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The identification of the sodium-pump as the membrane-bound Na^+/K^+-ATPase: a commentary by

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on 'The influence of some cations on an adenosine triphosphatase from peripheral nerves'
by J.C. Skou
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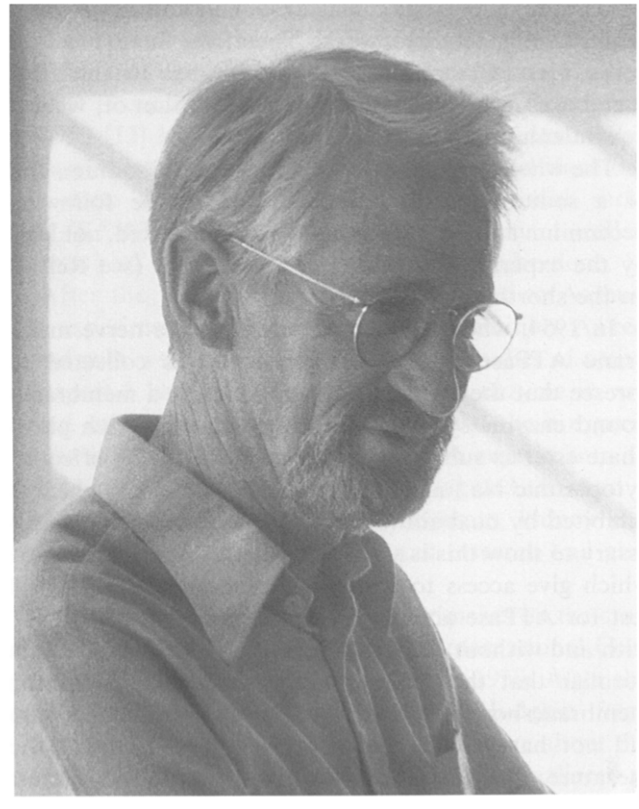
Introduction

The experiments published in the paper reprinted here [1] were started late 1954. I had spent July 1953 at the Woodshole Marine Biological Laboratory with Professor Nachmansohn, before I participated in the 19th International Conference of Physiology in Montreal. During my stay in Woodshole I read in a paper written by Nachmansohn that Libet [2] had shown that there is an ATPase in the sheath part of the giant axon. I wondered what the function was. I assumed that it was a lipoprotein, and as I needed a lipoprotein with enzyme activity for monolayer experiments I decided to have a look at this enzyme when I returned to Aarhus.

My background

I was a medical doctor who had taken a few years off from my clinical training to write a thesis on the mechanism of action of local anaesthetics. For this purpose I had obtained a position at the Institute of Physiology at the University of Aarhus. Thereafter, I intended to continue my medical training to become a surgeon.

I tested the effect of local anaesthetics on monomolecular layers of nerve membrane lipids and found that there is a correlation between anaesthetic potency and the ability of the local anaesthetics to penetrate into, and at a given area to increase the surface pressure in, the monolayer. This suggested to me that local anaesthetics block the conformational change, the opening, of the sodium channel, which I assumed was a protein, indirectly via an effect on the lipids in the membrane. In order to test this I planned to test the effect of surface pressure on the activity of a surface-spread enzyme, and take the enzyme activity as an indication of conformation. The idea was subsequently to take a monolayer of a mixture of the enzyme and lipids, and see if penetration of local anaesthetics into the lipids influenced the activity of the enzyme. For this purpose I needed a highly active enzyme, and one choice was acetylcholine esterase. This was not because of Nachmansohn's view on the role of this enzyme in nerve impulse transmission, but because it could be obtained with a high degree of purity from electric eel tissue. After the conference in Montreal I returned to Columbia University in New York with Professor Nachmansohn and prepared the enzyme.



Jens Chr. Skou

I considered the nerve membrane ATPase as a possible candidate for the monolayer experiments on the mixture of lipids and proteins. I did not at this point think in terms of active transport. The statement in the introduction to the paper on the nerve membrane ATPase, "A further study on the ATPase in nerves and its possible role in the active outward transport of sodium ions seem warranted", is thus a subsequent rationalization.

