J. Physiol. (1965), **180**, *pp.* 383–423
With 7 text-figures
Printed in Great Britain

PHOSPHORUS METABOLISM OF INTACT CRAB NERVE AND ITS RELATION TO THE ACTIVE TRANSPORT OF IONS

By P. F. BAKER

From the Physiological Laboratory, University of Cambridge

(*Received* 16 *February* 1965)

In 1957, Skou demonstrated that a microsomal fraction obtained from the leg nerves of the shore crab *Carcinus maenas* contained an ATPase, the properties of which bear a striking resemblance to those of the sodium pump. Thus, it requires Mg, Na and K for maximum activity and is inhibited by ouabain (Skou, 1960). Subsequent work has shown this enzyme in almost every tissue examined. The main problem in accepting Skou's enzyme as part of the pump mechanism lies in the fact that, in a broken cell preparation, it is not possible to decide whether the sites of action of Na and K are the same as in the living cell (that is, Na from inside and K from outside) or different. This has been solved for the red-cell ATPase where the site of sodium action is internal and potassium external (Glynn, 1962; Whittam, 1962*b*; Laris & Letchworth, 1962). Connelly (1959, 1962) has obtained essentially similar, but less direct, evidence for intact nerve. He showed that the extra oxygen uptake attendant on stimulation requires an increase in internal sodium, is much reduced in the absence of external potassium and is inhibited by ouabain. These changes in oxygen uptake can be linked with the operation of a transport ATPase through the observations of Chance & Williams (1955*a*, *b*) on the control of mitochondrial respiration. They showed that, in the presence of inorganic phosphate and substrate, the oxygen uptake of isolated mitochondria is controlled by the supply of ADP. Applying this to intact axons, an increase in the rate of ATP breakdown resulting from the operation of the sodium pump should raise the level of inorganic phosphate and ADP in the cell and these, in turn, should accelerate respiration and the resynthesis of ATP. The ability of Skou's enzyme to effect respiratory control in this way has been demonstrated in homogenates of brain cortex by Whittam (1962*a*). The purpose of this paper is to show that Skou's enzyme is present in intact crab nerve and that its activity is apparently inseparable from that of the sodium pump in this tissue.

A brief account of part of this work has already appeared (Baker, 1963).

METHODS

Material

The crab chosen for this work was the spider crab (*Maia squinado*) and not the shore crab (*Carcinus maenas*). The choice was made solely on grounds of size. The larger nerve trunks of *Maia* were preferred because they could be weighed more accurately and subsequent chemical analyses were easier. *Carcinus* was used in a few experiments and the results were very similar to those obtained on *Maia*.

Adult specimens, both male and female, were taken. Many of the experiments described were conducted at Plymouth, where the animals were frequently used within a few days of capture. For other experiments, crabs were transported to Cambridge and kept in an aquarium until required. The condition of the animals varied.

The bulk of the experiments were carried out during the early part of 1963. At this time, the sea temperature at Plymouth was well below the average for the time of year and a number of species were killed or severely reduced in numbers. Although *Maia* survived, they often appeared to be in rather poor condition. Nerves isolated from these animals gave apparently normal action potentials; but their resting orthophosphate (P_i) content was higher than in animals obtained under less severe conditions. Despite the quantitative differences, the responses of nerves obtained from crabs in very good and poor condition were always qualitatively similar. There was also an indication, evidence for which was the faster rate of Na accumulation in ouabain-poisoned nerves, that the ionic permeability was somewhat greater than normal in nerves obtained from crabs caught during this very cold spell. One other striking difference was the resistance to cyanide of nerves taken from well-fed aquarium crabs. This was never seen in freshly caught animals. The explanation was not clear, but the observation presumably reflected an increased ability of aquarium animals to obtain energy by cyanide-insensitive pathways. Most of the experiments described in this paper were performed on crabs which had been in captivity for less than a month and the resting P_i level in these nerves was reasonably constant. When tested, these nerves were always found to be cyanide sensitive.

Where experiments refer to crabs kept in captivity for longer periods, a note will be made to that effect.

Experimental procedure

Dissection of the nerve. Following removal of a leg from an animal, the nerve was extracted by cutting around the distal joint of the longest endopodite (endopodite II) and gently pulling the severed portion away from the rest of the leg. This left the nerve attached to the intact part of the limb (Furusawa, 1929). A further length of nerve was sometimes obtained by similar means from the next two segments (endopodites III and IV). In this way, a large leg yielded 15 cm or more of nerve and this was normally divided into three 5 cm lengths, enabling three separate determinations to be made on each leg. The cut ends of the nerve were tied with cotton and any strands of nerve which obviously did not run the whole length of the preparation were removed. The nerves were immersed in artificial sea water (10 K (Na) ASW, Table 1) contained in shallow Petri dishes exposed to the air. No further oxygenation of the solution was attempted. Nerves were always left for 1 hr before use and were never left longer than 5 hr. There was no obvious change in resting P_i level or subsequent behaviour over this period of time. A few experiments were made to test the survival of *Maia* nerve. After 4 days in 10 K (Na) ASW at 4° C, quite good action potentials were still obtained. After 24 hr at 4° C, the resting P_i level was normal; but there was a suggestion of deterioration in the pumping mechanism.

Weighing. All nerves were weighed during the initial soaking period in 10 K (Na) ASW and before any other experimental treatment. They were blotted carefully on Whatman No. 1 filter paper and weighed rapidly on a Stanton single-pan balance. Nerves of less than

10 mg were not used because of the inaccuracy in weighing. For larger quantities of material, consecutive weighings of the same nerve were highly reproducible and the procedure did not appear to harm the preparation.

Stimulation and subsequent treatments. Nerves were stimulated by withdrawing their ends into the air and applying a shock between the forceps holding the nerve and the solution. The action potential was monitored by recording between the other forceps and the solution. Nerves which failed to give an action potential were discarded. This was not a very critical test, as the loss of function of most of the very small nerve fibres would probably not have been noticed in this type of recording. A Grass stimulator was used and the strength of stimulus routinely given was a 30 V pulse of 2 msec duration. This large shock was used in an attempt to ensure stimulation of the majority of the small nerve fibres. Stimulation was always performed at room temperature (16–19° C); but all subsequent treatments were carried out in a water-bath maintained at 16·5° C.

These treatments usually consisted in transferring the nerve to a beaker containing the test solution equilibrated to the temperature of the water-bath and keeping this shaken throughout the course of the experiment. Because of the loose structure of *Maia* nerve, the extracellular space was rapidly flushed out by the test fluid and the cells would be bathed in the new medium within about a minute (see p. 388).

Nerves removed for analysis were blotted and rapidly frozen by placing them on a boat made of copper foil and floating on a mixture of acetone and solid carbon dioxide. Freezing of a nerve, 1 mm in diameter, was complete within 5 sec. Once thoroughly frozen, some 5–10 mm of nerve was severed from each end. This avoided those regions which may have been depolarized either owing to the tie or to the short period in air during stimulation. The ends were placed in artificial sea water before being weighed. The difference between initial weight and the weight of the ends was taken as the weight of nerve used. Some error in this value might have been introduced through changes in weight of the ends of the nerve and subsequent failure of this to be reversed on soaking in 10 K (Na)ASW. In the present experiments this source of error was probably small, as little change in weight occurred during the course of the experiment in the various test solutions used.

The middle portion of nerve was transferred to a small homogenizer containing 0·5 c.c. of 5% (w/v) trichloroacetic acid previously cooled until small crystals of ice were present in the solution. The nerve was immediately ground by two strokes of the plunger and then the homogenizer was dipped into the freezing mixture. This refroze both solution and nerve and homogenization was completed as the solution thawed. The homogenate was quantitatively transferred to a cold centrifuge tube, the homogenizer being washed twice with cold distilled water and the resulting suspension, which was about 1% with respect to the trichloroacetic acid, was placed in the refrigerator for an hour. The aim here was to ensure complete extraction of phosphate compounds. After an hour, the solution was either analysed immediately or stored in the deep freeze until analysis. This was always performed within 24 hr of the experiment and usually within 12 hr.

It was not possible to determine the extent of break-down of energy-rich phosphate compounds during homogenization and subsequent analysis. The low values for P_i observed in some experiments (the lowest was 24 μg P/g nerves) and the high reproducibility of similar determinations on the same crab, suggest that little break-down occurred. Even if the figure for P_i just quoted was all derived from hydrolysis of energy-rich phosphate compounds—which seems unlikely—it would only represent 6% of the total energy-rich phosphate in the nerve.

Analytical methods. The nerve homogenate was centrifuged and the cold supernatant quantitatively decanted. Analyses of P_i were made by the method of Berenblum & Chain (1938), as modified by Ennor & Stocken (1950). The extraction and development of the phosphomolybdate blue colour was performed in a 10 c.c. 'all-glass' syringe. During periods of shaking, the nozzle was covered with Parafilm. The extract was made up to 5 ml.

with absolute ethanol and, after gentle centrifugation, the optical density at 715 mμ measured over a suitable path length (10 cm was used in some instances). This method, although laborious, was very satisfactory. The colour was stable for more than 90 min and P_i could be determined to an accuracy of ±10% in amounts as low as 0·05 μg P. In general, about 0·1–0·5 μg P was present and the accuracy was better than ±2%. Both ATP and arginine phosphate (ArgP) were completely stable under the conditions of the phosphomolybdic acid extraction. Phosphate analyses were expressed as μg phosphorus/g of nerve. This includes a correction for extracellular space which was estimated at 30% (see p. 388). The formula used for this correction was μg P/g nerve = μg P/g wet nerve × 100/70.

Arginine was determined by the method of Rosenberg, Ennor & Morrison (1956).

Chromatographic techniques were similar to those used by Caldwell (1960). As the sensitivity of this method is low, a large quantity of nerve (50–100 mg) was required. To reduce the risk of break-down of energy-rich phosphate compounds, the cold neutralized supernatant was applied to the paper in a local atmosphere of ammonia.

Sodium, potassium and occasionally calcium were determined by means of an EEL flame photometer. Chloride was measured with a Cotlove chloridometer.

Design of experiments. The usual procedure for examining the effects of different experimental treatments on the P_i level was to compare nerves of the same crab, with two nerves as controls and two for each treatment. In general, the differences observed were very much larger than the variation between nerves from the same crab. As the effects were very reproducible, the results for any particular experimental treatment have been collected together and expressed as the arithmetic mean ± S.E. of the mean.

Solutions

The external solutions used were all bicarbonate–carbon dioxide buffered, artificial sea waters (Caldwell, 1960). Nerves survived in these media for up to 4 days at 4° C and so a special crab Ringer's solution was not used. The composition of the main experimental solutions is given in Table 1. Analar salts were used if available.

TABLE 1. Composition of external solutions. Concentrations in mg ions/l. solution

Solution	K	Na	Ca	Mg	Cl	SO_4	HCO_3
Sea water	10	470	10	54	550	29	—
10 K(Na)ASW	10	460	11	55	600	—	2·5
0 K(Na)ASW	—	470	11	55	600	—	2·5
100 K(Na)ASW	100	370	11	55	600	—	2·5
10 K(Na)SO_4ASW	10	920	ca.6	55	—	526	2·5

Solutions based on other univalent cations or anions were made by replacing Na or Cl on a one-to-one basis. 0 K (choline) ASW and 0 K (Li)ASW were buffered by the addition of 0·5 mM sodium bicarbonate. The pH of these solutions varied between 7·5 and 8·3 but was usually close to 8·0. Solutions will be referred to by the notation used in Table 1. This gives the potassium content, the major cation and the main anion in the solution. Unless otherwise indicated, ASW refers to a solution containing chloride as the major anion.

Cyanide-containing sea waters were made by adding a mixture of NaCN and HCN to give a final pH close to 8·0.

Electron microscopy

The fixation and preparation procedures used were similar to those described by Baker, Hodgkin & Shaw (1962).

RESULTS

Resting nerve

Structure. The leg nerves of the two crabs examined, *Carcinus maenas* and *Maia squinado*, were essentially similar in structure.

As dissected, the nerve trunk is loosely held together by connective tissue. Once this is broken, as commonly happens when nerves are obtained by pulling them out of the endopodites, the bundles of nerve fibres tend to fall apart (see Pl. 1 in Keynes & Lewis, 1951). This facilitates rapid equilibration of the extracellular space and also simplifies dissection of small bundles of nerves. Within the fibre bundles, two main cell types can be distinguished. These are the nerve cell processes or axons and the Schwann cells.

All the axons are enveloped in Schwann cell processes, although occasionally part of a fibre may be bare. Large axons, 10–20 μ in diameter, are usually surrounded by a number of Schwann cells, whereas axons of 1 μ or less are frequently crowded together, sometimes as many as 100 being located within the same Schwann-cell cleft. The mesaxons are well developed and rarely cross the Schwann-cell layer by the shortest possible path. For instance, in transverse sections, it is not uncommon to find that the length of the mesaxon is 10–40 times greater than the minimum thickness of the Schwann cell. Surrounding the Schwann cell and covering the outer ends of the mesaxon is a basement membrane. The largest fibres are often enveloped by other layers of cells and connective tissue.

