FURTHER INVESTIGATIONS ON A $Mg^{++} + Na^+$-ACTIVATED ADENOSINTRIPHOSPHATASE, POSSIBLY RELATED TO THE ACTIVE, LINKED TRANSPORT OF Na^+ AND K^+ ACROSS THE NERVE MEMBRANE

JENS CHR. SKOU

Institute of Physiology, University of Aarhus (Denmark)

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SUMMARY

The sodium activated ATPase prepared from crab nerve which was described in a previous paper[1] has been subjected to a more detailed study of the relation between enzyme, substrate, and a number of monovalent and divalent cations. It has also been investigated whether the enzyme catalyses an ATP-Pi or an ATP-ADP exchange reaction, and whether these reactions are cation-dependent. Finally, the effect of g-strophanthin on the ATPase activity and the exchange reaction has been investigated.

The simplest scheme by which the experimental results can be explained is as follows:

$$E + ATP + 2\,Mg + n\,Na + m\,K \rightleftharpoons \underset{n\,Na}{\overset{m\,K}{E}}Mg_2ATP \qquad (1)$$

$$\underset{n\,Na}{\overset{m\,K}{E}}Mg_2ATP \rightleftharpoons \underset{n\,Na}{\overset{m\,K}{E}} \sim P + 2\,Mg + ADP \qquad (2)$$

$$\underset{n\,Na}{\overset{m\,K}{E}} \sim P \rightarrow E + Pi + n\,Na + m\,K \qquad (3)$$

where n and m are unknown numbers, and where

$$\underset{n\,Na}{\overset{m\,K}{E}}Mg_2ATP$$

symbolises an enzyme–substrate–ion complex in which Mg^{++} is part of the enzyme–substrate complex and in which Na^+ and K^+ are attached to two different sites on the enzyme; these sites show high affinities for the respective ions.

Phosphorylation of the enzyme, steps (1) + (2), will take place also when Mg^{++} is the only cation present in the system, but under these circumstances the release of inorganic phosphate, step (3), is very slow. The rate of step (3) is increased by the addition of Na^+ to the system, but not by the addition of K^+. In the presence of $Mg^{++} + Na^+$, however, addition of K^+ leads to a considerable increase of the rate of step (3).

The binding of $Na^+ + K^+$ to the enzyme takes place during the attachment of ATP to the enzyme, *i.e.* during step (1).

G-strophanthin has no effect on the phosphorylation of the enzyme, steps (1) + (2). But g-strophanthin inhibits the activating effect of Na^+ and of $Na^+ + K^+$ on step (3), presumably by interfering with the binding of the cations to the enzyme during step (1).

The observations lend further support to the suggestion made previously that this enzyme is involved in the active, linked transport of Na^+ and K^+ across the nerve membrane.

INTRODUCTION

In previous experiments[1] it has been shown that from peripheral crab nerve can be prepared an enzyme which is activated by $Mg^{++} + Na^+$ and which in the presence of $Mg^{++} + Na^+$ is stimulated by K^+. The enzyme is an adenosintriphosphatase, it is located in submicroscopic particles, and it possesses such properties that the suggestion has been made that this enzyme is involved in the active transport of Na^+ and K^+ across the nerve membrane.

Experiments recently published by POST[2] lend support to this assumption. Also the red blood cell membrane contains an adenosintriphosphatase which is stimulated by $Na^+ + K^+$ and which has properties that make it reasonable to assume that the enzyme is involved in the active transport of Na^+ and K^+ across the cell membrane.

In the present experiments the properties of the nerve enzyme have been studied more closely, particularly with a view to its possible participation in active ion transport. The studies have been concerned with the relation between enzyme, substrate, and a number of monovalent and divalent ions. It has furthermore been investigated whether the enzyme catalyses an ATP–Pi or an ATP–ADP exchange reaction and how the exchange reaction is dependent on the cations; this was done to obtain information about the intermediate steps in the breakdown of ATP and the influence of the cations on these steps.

EXPERIMENTAL

The enzyme was prepared as previously described by homogenisation of crab nerve in 0.25 *M* sucrose and differential centrifugation. The enzyme is contained in the submicroscopic particle fraction which is obtained by centrifugation at 20,000 × *g* for 1–2 h of the supernatant after centrifugation for 15 min at 10,000 × *g*. In the present experiments the enzyme was cooled to −15° immediately after the preparation and stored at this temperature until use. By this method it is possible to preserve the activity of the enzyme for months. The enzyme was used immediately after thawing, and only once frozen enzyme was used.

The ATP and ADP solutions were prepared and the enzymic activity determined as described previously, except that the activity of the enzyme was measured at 30° instead of at 36°. The ITP solutions (sodium salt from Pabst) were prepared in the same way as the ATP solutions.

Abbreviations used: ADP and ATP, adenosine-5′-di- and tri-phosphate; IDP and ITP, inosine-5′-di- and triphosphate; Pi, inorganic orthophosphate; Versene, ethylenediaminetetraacetate; Amidol, 2,4-diaminophenol, dihydrochloride.

Apart from Cs^+, the added cations in the concentrations used did not influence the determinations of Pi under the experimental conditions. Nor did Cs^+ exert any influence when the reducing substance, Amidol, was added to the solution before molybdate. On the other hand, if Amidol was added to the solution after molybdate, high concentrations of Cs^+ in the solution led to precipitates. All cations were added as chlorides.

The enzymic activity is expressed in terms of μmoles Pi split off from ATP in 30 min.

Carrier-free ^{32}P was obtained from Philips, Amsterdam.

$AD^{32}P$ was prepared according to COOPER AND LEHNINGER[3].

The exchange reactions were determined in a total volume of 1 ml containing 0.1 ml of the enzyme solution. The reaction mixture was adjusted to pH 7.2 as in the determination of ATPase activity. The ATP concentration was 3 mmoles/l in the investigations of both the ATP–Pi and the ATP–ADP exchange reactions.

In the investigations of the ATP–Pi exchange reaction, Pi was added to the reaction mixture to final concentrations ranging from 1 to 10 mmoles/l; the Pi was labelled with ^{32}P, $5 \cdot 10^5$ counts/min. In some of the experiments ADP was further added to a final concentration of 1 mmole/l. In the investigations of the ATP–ADP exchange reaction, ADP was added to a final concentration of 1 mmole/l; the ADP was labelled with [^{32}P]ADP, $5 \cdot 10^5$ counts/min. In some of the experiments Pi was also added to final concentrations ranging from 1–10 mmoles/l.

The incubation time was 20 min at 30°. During this period the exchange reaction varied linearly with time. The reaction was stopped by heating the mixture to 80–85°. After cooling, 0.6 ml of the mixture was diluted to 10 ml and placed on a column of Dowex 1. The nucleotides were eluted according to COHN[4]. The radioactivity was measured on 10 ml of each eluate in a liquid counter (type M6, manufactured by 20th Century Electronics).

The amount of Pi or ADP exchanged was calculated by multiplying the average concentration of Pi or ADP during the incubation by the fraction of ^{32}P or $AD^{32}P$ incorporated into ATP. Correction was made for the small amount of breakdown of labelled ATP during the incubation; the correction was obtained by multiplying the average specific activity of ATP by the amount of ATP hydrolysed.