The sodium pump

The idea of a sodium pump in the cell membrane was introduced by Dean in 1941 [3] in a paper, 'Theories of electrolyte equilibrium in muscle'. Referring to experiments by Heppel (1938) by Heppel and Schmidt (1939) and by Steinbach (1940) (for references see Ref. 3), Dean concluded, "that the muscle can actively move potassium and sodium against concentration gradients... this requires work. Therefore there must be some sort of a pump, possibly located in the fiber membrane, which can pump out the sodium or, what is equivalent, pump in the potassium."

The whole concept of active transport of sodium, and of a sodium pump was developed in the following decennium and was convincingly demonstrated, not least by the experiments of Ussing and Zerahn (see Ref. 4) on the short-circuited frog skin.

In 1954, when I started to work on the nerve membrane ATPase, enough information was collected to foresee that the transport system must be a membrane-bound enzyme system, which has an energy-rich phosphate ester as substrate, and is activated by an effect of cytoplasmic Na^+ and of extracellular K^+, and which is inhibited by ouabain (see Ref. 5). The experiment necessary to show this is simple: take membrane fragments, which give access to both sides of the membrane, and test for ATPase activity as a function of Na^+ and K^+ with and without ouabain. Against this background it is peculiar that the sodium pump was identified as the membrane-bound Na^+/K^+-ATPase by someone who did not have the above-mentioned knowledge of the literature on the sodium pump, whose research interest was not active transport, and who met the pump more or less by accident. Had I not identified the pump, I am sure logical reasoning would soon have led someone in the transport field to the discovery; the time was ripe.

The Na^+/K^+-ATPase

After my return to Aarhus I continued the monolayer experiments, and at the end of 1954 I started to look for the nerve membrane ATPase. I had no access to giant axons, but used crab nerves instead. The experiments soon showed that membrane fragments isolated from a homogenate of the nerves contained a magnesium activated ATPase. In the presence of Mg^{2+} there was a low stimulation by Na^+. The results varied, however. It was excluded that this was due to Ca^{2+}. K^+ had no effect on the activity in the presence of Mg^{2+} but the combined effect of Na^+ and K^+ was not considered. The reason for the lack of reproducibility, I later realized, was that the concentration of Na^+ and K^+ in the test medium varied, partly because the nerves in some of the experiments were homogenized in 0.58 M KCl instead of sucrose, and partly because the Ba^{2+} salt of ATP which was used as a source for ATP sometimes was converted to the Na^+ and sometimes to the K^+ salt. After more than half a year of intermittent attempts to understand the lack of reproducibility I finally got the answer. It was an experiment in which the activity with Mg^{2+} and Na^+ of enzyme prepared in sucrose was higher when tested with the K^+ salt than with the Na^+ salt of ATP. This could obviously not be due to a difference in ATP but to an effect of K^+. The following experiments showed that K^+ in low concentrations relative to the concentration of Na^+ produced a pronounced increase in activity, whereas higher concentrations of K^+ not only reversed this activation, but also inhibited the low activity due to Na^+ alone. The results suggested that there were different sites for the activating effect of Na^+ and of K^+ and that the inhibition by K^+, was due to a competition for Na^+. This was in August, 1955.

Crab nerves were a lucky choice as a source of enzyme. With mammalian tissue most of the activity is hidden because of vesicle formation of the plasma membrane fragments when the cells are homogenized. The vesicles must be opened, for example with detergent, in order to see the combined effect of Na^+ and K^+. This is not the case with the crab nerve membranes. If I had used mammalian tissue as the enzyme source I would probably not have seen the combined effect of Na^+ and K^+.

What was the physiological function of the enzyme? Had I known the literature on the sodium pump I would probably not have had to spend more than half a year before I had tested and thereby observed the combined effect of Na^+ and K^+, and having observed the effect I guess that my first thought would have been that it was the sodium pump or part of it. However, I did not know the literature on the sodium pump. I was interested in blockage of nerve conduction, so I first thought that the enzyme was the sodium channel. However, this was soon rejected; it seemed unlikely from what was known about nerve conduction that the opening and closing of the sodium channel should be ATP-dependent. It seemed more likely that the enzyme was involved in the active transport of Na^+ and that ATP was the energy source for the transport.