Within the axons, mitochondria and a few small vesicles (or tubules) are the only prominent structures. A noticeable feature of the larger axons (from 1 μ in diameter upwards) is the concentration of mitochondria immediately beneath the axolemma. Frequently over 50% of an axon's mitochondria are located within 1000 Å of the cell membrane (see also Geren & Schmitt, 1954; Baker, 1965). The Schwann cell has a wider range of organelles, including a nucleus, mitochondria and many vesicles covering the size range 100–3000 Å.

Measurement of electron micrographs shows that, of the cellular elements in the whole nerve trunk, about 20% of the volume is contributed by Schwann cells, 75% by axons and 5% by unidentified cellular material. Comparable measurements of cell membrane contributed by the different cells (based on transverse sections of *Carcinus* nerve) show that about 50% is Schwann cell in origin, 35% axonal and the remaining 15% from the unidentified cellular material. If the latter is included with the Schwann cells, about twice as much membrane comes from the Schwann cells as from the axons. In an homogenized nerve, the mitochondria and nuclei can be separated and the 'microsomal' fraction remaining should contain cell membranes distributed as above and also various other membranous elements present in the axons and Schwann cells; but it seems certain that a large fraction of this preparation would be cell membrane in origin.

Determination of extracellular space. Throughout the course of this investigation, the wet weight of nerves was obtained after carefully blotting off any excess extracellular fluid. In order to express the results in terms of living tissue, it was necessary to estimate the contribution of the extracellular fluid to the wet weight. This was done by three methods: two indirect and one direct.

The direct approach utilized sea water containing ^{131}I. Nerves were soaked in this solution for different lengths of time and then removed, carefully blotted and frozen. The central portion was dissolved in a small volume of nitric acid and made up to 8 c.c. ^{131}I was counted in a gamma scintillation counter (Panax). The uptake of ^{131}I for a number of nerves is shown in Fig. 1. Extrapolating back to zero time this gives a value of 0·3 for the fraction of the wet weight occupied by extracellular fluid. There was no evidence for adsorption of ^{131}I. This was checked in a control experiment in which a nerve was soaked for 10 min in ^{131}I labelled sea water. Subsequent washing in an inactive solution removed 92% of the tracer in the first 2 min and after 5 min only 0·012% of the tracer remained in the nerve. (This experiment also serves to illustrate the rapidity with which the extracellular space equilibrated with the external medium. Independent confirmation of this fact was the short time taken for conduction block to develop after transferring a nerve from sodium ASW to choline ASW. Frequently, the action potential was lost within 30 sec.)

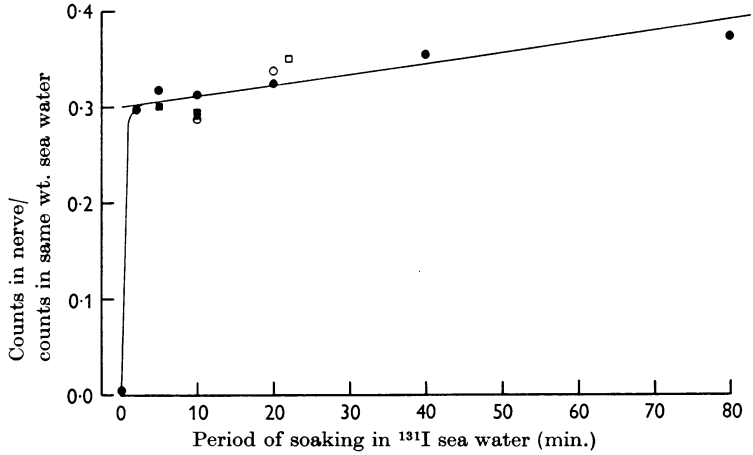

Fig. 1. Determination of the extracellular space of *Maia* nerve by measuring the uptake of ^{131}I- labelled sea water. Each symbol represents a different animal. ●, ◓, □ male crabs; ■, ○ female crabs.

The two indirect methods both gave values close to 0·3 for the extracellular space. They were based on estimation of total Na or Cl in the blotted nerve. The values obtained set an upper limit to the extracellular space as they assume that all the Na or Cl is extracellular, which is extremely unlikely. The values obtained by this approach gave for Na 0·34 and for Cl 0·32. An estimate of the internal Na and Cl can be made by washing the nerves in a medium lacking these ions. For Na the technique used was to wash the nerve in cold (4–8° C) 0 K (choline) ASW for 10 min, before freezing and analysing the middle portion of nerve. For Cl, the washing medium was 10 K (Na)NO_3ASW. The Na values obtained in this way were consistently around 18 m-moles/kg wet nerve. This would make the extracellular space 0·3, in complete agreement with the ^{131}I data. The Cl values were more variable: in general, the Cl content was higher the longer the nerve had been kept in 10 K (Na)ASW since dissection. This was presumably due to uptake of Cl into depolarized fibres. In nerves analysed shortly after dissection, the Cl content was 10 m-mole/kg wet nerve and the total Cl space 0·32. Correcting for the 'slowly equilibrating' chloride this gives a value of 0·3 for the extracellular space.

The remarkably similar results obtained by these three approaches leaves little doubt that the extracellular space of *Maia* nerve must

be close to 0·3 and this value has been used throughout the present work.

Much of the extracellular fluid is presumably held in the interstices between fibre bundles; but some must penetrate the tissue. The location of this extracellular space within the fibre bundle has been examined by Villegas & Villegas (1964) who showed that, in squid nerve, colloidal particles have access both to those regions containing collagen fibres and also to the mesaxon and space between Schwann-cell membrane and axolemma. In crab nerve, these same regions equilibrate rapidly with externally applied iodide ions (Baker, 1965). Thus it seems likely that extracellular fluid has rapid access to the outer face of the surface membranes of both Schwann cells and axons.

The phosphate compounds present in Maia nerve. In agreement with work on squid axoplasm (Caldwell, 1960) and *Maia* muscle (Caldwell & Walster, 1962), paper chromatography showed three main phosphate fractions in *Maia* nerve. These were ATP, ArgP and P_i. Free arginine was also present. If a sample of resting nerve homogenate was chromatographed in *n*-propanol–ammonia–water, dried and then the arginine phosphate hydrolysed by heating in a moist atmosphere at 100° for 1 hr, tests for arginine (Hobson & Rees, 1955) showed two orange spots—one corresponding with arginine phosphate and the other with free arginine. Adenosine diphosphate (ADP) was not detected.

On poisoning the nerves with 2 mM cyanide, the ATP and ArgP disappeared and the concentration of arginine and P_i increased. A faint spot which gave a positive adenine test (Gerlach & Döring, 1955) was normally visible travelling in the same region of the chromatogram as an adenosine monophosphate (AMP) marker. This suggested that in cyanide, ATP was broken down to AMP and P_i.

Quantitative analyses of the phosphate fractions on the chromatograms were not made. Instead, an estimate of the ATP and ArgP levels was obtained by measuring both P_i and arginine in the same homogenate. Subsequent calculations were based on the assumptions that the difference between the arginine content of resting and cyanide poisoned nerves solely represented arginine released from ArgP and that the corresponding changes in P_i level reflected break-down of ATP and ArgP. The argument as applied to arginine was probably valid, although some arginine may have been released from proteins. The total P_i released probably contained contributions from compounds other than ATP and ArgP. As no other phosphate compounds were detected on the chromatogram they were probably present in low concentration and so would not introduce a very large error into the calculation. In Table 2, values computed in this way are compared with Caldwell's data on the ATP, ArgP and P_i content of squid axoplasm.

The main difference between these two sets of determinations is in the ArgP content. As there is a tendency for ArgP to break down during chromatography, this might account for some of the difference; but it would have been surprising if the two sets of values had been identical as they are derived from animals in different phyla.

In a limited number of experiments, the resting arginine content of *Maia* nerve was found

to be 396 ± 20 μg arginine/g nerve. From these determinations it appears that the nerve contains three times as much ArgP as free arginine. Comparable measurements on the squid giant axon gave an arginine content of $132 \pm 6{\cdot}5$ μg arginine/g axoplasm and an ArgP/arginine ratio of between 4/1 and 6/1.

TABLE 2. Comparison of the content of various phosphate compounds in isolated squid axoplasm (Caldwell, 1960) and intact *Maia* nerve

Phosphate fraction	Mean amount of phosphorus present: Squid (μg P/g axoplasm)	Mean amount of phosphorus present: *Maia* (μg P/g nerve)
ATP	125	130
ArgP	105	180
P_i	117	91
Other cyanide-sensitive phosphate compounds	60	—
Total Pi after cyanide	407	401

TABLE 3. Variation in P_i level (μg P/g nerve) of resting and cyanide-poisoned nerves

Resting nerve	Poisoned nerve
75·5	461
47·5	398
72·0	392
57·5	377
62·0	434
74·0	429
70·5	369
65·5	418
Mean $\pm$ S.E. $65{\cdot}6 \pm 3{\cdot}4$	410 ± 11

The values for resting nerve were from a crab which had been kept in captivity for over a month: those for poisoned nerve were from a freshly caught animal. Exposure to 2 mM cyanide was for 4 hr at 20° C.

Variation in the resting inorganic phosphate level. There was some variation in P_i content between crabs, but the nerves from each of the eight walking legs of any particular animal were closely similar. This is illustrated in Table 3 in which the resting P_i level and the P_i content after cyanide poisoning are given for all eight legs of two crabs.

The resting sodium and potassium contents. The Na and K contents of *Maia* nerve were easily determined by flame photometry. Because of the low concentration of internal Na, more accurate measurements of internal Na were obtained if the extracellular space was first cleared of Na by washing in a Na-free medium such as choline or sugar. The technique used was to wash the nerves for 10 min at 4–8° C in 0 K(choline)ASW. Nerves were then frozen and the middle section analysed. The washing solution was kept cold to reduce the loss of Na through operation of the sodium pump. Nerves treated in this way were compared with unwashed nerves in which a correction was applied for the extracellular space (Table 4). The values

for washed nerves were consistently somewhat lower than the unwashed ones; but the loss of Na and K was small and results obtained by this method were not corrected for Na and K loss.

The Na content of nerves from the same crab was very constant and similar to that of nerves from other specimens. This was not always true for potassium where a wide variation in K content was sometimes observed. Abbott (in Abbott, Hill & Howarth, 1958) noted that the K content of *Maia* nerve fell progressively after dissection; but the scatter seen in the present experiments was not caused solely by this factor as analyses of freshly dissected material showed the same variation.

TABLE 4. Changes in ion content (m-mole/kg nerve) on washing with 0 K(choline)ASW at 4–8° C

	Unwashed nerve		Washed nerve	
Treatment	Na	K	Na	K
Resting nerve	32	211	28	187
Stimulated nerve	54	191	45	179
Stimulated and soaked in 0 K(Na)ASW	109	133	103	133

All the nerves were from the same crab. Values for unwashed nerve corrected for extracellular space of 0·3.

TABLE 5. The sodium and potassium content (m-mole/kg nerve) after soaking in different solutions

Solution 1	Solution 2	Sodium	Potassium
10 K(Na)ASW (1 hr)	—	28 ± 3·0	191 ± 8·5 (10)
0 K(Na)ASW (1 hr)	—	54 ± 4·4	134 ± 6·7 (4)
10 K(Na)ASW + 1 mM ouabain (1 hr)	—	75 ± 4·0	132 ± 11·7 (4)
10 K(Na)ASW + 2 mM cyanide (1 hr)	—	50 ± 8·1	159 ± 13·9 (4)
0 K(Na)ASW (1 hr)	10 K(Na)ASW (2 hr)	28 ± 2·6	173 ± 4·1 (2)
10 K(Na)ASW + 1 mM ouabain 1 (hr)	10 K(Na)ASW (2 hr)	34 ± 2·1	145 ± 22 (2)
10 K(Na)ASW + 2 mM cyanide (1 hr)	10 K(Na)ASW (2 hr)	31 ± 2·8	169 ± 10 (2)

Nerves were placed first in solution 1 and then in solution 2. Temperature 16·5° C. The number of nerves analysed is given in brackets.

Certain changes in the external medium resulted in alterations in the levels of Na and K in the cell. The Na content tended to rise and the K to fall following removal of external potassium or addition of ouabain or cyanide to the external medium (Table 5). All these conditions are known to inhibit the sodium pump in other tissues and the changes in ion content of *Maia* nerve suggest that the sodium pump was also inhibited in this preparation.