Adenylate kinase activity was determined by the method of KALCKAR[5].

RESULTS

ATPase activity

Monovalent cations: It has been shown previously[1] that the enzyme exhibits some ATPase activity in the presence of Mg^{++} alone, but the activity is only slight. With Mg^{++} present in the system, addition of Na^{++} results in an increase of the activity. If K^+, Rb^+, Cs^+, or NH_4^+ are added instead of Na^+ the activity shows only a very small increase, while addition of Li^+ leads to an increase in activity intermediate between activities produced by Na^+ and by the other monovalent cations (Fig. 1). Maximum activity with Na^+ is obtained at a concentration of 6 mmoles/l. The Na^+ concentration at half maximum activity is 1.4 mmoles/l, and it is independent of the MgATP concentration. The concentrations of the other monovalent cations, at half maximum activity is about 10 times as high. Na^+ seems accordingly to be the mono-

valent cation which gives the highest increase in activity and to which the enzyme has the highest affinity.

When the system contains both Mg^{++} and Na^{+}, addition of one of the other monovalent cations, NH_4^{+}, K^{+}, Rb^{+}, Cs^{+} or Li^{+}, leads to a considerable increase of the activity of the enzyme (Figs. 2 and 3). This effect is only obtained with Na^{+} in the solution. If Na^{+} is replaced, by, for example, Li^{+}, addition of one of the other cations does not lead to an increase in the activity, but to an inhibition of the activity due to Li^{+}.

The stimulation produced by K^{+}, Rb^{+}, Cs^{+}, NH_4^{+} and Li^{+} depends on the Na^{+}

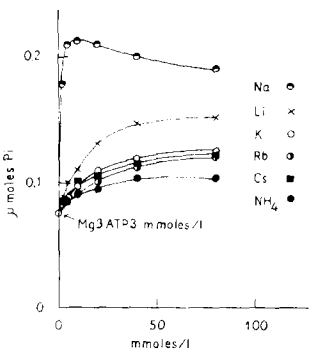


Fig. 1. Effect of monovalent cations on the enzyme activity in the presence of Mg^{++}. In Figs. 1–3 and 5–13 the enzyme activity is expressed as μmoles inorganic phosphate split off ATP within 30 min under the experimental conditions. ATP concentration: 3 mmoles/l. Cation present: 3 mmoles/l Mg^{++}.

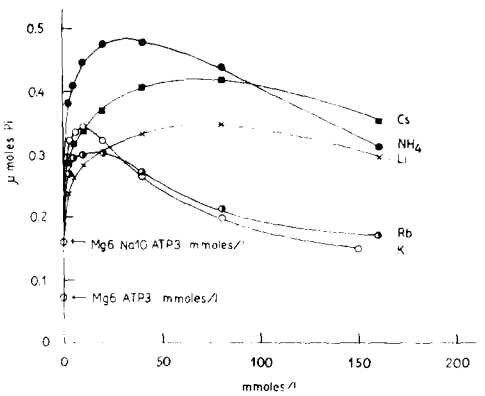


Fig. 2. Effect of monovalent cations on the enzyme activity in the presence of $Mg^{++} + Na^{+}$. ATP concentration: 3 mmoles/l. Cations present: 6 mmoles/l Mg^{++} + 10 mmoles/l Na^{+}.

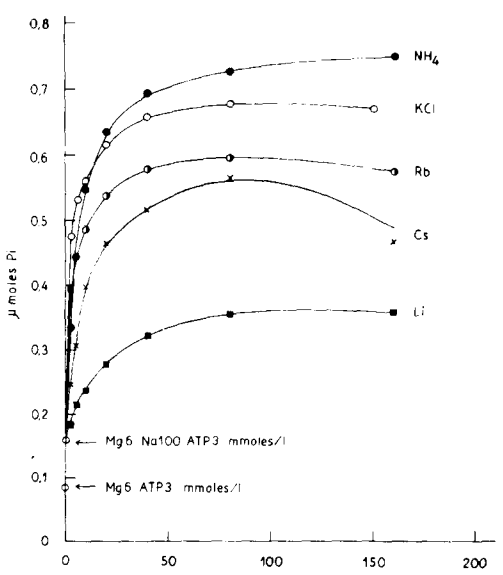


Fig. 3. Effect of monovalent cations on the enzyme activity in the presence of $Mg^{++} + Na^{+}$. ATP concentration: 3 mmoles/l. Cations present: 6 mmoles/l Mg^{++} + 100 mmoles/l Na^{+}.

concentration (Figs. 2 and 3). At low concentrations of Na^+, *e.g.* 10 mmoles/l (Fig. 2), the addition of low concentrations of these cations leads to an increase of the activity, while the activity decreases again when higher concentrations are added. As has been found previously with K^+, sufficiently high concentrations of the cations lower the activity to the level observed when only Mg^{++} is present in the system.

When the Na^+ concentration is raised, the maximum activity obtainable by addition of the other monovalent cations increases until the Na^+ concentration is equal to or higher than 100 mmoles/l (Fig. 3). The concentrations of the monovalent cations which correspond to the activity maxima also increase with the Na^+ concentration.

Table I shows the concentrations of K^+, Rb^+, Cs^+, NH_4^+ and Li^+ necessary to produce half maximum activity in the presence of 6 mmoles/l Mg^{++} and 100 mmoles/l Na^+; the values were read from Fig. 3. The concentrations are lower for K^+ and Rb^+ than for Cs^+, NH_4^+ and Li^+; the affinity of the enzyme is accordingly greater for the former than for the latter. On the other hand, the greatest activity is obtained with NH_4^+ (Fig. 3).

TABLE I

CONCENTRATIONS OF MONOVALENT CATIONS NECESSARY TO GIVE HALF MAXIMUM ATPASE ACTIVITY IN THE PRESENCE OF $Mg^{++} + Na^+$

The values were read from Fig. 3. Conditions: ATP 3 mmoles/l, Mg^{++} 6 mmoles/l, Na^+ 100 mmoles/l.

Cation	*Concentration (mmoles/l)*
Li^+	15.0
NH_4^+	5.0
K^+	1.8
Rb^+	2.1
Cs^+	8.0

Fig. 4 shows a kinetical analysis of the inhibition produced by K^+ at various concentrations of Na^+. The values are taken from Fig. 5 in the previously published paper[1]. The graph indicates[6] that the inhibitory effect of K^+ is due to a competitive displacement of Na^+. Similar analyses of the inhibitory effect of the other monovalent cations were not performed, but since their inhibition depends on the Na^+ concentration in the same way as that of K^+, one may assume that the inhibitory effect of these cations is also due to a competitive displacement of Na^+.

The experiments with the monovalent cations show accordingly that the enzyme has a high affinity for Na^+ and also, in the presence of $Mg^{++} + Na^+$, a high affinity for K^+ and Rb^+. They show further that in the presence of $Mg^{++} + Na^+$ the cations K^+, Rb^+, Cs^+, NH_4^+ and Li^+ exert two different actions on the enzyme. One action is a stimulation, and for K^+ the K_m value is 1.8 mmoles/l (Fig. 3). The other action is an inhibition which is due to a competitive displacement of Na^+; the latter has for K^+ a K_I value of 9 mmoles/l (Fig. 5).