I found support for this from a paper by Hodgkin and Keynes published in 1955 [6]. They poisoned giant

axons with dinitrophenol, cyanide or azide and found that this reduced the efflux of sodium, suggesting that energy-rich phosphate esters are the energy source for the active transport of Na^+. I was not aware, and apparently neither were they, as it is not cited in the paper, that Gardos in a Hungarian journal in 1954 [7] had published a paper in which he showed that ATP supports the accumulation of K^+ in red blood cells. However, in 1956 Hodgkin and Keynes reported that there was no dramatic recovery of the sodium efflux in cyanide-poisoned axons by a microinjection of ATP [8]. Fortunately, I did not see the paper till after I had sent the paper on the nerve membrane enzyme for publication. That ATP is the high-energy phosphate ester which supports the active extrusion of Na^+ in giant axons was shown by Caldwell and Keynes in 1957 [9].

Rereading the paper on the nerve membrane ATPase, I am surprised to see how little of the literature on active transport I have cited. Considering that the experiments suggested that there are two different sites for the activation by Na^+ and by K^+, I wonder why I did not cite papers which show that extracellular K^+ activates the Na^+ efflux. Apparently, I had not even read the literature after I had come to the conclusion that the enzyme was involved in active transport. An explanation is that I had very limited access to literature; the university was young, money was short, and we had access to very few journals. Another is that the nerve membrane ATPase experiments were, so to say, left-hand work; my main interest was still at that time the effect of local anaesthetics. The results of the experiments with local anaesthetics were published in a series of papers (for references see Ref. 10).

At an international Neurochemistry meeting in Aarhus in 1956 I presented the observations on the crab nerve ATPase in a discussion after a paper by R.D. Keynes, 'Electrolytes and Nerve Activity'. A. Pope, who was at the meeting, looked for the enzyme in rat brain tissue after his return home and observed the combined activation of Na^+ and K^+ on an Mg^{2+}-activated ATPase activity [11].

The paper on the nerve membrane enzyme was written up in 1956 and published in 1957. Mogens Schou helped me with the English writing. He had spent a year in the USA and was more experienced in English than I was. We had spent 2 years together at the Institute of Physiology and he had then moved to the Psychiatric University Hospital in Aarhus and had started his important work on the effect of lithium on mania. We discussed whether the words 'sodium pump' should be in the title, but I found it too provocative. The final title became, 'The influence of some cations on an adenosine triphosphatase from peripheral nerves'. It is no wonder that it took some time before it was realized that the enzyme had something to do with the transport of Na^+ and K^+.

In 1958 I presented a paper entitled 'The influence of the degree of unfolding and the orientation of side chains on the activity of a surface spread enzyme' at the Fourth International Congress on Biochemistry in Vienna. These were the results of the experiments with the surface-spread acetylcholine esterase. At the conference I met Robert Post, whom I knew from Woodshole. Nachmansohn had shared a laboratory with Harry Grundfest, who had Robert Post as a visitor while I was there. He and his wife Elisabeth had taken me in their car from Woodshole to the conference in Montreal. Robert told me that he had measured the stoichiometry of the Na^+–K^+ exchange through the pump in red blood cells. He had found that 3 Na^+ are transported out for each 2 K^+ transported into the cells. I told him that I had identified what seemed to be the pump and that it was a membrane-bound Na^+/K^+-ATPase. He had not seen the paper, but I understood from his reaction that this interested him more than surface-spread enzymes. "Is it inhibited by ouabain?" was his first question, and to this I could only reply, "What is ouabain?" I was not aware that Schatzmann in 1953 [12] had shown that cardiac glycosides inhibit the active transport of Na^+ and K^+ and I had therefore not done the crucial experiments that could show Na^+/K^+-ATPase as the transport system. I called Aarhus and arranged for the experiment to be done.

After the Vienna conference, Post came to Aarhus. The experiment with ouabain showed inhibition. As red blood cells were a much used tissue for studies of active transport I had started to look for the enzyme in red blood cells. Post asked if I would leave these experiments to him. I had no experience with experiments on red blood cells, and as he had, I agreed. Instead I turned to other tissues, mammalian brain, kidney and muscle. I had decided to continue in research and give up surgery.