The alterations in Na and K content probably resulted from passive movement down the concentration gradients of these ions. As measurements of the unidirectional fluxes were

not made, no evidence is available to decide whether the permeability to Na and K was altered by these treatments. For the squid, Hodgkin & Keynes (1955) showed that removal of external potassium or poisoning with cyanide did not alter the Na influx or K efflux and similar results have been obtained for the action of ouabain on the isolated frog ventricle (Schreiber, 1956). At first glance, the observation that, following soaking in ouabain, the changes in ion content were greater than in either 0 K (Na)ASW or cyanide (Table 5) might be taken to favour an effect of these solutions on the passive permeability. But a more likely explanation is that the sodium pump was inhibited to different extents. Thus the difference was probably due to a more rapid inhibition of the pump by ouabain than by cyanide. The slow action of cyanide, arising from the time taken for all the energy-rich phosphate compounds in the cell to be broken down, would enable some pumping to continue for part of the experimental period. In 0 K (Na)ASW, it is probable that the region immediately external to the cell membrane was never completely K-free because of a slow leak of K from the cell. This might have enabled some pumping to continue.

Abbott *et al.* (1958) have estimated the area of axon membrane in *Maia* nerve to be about $1{\cdot}57 \times 10^4$ cm^2/g 'axons'. If this value is corrected for an extracellular space of 0·3 and the presence of 20% Schwann cells in whole nerve, the amount of membrane in intact nerve will be about 9×10^3 cm^2/g wet nerve. Because of the possible errors in this estimate, the value of 10^4 cm^2/g wet nerve has been used. (This value was also taken by Abbott *et al.* 1958). If the net gain of Na during soaking in ouabain is expressed in terms of the axon membrane, the resting Na influx was 0·9 pmoles/cm^2 per second. This value is probably too high as it is unlikely that the axons were the only cells into which Na was penetrating. The area of Schwann cell and other membrane is roughly twice that of the axons, so if the permeabilities of these membranes were similar, the net rate of Na entry was probably around 0·3 pmoles/cm^2 per second. This is very low, but not incompatible with recent work of Brinley (1964) on lobster giant axons. He has determined the unidirectional fluxes of Na and K and his values are considerably lower than those for cephalopod nerve (Keynes, 1951; Hodgkin & Keynes, 1955). This probably results from the much higher electrical resistance of the axolemma of crustacean nerve (Hodgkin, 1947).

These changes were largely reversible. The Na was pumped out and the K reabsorbed on replacing the nerves in 10 K(Na)ASW. The surprising feature was that the effects of ouabain were reversible, as Caldwell & Keynes (1959) reported that it was an irreversible inhibitor of the sodium pump in squid nerve. There was some suggestion that following ouabain poisoning, although the original sodium level was restored, there was only a small net increase in potassium (Table 5).

Effects of different bathing media on the resting P_i level. Inclusion of 2 mM cyanide in the 10 K(Na)ASW resulted in a rapid increase in the P_i level of the cell. When the release of P_i ceased, ArgP and ATP were no longer detectable. On removing the cyanide, the P_i level fell towards its initial value and the ion content returned towards normal. Taken together, these observations suggest that the operation of the sodium pump in *Maia* nerve required the presence of energy-rich phosphate. This is in agreement with the well established role of energy-rich phosphate in the sodium pump (Maizels, 1951; Hodgkin & Keynes, 1955; Caldwell, Hodgkin, Keynes & Shaw, 1960). The sensitivity of *Maia* nerve to cyanide suggests that oxidative phosphorylation was the main source of energy-rich

phosphate. Caldwell (1960) reached the same conclusion for squid nerve; but other tissues, notably the red cell, rely on glycolysis.

The P_i level of unstimulated nerve, which had been soaked in 10 K(Na)-ASW for at least 1 hr was 91 ± 4 μg P/g nerve. After soaking in either 0 K(Na)ASW or 10 K(Na)ASW + 1 mM ouabain it was reduced to about 70 μg P/g nerve. Thus, although both these treatments inhibit the sodium

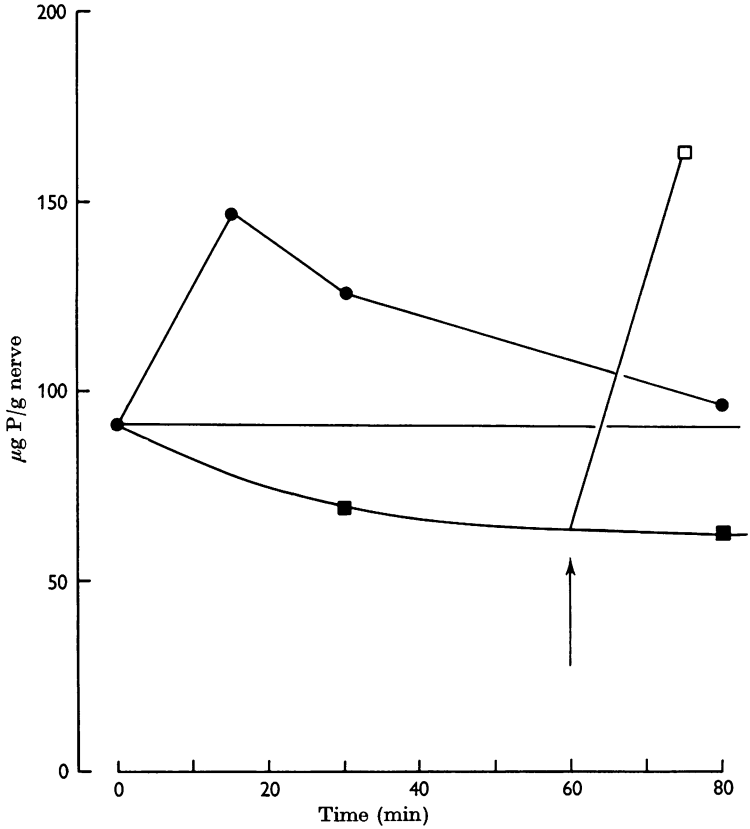

Fig. 2. Changes in P_i content of unstimulated *Maia* nerves during soaking in different solutions. The straight line at 91 μg P/g nerve represents the P_i level in the control nerves in 10 K(Na)ASW; (●) nerves immersed in 100 K(Na)ASW; and (■) nerves in 0 K(Na)ASW. At the arrow, nerves were transferred from 0 K(Na)ASW to 10 K(Na)ASW (□). The points are averages of a number of determinations on different crabs. Temperature 16·5° C.

pump, they do not act by causing break-down of energy-rich phosphate compounds. On returning the nerve to 10 K(Na)ASW there was a large increase in P_i in the nerve pretreated with 0 K(Na)ASW (Fig. 2) and a small one in the ouabain-treated nerve. The increases in P_i occurred at the time the nerve was pumping out Na and so might well have reflected

increased activity of the sodium pump. Figure 2 also shows the effect of soaking in 100 K(Na)ASW. There was a transient increase in P_i, which can also be correlated with increased activity of the pump mechanism.

This series of experiments strongly suggests that the sodium pump in *Maia* nerve is closely associated with an intracellular energy-rich phosphatase, an increase in the activity of this system resulting in an elevation in the P_i level. It was not possible to decide from these experiments whether the increase in P_i during pumping was triggered by the fall in internal potassium or rise in internal sodium or both factors; but a dependence on external potassium and inhibition by external application of ouabain was already clear.

Stimulated nerve

Effects of stimulation on the Na and K contents of nerve. A convenient way of changing the intracellular Na and K was to stimulate the nerves electrically. Nerves in which the sodium pump had been inactivated by removal of external potassium, or addition of cyanide or oubain to the 10 K(Na)ASW continued to give action potentials for some time. The

TABLE 6. The ion content (m-mole/kg nerve) immediately after stimulation in different solutions

Solution	Sodium	Potassium
10 K(Na)ASW (unstimulated)	28 ± 3·0	191 ± 8·5 (10)
10 K(Na)ASW	49 ± 2·4	182 ± 5·3 (10)
10 K(Na)ASW + 1 mM ouabain	102 ± 3·1	113 ± 11·2 (2)
0 K(Na)ASW	60	151 (1)
10 K(Li)ASW	12 ± 2·0	161 ± 17·0 (2)

Stimulation rate 30 impulses/sec for 10 min. Temperature 17–19° C. The number of nerves analysed is given in brackets.

compound action potential became smaller as the nerves filled with sodium and lost potassium. Table 6 shows the changes in internal Na and K following stimulation in various solutions. In those instances where the sodium pump was inactivated, the increase in internal Na and fall in internal K was greater than in the control nerves in which the sodium pump could operate during the stimulation period.

By first inactivating the sodium pump, it was possible to estimate the sodium uptake and potassium loss per impulse. This was done by stimulating nerves in 0 K (Na)ASW containing 1 mM ouabain and the results of a single experiment are shown in Table 7. In calculating the ion transfer/cm² per impulse, the area of nerve membrane was taken as 10^4 cm²/g wet nerve and a small correction was applied for the passive increase in Na in a ouabain poisoned nerve (Table 5). This gave values of 1·6 pmoles/cm² per impulse for the net influx of sodium and 1·1 pmoles/cm² per impulse for the net efflux of potassium. Considering the errors likely to have arisen in measuring the total area of nerve membrane/g of tissue and also the difficulty of stimulating all the small nerve fibres, these values are in good agreement with published data. Hodgkin & Huxley (1947) and Keynes (1951) obtained values of 1·7 and

2·4 pmoles/cm² per impulse respectively for net K loss from *Carcinus* nerve and values ranging from 3 to 9 pmoles/cm² per impulse have been obtained for the net Na and K fluxes in cephalopod giant axons (summarized in Hodgkin, 1957).

It is unlikely that the increase in Na content was evenly distributed throughout the tissue. The Na concentration in small fibres probably exceeded that in larger ones; but, while the Na content of all the nerve fibres would be increased to some extent, it is unlikely that much change occurred in the Schwann cells. In the only case where it has proved possible to insert micro-electrodes into the Schwann cell, no response to electrical stimulation was detected (Villegas, Gimenez & Villegas, 1962), and so it is likely that the changes in ion content on stimulation were restricted to the axons.

From an analysis of electron micrographs of fixed nerve, it was estimated that of the total volume occupied by cells, 70–80% was axons and 20% Schwann cells (p. 387). Provided the resting Na concentration in both axons and Schwann cells is similar, which seems a reasonable assumption, this means that changes occurring solely in the axons will be diluted to some extent by the presence of Schwann cells. No attempt was made to correct for this.

TABLE 7. Changes in ion content (m-mole/kg nerve) on stimulation

Duration of stimulation	Frequency	Sodium	Potassium
—	—	28·0	209
2 min	10/sec	56·5	189
5 min	10/sec	83·0	165

All nerves were soaked for 10 min in 0 K(Na)ASW containing 1 mM ouabain before stimulation in this solution. All values were obtained on the same crab, two nerves being used for each treatment. Temperature 18° C.

Changes in P_i level on stimulation. One effect of electrical stimulation of nerve in a physiological medium should be an intensification of pumping activity which might also be expected to elevate the P_i level. This was first observed by Gerard (1932), who described an increase in P_i, together with a fall in creatine phosphate and adenyl pyrophosphate, during tetanization of myelinated nerve. Stimulation of *Maia* nerve in 10 K(Na)-ASW also resulted in an increase in the P_i content of the tissue (Table 8). The main ion movements associated with electrical activity in nerves are a gain in internal sodium and calcium and a loss of internal potassium. If one or all of these changes acts as a trigger for the alteration in P_i level, it follows that an increase in P_i should be preceded by considerable changes in internal ion concentrations. Figure 3 shows this to be the case for the increase in internal sodium and fall in internal potassium. The time course of calcium movement has not been examined.

The increase in P_i level during stimulation was abolished by inclusion of ouabain in the 10 K(Na)ASW. This drug is a potent inhibitor of the sodium pump in *Maia* nerve and the absence of any change in P_i level during tetanization of nerves in its presence indicates that much of the P_i increase was due to the sodium pump. These observations are interesting from another point of view, because the failure to detect any increase in P_i in nerves stimulated in ouabain suggests that energy-rich phosphate

is not immediately involved in the transmission of the nerve impulse. This is in agreement with the now generally accepted ionic basis of the nerve impulse. However, the complete absence of any change is surprising. Some ouabain-insensitive increase in the rate of resynthesis of membrane material or pumping of ions other than Na and K might have been expected to follow massive stimulation; but in a number of experiments, no significant difference was found between unstimulated and stimulated

TABLE 8. Change in P_i content (μg P/g nerve) following stimulation

Test solution	Unstimulated nerve (20 min soak)	Stimulated nerve	% increase on stimulation
10 K(Na)ASW, pH 8·1	91 ± 4·1 (48–133)	162 ± 4·7 (126–207)	78
10 K(Na)ASW, pH 6·1	74	102	38
10 K(Na)ASW, pH 8·1 + 1 mM ouabain	78 ± 2·7 (66–87)	78 ± 3·0 (62–89)	0
10 K(Li)ASW, pH 8·1	93 ± 4·1 (89–97)	90 ± 5·8 (71–126)	−3
10 K(Li)ASW + 10 K(Na)ASW (1:1)	118 ± 4·8 (109–130)	159 ± 14·5 (131–210)	35
10 K(Na)ASW + 10 K(choline) ASW (1:1)	92	162	76
10 K(Na)ASW + 10 K(Ba) ASW (1:1)	107	177	65
10 K(NH_3^+OH)ASW, pH 6·3*	236	290	23

* This solution was toxic and the increase in P_i during stimulation was also seen in unstimulated controls. The initial soak was only 15 min.

