THE INFLUENCE OF SOME CATIONS ON AN ADENOSINE TRIPHOSPHATASE FROM PERIPHERAL NERVES

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Stimulation of a nerve leads to an influx of sodium ions into the fibre and hence to an increase in the intra-axonal sodium concentration[1]. Normal conditions are restored by an outward transport of the sodium ions, and this process requires energy because the efflux takes place against an electrochemical gradient. The mechanism of this transport is not known.

In experiments with giant axons from *Sepia officinalis* and from *Loligo forbesi*, HODGKIN AND KEYNES[2] found that dinitrophenol, azide and cyanide inhibit the active transport of sodium ions out of the nerve; this inhibition is reversible. In the concentrations used all three substances also inhibit the oxydative phosphorylation which takes place in mitochondria; dinitrophenol and azide do so through an uncoupling of the phosphorylation[3,4], and cyanide through an inhibition of the oxydation[5]. CALDWELL[6] observed correspondingly that addition of these substances, in the concentrations used by HODGKIN AND KEYNES, led to a reduction of the content of energy-rich phosphate esters in the axoplasm of giant axons. This seems to indicate that energy-rich phosphate esters are somehow involved in the active transport of sodium ions out of the nerve fibres.

In this connexion it is of interest that LIBET[7] and ABOOD AND GERARD[8] were able to demonstrate an adenosine triphosphatase (ATPase) in the sheath of giant axons. A further study on the ATPase in nerves and its possible role in the active outward transport of sodium ions seems warranted.

According to LIBET, the ATPase in the sheath of giant axons is calcium-activated, while the experiments by ABOOD AND GERARD suggest that it is activated by magnesium and located in submicroscopic particles. In peripheral nerves from the rat the latter authors found both a calcium- and a magnesium-activated ATPase. The calcium-activated enzyme was predominantly located in the mitochondria, while the magnesium-activated, as in giant axons, was mainly located in the submicroscopic particles.

Giant axons were not available to us. In preliminary experiments we found that a homogenate of leg nerves from the shore crab (*Carcinus maenas*) contained both a calcium- and a magnesium-activated ATPase, and that their localization was similar to that of the ATPase found by ABOOD AND GERARD in rat-nerve homogenates. For our study we have chosen the magnesium-activated enzyme, because it resembles the magnesium-activated ATPase from the sheath of giant axons in that it is located in submicroscopic particles.

The present investigation is concerned with the effect on the enzyme activity exerted by the cations normally present in the tissue–sodium, potassium, magnesium and calcium.

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EXPERIMENTAL

The ATPase was prepared by homogenization and subsequent differential centrifugation[8] of leg nerves from the shore crab (*Carcinus maenas*).

The isolated nerves were washed and homogenized in 10 volumes of 0.25 *M* ice-cold sucrose buffered with histidine, 30 m*M*/l, pH 7.6. The admixture of alkali metal ions was avoided by using the histidine base, and the pH was adjusted by addition of 1 *N* HCl. The homogenate was centrifuged in a Servall angle centrifuge at 0° C. Fragments and stroma were removed by centrifugation at 2,000 × *g* for 15 minutes, mitochondria and some submicroscopic particles by centrifugation at 10,000 × *g* for 15 minutes. The supernatant was centrifuged at 20,000 × *g* (maximum available) for 3 hours. During this centrifugation the temperature rose from 0° to 8–10° C.

The sediment after this last centrifugation was suspended in a volume of 0.25 *M* ice-cold buffered sucrose corresponding to one half of the volume of the original homogenate. This supension was centrifuged at 10,000 × *g* for 10 minutes in order to remove any remaining mitochondria. The final supernatant was used as enzyme solution in the experiments; it contained from one half to two thirds of the activity originally present in the homogenate.

The enzyme was relatively unstable; when it was stored in a refrigerator at 4–5° C, its activity fell to one half in 3–4 days.