Divalent cations. Mg^{++}: In the previous investigation[1] it was found that at an ATP concentration of 3 mmoles/l, the optimum Mg^{++} concentration was also of the order of 3 mmoles/l. This was so in a system containing Mg^{++} alone or $Mg^{++} + Na^+$ or $Mg^{++} + K^+$. When the concentration of Mg^{++} exceeded that of ATP the activity

was inhibited, and the same was observed when the ATP concentration exceeded the Mg^{++} concentration. This optimum 1/1 ratio between Mg^{++} and ATP is also found at other concentrations of Mg^{++} and ATP (Fig. 5).

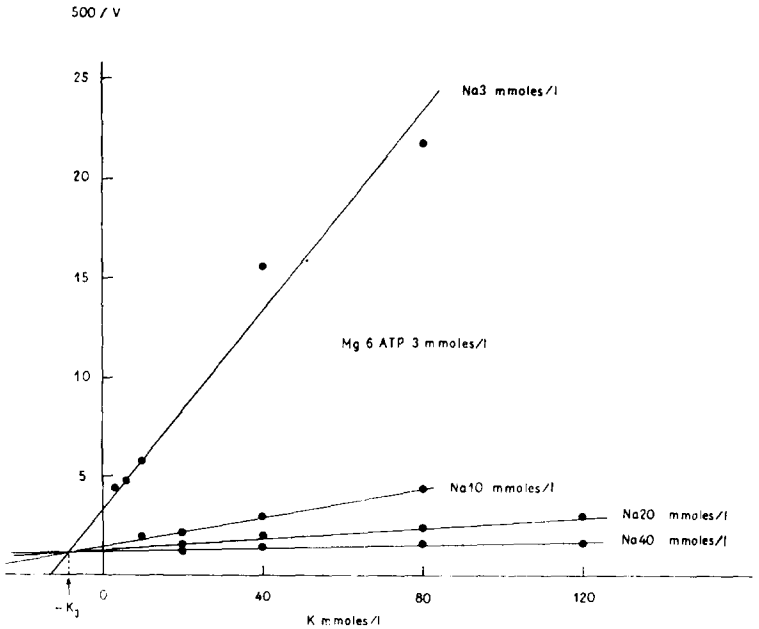


Fig. 4. Plots of reciprocal rate of enzyme activity versus K^{+} concentration. Data from Fig. 5 in ref. 1. ATP concentration: 3 mmoles/l. Cations present: 6 mmoles/l Mg^{++} + 3, 10, 20 and 40 mmoles/l Na^{+}.

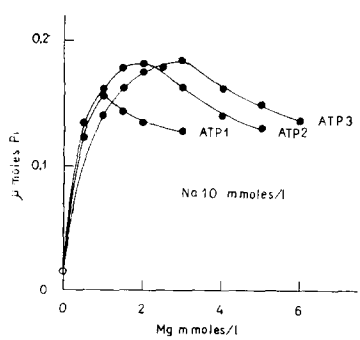


Fig. 5. Effect of the Mg^{++} concentrations on the enzyme activity at various ATP concentrations in the presence of Na^{+}. ATP concentration: 1, 2 and 3 mmoles/l. Cation present: 10 mmoles/l Na^{+}.

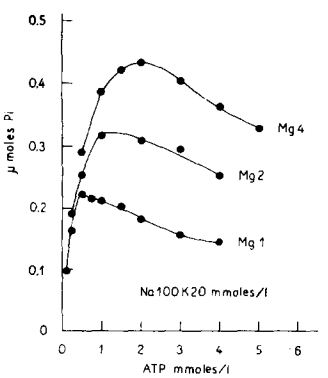


Fig. 6. Effect of the ATP concentration on the enzyme activity at various Mg^{++} concentrations in the presence of Na^{+} + K^{+}. Cations present: 1, 2 and 4 mmoles/l Mg^{++} + 100 mmoles/l Na^{+} + 20 mmoles/l K^{+}.

If the system contains Mg^{++} and Na^{+} + K^{+} in concentrations giving maximum activity of the enzyme, the concentration of Mg^{++} which is necessary for maximum activity with an ATP concentration of 3 mmoles/l is around 6 mmoles/l, *i.e.* the optimum Mg^{++}/ATP ratio is now of the order of 2/1 (Fig. 6). A further increase of the

Mg^{++} concentration leads to a slight inhibition of the activity. Further addition of ATP results in a more marked inhibition of the activity.

The activation of the enzyme with $Na^+ + K^+$ accordingly changes the optimum Mg^{++}/ATP ratio from 1/1 to 2/1.

Calcium: Ca^{++} inhibits the activity of the enzyme, and the inhibition in a system containing Mg^{++} or $Mg^{++} + Na^+$ or $Mg^{++} + K^+$ is different from that obtained in a system containing $Mg^{++} + Na^+ + K^+$ (Fig. 7).

In the presence of Mg^{++} alone or $Mg^{++} + Na^+$ or $Mg^{++} + K^+$, *i.e.* when the optimum Mg^{++}/ATP ratio is 1/1, the Ca^{++} inhibition is accompanied by a decrease in the optimum Mg^{++} concentration.

In the presence of $Mg^{++} + Na^+ + K^+$, *i.e.* when the optimum Mg^{++}/ATP ratio is 2/1, the Ca^{++} inhibition is accompanied by an increase in the optimum Mg^{++} concentration. The inhibition is counteracted by an increase in the Mg^{++} concentration, but the original activity is not fully restored even with high concentrations of Mg^{++}.

The two different ways in which Ca^{++} influences the optimum Mg^{++}/ATP ratio indicates two different types of Mg^{++} activation.

One type of activation is obtained in the presence of Mg^{++} alone or $Mg^{++} + Na^+$ or $Mg^{++} + K^+$; it is non-competitively inhibited by Ca^{++}, and the optimum Mg^{++}/ATP ratio is under these circumstances 1/1.—To this is, when the system contains $Mg^{++} + Na^+ + K^+$, added another type of activation, which is inhibited competitively by Ca^{++}. The optimum Mg^{++}/ATP ratio is now 2/1.

ITP as substrate: In order to explore this hypothesis of two types of Mg^{++} activation, experiments were performed with ITP as substrate instead of ATP.