All nerves were presoaked in 10 K(Na)ASW for at least 1 hr. Before stimulation, nerves were washed in the test solution for 20 min. Stimulation was for 10 min at 30 impulses/sec. Temperature 18–19° C. The mean, S.E. of mean and range are given where more than two sets of determinations were made.

nerves from the same crab immersed in 10 K(Na)ASW containing 0·1 or 1 mM ouabain or between nerves stimulated in 10 K(Na)ASW and then transferred to 10 K(Na)ASW containing 0·1 mM ouabain and those immersed directly in the latter solution. These results indicate that the main function of energy-rich phosphate—during and after stimulation—is to supply energy for the restoration and maintenance of the steep activity gradients of sodium and potassium.

Further information on the nature of the process involved in triggering the increase in P_i level was obtained by substituting lithium for sodium in the external solution. Lithium can replace sodium in the action potential mechanism of most nerves—including *Maia*—and so it was possible to stimulate nerves in this solution. Prolonged stimulation resulted in the usual fall in internal potassium; but there was an increase in lithium content and a fall in internal sodium (Table 6). The Na content fell as Na exchanged with Li during the action potential. There was no significant increase in P_i during stimulation in 10 K(Li)ASW. As this could have

been due to an inhibitory effect of Li on the mechanism of energy-rich phosphate consumption, a control experiment was performed. Nerves were stimulated in a solution containing equal quantities of 10 K(Na)ASW and 10 K(Li)ASW and the usual increase in P_i occurred (Table 8). Also, when two nerves stimulated in 10 K(Li)ASW were transferred to 10 K(Na)-ASW for a further 5 min stimulation, an increase in P_i was observed. These results indicate that internal lithium does not immediately inhibit the sodium pump.

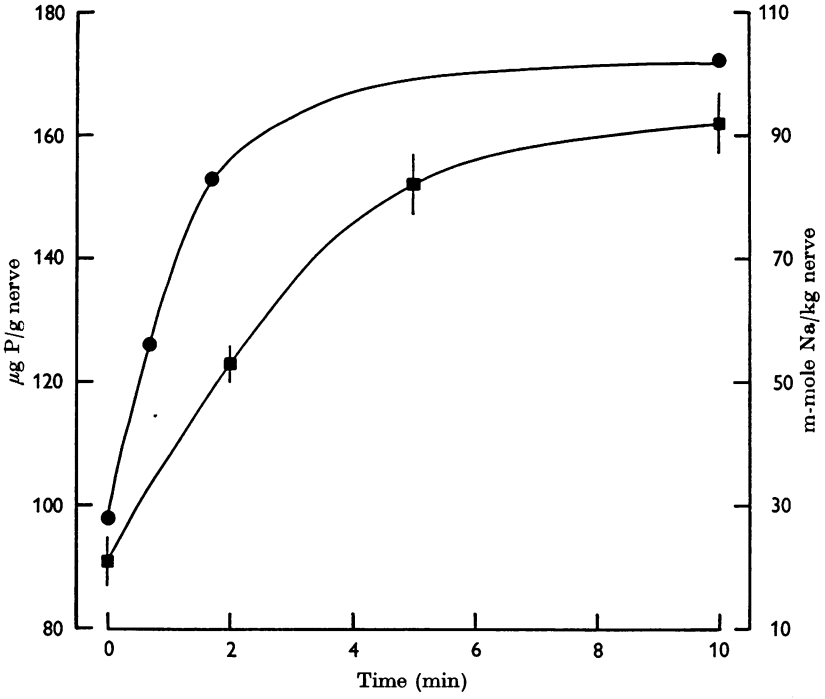

Fig. 3. Comparison of rate of increase in sodium and P_i contents of stimulated *Maia* nerve. P_i (■) was determined on nerves stimulated at 30 impulses/sec throughout the experimental period. The vertical lines on the P_i curve represent 2 × S.E. of the mean. The increase in sodium (●) was determined as in Table 7 and the time scale adjusted to 30 impulses/sec. The fall in potassium followed a curve whose time course was almost identical to that of the increase in internal sodium. Temperature 16–18° C.

In all these experiments, stimulation resulted in a fall in internal potassium; but an increase in P_i was only observed when the medium in which stimulation was performed contained sodium. This might be because the fall in internal K only increased the release of P_i when the external medium contained both Na and K: or it could be that the operative factor was the increase in internal Na. The latter seems more probable. The

following two groups of experiments were designed to help decide between the two possible modes of action of sodium. In the first, nerves stimulated in 10 K(Li)ASW were transferred to 10 K(Na)ASW for 15 min and, in the second, unstimulated nerves were soaked for 2 hr in 0 K(dextrose)ASW and subsequently returned to 10 K(Na)ASW. Both these treatments ensured a reduced internal potassium and sodium concentration. In neither case was an increase in P_i observed on resoaking in an external solution containing both sodium and potassium. The P_i content of nerves stimulated in 10 K(Li)ASW averaged 84 μg P/g nerve and after soaking in 10 K(Na)ASW it was 88 μg P/g nerve. Immersion in 0 K(dextrose)ASW raised the P_i level to 143 μg P/g nerve but, on returning the nerves to 10 K(Na)ASW, the level fell rapidly to 89 μg P/g nerve. The reason for the high P_i level in sugar is discussed later (p. 402).

These results strongly suggest that the mechanism which produced an elevation in P_i was triggered by an increase in internal sodium ions. However, sodium cannot be the sole activator, because in nerves whose Na content had been increased by soaking in 0 K(Na)ASW, the P_i level remained unaltered, or even depressed, unless potassium was added to the external solution (Fig. 2). The role of external potassium is discussed in the following section; but it is worth noting at this point that, during tetanization in 10 K(Na)ASW, the presence of a somewhat elevated K concentration in the vicinity of the axolemma may have contributed to the raised content of P_i in the nerve.

Other substitutes for Na in the action potential mechanism were sought. A few organic cations and barium were tested. Of these choline, tris(hydroxymethyl)aminomethane, tetraethylammonium, guanidinium and barium were all unable to replace Na completely; but nerves which had been rendered electrically inexcitable by soaking in choline ASW gave action potentials for some time following transfer to a Na-free artificial sea water based on hydroxyammonium chloride. (The Na content of this solution as measured by flame photometry was 1 mM.) As this salt is very acidic, the pH of the solution was brought to pH 6·3 by addition of choline hydroxide. The solution proved to be poisonous, resulting in a rapid break-down of P_i and gain in internal sodium (Table 9). This precluded any possibility of testing whether it was able to substitute for Na in the pump mechanism.

Recovery from the effects of stimulation. After stimulation in 10 K(Na)-ASW, the P_i level declined towards its resting value in a roughly exponential manner (Fig. 4). At 16·5° C, the recovery process was half complete in 25 min. Although a detailed comparison is not possible because of the differences in experimental technique, this time course is similar to, but somewhat longer than that obtained by Beresina & Feng (1933) for recovery heat production in tetanized *Maia* nerve. (But as the P_i level is not directly proportional to the rate of recovery (see p. 408), this discrepancy is not surprising.) During the first hour after stimulation and soaking in 10 K(Na)ASW, the Na and K contents of the nerve returned to

their original values (Table 9). The time course of this process was not determined.

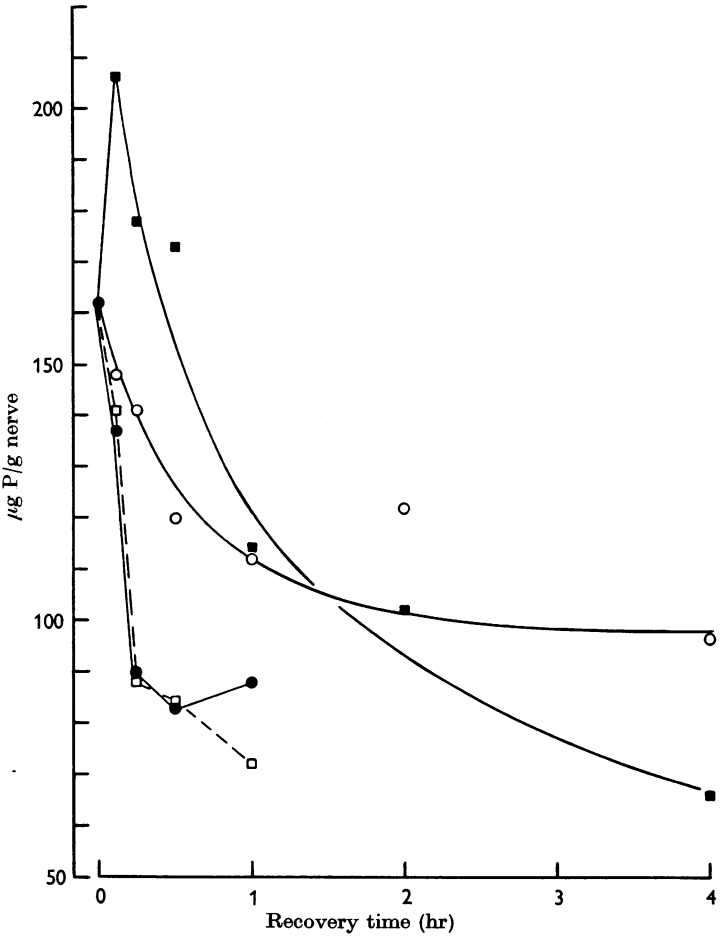

Fig. 4. Changes in P_i content of *Maia* nerves during recovery from stimulation. All nerves were stimulated in 10 K(Na)ASW for 10 min at 30 impulses/sec before transfer to the recovery solution: 10 K(Na)ASW (○); 100 K(Na)ASW (■); 0 K(Na)ASW (●); 10 K(Na)ASW containing 1 mM ouabain (□). The points are averages of a number of determinations on different crabs. Temperature 16·5° C.

Recovery in solutions of different potassium content. Following stimulation in 10 K(Na)ASW, alteration in the potassium content of the artificial sea water produced changes both in P_i level and pumping activity. If a nerve was transferred to 100 K(Na)ASW, the P_i level rose, reaching a peak during the first 15 min, and then fell to a level somewhat lower than the unstimulated value (Fig. 4). (Too few experiments were made to determine the reproducibility of this apparent depression of the final P_i

below that of the resting nerve; but comparable measurements of the oxygen uptake of *Libinia* nerve (Oberholzer, unpublished) and frog nerve (Oberholzer, Brink & Bronk, 1951), show a similar depression. The figures given in Table 9 show that the ionic content returned to its resting value during soaking in 100 K(Na)ASW. The final K concentration was somewhat higher than the unstimulated level which might indicate that K reabsorption was favoured by the reduced potassium concentration gradient.

TABLE 9. The ion content (m-mole/kg nerve) of stimulated nerves left to recover in various solutions

Recovery solution	Sodium	Potassium
—	49 ± 2·4	182 ± 5·3 (10)
10 K(Na)ASW	22 ± 4·7	192 ± 14·5 (5)
10 K(Na)ASW (2 hr)	26 ± 3·0	212 ± 4·1 (2)
100 K(Na)ASW (2 hr)	21	228 (2)
0 K(Na)ASW	106 ± 7·8	133 ± 10·1 (8)
10 K(Na)ASW + 1 mM ouabain	121	115 (2)
10 K(Na)ASW + 2 mM cyanide	73 ± 2·7	135 ± 7·6 (4)
10 K(Na)ASW 0° C	55	164 (2)
10 K(Na)ASW + 10K (NH_4^+OH)ASW (1:1)	65	94 (2)

Nerves were stimulated in 10 K(Na)ASW for 10 min at 30 impulses/sec and left to recover for 1 hr unless otherwise stated. The number of nerves analysed is given in brackets. Where more than two experiments were made, results are expressed as mean ± S.E. of mean. Temperature 16·5° C. The first line refers to nerves analysed immediately after stimulation.

Complete removal of potassium from the external medium slowed the operation of the sodium pump to such an extent that the nerves accumulated sodium and lost potassium (Table 9). This was reversed on returning the nerves to 10 K(Na)ASW. The P_i level of stimulated nerve fell rapidly on transfer from 10 K(Na)ASW to 0 K(Na)ASW (Fig. 4), and it remained low as long as the nerve was kept in this solution. However, addition of potassium to the external medium resulted in a rapid increase in P_i level and this was correlated with a period of intense pumping activity. Other K concentrations produced intermediate effects (Fig. 7).