The disodium or dibarium salts of ATP and the barium salt of ADP (Sigma products) were converted into free acids, the Na salt by passage through an Amberlite 120 (H^+ form) column and the barium salts by precipitation with equimolar amounts of sulphuric acid and subsequent passage through an Amberlite 120 column to remove residual barium ions. The free acids were neutralized to pH 7 with a 1 *M* solution of 2-amino-2-methyl-1,3-propanediol. In the concentrations used, this substance does not affect the enzyme activity and is without influence on the phosphate determination.

ATP and ADP were determined spectrophotometrically[9] and by measurement of 7'P; Na and K were determined with a flame spectrophotometer, Beckman DU, flame attachment No. 9200. The pH was measured with a glass electrode, Radiometer PHM 3. Inorganic phosphate was determined by the method of FISKE AND SUBBAROW[10] with amidol as the reducing agent.

The activity of the enzyme was determined in a volume of 1.0 ml containing 0.1 ml of the above-mentioned enzyme solution. The reaction mixture was buffered with histidine, 30 m*M*/l, pH 7.2. Unless otherwise stated, the mixture contained 3 m*M* ATP/l. The cations were added as solution of their chloride. All experiments were performed at 36° C. After a 10-minute temperature equilibration the experiment was started by the addition of enzyme, and the mixture was incubated for 15 or 30 minutes, according to the reaction velocity. The hydrolysis of ATP was linear within the experimental period. The reaction was stopped by the addition of 0.1 ml of 50 % trichloroacetic acid, and after centrifugation aliquots of 0.4 ml were taken out for determination of inorganic phosphate.

In the absence of added cations, the reaction mixture contained small amounts (about 0.1 m*M*/l) of sodium and potassium, which originated from the enzyme solution and from the ATP.

In all the diagrams except Fig. 1, the enzyme activity is expressed as μg of P split off from ATP in 30 minutes.

RESULTS

When the substrate is ADP, no inorganic phosphate is liberated (data not presented). In the presence of ATP the hydrolysis stops when inorganic phosphate corresponding to one phosphate group has been split off (Fig. 1).

Fig. 2. shows that under the experimental conditions used the pH optimum is 7.2.

The enzyme activity is zero when no cations are added or when K^+ or Ca^{++} alone are added to the reaction mixture. The addition of Na^+ gives rise to a just measurable activity, which is independent of the sodium concentration. When Mg^{++} is added, the enzyme shows a slight activity with a maximum at 3 m*M*/l (Fig. 3).

If sodium ions are added to a reaction mixture which already contains magnesium ions, the enzyme activity increases (Fig. 4). When the concentrations of Mg^{++} and of ATP are 3 m*M*/l, a maximum activity is observed when the sodium concentration is 6 m*M*/l. At higher sodium concentrations, the activating effect of Na^+ decreases.

Addition of potassium ions to a mixture containing magnesium ions does not affect the enzyme activity; addition of calcium ions results in an inhibition.

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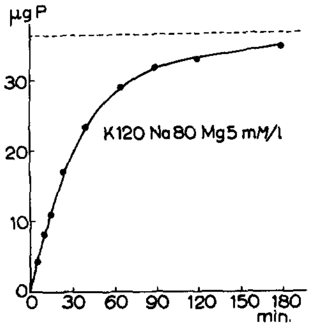

Fig. 1.

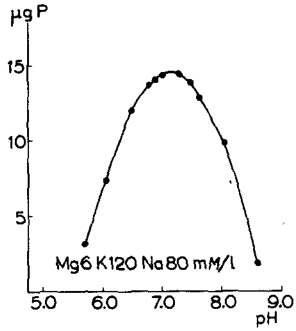

Fig. 2.

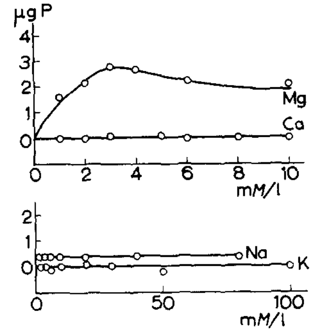

Fig. 3.

