations of oligomycin inhibit (NaK)-ATPase at 27° more than at 37° . Prompted by this observation, we studied the effect of oligomycin over a wider range of temperatures. The results presented in Fig. 2 indicate that the inhibitory effect of oligomycin on the increment in activity induced by Na^+ plus K^+ decreased gradually between 6° and 37° . The oligomycin concentration required for 50% inhibition is about 40 times as high at 37° as at 6° . Fig. 2 also shows that very low concentrations

Table 1. *Effect of ouabain on the (NaK)-ATPase of rat-brain microsomes at different temperatures*

The composition of the reaction mixture and conditions were as described in the Methods section. Ouabain was added where indicated at a concentration of $50 \mu\text{M}$. Inhibition is expressed as a percentage of the activity increment induced by Na^+ plus K^+ . A negative inhibition indicates acceleration.

Temp.	ATPase activity (mμmoles of P _i liberated/min./mg. of protein)			Inhibition (%)
	Mg ²⁺	Mg ²⁺ + Na ⁺ + K ⁺	Mg ²⁺ + Na ⁺ + K ⁺ + ouabain	
0°	10.0	16.8	17.2	—5.9
6	14.4	38.4	41.6	—13.7
10	32.8	97.6	87.2	16.1
20	94.4	368	265	37.2
26	192	932	532	55.4
37	240	1680	800	61.1

cause a small but still significant increase in ATPase activity. The stimulatory effect of oligomycin was also found to depend on temperature.

Effect of oligomycin on labelling of an acid-precipitable fraction with $[^{32}\text{P}]\text{ATP}$. It has been reported by various investigators (Albers, Fahn & Koval, 1963; Whittam, Wheeler & Blake, 1964; Post *et al.* 1965) that, when the membranal particles in which the (NaK)-ATPase is located are incubated with ATP labelled in the γ -phosphate group, the label appears in an acid-precipitable fraction. The protein-bound phosphate behaves in many respects like an intermediate in the ATPase. Whittam *et al.* (1964) found that, in kidney-cortex microsomes, oligomycin does not interfere with the stimulatory

effect of Na^+ on the labelling, but counteracts the effect of K^+ on the dephosphorylation. It was decided to test whether oligomycin would also, at a stimulatory concentration, affect the pattern of labelling. Results of an experiment, shown in Table 2, in which the effect of inhibitory and stimulatory concentrations of oligomycin on the transphosphorylation reaction were compared indicate that $1.0\text{ }\mu\text{g.}$ of oligomycin/mg. of protein blocks the K^+ -dependent dephosphorylation step. This concentration of oligomycin, which is sufficient to inhibit ATPase at room temperature, also increases the labelling moderately when only Mg^{2+} and Na^+ are added to the reaction mixture. By contrast, $0.05\text{ }\mu\text{g.}$ of oligomycin/mg. of protein, which stimulates ATPase, nearly doubles the labelling increment caused by Na^+ , but does not interfere with the K^+ -mediated dephosphorylation.

Effect of ouabain and oligomycin on membranal brain phosphatase at various temperatures. It has been shown that preparations from erythrocyte membrane (Judah, Ahmed & McLean, 1962) and from brain microsomes (Ahmed & Judah, 1964) that possess (NaK)-ATPase activity can also

Table 2. *Effect of low and high concentrations of oligomycin on the labelling of an acid-precipitable fraction by $[^{32}\text{P}]\text{ATP}$*

The procedure for labelling and other conditions were as described in the Methods section.

Additions to assay medium	Radioactivity in acid-precipitable fraction (counts/min.)		
	Concn. of oligomycin ($\mu\text{g./mg. of protein}$)... 0	0.05	1.00
Mg^{2+}	370	370	—
$\text{Mg}^{2+} + \text{Na}^+$	960	1720	1170
$\text{Mg}^{2+} + \text{Na}^+ + \text{K}^+$	390	380	660

Fig. 2. Temperature-dependence of the action of oligomycin on the membranal ATPase. The composition of the reaction mixture for measuring (Mg)-ATPase and (NaK)-ATPase activities and all other conditions were as given in Fig. 1. The concentration of oligomycin in the reaction mixture was as indicated in the Figure. The activity increment induced by Na^+ plus K^+ in the absence of oligomycin is represented on the ordinate as 100%. Temperature: $\circ$, 6° ; $\triangle$, 16° ; $\square$, 27° ; $\bullet$, 37° .

Table 3. *Effect of ouabain on the hydrolysis of p-nitrophenyl phosphate catalysed by rat-brain membranal ATPase preparation*

The composition of the reaction mixture and the conditions of the reaction were as described in the Methods section. Ouabain was added where indicated at a concentration of $100\text{ }\mu\text{M}$. Inhibition is expressed as a percentage of the activity increment induced by K^+ .