Changes in the composition of the artificial sea water and their effect on the K-sensitivity of the sodium pump. In artificial sea waters containing 10 mM potassium, the P_i level of stimulated nerve was not appreciably affected by removal—either singly or together—of calcium and magnesium; by alteration in pH from 6 to 9 or by replacement of chloride by other anions (nitrate, acetylglycine, propionate and sulphate). But removal of potassium from any of these solutions produced a marked reduction in P_i level (Table 10). Thus the changes in P_i level are very specific for external potassium.

The P_i level in both 10 K (Na)ASW and 0 K (Na)ASW was increased in the complete absence of divalent ions. This probably resulted from an increase in the permeability to both

sodium and potassium. The effect of this would be to increase the internal Na concentration and also, by increasing K leakage (Shanes & Hopkins, 1948), to elevate the amount of K in the space immediately external to the cell membrane. Both these effects would tend to increase the activity of the sodium pump. Gerard (1932) notes that substances lowering the concentration of ionic calcium increase the respiration rate of nerve, which is in agreement with the present results.

The explanation of the elevated P_i content observed in propionate ASW is not clear.

The absence of any appreciable change in P_i level over a wide range of external pH, in both 10 K(Na)ASW and 0 K(Na)ASW, indicates that hydrogen ions cannot replace external potassium to any extent. Page, Goerke & Storm (1964) have suggested that the fall in K content of cardiac cells subjected to a low external pH (5·4) might be interpreted in terms of competition between hydrogen and potassium ions; but the pH at which this was observed was lower than any used in the present experiments. The extent to which changes in external pH may have altered the intracellular pH is not certain; but, in short-term experiments, it seems probable that little change occurred (Caldwell, 1958).

TABLE 10. External potassium and the level of P_i (μg P/g nerve) under different conditions

Test solution	Potassium content of solution (mM) 0	10	% reduction in 0 K ASW
pH			
(Na)ASW, pH 6·1	80	124	35·5
(Na)ASW, pH 7·1	87	118	26
(Na)ASW, pH 8·1	74	128	42
(Na)ASW, pH 9·1	75	127	41
Divalent cations			
0 Mg, 11 mM-Ca(Na)ASW	55	113	51
55 mM-Mg, 0 Ca(Na)ASW	69	148	53·5
0 Mg, 0 Ca(Na)ASW	107	189	43·5
Anions			
(Na) chloride ASW	90	141	36
(Na) propionate ASW	125	180	30·5
(Na) nitrate ASW	77	135	43
(Na) acetylglycine ASW	80	122	34·5
(Na) sulphate ASW	83	128	35
Replacement of sodium			
(Li)ASW	162	204	20·5
(Choline)ASW	193	208	6·5

All nerves stimulated in 10 K(Na)ASW for 10 min at 30 impulses/sec before treatment for 15 min at 16·5° C in the test solution. All values are averages of at least two determinations.

Complete replacement of the sodium in 10 K(Na)ASW by choline, lithium or an osmotically equivalent amount of sucrose resulted in a marked rise in P_i content which was only slightly reduced in the absence of external potassium (Table 10). The rise in P_i was not maintained, but fell towards the basal (10 K(Na)ASW) level over a period of 2 hr. In 1K-(choline)ASW, the P_i content after 15 min was 178 μg P/g nerve and after 2 hr it was 122 μg P/g nerve. This fall in P_i level was associated with a decrease in the sodium content of the nerves. These changes were much decreased when ouabain was included in the Na-free medium or when unstimulated nerves were used. The requirement for internal sodium and

the inhibitory effect of ouabain suggest that the increase in P_i was in some way associated with an alteration in the operation of the sodium pump.

One explanation of the reduction in potassium sensitivity and high P_i level observed in Na-free solutions might be that the external potassium concentration required to activate the pump is much lower in the absence of external sodium. Thus, even in nominally K-free solutions, enough potassium would be present in the space immediately external to the cell membrane to enable some pumping to continue. Further evidence about the action of potassium was obtained using de-ionized sucrose. (Nerves presoaked for 30 min in this solution, buffered by 2 mM-TRIS-HCl at pH 8·0, gave apparently normal action potentials on being returned to 10 K(Na)ASW.) In isotonic de-ionized sucrose, the P_i level of stimulated nerves was 75–85 μg P/g nerve compared with 135 μg P/g nerve in untreated isotonic sucrose. This difference did not stem from the reduction in ionic strength of the de-ionized sucrose as addition of 10 mM-NaCl was without effect. Addition of 0·5 mM potassium to de-ionized sucrose, containing 10 mM-NaCl, increased the P_i content of stimulated nerves to 100 μg P/g nerve; but this was still well below that observed in nerves immersed in untreated sucrose, the measured potassium content of which was only 0·04 mM. These experiments suggest that potassium was required for the increase in P_i content of nerves immersed in Na-free solutions; but the discrepancy between de-ionized sucrose containing potassium and K-free isotonic sucrose indicates that some other factor was also involved. The nature of this factor is unknown.

Replacement of potassium by other cations. A few experiments were made to determine whether potassium could be replaced by other ions. Solutions were used in which K was replaced by an equal concentration of the test ion. The nerves were first stimulated in 10 K(Na)ASW and then transferred to the test solution. In some experiments, the stimulated nerve was soaked for 15 min in 0 K(Na)ASW before transfer to the test solution. This ensured a low initial P_i level and so facilitated detection of any slight increase in P_i content due to the K-like effect of the test solution.

The main results obtained are shown in Table 11. The ions tested can be grouped in order of effectiveness at maintaining an elevated P_i level. The most K-like in this respect were rubidium and thallous ions, while caesium, lithium and sodium were almost without effect. Ammonium was intermediate between these two groups. Measurement of the Na content of the nerves showed that, in general, where the P_i level was elevated, there was a net efflux of sodium; whereas in those instances where no increase in P_i occurred, the nerve continued to gain sodium (Table 11). The exceptions were solutions containing ammonium or thallous ions. In both of these cases, the P_i level was elevated and some net loss of Na occurred when compared with a control in 0 K(Na)ASW; but the Na content after 1 hr was still well above normal. This suggested that these two ions might increase the passive Na influx as well as stimulate the sodium pump. Mullins & Moore (1959) showed that thallous ions behaved like potassium in depolarizing muscle and they also noted that thallium-treated muscles gained sodium, presumably due to an increase in sodium permeability.

The uptake of these cations was not studied. The flame photometer used did not discriminate between rubidium and potassium and so the 'potassium' content of fibres left to recover in 10 Rb(Na)ASW included any rubidium which was present. The value obtained was 165 m-mole/kg nerve which is somewhat less than the comparable value in 10 K(Na)ASW; but is considerably more than in nerves left in 0 K(Na)ASW. This suggests that Rb was absorbed by the nerve. Rubidium has been shown to replace potassium in the sodium pump in other tissues (Bonting, Caravaggio & Hawkins, 1963; Hashimoto & Yoshikawa, 1963; Adrian & Slayman, 1964).

TABLE 11. Replacement of potassium by other cations and their effect on the P_i (μg P/g nerve) and sodium (m-mole/kg nerve) contents of stimulated nerves

Test solution	P_i level	P_i level in nerves presoaked in 0 K(Na)ASW	Sodium content
0 K(Na)ASW	90 (12)	83 (2)	73 (4)
0 K(Na)NO_3ASW	77 (2)	—	74 (2)
10 Li(Na)ASW	76 (4)	72 (2)	81 (2)
10 Cs(Na)ASW	91 (2)	77·5 (2)	58 (2)
10 NH_4(Na)ASW	107 (2)	105 (2)	59 (4)
10 Rb(Na)ASW	125 (4)	122 (2)	27 (2)
10 Tl(Na)NO_3ASW	126 (2)	—	39 (2)
10 Tl(Na)NO_3ASW (1 hr)	135 (2)	—	39 (2)
10 K(Na)ASW	141 (24)	143 (2)	22 (5)
10 K(Na)NO_3ASW	135 (2)	—	—
10 Rb(Na)ASW + 1 mM ouabain	87 (2)	83 (2)	—
10 Tl(Na)NO_3ASW + 0·2 mM ouabain	61 (2)	—	—

Nerves stimulated in 10 K(Na)ASW for 10 min at 30 impulses/sec, before transfer to the test solution. P_i determined after 15 min, unless stated to the contrary. Nerves analysed for sodium after 1 hr. All values are averages. The number of determinations made is given in brackets. Temperature 16·5° C.

The effect of ouabain on the P_i level. Addition of ouabain to the 10 K(Na)-ASW recovery medium caused a rapid fall in the P_i level (Fig. 4). Under these conditions, the sodium pump was inhibited and the nerve accumulated sodium and lost potassium. The ouabain concentration normally used was 0·1 or 1 mM; but 0·01 mM was partially effective (Table 12). Raising the potassium concentration tended to counteract the inhibitory effect of the glycoside (Table 12 and Fig. 7). This observation is similar to that of Glynn (1957) on the red blood cell and Page *et al.* (1964) on cardiac muscle. Ouabain also inhibited the stimulatory effect of rubidium and thallous ions (Table 11).

On returning a ouabain-poisoned nerve to 10 K(Na)ASW, the P_i level increased, but much less than in a comparable nerve pretreated with 0 K(Na)ASW. During the period of P_i elevation, there was some recovery towards a normal ion content. After 1 hr in 1 mM ouabain, the internal sodium and potassium concentrations were, respectively, 121 and 115

m-mole/kg nerve. A further 2 hr in 10 K(Na)ASW reduced these values to 48 m-mole/kg nerve for Na and 107 m-mole/kg nerve for potassium. There was clearly a large fall in internal sodium, but very little change in internal potassium. The nature of the ions involved in maintaining electroneutrality is not known. These observations on the partial reversibility of ouabain poisoning confirm those described for resting nerve (p. 392).

TABLE 12. Ouabain inhibition of the effects of potassium on the level of P_i (μg P/g nerve)

Test solution	Potassium concentration in ASW (mM) 0	10	100
(Na)ASW	90 ± 6·3 (12)	141 ± 4·9 (24)	178 ± 7·7 (15)
(Na)ASW + 0·01 mM ouabain	—	121 ± 7·0 (2)	148 ± 13·0 (2)
(Na)ASW + 0·1 mM ouabain	—	85 ± 6·1 (2)	117 ± 1·6 (2)
(Na)ASW + 1 mM ouabain	81 ± 4·7 (4)	89 ± 4·1 (11)	113 ± 3·7 (4)

Nerves stimulated in 10 K(Na)ASW for 10 min at 30 impulses/sec and then soaked for 15 min in the test solution at 16·5° C. The number of analyses made is given in brackets.

TABLE 13. Reproducibility of changes in P_i when compared on nerves obtained from the same crab

Treatment 1	Treatment 2	(Treatment 2 – treatment 1) mean ± S.E. of the mean
10 K(Na)ASW unstimulated	10 K(Na)ASW	+52 ± 4·5 (12)
10 K(Na)ASW + 1 mM ouabain, unstimulated	10 K(Na)ASW + 1 mM ouabain	+3 ± 1·4 (6)
10 K(Na)ASW	10 K(Na)ASW + 1 mM ouabain	−49 ± 4·7 (5)
10 K(Na)ASW	0 K(Na)ASW	−42 ± 8·1 (5)
0 K(Na)ASW	100 K(Na)ASW	+65 ± 8·4 (3)
10 K(Na)ASW	100 K(Na)ASW	+31 ± 5·2 (15)
0 K(Na)ASW	10 K(Na)ASW + 1 mM ouabain	−9 ± 1·4 (7)

The first two lines refer to differences between stimulated and unstimulated nerves. In all other cases, the nerves were first stimulated in 10 K(Na)ASW for 10 min at 30 impulses/sec and then left to recover in the test solution for 15 min at 16·5° C. The number of analyses is given in brackets. No experiments have been omitted.

Reproducibility of the effects of internal sodium, external potassium and ouabain. The changes in P_i content attendant on stimulation in 10 K(Na)-ASW and subsequent recovery in (Na)ASWs of different potassium concentration or ouabain content were very consistent, especially when compared on nerves obtained from the same crab (Table 13).