Fig. 1. Liberation of inorganic phosphate with ATP as substrate. The dotted line indicates first P of ATP. Abscissa, time in minutes; ordinate, µg P removed from ATP. The reaction mixture contained 5 m*M* Mg, 80 m*M* Na and 120 m*M* K per litre. The initial concentration of ATP was 1.16 m*M*/l.

Fig. 2. Relation between enzyme activity and pH. Abscissa, pH; ordinate, µg P removed from ATP in 30 minutes. The reaction mixture contained 6 m*M* Mg, 80 m*M* Na, 120 m*M* K and 3 m*M* ATP per litre.

Fig. 3. Enzyme activity in relation to the concentration of Mg^{++}, Ca^{++}, Na^{+}, or K^{+}. Abscissa, ion concentration in m*M*/l; ordinate, µg P removed from ATP in 30 minutes.

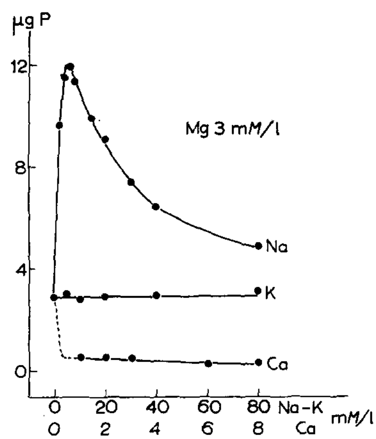

Fig. 4.

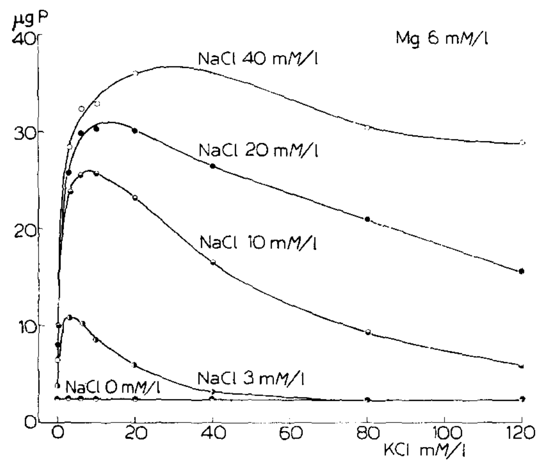

Fig. 5.

Fig. 4. Enzyme activity in relation to the concentration of Na^{+}, K^{+}, Ca^{++} in the presence of Mg^{++}, 3 m*M*/l. Abscissa, ion concentration in m*M*/l; ordinate, µg P removed from ATP in 30 minutes.

Fig. 5. Enzyme activity in relation to the concentration of K^{+} in the presence of Mg^{++}, 6 m*M*/l, and different concentrations of Na^{+}. Abscissa, potassium concentration in m*M*/l; ordinate, µg P removed from ATP in 30 minutes.

If both magnesium and sodium ions are present in the mixture, addition of potassium ions results in a further increase in the enzyme activity (Fig. 5). When the potassium concentration is raised, the enzyme activity reaches a maximum and then decreases, and at high potassium concentrations it is smaller than it was when no potassium ions were added. The latter phenomenon is seen only from the curves representing 3 and 10 m*M*/l. The curve for 3 m*M*/l shows that high concentrations of potassium inhibit only that part of the activity which is due to Na^{+}, but not the part which is due to Mg^{++}.

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It is further seen from Fig. 5 that the maximum activity obtained in the presence of potassium increases with the sodium concentration. The potassium concentration required for maximum enzyme activity also depends on the sodium concentration; it increases when the sodium concentration is increased. Maximum enzyme activity is obtained when the potassium concentration is roughly equal to that of sodium. Finally, it should be observed that the inhibition due to high potassium concentrations decreases when the concentration of sodium is increased. The effect of potassium ions depends accordingly not only on the presence of magnesium and sodium ions, but also on the concentration of sodium ions.