Studies of LEVEDAHL AND JAMES[7] on the rotatory dispersion of ATP seem to show that the ATP molecule is folded back upon itself, and that the NH_2 group of

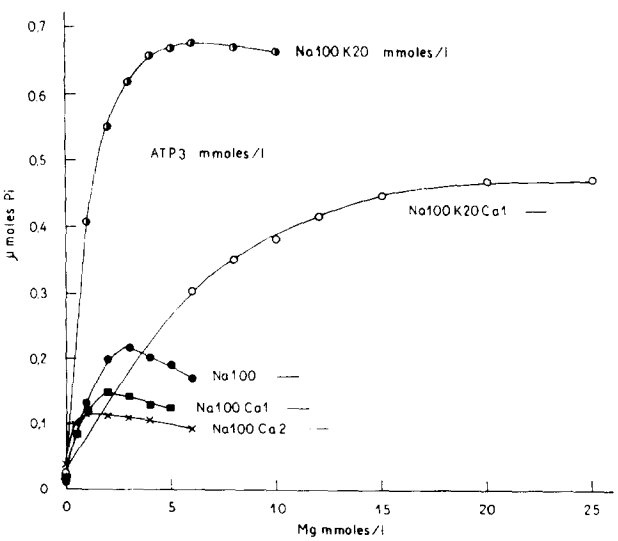


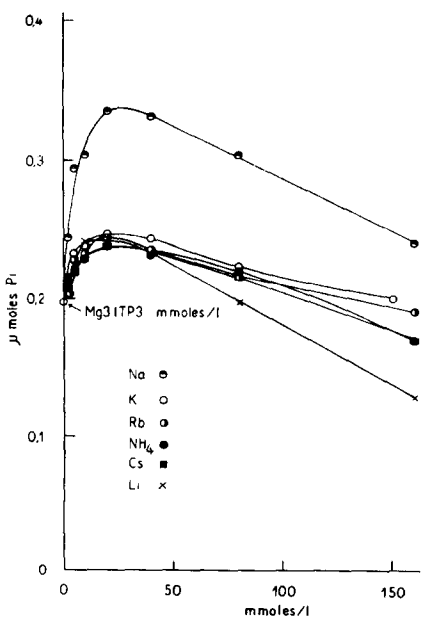
Fig. 7. Effect of Mg^{++} in the presence of Na^+ and $Na^+ + K^+$ and in the absence and presence of Ca^{++}. ATP concentration: 3 mmoles/l. Cations present: 100 mmoles/l $Na^+ + 0$, 1 and 2 mmoles/l Ca^{++}. 100 mmoles/l $Na^+ + 20$ mmoles/l $K^+ + 0$ and 1 mmole/l Ca^{++}.

adenine together with the two terminal phosphate groups play a role in this folding. LEVEDAHL AND JAMES proposed that this folded structure is important for the activity of ATP in biological systems. SZENT-GYÖRGYI[8] has suggested that since Mg^{++} invariably seems necessary for the activity of ATP, it is this ion which connects the two ends of the folded molecule through a quadridentate chelate formation between the two terminal phosphate groups of the phosphate chain and the NH_2 group and the N7 group of the adenine part of the ATP molecule.

One or the other of the two effects of Mg^{++} on the Na^+ + K^+ activated ATPase may conceivably be due to such a chelate formation. In either case, one should expect to find a change of the optimum Mg^{++}/ATP ratio if ATP were replaced as substrate by ITP, which lacks the NH_2 group in the purine, but which is otherwise structurally identical with ATP.

The enzyme catalyses the hydrolysis of ITP to IDP + Pi, and also with ITP as substrate Mg^{++} is an obligatory requirement. It is observed that the activity with Mg^{++} alone is higher with ITP than with ATP as substrate (see Fig. 9) and that the optimum Mg^{++}/ITP ratio is 1/1.

In the presence of Mg^{++}, addition of one of the monovalent cations, Na^+, Li^+, K^+, Rb^+, Cs^+ or NH_4^+, leads to an increase of the activity (Fig. 8), and it is noted, as in the experiments with ATP, that the increase is highest when the added monovalent cation is Na^+ and lower with Li^+ and the other monovalent cations. Contrary to what was found with ATP as substrate, the activating effect of Li^+ and of the other monovalent cations is in these experiments almost the same. The increase of activity due to Na^+ is smaller when the substrate is ITP than when it is ATP (Fig. 9); but since the activity due to Mg^{++} alone is higher with ITP as substrate, the total activity with Mg^{++} + Na^+ becomes higher. The concentration of Na^+ which gives



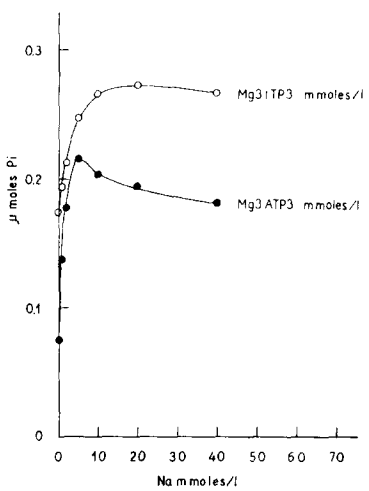


Fig. 9. Effect of Na^+ on the enzyme activity in the presence of Mg^{++} and with ATP and ITP as substrate. ATP and ITP concentrations: 3 mmoles/l. Cation present: 3 mmoles/l Mg^{++}.

Fig. 8. Effect of monovalent cations on the enzyme activity in the presence of Mg^{++} and with ITP as substrate. ITP concentration: 3 mmoles/l. Cation present: 3 mmoles/l Mg^{++}.

half maximum activity with ITP as substrate is 2.5 mmoles/l compared to 1.4 mmoles/l with ATP as substrate. The optimum Mg^{++}/ITP ratio in the presence of Mg^{++} + Na^+ is 1/1.

When the system contains Mg^{++} + Na^+, the addition of one of the other monovalent cations, Li^+, K^+, Rb^+, Cs^+ or NH_4^+, leads to an increase of the activity, and the effect is dependent on the Na^+ concentration (Fig. 10). It should be noted that this stimulating effect is much smaller when ITP than when ATP is the substrate, and also that at a Na^+ concentration of 100 mmoles/l the added cations lead to a decrease of the activity at lower concentrations than in the systems containing ATP as substrate. This is even more pronounced when the Na^+ concentration is low, for example 20 mmoles/l (Fig. 11); in fact, under these circumstances K^+ exerts only a very slight stimulating effect.

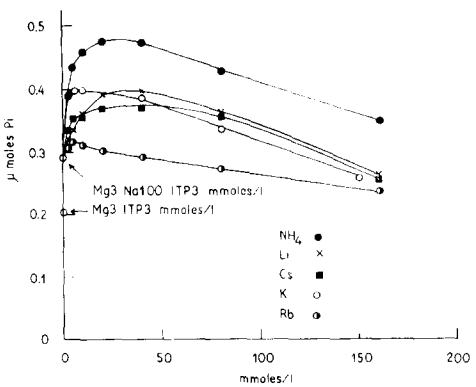


Fig. 10. Effect of monovalent cations on the enzyme activity in the presence of Mg^{++} + Na^+ and with ITP as substrate. ITP concentration: 3 mmoles/l. Cations present: 3 mmoles/l Mg^{++} + 100 mmoles/l Na^+.