I presented the results of the experiments on the crab nerve membrane ATPase at the 21st International Congress of Physiology in Buenos Aires in 1959. After the presentation of the paper, Professor Hodgkin from Cambridge invited me for lunch to hear more about the enzyme. This impressed me and I began to realize that the identification of the sodium pump was of a certain importance.

The results of the experiments on red blood cells by Post and co-workers which was published in 1960 [13] showed a close correlation between cation effects on fluxes and on the Na^+/K^+-ATPase activity in the broken membranes. It was this paper, more than my first paper, which aroused the interest in the Na^+/K^+-ATPase among people in the transport field.

In the early 1960's so much evidence was collected from a number of laboratories that it was possible in 1965 in a review paper [14] to conclude that the enzyme system fulfils the following requirements to a system

responsible for the active transport of Na^+ and K^+ across the cell membrane.

(1) It is located in the cell membrane;
(2) on the cytoplasmic sites it has a higher affinity for Na^+ than for K^+;
(3) it has an affinity for K^+ on extracellular sites which is higher than for Na^+;
(4) it has enzymatic activity and catalyzes ATP hydrolysis;
(5) the rate of ATP hydrolysis depends on cytoplasmic Na^+ as well as extracellular K^+;
(6) it is found in all cells that have coupled active transport of Na^+ and K^+;
(7) the effect of Na^+ and of K^+ on transport in intact cells and on the activity of the isolated enzyme correlates quantitatively; and
(8) the enzyme is inhibited by cardiac glycosides; the inhibitory effect on the active fluxes of the cations correlates with the inhibitory effect on the isolated enzyme system.

The enzyme system was named the Na^+ and K^+ activated ATPase or Na^+/K^+-ATPase.

However, from the point of view of identification of the pump there was still an unanswered question, namely, is the Na^+/K^+-ATPase only part of the transport system, the engine, so to speak, or is it the complete transport system? Can the Na^+/K^+-ATPase activity measured in the test-tube be taken as an indication of transport activity through the membrane fragments?

This answer could not be given until it became possible to dissolve purified fully active enzyme in detergent, reincorporate the enzyme in the membrane of liposomes with no loss of activity, and measure transport as well as ATP hydrolysis. This was a number of years in the future, at which time studies showed that the Na^+/K^+-ATPase is the transport system (see Ref. 15).

The identification of the Na^+-pump as the membrane-bound Na^+/K^+-ATPase opened up for the following years the possibility of clarifying the structure and the molecular mechanism by which the hydrolysis of ATP is coupled to the transport of Na^+ and K^+ against electrochemical gradients. This is a long story, not yet ended, in which the first step was to find a way to handle and to purify a membrane-bound enzyme. Interested readers are referred to the recently published Vol. 156 of Methods in Enzymology, which is devoted to the Na^+,K^+-pump [16], or to the proceedings of the Fifth International Conference on the Na^+,K^+-pump [17].

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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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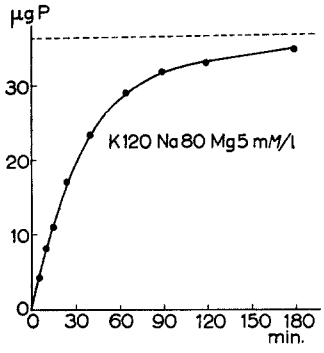

Fig. 1.

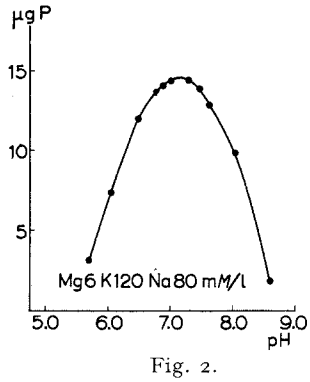

Fig. 2.

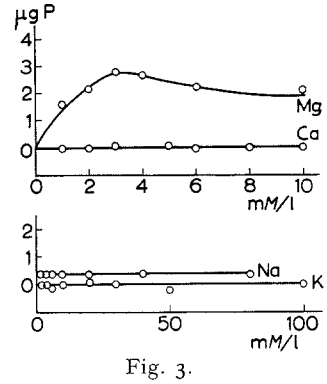

Fig. 3.