The addition of K^+ has also an effect on the relation between enzyme activity and sodium concentration (Fig. 6). In the absence of K^+, the activity reaches a maximum at 6 m*M* Na/l; when more sodium is added, the activity decreases. When as little as 3 m*M* K/l is added to the system, the activity is not only enhanced but shows a steady rise with the sodium concentration until it finally levels off. The sodium concentration at which this level is reached increases with the amount of potassium added.

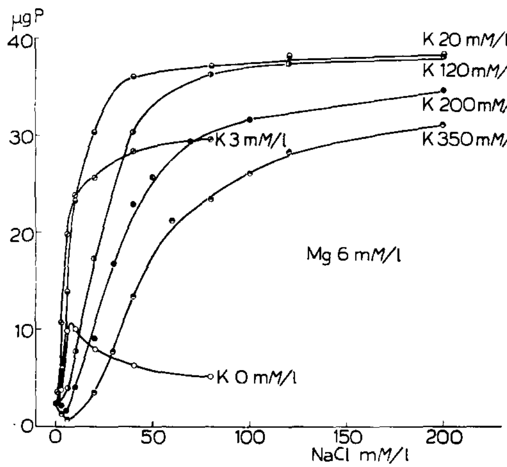

Fig. 6. Enzyme activity in relation to the concentration of Na^+ in the presence of Mg^{++}, 6 m*M*/l, and different concentrations of K^+. Abscissa, sodium concentration in m*M*/l; ordinate, µg P removed from ATP in 30 minutes.

A certain, low activity in the absence of sodium is observed in Fig. 6. It is, as previously pointed out (*cf.* Fig. 4), due to the presence of magnesium, and it is independent of the potassium concentration. In the presence of high concentrations of potassium, 200 and 350 m*M*/l, the addition of small amounts of sodium, 3 and 6 m*M*/l, leads to an inhibition of this low, magnesium-dependent activity. Higher concentrations of sodium have, as usual, an activating effect.

It appeared from Fig. 4 that calcium ions inhibit the activity which is due to the presence of Mg^{++}. An inhibition is also seen when calcium ions are added to a system containing magnesium + sodium ions or magnesium + sodium + potassium ions (Fig. 7).

When Mg^{++} or Mg^{++} + Na^+ are the only cations present, the optimum magnesium concentration is 3 m*M*/l. In the presence of potassium the optimum concentration of magnesium is 6 m*M*/l, and this value is independent of the potassium concentration. If calcium ions are also added, the optimum magnesium concentration becomes still higher, and it increases with the calcium concentration (Fig. 8).

In Figs. 9 and 10 is shown the relation between enzyme activity and sodium concentration in the absence and presence of calcium ions at various concentrations of potassium and magnesium. It appears that the activating effect of Na^+ is proportional to the Mg:Ca ratio.

It was previously noted (Fig. 6) that low concentrations of sodium in the presence

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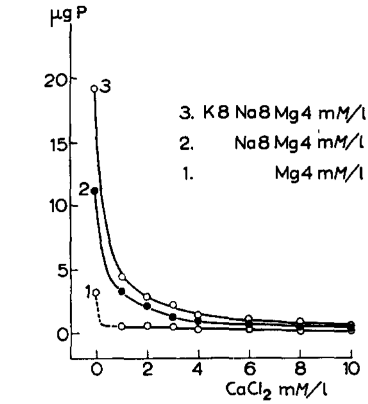

Fig. 7. Enzyme activity in relation to the concentration of Ca^{++} in the presence of Mg^{++}, $Mg^{++} + Na^{+}$, or $Mg^{++} + Na^{+} + K^{+}$. Abscissa, calcium concentration in m*M*/l; ordinate, µg P removed from ATP in 30 minutes.