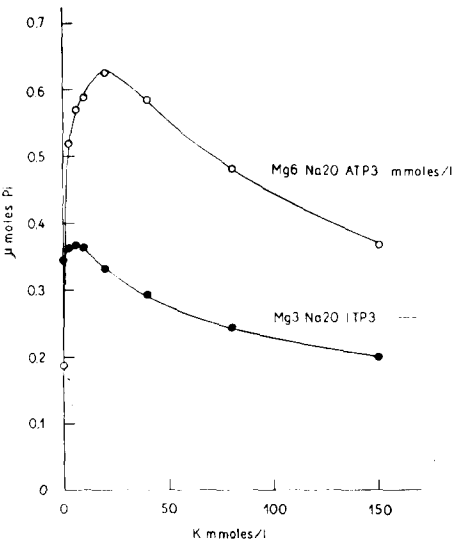


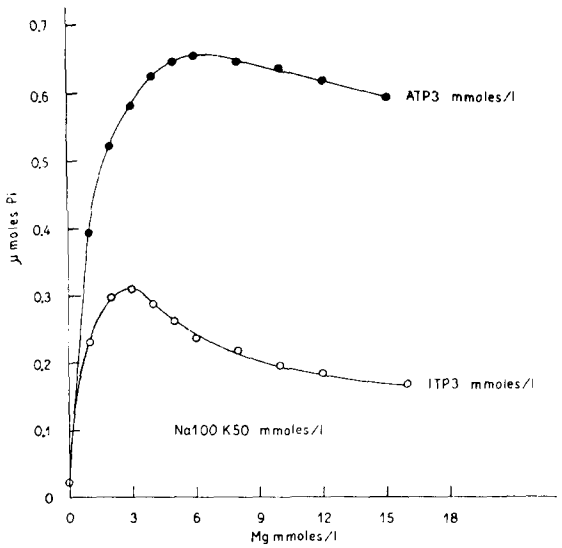
Fig. 11. Effect of K^+ on the enzyme activity in the presence of Mg^{++} + Na^+ and with ATP and ITP as substrate. ATP and ITP concentrations: 3 mmoles/l. Cations present: 3 and 6 mmoles/l Mg^{++} + 20 mmoles/l Na^+.

The optimum Mg^{++}/ITP ratio which is 1/1 with Mg^{++} + Na^+ in the system is not increased by adding K^+, it is still 1/1, contrary to the optimum Mg^{++}/ATP ratio, which in the presence of Na^+ + K^+ is 2/1 (Fig. 12).

The experiments with ITP accordingly support the idea of two different effects of Mg^{++} when ATP is the substrate. The activation which is observed in the presence of Mg^{++} alone or Mg^{++} + Na^+ or Mg^{++} + K^+ can be demonstrated with ITP as well as with ATP as substrate. But the second type of activation, that observed in the presence of Mg^{++} + Na^+ + K^+, is found only with ATP as substrate. This type of activation must consequently depend on the configuration of the adenine part of the ATP molecule.

G-strophanthin: Since g-strophanthin is known to inhibit active cation transport in a number of biological systems[9,10], the effect of this compound was also studied (Fig. 13).

Fig. 12. Effect of Mg^{++} on the enzyme activity in the presence of Na^+ + K^+ and with ATP and ITP as substrate. ATP and ITP concentrations: 3 mmoles/l. Cations present: 100 mmoles/l Na^+ + 50 mmoles/l K^+.



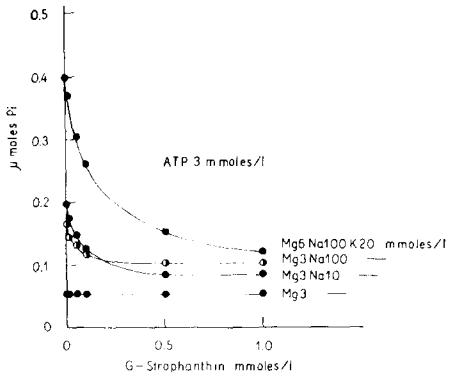


Fig. 13. Effect of g-strophanthin on the enzyme activity in the presence of Mg^{++}, Mg^{++} + Na^+ and Mg^{++} + Na^+ + K^+. ATP concentration: 3 mmoles/l. Cations present: 3 mmoles/l Mg^{++}; 3 mmoles/l Mg^{++} + 10 and 100 mmoles/l Na^+ 6 mmoles/l Mg^{++} + 100 mmoles/l Na^+ + 20 mmoles/l K^+.

Within the concentration range studied (up to 1 mmole/l), g-strophanthin does not influence the activity when the system contains only Mg^{++}. However, g-strophanthin inhibits the increase of activity produced by addition of Na^+. Maximum inhibition is obtained at a g-strophanthin concentration of 0.5 mmole/l; the inhibition is only partial and decreases with increasing Na^+ concentration.

G-strophanthin also inhibits the increase of activity produced by addition of Na^+ + K^+. The concentration required to give maximum inhibition is somewhat higher, around 1 mmole/l, than when the system contains only Mg^{++} + Na^+.

When Mg^{++} + Na^+ + K^+ are present in the system, addition of g-strophanthin leads to a reduction of the optimum Mg^{++}/ATP ratio from 2/1 to 1/1.

ATP–Pi and ATP–ADP exchange reactions

The intermediary reaction steps were investigated by studying the ATP–Pi and the ATP–ADP exchange reactions.

Addition of [^{32}P]phosphate to the system did not in any instance lead to a measurable labelling of ATP. The concentration of Pi varied from 1 to 10 mmoles/l,

and the experiments were carried out in the presence of Mg^{++} alone, $Mg^{++} + Na^+$, $Mg^{++} + K^+$ and $Mg^{++} + Na^+ + K^+$. Neither were the results influenced by addition of ADP in a concentration of 1 mmole/l.

If, on the other hand, ADP labelled with $AD^{32}P$ was added to the system, a labelling of ATP was observed. The extent of the labelling of ATP depends on the concentration of ADP (Table II); it is independent of whether or not Pi is added to the system. This incorporation of labelled ADP into ATP cannot be due to the action of adenylate kinase ("myokinase"), since the submicroscopic particles were shown to be devoid of such activity.

TABLE II

THE DEPENDANCE OF THE ATP–ADP EXCHANGE RATE ON THE ADP CONCENTRATION

Conditions: ATP 3 mmoles/l, Mg^{++} 3 mmoles/l.

ADP concentration mmoles/l	*ADP exchanged mμmoles/20 min*
0.08	40
0.16	116
1.16	260
2.66	238

The ATP–ADP exchange reaction requires Mg^{++} (Fig. 14), and the optimum Mg^{++}/ATP ratio is 1/1. This is so also when the system contains $Mg^{++} + Na^+$ and when it contains $Mg^{++} + Na^+ + K^+$; in the latter respect the ATP–ADP exchange reaction differs from the ATPase activity.