The relation between changes in P_i and cellular metabolism

The rate of energy-rich phosphate break-down. As the cell is a closed system, in the absence of an external source of P_i the intracellular level of P_i must depend on the relative rates of synthesis and break-down of phosphate compounds. In *Maia* nerve, the majority of the phosphate is

present as ATP and ArgP. Any increase in the rate of synthesis of these energy-rich phosphate compounds, without a comparable increase in their rate of break-down, will result in a decrease in the level of P_i in the cell. The reverse, an increase in energy-rich phosphate break-down without an increase in synthesis, would elevate the P_i level. In practice, an increase in the rate of energy-rich phosphate break-down in normal nerve might be expected to release more ADP which, in turn, would accelerate ATP regeneration by stimulating oxidative phosphorylation and glycolysis. Although this will tend to oppose the increase in P_i level, a new steady state will be reached with the P_i level somewhat higher than normal. In the absence of other factors, the amount by which the steady state is shifted will depend on the extent to which break-down processes are activated.

The increase in P_i observed during pumping could have resulted either from an acceleration of energy-rich phosphate break-down or a reduction in the rate of synthesis. As the majority of the energy-rich phosphate in *Maia* nerve seems to be produced by oxidative phosphorylation, the break-down reactions can largely be separated from synthesis by poisoning the cell with 2 mM cyanide. This markedly reduces the rate of synthesis of energy-rich phosphate compounds and, under these conditions, the initial rate of P_i release gives a measure of the total energy-rich phosphatase activity of the tissue. Figure 5 illustrates the type of results obtained. Comparison of two treatments was always made using nerves obtained from the same crab. The results are collected together in Table 14.

Although rather few experiments were made, a clear correlation was found between elevation of the P_i level and increased rate of break-down of energy-rich phosphate compounds in the tissue (Fig. 6). The smooth curve in Fig. 6*A* was drawn according to the equation

$$v = c[\alpha]^2,$$

where v is the initial rate of energy-rich phosphate break-down (measured as P_i released), α the fraction of total cyanide-sensitive P present as P_i and c is a constant.

This relation can be derived from a reaction of the type:

$$XP \underset{E_2}{\overset{E_1}{\rightleftharpoons}} X + P_i$$

which is a considerable simplification of the phosphate cycle present in the cell. If, as seems likely, ATP is the main utilizable source of energy in the cell, the above scheme assumes that the rate of transfer of ~P from ArgP to ADP is much faster than the rate of ATP break-down. Some evidence for this has been obtained with squid axons (Caldwell, Hodgkin, Keynes & Shaw, 1964).

If both the reverse reactions can be neglected, the rate of conversion of XP into $X + P_i$ by enzymes E_1 is given by

$$v_1 = k_1[E_1][XP]$$

and the rate of resynthesis of XP by enzymes E_2 is given by:

$$v_2 = k_2[E_2][X][P_i],$$

where k_1 and k_2 are rate constants and [ ] activities.

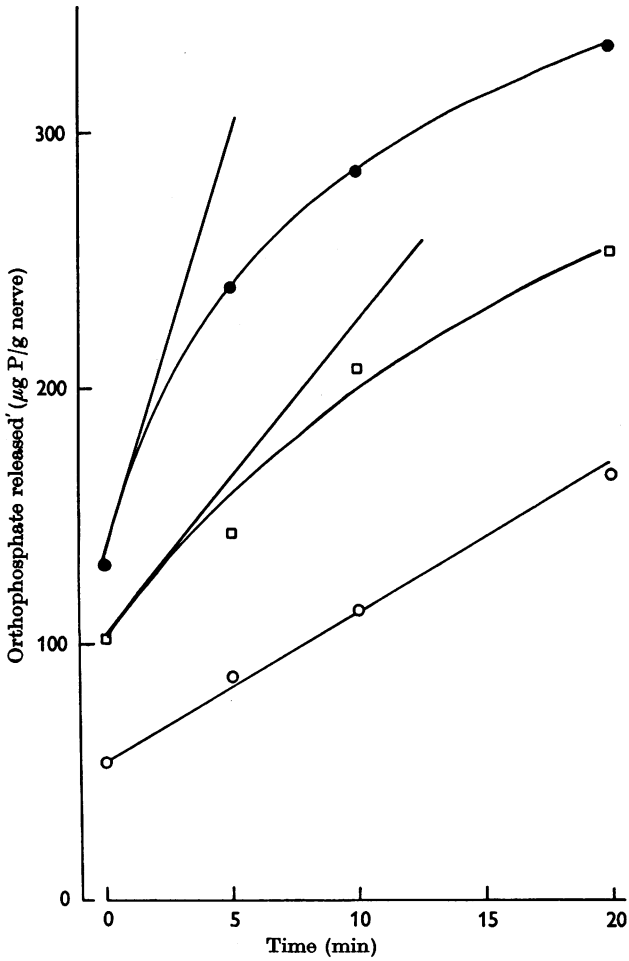

Fig. 5. Rate of P_i release following addition to 2 mM cyanide to the test solution. Stimulation, where used, was for 10 min at 30 impulses/sec in 10 K(Na)ASW. Nerves were presoaked for 15 min in the test solution before application of cyanide. The curves for stimulated (●) and unstimulated (□) nerves in 10 K (Na) ASW were obtained on the same crab. The curve for stimulated nerve immersed in 0 K(Na)ASW (○) was obtained on a separate animal. Temperature 16·5 °C. The curves were drawn by eye and the initial rate of P_i release was obtained by drawing tangents as shown. This process was somewhat arbitrary.

If the total P in the system is 1 and the fraction of P as P_i is α, these equations may be rewritten

$$v_1 = k_1[E_1][1-\alpha] \quad (1)$$

and

$$v_2 = k_2[E_2][\alpha]^2. \quad (2)$$

For any set of values of k_1, k_2, E_1 and E_2, a steady state will be reached when $v_1 = v_2$. A change to a new steady state can be considered as a change in the concentration of E_1, E_2 remaining constant but not rate limiting. (The activation of an enzyme by a non-metabolized activator is formally similar to increasing the concentration of enzyme.)

From equations 1 and 2, for any particular steady state

$$v_1 = k_2[E_2][\alpha]^2$$

and as $k_2[E_2]$ is constant, this reduces to $v_1 = c[\alpha]^2$, which was the equation used to construct the smooth curve in Fig. 6. The reasonable fit between curve and experimental points

TABLE 14. Initial rates of P_i release (μg P/g nerve per min) in cyanide poisoned nerves

Unstimulated nerve	Rate of P_i release
10 K(Na)ASW	12·5[1]
0 K(Na)ASW + 1 mM ouabain	3·2[4]
Stimulated nerve	
10 K(Na)ASW	31[1]; 25[2]; 20; 31[3]; 16[5]
0 K(Na)ASW	6·4[4]
10 K(Na)ASW + 1 mM ouabain	9[2]; 8[3]
100 K(Na)ASW	22[5]

Nerves were left for 15 min in the test solution before application of cyanide. Stimulation was for 10 min at 30 impulses/sec in 10 K(Na)ASW. Temperature 16·5° C. Superscript numbers refer to the crab used.

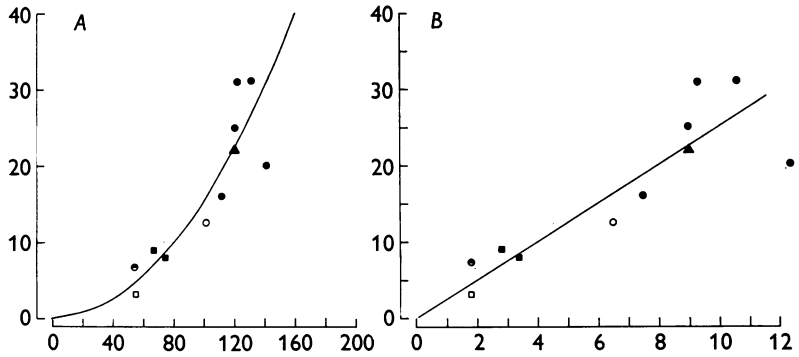

Fig. 6. Relation between P_i level and the rate of break-down of energy-rich phosphate compounds in *Maia* nerve.

A. Abscissa, P_i level before poisoning (μg P/g nerve): ordinate, initial rate of P_i release (μg P/g nerve per min) following application of cyanide. Smooth curve drawn according to the formula $v = c[\alpha]^2$ (see text): c taken as 250 from *B*.

B. Ordinate, as for *A*: abscissa α^2, where α is the fraction of total energy-rich phosphate present as P_i. The total cyanide-sensitive phosphate was taken as 400 μg P/g nerve. The straight line was drawn through the points by eye.

The symbols used in both *A* and *B* refer to the treatment of the nerve. Stimulated nerves in 10 K(Na)ASW (●); 100 K(Na)ASW (▲); 0 K(Na)ASW (◓); 10 K(Na)ASW + ouabain (■) and unstimulated nerves in 10 K(Na)ASW (○); 0 K(Na)ASW + ouabain (□). Temperature 16·5° C.

is surprising considering the simplifying assumptions made; but it supports the view that the level of P_i in the nerve cell depends in a relatively simple way on the rates of energy-rich phosphate synthesis and break-down.

The most important conclusion from the point of view of the present work is that, in the absence of other factors, the rate of break-down of energy-rich phosphate compounds did not increase in direct proportion to the level of P_i, but as the square of the P_i concentration. This means that small increases in P_i level may have represented much larger changes in energy-rich phosphatase activity.

When the P_i content was already high, the initial rate of P_i release on adding cyanide was difficult to measure accurately. This might explain the apparent fall in energy-rich phosphatase activity at high initial P_i values; but, in the absence of further experiments, other factors—for instance, a fall in the effective concentration of E_2—cannot be excluded.

From Fig. 6*A* or the more convenient straight line obtained by plotting v against $[\alpha]^2$ (Fig. 6*B*), it is possible to convert the observed levels of P_i directly into rates of energy-rich phosphate break-down in the cell. This has not been done as a general practice, because too little information is available to assess the general validity of the relation between P_i level and rate of energy-rich phosphate break-down; but in some instances a knowledge of the rate of break-down is more useful than the P_i level. Two examples of this are given below:

(1) The rate at which energy-rich phosphate is broken down is more directly comparable with recovery heat production and oxygen consumption than is the P_i level. From the data of Fig. 4, the rate of energy-rich phosphate utilization falls to half in 18 min, whereas the P_i takes 25 min. This helps to explain the discrepancy in time course between the present experiments and those of Beresina & Feng on recovery heat production (p. 398); but, even so, the P_i content (and energy-rich phosphatase ?) remained elevated above the resting level for longer than either the recovery heat production or oxygen uptake. One possible explanation of this apparently slow recovery might be a progressive impairment in the ability of the isolated nerves to resynthesize ATP.

(2) An estimate of the affinity for external potassium of the ouabain-sensitive energy-rich phosphatase was obtained by plotting the ouabain-sensitive phosphatase activity of stimulated nerve against the external potassium concentration (Fig. 7).

For stimulated nerves immersed in (Na)ASW, the apparent Michaelis constant for external potassium is 9·3 mM and the maximum rate of energy-rich phosphate consumption by ouabain-sensitive pathways 42 μg P released/g nerve per min. From a few experiments on unstimulated nerve immersed in (Na)ASW, the apparent Michaelis constant for external potassium is about 15 mM. Figure 7 also suggests that ouabain acts as a mixed inhibitor of the energy-rich phosphatase, decreasing both the affinity for potassium and also the maximum velocity.

Apparent Michaelis constants can also be calculated for the various cations which can replace external potassium. As rather few P_i determinations were made, it is necessary to assume that the maximum velocity of the energy-rich phosphatase is the same for all the ions. This is true for the red cell ATPase (Whittam & Ager, 1964). For stimulated nerve immersed in (Na)ASW, the concentrations required for half maximum activation were 13 mM for thallium, 20·5 mM for rubidium, 50 mM for ammonium and 160 mM for caesium.

The method of calculation makes the assumption that the ouabain-sensitive energy-rich phosphatase was totally inhibited in 0 K(Na)ASW containing 1 mM ouabain. This seems reasonable, because the sodium efflux from squid nerve immersed to 10 K(Na)ASW was effectively abolished by 0·01 mM ouabain (Caldwell & Keynes, 1959).

Basically similar results to the present ones were obtained by Gerard & Tupikova (1938, 1939). They observed that during stimulation of sciatic nerve the P_i content was elevated. If metabolism was impaired by anoxic conditions creatine phosphate broke down at a rate which was increased by stimulation. These results indicate that an increase in P_i level during tetanization was associated with an acceleration of energy-rich phosphatase reactions in the nerve.

It should be stressed that the numerical relation between P_i level and rate of break-down of energy-rich phosphate compounds derived in the present studies may not apply in other experiments even on the same species. Any variation in the concentration of E_2 will alter the constant c and hence the rate of energy-rich phosphate break-down equivalent to a particular value of P_i. All the experiments described were performed in the winter on crabs which appeared to be in much the same physiological condition. Crabs which had been kept for a long period in the Plymouth aquarium had a much lower resting P_i content—although the level was increased by stimulation—and it seems likely that in these cases c was greater than 250, the figure derived from fresh crabs.