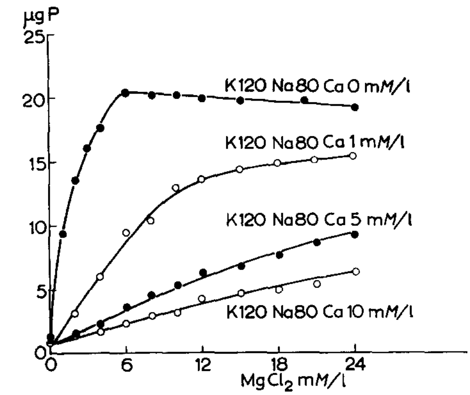

Fig. 8. Enzyme activity in relation to the concentration of Mg^{++} in the presence of K^{+}, 120 m*M*/l, Na^{+}, 80 m*M*/l, and different concentrations of Ca^{++}. Abscissa, magnesium concentration in m*M*/l; ordinate, µg P removed from ATP in 30 minutes.

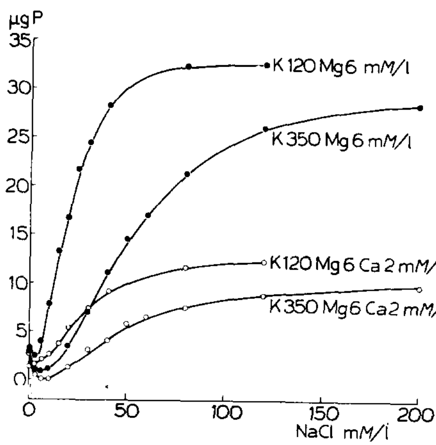

Fig. 9.

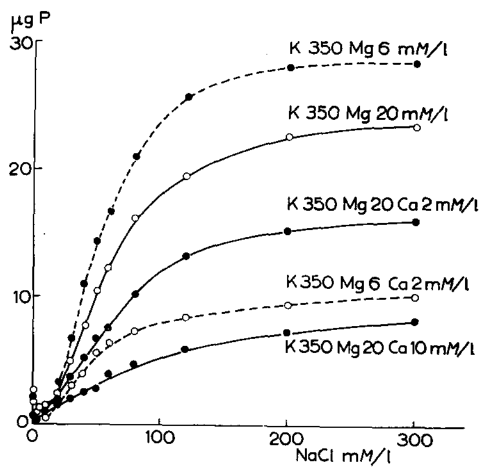

Fig. 10.

Fig. 9. Enzyme activity in relation to the concentration of Na^{+} in the presence of Mg^{++}, 6 m*M*/l, and different concentrations of K^{+} and Ca^{++}. Abscissa, sodium concentration in m*M*/l; ordinate, µg P removed from ATP in 30 minutes.

Fig. 10. Enzyme activity in relation to the concentration of Na^{+} in the presence of K^{+}, 350 m*M*/l, and different concentrations of Mg^{++} and Ca^{++}. Abscissa, sodium concentration in m*M*/l; ordinate µg P removed from ATP in 30 minutes.

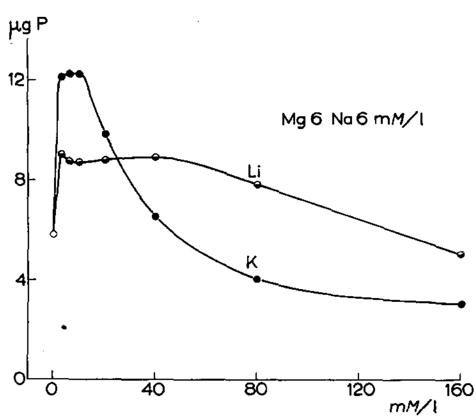

Fig. 11.

Fig. 11. Enzyme activity in relation to the concentration of K^{+} or Li^{+} in the presence of Mg^{++}, 6 m*M*/l, and Na^{+}, 6 m*M*/l. Abscissa, ion concentration in m*M*/l; ordinate, µg P removed from ATP in 30 minutes.

of high potassium concentrations inhibited the enzyme activity due to magnesium. Figs. 9 and 10 show that this is also the case in the presence of calcium.