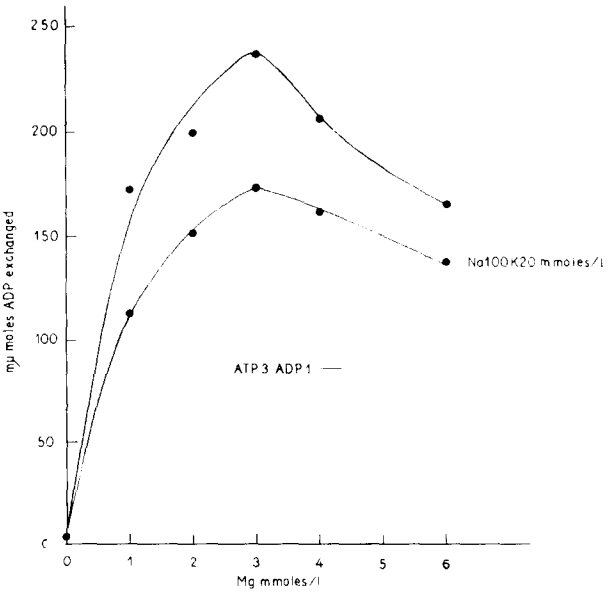


Fig. 14. Effect of Mg^{++} on rate of ATP–ADP exchange in the absence and presence of $Na^+ + K^+$. In Fig. 14–17 the exchange rate is expressed as mμmoles ADP exchanged per 20 min. ATP concentration: 3 mmoles/l. Cations present: 0 mmole/l Na^+ + 0 mmole/l K^+; 100 mmoles/l Na^+ + 20 mmoles/l K^+.

It appears from Fig. 15 that the ATP–ADP exchange is reduced by addition of Na^{+} to the system. The addition of K^{+} does not influence the exchange reaction, but in the presence of Na^{+} the addition of K^{+} leads to a further decrease of the exchange reaction (Fig. 16). It should be noted that when the Na^{+} concentration is low (10 mmoles/l), this inhibition disappears when the K^{+} concentration is raised.

Fig. 17 presents experiments on the effect of Ca^{++} in a system containing Mg^{++} alone. It may be noticed that the optimum Mg^{++}/ATP ratio in this experiment is somewhat lower than 1/1; similar observations have occasionally been made in determinations of the ATPase activity. Since the optimum Mg^{++}/ATP ratio may be raised somewhat by addition of small amounts of Versene, the optimum Mg^{++}/ATP ratio lower than 1/1 may presumably be caused by the presence of traces of Ca^{++} in the system.

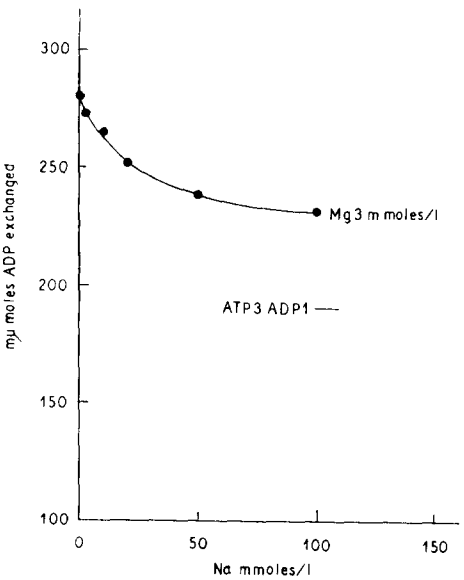


Fig. 15.

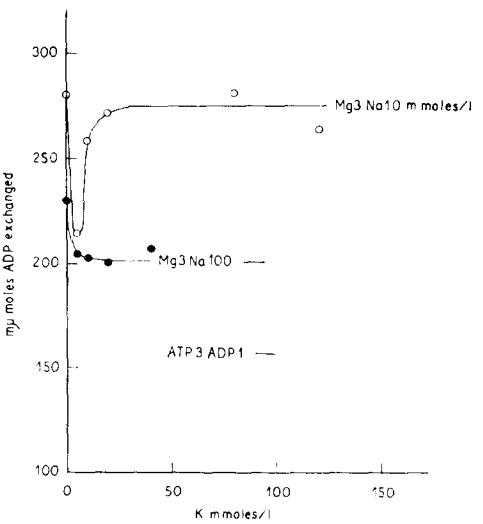


Fig. 16.

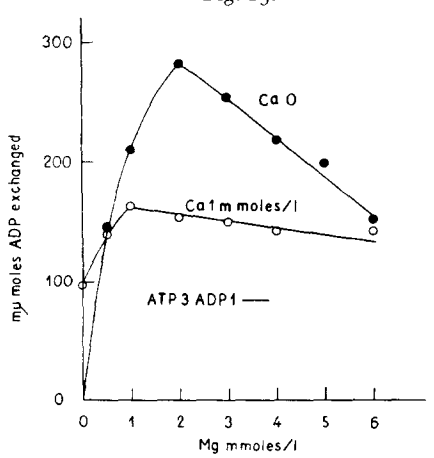


Fig. 17.

Fig. 15. Effect of Na^{+} on rate of ATP–ADP exchange in the presence of Mg^{++}. ATP concentration: 3 mmoles/l. Cation present: 3 mmoles/l Mg^{++}.

Fig. 16. Effect of K^{+} on rate of ATP–ADP exchange in the presence of Mg^{++} + Na^{+}. ATP concentration: 3 mmoles/l. Cations present: 3 mmoles/l Mg^{++} + 10 and 100 mmoles/l Na^{+}.

Fig. 17. Effect of Mg^{++} on rate of ATP–ADP exchange in the absence and presence of Ca^{++}. ATP concentration: 3 mmoles/l. Cation present: 0 and 1 mmole/l Ca^{++}.

When Ca^{++} is added to the system, the exchange reaction is inhibited, and the optimum Mg^{++}/ATP ratio is markedly lowered. The effect of Ca^{++} on the exchange reaction thus corresponds to its effect on the ATPase activity when the optimum Mg^{++}/ATP ratio is 1/1.

G-strophanthin in the concentrations tested (up to 0.5 mmole/l), does not influence the ATP–ADP exchange reaction when the system contains only Mg^{++}, whereas in the presence of $Mg^{++} + Na^+$ or $Mg^{++} + Na^+ + K^+$ the addition of g-strophanthin counteracts to some extent the inhibition due to Na^+ or to $Na^+ + K^+$.

The enzyme is also able to catalyse an exchange reaction between ITP and ADP; the rate of exchange amounts to 75–80 % of that between ATP and ADP. The ITP–ADP exchange also requires Mg^{++} and is slightly inhibited by Na^+ and by $Na^+ + K^+$.

DISCUSSION

A few small quantitative differences have been observed between the results of the present experiments and those obtained in the previous study[1]. An explanation might be that in the previous experiments the enzyme had been stored at 0° and never longer than a week, whereas in the present work the enzyme was stored at −15° up to several months. However, since the various enzyme preparations in the present experiments also revealed small quantitative differences, the cause of the variation must presumably be found in the existence of an unstable factor, which is affected in a slightly different way in the various preparations. This problem is being subjected to further investigations.

It appears from the experiments that when $Mg^{++} + Na^+$ are present in the system K^+ and the other monovalent cations exert two different actions on the activity of the enzyme: in lower concentrations a stimulation and in higher concentrations an inhibition. The kinetical analysis indicates that the inhibition is due to a competitive displacement of Na^+ from the enzyme, and this assumption is supported by the observation that the cations in high concentrations lower the activity to the level observed in the presence of Mg^{++} alone. The competitive nature of the inhibition indicates that the point of action of the inhibitory effect of K^+ and the other monovalent cations is identical with the point of action of the activating effect of Na^+. The affinity for Na^+ at this site is about 7–10 times higher than it is for K^+.