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 mM Mg, 80 mM Na and 120 mM K per litre. The initial concentration of ATP was 1.16 mM/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 mM Mg, 80 mM Na, 120 mM K and 3 mM ATP per litre.

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

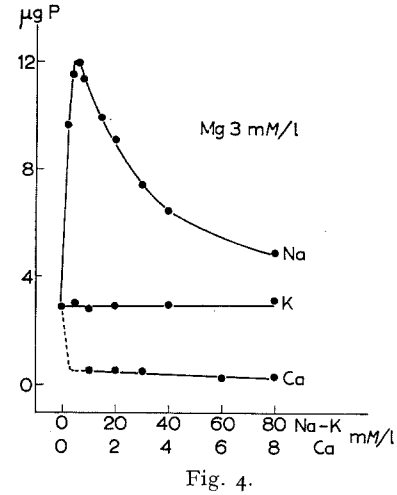

Fig. 4.

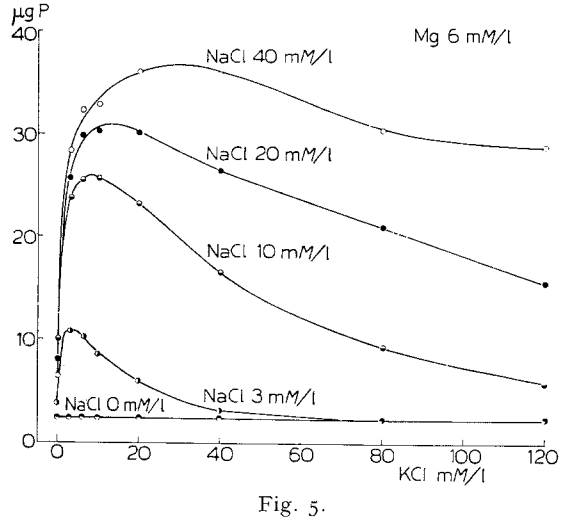

Fig. 5.

Fig. 4. Enzyme activity in relation to the concentration of Na^{+}, K^{+}, Ca^{++} in the presence of Mg^{++}, 3 mM/l. Abscissa, ion concentration in mM/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 mM/l, and different concentrations of Na^{+}. Abscissa, potassium concentration in mM/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 mM/l. The curve for 3 mM/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 mM Na/l; when more sodium is added, the activity decreases. When as little as 3 mM 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.

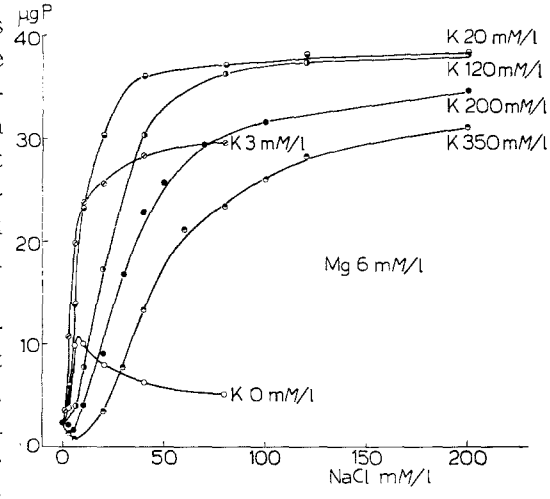

Fig. 6. Enzyme activity in relation to the concentration of Na^+ in the presence of Mg^{++}, 6 mM/l, and different concentrations of K^+. Abscissa, sodium concentration in mM/l; ordinate, μg P removed from ATP in 30 minutes.

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 mM/l, the addition of small amounts of sodium, 3 and 6 mM/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 mM/l. In the presence of potassium the optimum concentration of magnesium is 6 mM/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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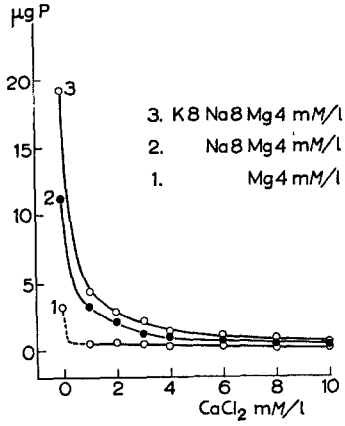

Fig. 7. Enzyme activity in relation to the concentration of Ca^{++} in the presence of Mg^{++}, $Mg^{++} + Na^{+}$, or $Mg^{++} + Na^{+} + K^{+}$. Abscissa, calcium concentration in mM/l; ordinate, μg P removed from ATP in 30 minutes.