The effect of Li^+ was studied in a few experiments. Lithium ions do not affect the activity due to magnesium, but if the system contains both magnesium and sodium, the addition of Li^+ results in an increase of the activity. The action of lithium is consequently similar to that of potassium, but it should be noted that at low concentrations lithium has a weaker effect than potassium, while its effect is stronger at high concentrations (Fig. 11).

DISCUSSION

In a brain-tissue homogenate, UTTER[12] demonstrated a magnesium-activated apyrase, the activity of which varied with the sodium concentration. Homogenates of rat nerves[8] and of crab nerves contain not only a magnesium-activated ATPase, but also an adenylic kinase. It seems possible that the apyrase activity demonstrated by UTTER was due to the combined effects of a magnesium-sodium-activated ATPase and an adenylic kinase.

The ATPase from crab nerve studied here is magnesium-activated and located in submicroscopic particles. In these respects it resembles the ATPase isolated from rat nerve and from the sheath of giant axons by ABOOD AND GERARD[8] and also the ATPase isolated from muscle by KIELLY and MEYERHOF[11]. There are further points of similarity between the magnesium-activated ATPases from crab nerve and from muscle. Their pH optima are roughly identical, 7.2 and 6.8, respectively, and they are both strongly inhibited by calcium ions. The effect of sodium ions on the activity of the magnesium-activated ATPase from muscle has not been studied.

The experiments reported here show that the activity of the ATPase from crab nerve is highly dependent on the relative concentrations of the four cations Na^+, K^+, Mg^{++} and Ca^{++}. The presence of magnesium ions is an obligatory requirement for the activity of the enzyme; sodium ions increase the activity when magnesium ions are present; potassium ions increase the activity when the system contains both magnesium and sodium ions. In high concentrations potassium ions inhibit that part of the activity which is due to Na^+, while the activity due to Mg^{++} is not affected. Calcium ions inhibit the activity under all conditions.

In the presence of magnesium or of magnesium + sodium the optimum magnesium concentration was found to be 3 mM/l when the ATP concentration was also 3 mM/l. Preliminary studies indicate that the optimum concentration of magnesium is equal to the ATP concentration also at other ATP levels. From this, one might infer that the substrate for the enzyme is magnesium-ATP.

The differences between the effects of sodium and potassium suggest that the *activating* effects of sodium and potassium differ in their point of attack, and that the *inhibitory* effect of potassium is due to an interference with the sodium activation. An hypothesis to fit this would be that the substrate most readily attacked by the enzyme is sodium-magnesium-ATP. Addition of K^+ might then lead, on the one hand, to a direct stimulation of the enzyme in the presence of Mg^{++} and Na^+ and, on the other, to a displacement of sodium from the substrate, resulting in an inhibition.

This assumption would explain, firstly, why potassium is activating only in the presence of magnesium + sodium.

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Secondly, it would explain the relation between enzyme activity and potassium concentration (*cf.* Fig. 5). The rise of activity on addition of K^+ must then be due to a direct stimulation of the enzyme by this ion. With an increase in the potassium concentration this stimulation must be counteracted by an inhibition due to displacement of sodium from the substrate. The activity must consequently pass through a maximum and eventually approach the level observed when no sodium was added to the system.

Thirdly, it would explain the dependence of the potassium effect on the sodium concentration. The higher the sodium concentration, the higher must be the potassium concentration required to give a certain displacement of sodium from the substrate. Since a higher potassium concentration means a stronger activation of the enzyme, it follows that the maximum enzyme activity obtained by the addition of potassium must increase with the sodium concentration. The potassium concentration required to give maximum activity must also increase with the sodium concentration. Fig. 5 shows that maximum activity was obtained when the concentration of potassium was approximately equal to that of sodium. The concentration of potassium required to reduce the enzyme activity to the level observed when no sodium was added must accordingly also increase with the sodium concentration.

Although the above-mentioned assumption could explain a majority of the data, it should be noted that a few observations are not accounted for in this way, *e.g.* the decrease in the activating effect of Na^+ above a certain concentration in a system which does not contain K^+, and the inhibitory effect of small amounts of sodium in the presence of a high potassium concentration. Further studies may clarify these points.