With $Mg^{++} + Na^+$ in the system the K_m value for K^+ was found to be 1.8 mmoles/l and the K_I value to be 9 mmoles/l. One must therefore conclude that the point of action of the K^+ stimulation must differ from that of the K^+ inhibition and thereby also from that of the activating effect of Na^+.

There seem accordingly to be two different sites on the enzyme or enzyme complex with high affinities for monovalent cations. At one site (indicated by 1 in Fig. 18) the affinity for Na^+ is higher than for K^+ and the other monovalent cations, and at this site these cations can displace Na^+ by competition. At another site (indicated by 2 in Fig. 18) the affinity for K^+ is higher than for any of the other monovalent cations.

When ITP is substituted for ATP as substrate, the affinity of the enzyme for Na^+ is lowered (Fig. 9). Under these circumstances one should expect the other monovalent cations to displace Na^+ more readily from the enzyme, and it was correspondingly found that in a system with ITP as substrate the inhibition produced by K^+ or one

of the other monovalent cations is stronger and commences at lower concentrations than when the substrate is ATP. It should be noted, however, that the lower enzymic activity with ITP in the presence of $Na^+ + K^+$ is due, not only to a more pronounced K^+ inhibition, but also, and primarily, to a lower K^+ stimulation; this appears from experiments (not presented) in which Na^+ was present in sufficiently high concentration (350 mmoles/l) to counteract effectively the competitive K^+ inhibition.

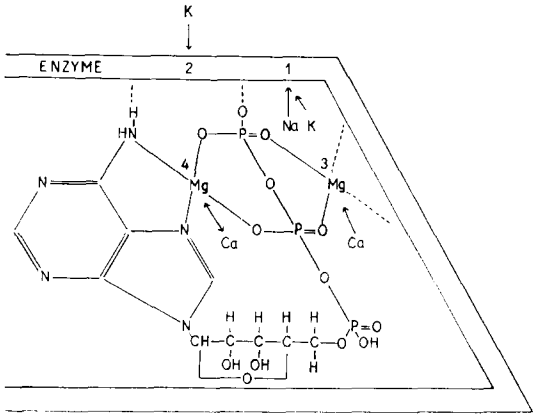


Fig. 18. Hypothetical scheme for the relation between enzyme, ATP, Mg^{++}, Ca^{++}, Na^+ and K^+.

It was pointed out above that Mg^{++} seems to exert two different actions; this may be visualized in the following way.

Under certain experimental conditions (in the presence of Mg^{++} alone or $Mg^{++} + Na^+$ or $Mg^{++} + K^+$) one molecule of Mg^{++} is attached to the complex for each molecule of ATP, giving an optimum Mg^{++}/ATP ratio equal to 1/1. The presence of this Mg^{++} (the position of which is indicated by 3 in Fig. 18) is necessary for the ATPase activity as well as for the ATP–ADP exchange reaction, and its action is inhibited non-competitively by Ca^{++}.

If the system contains K^+ in addition to $Mg^{++} + Na^+$, a second Mg^{++} (indicated by 4 in Fig. 18) is added to the complex, giving an optimum Mg^{++}/ATP ratio of 2/1. The action of this Mg^{++} is inhibited competitively by Ca^{++}, and it is observed only with ATP, and not with ITP, as substrate, which shows that it is dependent on the adenine part of the ATP molecule.

The experiments with labelled ADP and Pi showed an ATP–ADP exchange reaction but no ATP–Pi exchange reaction. Since the preparation does not exhibit adenylate kinase activity, one may assume that an intermediate step in the breakdown of ATP is the formation of a phosphorylated enzyme in which the phosphate is bound to the enzyme by an energy-rich bond. The simplest scheme which can explain the results is as follows:

$$E + ATP \rightleftharpoons EATP \quad (1)$$

$$EATP \rightleftharpoons E \sim P + ADP \quad (2)$$

$$E \sim P + H_2O \rightarrow E + Pi \quad (3)$$

$$\overline{ATP + H_2O \rightarrow ADP + Pi}$$

where E is the enzyme.

An ATP–ADP exchange reaction might also have been obtained if, instead of a

phosphorylated enzyme, an $E \sim ADP$ were formed, but in this case the reaction should proceed as follows[11]:

$$E + ATP \rightleftharpoons EATP \quad (4)$$

$$EATP \rightleftharpoons E \sim ADP + Pi \quad (5)$$

$$E \sim ADP + x \rightleftharpoons E \sim x + ADP \quad (6)$$

where x is an unknown compound.

However, according to this scheme, it is a prerequisite for an ATP–ADP exchange reaction that the solution contains Pi, and since the ATP–ADP exchange reaction was found to be independent of the presence of Pi, this reaction scheme can be disregarded.

Even if no ATP–Pi exchange reaction has been obtained, the breakdown of the phosphorylated enzyme might have proceeded in the following way[11]:

$$E \sim P + x \rightleftharpoons E \sim x + Pi \quad (7)$$

$$E \sim x \rightarrow E + x \quad (8)$$

In this case it should be possible to obtain an ATP–Pi exchange reaction by adding ADP, and since this was not the case, it must be concluded that the reaction proceeded as shown in expression (3).

In all systems capable of catalysing an ATP–ADP exchange reaction Mg^{++} has been found to be an obligatory requirement[11–13], and it has also been found that the optimum Mg^{++} concentration is equal to the concentration of ATP[13]. This has been confirmed in the present experiments. No evidence is available as to how this Mg^{++} exerts its effect, but the 1/1 ratio between Mg^{++} and ATP suggests that it is involved in the formation of the enzyme–substrate complex.

Na^+ has a moderate inhibitory effect on the ATP–ADP exchange reaction, whereas K^+ is without effect. With $Mg^{++} + Na^+$ present in the system, addition of K^+ gives rise to two different effects. A low concentration of K^+ relative to that of Na^+ leads to an intensification of the Na^+ inhibition, while a K^+ concentration which is high relative to the Na^+ concentration restores the rate of the reaction which was observed in the presence of Mg^{++} alone.

There is thus a qualitative similarity between the effects of K^+ on the ATPase activity and on the exchange reaction. In both cases the effect is dependent on the presence of Na^+, and in both cases high concentrations of K^+ relative to Na^+ abolishes the effect. In experiments on the ATPase activity the latter phenomenon has been found to be due to a displacement of Na^+, and the same mechanism appears likely also in the case of the exchange reaction. This similarity between the K^+ effects indicates that it is the same enzyme which is responsible for the hydrolysis of ATP and for the ATP–ADP exchange reaction.

Na^+ and, still more pronounced, $Na^+ + K^+ +$ an extra molecule of Mg^{++} increase the liberation of Pi from ATP, *i.e.* step (3) of the reaction. It is important to note, however, that according to the experiments with ITP the configuration of the adenine part of the ATP molecule is of importance for the activating effect of $Na^+ + K^+ + Mg^{++}$, and it must consequently be assumed that the binding of the cations to the enzyme takes place already during the attachment of ATP to the enzyme, *i.e.* already during process (1).