Temp.	Phosphatase activity ($\mu\text{moles of P}_i$ liberated/hr./mg. of protein)			Inhibition (%)
	Additions..... Mg^{2+}	$\text{Mg}^{2+} + \text{Na}^+ + \text{K}^+$	$\text{Mg}^{2+} + \text{Na}^+ + \text{K}^+$ + ouabain	
0°	0.4	1.3	1.3	0.0
7	0.6	2.7	2.4	14.3
13	1.3	5.3	4.5	18.6
16	1.2	8.0	5.6	35.1
23	1.5	14.0	7.5	52.0

hydrolyse *p*-nitrophenyl phosphate. This phosphatase activity is stimulated by K^+ and inhibited by ouabain. The above authors suggested that the K^+ -stimulated phosphatase reaction may represent an intermediate stage in the hydrolysis of ATP. This raised the question whether a temperature effect similar to the one found for ATPase could also be established with *p*-nitrophenyl phosphate as substrate. The results in Table 3 indicate that the effect of temperature on the K^+ -induced activation of the phosphatase is much less pronounced than that with ATP as substrate. More significant, however, is the temperature change in the effectiveness of ouabain and oligomycin: 0.1mm-ouabain, which suppresses the K^+ -induced activation at 23°, is practically ineffective below 16°. The temperature-dependence of oligomycin action is also qualitatively similar to that found for (NaK)-ATPase. The results in Fig. 3 indicate that a nearly fourfold increase in the concentration of oligomycin is required to cause 50% inhibition at 37° as compared with that at 5°. It is also apparent that, as with ATPase, low oligomycin concentrations have a stimulating effect, whereas higher concentrations have an inhibitory effect. In spite of the great similarity between the effects of ouabain and oligomycin on ATPase and phosphatase activities respectively, however, the temperature effect in activation and inhibition of the phosphatase is much less striking than that for ATPase.

Fig. 3. Temperature-dependence of the action of oligomycin on membranal phosphatase. The reaction mixture contained tris buffer (50mm) adjusted to pH 7.4 with HCl, $MgCl_2$ (5mm), *p*-nitrophenyl phosphate (5mm) and the membranal preparation in an amount equivalent to 0.4mg. of protein/ml.; KCl, where added, was present in a concentration of 10mm. The total volume was 1.0ml. The method of assay and all other conditions were as described in the Methods section. The activity increment induced by K^+ in the absence of oligomycin is represented on the ordinate as 100%. Temperature: ○, 5°; △, 21°; □, 37°.

DISCUSSION

The results presented above show that certain characteristic properties of the membrane ATPase, such as its response to alkali-metal ions and to inhibitors, undergo striking changes with temperature. Configurational changes of the polypeptide chain, as visualized by Linderström-Lang & Schellman (1959), are believed to underlie the change resulting from exposure of an enzyme to ions or other environmental influences. Since these changes are temperature-dependent, the temperature effect may be expected to be especially strong for enzymes requiring excessive 'reshaping' for their activation. The molecular models developed for the visualization of K^+ -dependent Na^+ transport across the cell membranes (Opit & Charnock, 1965; Whittam & Ager, 1965) are based on sequential changes in the folding of membranal enzymes, related to the various phases of the process of ion transport. There is also some more direct evidence in favour of the view that Na^+ -plus- K^+ -induced activation of membrane ATPase is connected with changes in the conformation of the enzyme (Squires, 1965).

In the light of the above considerations, it is tempting to assume that the drastically decreased activation of membrane ATPase at low temperatures by Na^+ plus K^+ is due to 'freezing out' of the conformational changes. The considerably higher energy of activation of the reaction catalysed by (NaK)-ATPase in the 0–6° range (39500 cal./mole) compared with the values calculated for the 20–37° range (7800 cal./mole) may also be connected with increased rigidity of the enzyme structure in the lower range. It may be pointed out that one of the explanations offered for the discontinuities in the Arrhenius plot is based on changes in enzyme conformation at the point where the break is apparent (Levi *et al.* 1959).

One of the conclusions of the present investigation is the inverse relationship observed between the temperature coefficient of ouabain-induced inhibition and that of oligomycin inhibition. The ineffectiveness of ouabain at low temperatures may be explained by assuming that ouabain acts by decreasing the flexibility of the enzyme molecule, which is inherently low at low temperatures. Since ouabain ceased to act below 10°, whereas activation by Na^+ plus K^+ was observed at even lower temperatures, it is possible that a certain amount of flexibility of the enzyme molecule is essential to allow access of the relatively large ouabain molecule to the site of its action. By contrast, oligomycin, by analogy with its action on oxidative phosphorylation, may act on one of the intermediate stages in the sequence of reactions leading to the liberation of P_i from ATP (cf. Jöbsis & Vreman,

1963). This stage probably has a larger temperature coefficient than other intermediate stages in the reaction and, as a corollary, the inhibitory action of oligomycin should become more pronounced at lower temperatures. Since K^+ -stimulated membrane phosphatase showed an inhibition pattern qualitatively similar to that of ATPase, it is plausible that oligomycin should interfere with the K^+ -mediated dephosphorylation step as suggested by Whittam *et al.* (1964).

It is noteworthy that oligomycin should, in very low concentration, stimulate the Na^+ -plus- K^+ -dependent hydrolysis of ATP, increase the transfer of labelled phosphate in the presence of Na^+ to an acid-precipitable fraction, but fail to block K^+ -dependent dephosphorylation of the intermediate. These findings recall the observations reported by Lee & Ernster (1965) on the action of low oligomycin concentrations on certain energy-linked reactions in mitochondria.

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