The effect of calcium on the enzyme is purely inhibitory. The inhibition is counteracted by the addition of extra magnesium and may therefore be due to a competition between calcium and magnesium.

It may now be asked whether the present studies have produced any evidence of a connexion between this enzyme and the active extrusion of sodium ions from the axon.

The process responsible for this transport must presumably be located in or in close proximity to the nerve membrane. In homogenates of crab nerve the enzyme studied here is located in submicroscopic particles, and we do not know its localization in the intact nerve, but it is suggestive that ABOOD AND GERARD were able to isolate from the sheath of giant axons an ATPase which was also magnesium-activated and located in submicroscopic particles.

As previously pointed out, studies by HODGKIN AND KEYNES and by CALDWELL indicate a connexion between the sodium extrusion from nerve and the metabolism of energy-rich phosphate esters. The substrate of ATPase is an energy-rich phosphate ester.

HODGKIN AND KEYNES[13] have shown that the sodium efflux from giant axons depends on and is directly proportional to the intra-axonal sodium concentration; they injected sodium into the axons, and the intra-axonal sodium concentrations in their experiments varied from the 40 mM/l normally present to 130 mM/l.

In the crab nerve the intra-axonal potassium concentration is 342 mM/kg axoplasm[14]; this is calculated from determinations of the potassium and sodium concentrations in whole crab nerves on the assumption that the intra-axonal sodium concentration is the same as in giant axons, *viz.* 40 mM/kg axoplasm.

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The present experiments showed that in the presence of 350 m*M* K/l the activity of the magnesium-activated ATPase is highly dependent on the sodium concentration, and there is an approximate direct proportionality between the enzyme activity and sodium concentration within the concentration range used in the experiments of HODGKIN AND KEYNES (*cf.* Fig. 10). The extent of the changes in enzyme activity is influenced by the Mg:Ca ratio in the system, but the linearity is observed at all magnesium and calcium concentrations used. If, in analogy to what happens in the nerve after stimulation, a rise in the sodium concentration is accompanied by a decrease in the potassium concentration, the ATPase activity is further enhanced (*cf.* Fig. 9).

The above considerations show that the crab-nerve ATPase studied here seems to fulfil a number of the conditions that must be imposed on an enzyme which is thought to be involved in the active extrusion of sodium ions from the nerve fibre. Further studies on the enzyme and its relation to the cations may serve to throw light on the nature of this process.

SUMMARY

Leg nerves from the shore crab (*Carcinus maenas*) contain an adenosine triphosphatase which is located in the submicroscopic particles. The influence of sodium, potassium, magnesium and calcium ions on this enzyme has been investigated.

The presence of magnesium ions is an obligatory requirement for the activity of the enzyme. Sodium ions increase the activity when magnesium ions are present. Potassium ions increase the activity when the system contains both magnesium and sodium ions. Potassium ions in high concentration inhibit that part of the activity which is due to Na^+, while the activity due to Mg^{++} is not affected. Calcium ions inhibit the enzyme under all conditions.

When Mg^{++} or $Mg^{++} + Na^+$ are present in the system, the optimum magnesium concentration is equal to the concentration of ATP. If potassium ions are added, the optimum magnesium concentration is doubled. If calcium ions are also added, the optimum magnesium concentration becomes still higher, and it increases with the calcium concentration.

A majority of these observations may be explained by assuming (a) that the substrate most readily attacked by the enzyme is sodium-magnesium-ATP, (b) that potassium ions stimulate the enzyme directly, and (c) that an increase in the concentration of potassium ions leads to a displacement of sodium ions from the substrate and accordingly to an inhibition of the reaction.

If the system contains the four cations in concentrations roughly equal to those in the crab-nerve axoplasm, an increase in the sodium concentration as well as a decrease in the potassium concentration will lead to an intensification of the enzyme activity. This observation, as well as some other characteristics of the system, suggest that the adenosine triphosphatase studied here may be involved in the active extrusion of sodium from the nerve fibre.

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