The reaction sequence seems accordingly to be as follows:

$$E + ATP + 2\,Mg + n\,Na + m\,K \rightleftharpoons \underset{n\,Na}{\overset{m\,K}{E\,Mg_2\,ATP}} \tag{9}$$

$$\underset{n\,Na}{\overset{m\,K}{E\,Mg_2\,ATP}} \rightleftharpoons \underset{n\,Na}{\overset{m\,K}{E}} \sim P + 2\,Mg + ADP \tag{10}$$

$$\underset{n\,Na}{\overset{m\,K}{E} \sim P} \rightarrow E + Pi + n\,Na + m\,K \tag{11}$$

where n and m are unknown numbers, and the expression

$$\underset{n\,Na}{\overset{m\,K}{E\,Mg_2\,ATP}}$$

symbolises an enzyme–substrate–ion complex in which Mg^{++} constitutes part of the enzyme–substrate complex and Na^+ and K^+ are attached to two different sites on the enzyme (see Fig. 18).

G-strophanthin inhibits that part of step (11) which is due to the presence of Na^+ or $Na^+ + K^+$, while it has no effect on the splitting of the phosphorylated compound which is obtained with Mg^{++} alone. With $Na^+ + K^+$ in the system the inhibition is accompanied by a decrease of the optimum Mg^{++}/ATP ratio from 2/1 to 1/1. Furthermore, g-strophanthin counteracts to some extent the inhibition of the exchange reaction due to Na^+ or $Na^+ + K^+$. The effect of g-strophanthin seems accordingly to be due to an inhibition of the binding of Na^+ and K^+ to the enzyme in step (9) and thereby indirectly to an inhibition of that part of step (11) which is due to Na^+ or $Na^+ + K^+$.

The 1/1 ratio of Mg^{++}/ATP suggests that Mg^{++} is involved in the formation of the enzyme substrate complex. It seems possible that Mg^{++} (3 in Fig. 18), in analogy with SMITH's[14] theory for the role of metal ions for the peptidase activity, by a chelate formation between the two terminal phosphate groups and groups in the enzyme binds ATP to the enzyme. Mg^{++} acts as an electronic bridge and is thereby able to establish a connection between the electronic system in the phosphate chain and that in the protein. A displacement of electrons is hereby made possible, so that the P–O–P bond is weakened with the resultant possibility of hydrolytic splitting.

It is tempting to assume that the second Mg^{++}, which is involved in the activating effect of $Na^+ + K^+$ and the effect of which is dependent on the NH_2 group in the adenine part of ATP, becomes attached to the ATP molecule as suggested by SZENT-GYÖRGYI[8] (4 in Fig. 18). As pointed out by SZENT-GYÖRGYI, such a chelate formation will make one common electronic system of the phosphate chain and of the adenine with common non-localised electrons which could transport energy.

This model with two Mg^{++} ions in the enzyme-ATP complex opens the possibility that this common electronic system of ATP may, through the Mg^{++} placed as shown in position 3 in Fig. 18 be connected with and thereby able to act on the electronic system of the enzyme. It may be through such an interaction of the two electronic systems that the enzyme develops the peculiar and specific affinities for Na^+ and K^+.

In the previous study it was pointed out that the enzyme investigated possesses properties that make it reasonable to assume that it is involved in the active transport

of cations across the nerve membrane. The present experiments seem to lend further support to this idea.

An enzyme involved in transport across the nerve membrane must be expected to be located in the membrane, and although no direct evidence is available on this point concerning the enzyme studied, various observations may indicate that in the intact nerve it is, in fact, located here. LIBET[15] and ABOOD AND GERARD[16] investigated an ATPase in peripheral nerves which, like the one studied here, was activated by Mg^{++} and which after homogenisation and differential centrifugation was located in submicroscopic particles. This enzyme could be isolated from the sheath but not from the axoplasm of giant axons. Furthermore, HANZON AND TOSCHI[17] studied by the electron microscope a submicroscopic particle fraction from nerve tissue, isolated under conditions similar to those used here, and found that the appearance of these particles indicated that they were fragments of disintegrated membrane.

HODGKIN AND KEYNES[18] have demonstrated that in peripheral nerve the outward transport of Na^+ is linked to a transport of K^+ into the fibre. One might therefore expect an enzyme involved in this transport to have a high affinity for both Na^+ and K^+, and one might also expect that the location of these affinities differed from one another. As appears from the experiments reported above, these are the properties shown for the enzyme studied.

One may visualize an arrangement of the system in the membrane according to which the site of the Na^+ affinity faces the intracellular water phase, while the site of the K^+ affinity is in contact with the extracellular water phase. At the site with highest Na^+ affinity the saturation of the enzyme with Na^+ depends on both the Na^+ and the K^+ concentration, and it appears from Fig. 9 in the previously published paper that the Na^+ saturation is about 40 % when the Na^+ and K^+ concentrations are 40 and 350 mmoles/l, respectively, *i.e.* identical with the intracellular concentrations of these ions in crab nerve. Since the activity varies with the Na^+ saturation, a change in Na^+ concentration above and below that corresponding to the intracellular level must consequently lead to a change in the enzyme activity. Moreover, due to the competition between Na^+ and K^+ a decrease in the K^+ concentration will increase the activity of the enzyme, while an increase in the concentration of K^+ will decrease the activity.

At the site with highest K^+ affinity the saturation with K^+ is equal to 80–90 % when the K^+ concentration is around 12 mmoles/l, *i.e.* identical with the extracellular K^+ concentration in the crab.

The enzyme has accordingly such affinities for Na^+ and K^+ as would be appropriate if the function of the enzyme were to control the magnitude of and the ratio between the intracellular Na^+ and K^+ concentrations.

It may finally be mentioned that the enzyme activity due to Na^+ and $Na^+ + K^+$ is inhibited by g-strophanthin. No data are available concerning the effect of g-strophanthin on active cation transport across the nerve membrane, but it is known that g-strophanthin inhibits the active cation transport in red blood cells[9] and in frog skin[10].

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A TECHNIQUE FOR ELUTION OF PROTEINS FROM STARCH GEL

A. H. GORDON
National Institute for Medical Research, Mill Hill, London (Great Britain)

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SUMMARY

A technique for recovery of separated plasma proteins after electrophoresis in starch gel is described. By means of a second electrophoresis applied to sections which have been cut from the main gel almost quantitative recoveries of these proteins, except for γ-globulin were obtained. The trace amounts of protein originating from the gel itself under these conditions were measured. Considerable amounts of starch are eluted at the same time but do not interfere with the estimation of the plasma proteins.

INTRODUCTION

With the increasing use of electrophoresis in gels the need has become apparent for a simple and quantitative method for recovery of the separated materials. A technique involving lifting an agar gel on to a cellophane membrane placed so as to isolate pools of buffer at anode and cathode ends of the slab followed by a period of elution by electrophoresis into these pools has been described[1]. Since the introduction of electrophoresis in starch gel by SMITHIES[2], several methods for elution from such gels have been worked out. That of JARRIGE AND LAFOSCADE[3] depends on cutting a slot in the gel in front of the band of material to be eluted, filling this with buffer and cotton wool and eluting by electrophoresis into the buffer. MORETTI,