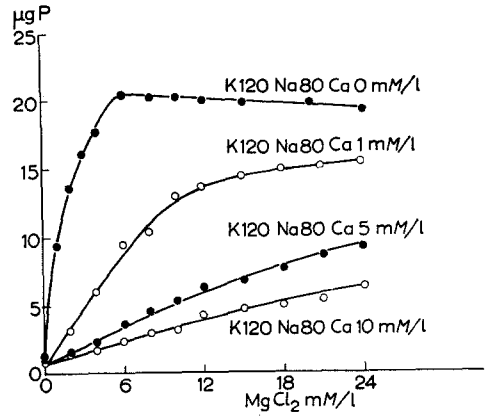

Fig. 8. Enzyme activity in relation to the concentration of Mg^{++} in the presence of K^{+}, 120 mM/l, Na^{+}, 80 mM/l, and different concentrations of Ca^{++}. Abscissa, magnesium concentration in mM/l; ordinate, μg P removed from ATP in 30 minutes.

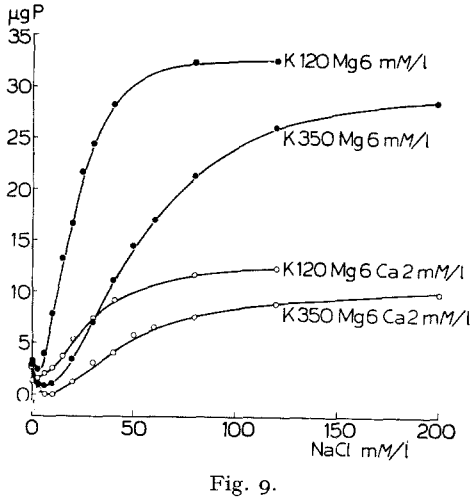

Fig. 9.

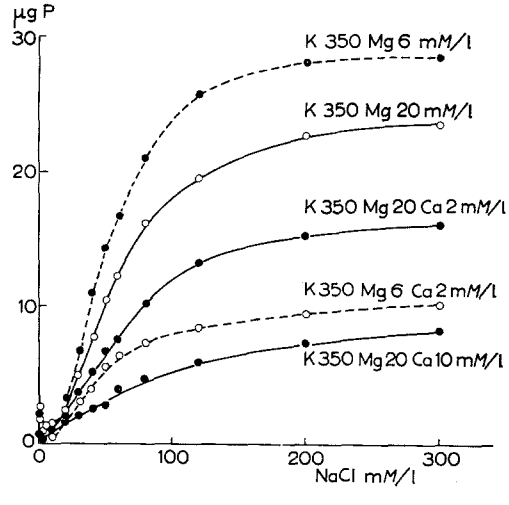

Fig. 10.

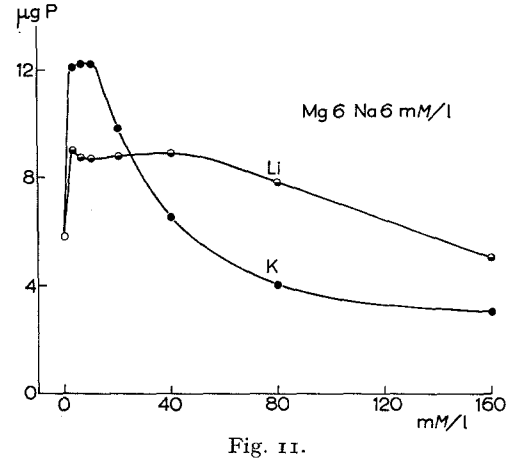

Fig. 11.

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

Fig. 11. Enzyme activity in relation to the concentration of K^{+} or Li^{+} in the presence of Mg^{++}, 6 mM/l, and Na^{+}, 6 mM/l. Abscissa, ion concentration in mM/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 mM 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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