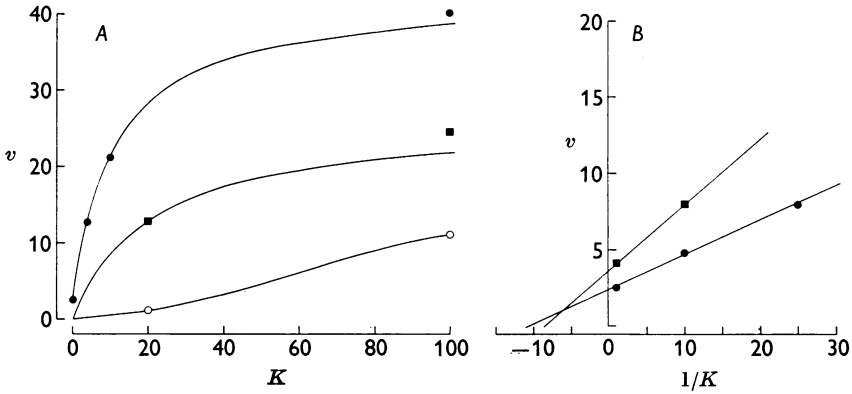

Fig. 7. Ouabain-sensitive energy-rich phosphate break-down in stimulated nerve as a function of the external potassium concentration. Stimulation was for 10 min at 30 impulses/sec in 10 K(Na)ASW before transfer to the test solution. The external media were (Na)ASW (●); (Na)ASW + 0·01 mM ouabain (■) and (Na)ASW + 0·1 mM ouabain (○). Total energy-rich phosphatase activity was obtained from the P_i level and the rate of energy-rich phosphate break-down by ouabain-sensitive pathways (v) was taken as (total phosphatase—the residual phosphatase in 0 K(Na)ASW containing 1 mM ouabain). v is expressed as μg P released/g nerve per min. K is the external potassium concentration (mM). Temperature 16·5° C. In *A*, the two upper curves are rectangular hyperbolae, the lowest curve was drawn by eye. *B* is a Lineweaver & Burk plot of the two upper curves in *A*.

It is not clear whether the relation between P_i level and energy-rich phosphatase activity derived for nerves immersed in (Na)ASW is directly applicable to nerves bathed in Na-free solutions. Some of the energy-rich phosphatase activities calculated from the high P_i levels observed in these solutions are above the maximum rate of phosphate utilization by the sodium pump in (Na)ASW. Measurements of the oxygen uptake of *Libinia* nerve (P. F. Baker & C. M. Connelly, unpublished) show that, although the affinity of the pump for external potassium is increased as the external sodium concentration is decreased, the maximum rate of oxygen uptake associated with pumping remains constant. This suggests

that the high P_i levels obtained in Na-free media probably resulted from at least two factors: a high rate of energy-rich phosphate break-down due to an increased affinity of the pump for external potassium and also a slight impairment in the phosphorylating efficiency of the mitochondrion. The over-all effect of this last factor would be to shift the curve in Fig. 6*A* to the right. A possible link between low external sodium and the mitochondrion might be an increased influx of calcium into the cell from the low sodium media (Lüttgau & Niedergerke, 1958). The low P_i level observed in nerves immersed in de-ionized sucrose (p. 402) might have resulted from removal of the last traces of calcium from the medium.

A few experiments were performed with other poisons. Application of azide (5 mM) or dinitrophenol (0·2 mM) at pH 6·8 caused appreciable break-down (65–100 %) of energy-rich phosphate over 1–2 hr. Dinitrophenol (0·2 mM), at pH 8·0 applied for 2 hr, only caused about 50 % of the energy-rich phosphate to break-down. These observations resemble those of Caldwell (1960) on squid nerve. Neither iodoacetate (2 mM) nor fluoride (5 mM) caused any detectable break-down over a 15 min period. The P_i level in stimulated nerves soaked in 10 K(Na)ASW containing iodoacetate or fluoride was higher than in 0 K(Na)ASW controls, suggesting that in these concentrations neither iodoacetate nor fluoride markedly inhibited the energy-rich phosphatase reaction. Although direct measurements of the effect of these poisons on the Na and K content of *Maia* nerve were not made, these observations are not opposed to those of Shanes & Berman (1955) who found that iodoacetate did not reduce the Na efflux from squid nerve and also Glynn's (1963) finding that 1 mM-iodoacetamide failed to inhibit the electric organ transport ATPase.

Changes in arginine level. Because of the rapid equilibration between the various phosphate fractions of the cell, it was not possible to identify the substrate for the energy-rich phosphatase activated during recovery. The main energy-rich phosphate compounds in *Maia* nerve are ATP and ArgP and from measurements of arginine and P_i in the same homogenate it was found that about two-thirds of the change in P_i following stimulation in 10 K(Na)ASW was accounted for by ArgP and the rest was presumably derived from ATP.

Changes in respiration rate. In intact muscle, evidence has been presented that the supply of ADP is the rate limiting factor in respiration (Chance & Connelly, 1957; Chance & Weber, 1963). If this is also true of nerve, an acceleration in the rate of energy-rich phosphate break-down ought, by providing more ADP, to stimulate respiration. This has been confirmed for desheathed frog nerve by Brink, Bronk, Carlson & Connelly (1952) and Connelly (1959, 1962). A stimulation of oxygen consumption was only observed where, from the present work, there should have been activation of an intracellular energy-rich phosphatase and hence increased supply of phosphate acceptor.

A few experiments were made to determine whether *Maia* nerve behaved

similarly. The oxygen consumption was not measured during stimulation, but only during the recovery period. The resting oxygen consumption in 10 K(Na)ASW at 20° C was 1·36 μl./g wet nerve per min. Meyerhof & Schulz (1929) give a value of 0·8 c.c./g dry nerve per hr at 16° C for the uptake of oxygen by unstimulated *Maia* nerve. Assuming that the nerve contains 88 % water (Chang, 1931) and that the Q_{10} is 3, this gives a value of 3·5 μl./g wet nerve per min at 20° C. This is considerably higher than in the present experiments; but the discrepancy may stem from some difference in the treatment of the fibres. Gerard (1932), presumably using *Libinia*, gives a value of 2·1 μl./g wet nerve per min at 22° C, which is close to the present results.

Stimulation in 10 K(Na)ASW and subsequent recovery in the same solution increased the oxygen consumption 2–3 times, which is also in agreement with other workers. The oxygen uptake was highest during the first 15 min and subsequently declined becoming indistinguishable from the resting rate after 30–40 min. No increase was seen in nerves stimulated in 10 K(Li)ASW or in nerves stimulated in 10 K(Na)ASW and left to recover in 0 K(Na)ASW. These results are in complete agreement with those of Connelly.

The steady state. At rest, with a constant intracellular P_i level, the rate of energy-rich phosphate break-down must equal the rate of resynthesis. The rate of break-down has been estimated from a single cyanide poisoning experiment to be 12·5 μg P released/g nerve per min and the rate of synthesis can be calculated from the oxygen consumption data. If a P/O ratio of 3 is assumed, and correcting for the extracellular space and difference in temperature (assuming a Q_{10} of 3), the resting oxygen uptake of 1·36 μl./g wet nerve per min at 20° C is equivalent to a value of 10·7 μg P released/g nerve per min at 16·5° C. This is surprisingly close to the observed value for the initial rate of P_i release. However, the latter rests on a single determination and it would seem more reasonable to take the energy-rich phosphatase activity which, from Fig. 6, is equivalent to the mean resting P_i level of 91 μg P/g nerve. This gives a mean resting rate of break-down of energy-rich phosphate of 12 μg P/g nerve per min. The similarity between the rates of synthesis and break-down is still striking.

A similar argument can be applied to the new steady state achieved after stimulation in 10 K(Na)ASW. The measured rate of P_i release following application of cyanide averaged 25 μg P released/g nerve per min and, making the same assumptions as for resting nerve, the average oxygen uptake after stimulation (3·6 μl./g wet nerve per min at 20° C) is equivalent to a rate of P_i utilization of 28 μg P/g nerve per min at 16·5° C. The two values are again in close agreement. These calculations support the view that in predominantly aerobic tissues the rate of oxygen consumption,

in many instances, is proportional to the rate of energy-rich phosphate break-down (Whittam & Willis, 1963).

The Na:~P ratio. In resting nerve the sodium content remained constant. Yet, addition of ouabain to the medium produced a net increase in internal sodium of 47 m-moles/kg nerve per hr. A similar, but smaller, net increase occurred on transferring the nerve to 0 K(Na)ASW (Table 5). If it is assumed that the action of these solutions is simply to inhibit the sodium pump, without altering the passive permeability to sodium, it follows that, under resting conditions, in order to keep pace with the rate of entry, 47 m-moles/kg nerve of sodium must have been extruded from the fibres every hour. If all the energy-rich phosphate consumed (12 μg P/g nerve per min) was used for this purpose, the calculated Na:~P ratio is 2·0. This is a minimal value as it is unlikely that all the energy-rich phosphate was used to pump out sodium. A measure of the energy-rich phosphate used under resting conditions for processes other than the sodium pump was obtained by first inhibiting the pump. The initial rate of P_i release following application of cyanide to an unstimulated nerve in 0 K(Na)ASW containing 1 mM ouabain was 3·2 μg P released/g nerve per min (Table 14); but this is based on a single determination. However, it indicates that three-quarters of the basal energy-rich phosphatase activity was used for pumping, giving a Na:~P ratio of 2·7. In other experiments, the values for the Pi content of unstimulated nerves in 0 K(Na)ASW averaged 65 μg P/g nerve, which is equivalent to a residual rate of hydrolysis of energy-rich phosphate of 6 μg P released/g nerve per min. This would mean that only half of the total energy-rich phosphatase was used in pumping out sodium and the Na:~P ratio should, accordingly, be increased to 4·0. The correct value for the ratio probably lies between 2·7 and 4·0.

The site of energy-rich phosphate break-down and P_i release. As the energy-rich phosphate is synthesized within the cell, the substrate-binding site of the enzyme must, presumably, also be available to the cell interior. The P_i released did not appear in the external medium, suggesting that the process of ATP break-down occurs inside the cell membrane. Evidence for the slow loss of P_i from *Maia* nerve was obtained by direct measurement of the P_i content of the bathing medium. The net loss of P_i, 2×10^{-3} pmole/cm^2 per sec, was more rapid than the Pi efflux from squid nerve (Caldwell *et al.* 1964); but was only 1 % of the P_i released per second by the intracellular energy-rich phosphatase under resting conditions.

A few experiments were also performed to test whether externally applied ATP was broken down by *Maia* nerve. Some hydrolysis occurred; but the quantity was small and very variable and amounted to only 2 % of the rate of energy-rich phosphate breakdown in a cyanide poisoned resting nerve. It was unaltered by stimulation and was unaffected by

ouabain; but was reversibly inhibited by fluoride (Table 15). The most likely cause of the rather large net loss of P_i and the break-down of externally applied ATP was the presence of a few damaged cells in the preparation; but the existence of an 'ecto-ATPase', similar to that described by Cummins & Hydén (1962) in glial cells, cannot be excluded.

TABLE 15. The loss of P_i from intact *Maia* nerve and the hydrolysis of externally applied ATP

Bathing solution	P_i release (μg P/g wet never per min)
10 K(Na)ASW	0·13 ± 0·02
10 K(Na)ASW + 3 mM-ATP	0·34 ± 0·08
0 K(Na)ASW + 3 mM-ATP	0·26
10 K(Na)ASW + 3 mM-ATP nerve stimulated throughout	0·27
10 K(Na)ASW + 1 mM ouabain + 3 mM ATP	0·35
10 K(Na)ASW + 5 mM fluoride + 3 mM-ATP	0·14

The ends of the nerve were held by forceps and only the middle portion of the nerve trunk dipped into the test fluid. The solution was gently agitated and P_i loss measured over 10 min periods. Temperature 17–18° C.

DISCUSSION

P_i level and the rate of energy-rich phosphate break-down

The main conclusion from these experiments is that intact crab nerve contains an enzyme system which can utilize energy-rich phosphate compounds but requires the presence of both internal sodium and external potassium for activity. When stimulated, the system accounts for a substantial fraction of the energy-rich phosphatase activity of the tissue and, under normal conditions, its operation is associated with a net outward movement of sodium and a net uptake of potassium. It seems likely that this sodium and potassium-sensitive energy-rich phosphatase forms part of the sodium-pump mechanism. During periods of intense pumping activity, when the energy-rich phosphatase is activated, the concentration of P_i in the cell is raised and the oxygen consumption increased.

These results bear a close resemblance to those of Gore & McIlwain (1952) on slices of mammalian cerebral cortex. In their experiments, electrical stimulation of the slices in media containing sodium resulted in an increase in respiration, a rise in P_i and a fall in creatine phosphate. Reducing the calcium or increasing the potassium (or ammonium) content of the bathing fluid had a similar effect. The main problem in working with slices is that the cells tend to be somewhat damaged and have a high cation permeability. This makes it difficult to decide whether the site of action of an ion is inside or outside a cell. However, it seems likely that the present experiments and those of Gore & McIlwain have an essentially similar explanation. Changes in P_i content during pumping have

also been observed in intact brain (Dawson & Richter, 1950; Shapot, 1957); myelinated nerve (Gerard & Tupikova, 1939); mammalian unmyelinated nerve (Greengard & Straub, 1959); the red cell (Laris & Letchworth, 1962); yeast (Rothstein, 1946) and frog muscle (Conway, 1963).

Substrate and site of energy-rich phosphate break-down

Although the substrate of the energy-rich phosphatase is not known, it is most likely to be either ATP or ArgP. In experiments with perfused squid axons, the break-down of internally perfused ATP, in the presence of potassium and magnesium, was greatly stimulated by addition of sodium to the perfusion fluid and was inhibited by external application of ouabain (Baker & Shaw, 1965). By analogy with these experiments, it seems probable that ATP is the substrate for the ouabain-sensitive energy-rich phosphatase of *Maia* nerve. There was a small net loss of P_i from the nerve and some break-down of externally applied ATP; but these were unaffected by inhibition of the sodium pump and probably resulted from damaged fibres. It is concluded that the site of energy-rich phosphate break-down is internal to the cell membrane, as is the point of activation by sodium; but potassium is only effective when applied externally.

As the mesaxon and space between the axon and Schwann cell are both filled with extracellular fluid, the main structures which are bathed on one side by intracellular fluid and on the other by the external medium are the axolemma and Schwann cell membranes. It seems likely that the machinery of the sodium pump is located within these membranes. In those instances where the morphological origin of an isolated membrane fraction is known, ouabain-sensitive ATPases have been found in the surface membrane of neurones (Cummins & Hydén, 1962) and the external membrane of synaptosomes (Hosie, 1964). Histochemical evidence has also been obtained for the location of a ouabain-sensitive ATPase at the surface of nerve cells (McClurkin, 1964). The accumulation of mitochondria immediately below the axolemma of *Maia* nerve also fits with a requirement for ATP at the cell surface.

Activation by ions and inhibition by ouabain

Thallous, rubidium and to some extent caesium and ammonium ions were able to substitute for potassium in the external medium; but lithium was unable to replace internal sodium. External application of ouabain inhibited both the sodium pump and the energy-rich phosphatase.

The selectivity of the sodium pump against lithium is in agreement with other work on nerve (Connelly, 1959; Ritchie & Straub, 1957) and muscle (Keynes & Swan, 1959); but the red cell is less selective (Whittam & Ager, 1964). Although the selectivity of the internal activation site of the pump

is markedly different from that of the sodium channel of the action potential, a similar difference between pump and electrical properties is not found for the external activation site. The order of effectiveness of various ions in replacing potassium in the external solution is the same as their ability to depolarize *Maia* nerve (Wilbrandt, 1937; Baker, 1964). The importance of this observation is not clear. The depolarization attendant on transferring a nerve from 0 K(Na)ASW to 10 K(Na)ASW is not large and Hodgkin & Keynes (1954) showed that electrical hyperpolarization, by up to 28 mV, of a nerve immersed in 10 K(Na)ASW did not affect the sodium efflux, which indicates that at low potassium concentrations depolarization *per se* is not the activator of the pump. But the similarity in selectivity for monovalent cations between the resting-potential mechanism and the potassium-activation site of the sodium pump suggests that these processes may share certain features in common.

Very little residual ouabain-sensitive energy-rich phosphatase was detected in nerves which had been vigorously agitated in 0 K(Na)ASW. This observation is interesting for two reasons. It indicates that external sodium is unable to replace potassium, to any extent, at the outside activation site of the pump and, also, if ouabain-sensitive exchange diffusion of sodium occurs in the absence of external potassium, as happens in the red cell (Glynn, 1957), this process does not effect much net break-down of energy-rich phosphate.

In the presence of 460 mM external sodium, the ouabain-sensitive energy-rich phosphatase of stimulated nerve was half maximally activated by an external potassium concentration of 9 mM. The rate of ouabain-sensitive energy-rich phosphate break-down in unstimulated nerves immersed in 100 K(Na)ASW was about half that of stimulated nerves immersed in the same solution. This indicates that under resting conditions the internal sodium-requiring site is about half maximally activated, defining maximum in this instance as the highest rate attainable following massive stimulation. Thus, the energy-rich phosphatase of unstimulated nerve immersed in 10 K(Na)ASW should be about half maximally activated with respect to both internal sodium and external potassium. Under these conditions, the observed rate of P_i release by ouabain-sensitive routes varied between 1/4 and 1/6 of that seen in stimulated nerves immersed in 100 K(Na)ASW.

The Na:~P ratio and efficiency of the pump

Intact nerves immersed in 10 K(Na)ASW maintained a constant resting sodium content of about 30 m-mole/kg nerve. Under these conditions there was a net extrusion of sodium against a steep electrochemical gradient and the Na:~P ratio determined for this process varied between

2·7 and 4·0. This range is in good agreement with Na: ~ P ratios calculated for other tissues. A Na: ~ P ratio close to 3 has been demonstrated for both 'downhill' and 'on-the-level' movement of sodium in the red blood cell (Glynn, 1962; Sen & Post, 1964) and Bonting & Caravaggio (1963) have derived Na: ~ P ratios of approximately 3 for a variety of tissues by comparing published data for the rates of sodium movement with their own values for the ouabain-sensitive ATPase activity in homogenates of the same tissue. A Na: ~ P ratio of 3 was also obtained from the extra oxygen consumption associated with sodium transport across frog skin and toad bladder. These calculations assumed a P/O ratio of 3 (Zerahn, 1956; Leaf, Page & Anderson, 1959). Caldwell *et al.* (1960) obtained a value of 0·7 for the Na: ~ P ratio in squid nerve but, as they pointed out, no allowance was made for break-down of ATP by processes other than the pump and, if these are taken into account, the Na: ~ P ratio is close to 3 (Baker & Shaw, 1965).

The electrochemical potential difference against which the sodium is moved in intact nerve immersed in sea water is about 120 mV and a process extruding three sodium ions per energy-rich phosphate bond split must be reasonably efficient in converting chemical energy into work. Depending on the value taken for the free energy of hydrolysis of the terminal pyrophosphate bond of ATP, the efficiency of the sodium pump in utilizing chemical energy is about 80 %.

Comparison of the energy-rich phosphatase of intact nerve with Skou's enzyme

Similarity between Maia and Carcinus. The properties of the ouabain-sensitive phosphatase of intact *Maia* nerve are strikingly similar to those of the ATPase isolated by Skou from *Carcinus* nerve. In a single experiment, the resting P_i content of *Carcinus* nerve was increased following stimulation and depressed after soaking in 0 K(Na)ASW or 10 K(Na)ASW containing 1 mM ouabain. These observations, together with the similarity between *Carcinus* and *Maia* nerves both in structure and resting Na and K content, suggest that the sodium pump mechanism in *Carcinus* and *Maia* nerves is very similar.

Site of origin of ATPase. Although Skou's preparation has not been examined in the electron microscope, it was obtained by a differential centrifugation routine which is well known to give a membrane-rich fraction. In the case of crab nerve, this would be largely cell membrane in origin (p. 387). The ouabain-sensitive phosphatase of intact nerve is also located in the surface membrane.

Ion activation. The preparation of Skou was largely an ATPase, the preferred substrate being Mg-ATP. Both sodium and potassium ions were

required for maximum activity. In a broken-cell preparation, added ions will have access to both sides of the membrane and this must be borne in mind when interpreting the results. At constant sodium concentration, raising the potassium concentration first activated and then inhibited the enzyme. This was interpreted as a displacement of sodium from its activating site (internal in the living cell) by potassium. The data fitted a 1:1 competition between Na and K with a Michaelis constant (K_m) for sodium of 1·4 mM and an inhibitor constant (K_i) for potassium of 9·0 mM. For simple competitive inhibition, Dixon & Webb (1964) give the relation between K_m, K_i and the effective Michaelis constant (K_p) as

$$K_p = K_m(1+i/K_i);$$

where i is the concentration of inhibitor. Applying Skou's data to intact resting nerve, with an internal potassium concentration of 200 mM, the effective Michaelis constant for sodium should be 33 mM. As the internal sodium concentration is about 30 mM, the sodium binding site of the phosphatase should be about half saturated with sodium. This prediction has been confirmed.

At constant potassium concentration, Skou observed that raising the sodium concentration increased the activity of the ATPase to a maximum value. No subsequent inhibition was seen except in nominally K-free solution; but Skou did not use sodium concentrations as high as those which normally bathe intact nerve. In other ouabain-sensitive ATPase preparations, at high sodium concentrations, sodium appears to displace potassium from its activating site (Post *et al.* 1960; Whittam & Wheeler, 1961; Whittam & Ager, 1962; Green & Taylor, 1964). In the presence of 100 mM sodium, Skou's enzyme was half maximally activated by a potassium concentration of 1·8 mM: whereas intact nerve immersed in sea water containing 460 mM sodium required 9 mM potassium for half maximum activation. This difference is readily explained if external sodium is inhibitory. Thus, if K_m for potassium is 0·8 mM and K_i for sodium is 50 mM, the effective Michaelis constant (K_p) for potassium will be 2·4 mM in the presence of 100 mM sodium and 8·2 mM in the presence of 460 mM sodium. These values are close to those observed.

The high P_i level and reduction in potassium sensitivity of stimulated *Maia* nerves immersed in Na-free solutions can be explained in terms of a sodium-potassium competition at the potassium activation site of the pump; but the interpretation of these experiments is complicated (see p. 409). However, recent experiments with *Libinia* nerve have produced clear-cut evidence for sodium-potassium competition (P. F. Baker & C. M. Connelly, unpublished). The extra oxygen consumed during pumping was measured and the affinity of this process for external potassium in-

creased as the external sodium concentration decreased. K_i for external sodium was 60 mM and K_m for potassium was 1 mM. Numerically similar constants have been obtained for the red cell ATPase (Whittam & Ager, 1964).

Skou observed that in nominally K-free solution, sodium activated and then inhibited the ATPase. This may have resulted from some potassium contamination which enabled activation at low sodium concentrations, followed by inhibition as the potassium was displaced from its activation site by sodium; but other explanations cannot be excluded (Baker 1964).

Both the energy-rich phosphatase and Skou's enzyme were activated by cations other than potassium. The effectiveness of thallium in activating the ATPase has not been examined, but where comparison is possible, the action of different cations on the two phosphatases is very similar. The only marked difference is seen with lithium which has some K-like action on the ATPase but none on the intact nerve. As isolation of the ATPase involves disruption and depolarization of the axolemma, it would be surprising if the properties of the isolated enzyme were identical in all respects, with those in the intact nerve. Activation by lithium might be an example of such a change in ion selectivity resulting from the isolation procedure.

The Na: ~P ratio. Transport of three sodium ions per energy-rich phosphate bond cleaved suggests that the relation between ATP break-down and sodium concentration ought not to be linear at low sodium concentrations. This has not really been looked for in the isolated ATPase, although at high potassium concentrations Skou observed that the curve relating the rate of ATP break-down to sodium concentration was S-shaped, which is the shape expected if more than one sodium ion is required to activate the ATPase.

Inhibition by ouabain. Both the energy-rich phosphatases of intact nerve and Skou's enzyme were inhibited by ouabain; but, compared with the red cell, crab nerve is very resistant to this inhibitor.

Conclusions. It is concluded that the ouabain-sensitive phosphatase of *Maia* nerve and the ouabain-sensitive ATPase isolated by Skou (1957) from *Carcinus* nerve are identical and form part of the sodium pumping mechanism in intact crab nerve. It is not clear whether the ions activating the phosphatase are also the ions pumped or whether other binding sites are required for pumping to occur.

SUMMARY

1. A brief description is given of the structure of the walking leg nerves of the crabs *Maia squinado* and *Carcinus maenas*. In a freshly blotted nerve trunk, the fraction of the wet weight occupied by extracellular fluid is 0·3.

2. The main phosphate compounds present in *Maia* nerve were ATP, ArgP and P_i. Free arginine was also present. Poisoning the nerves with cyanide resulted in a rapid break-down of ATP and ArgP and an increase in the level of arginine, P_i and AMP.

3. The Na and K contents of the nerve trunk were measured by flame photometry. Internal Na increased and K decreased following electrical stimulation of the nerves or inhibition of the sodium pump by removal of external potassium or addition of ouabain or cyanide to the medium. These changes were reversed on returning the nerves to sea water.

4. Experiments are described which show that the P_i level was elevated during periods of intense pumping activity. Both pump and P_i elevation required the presence simultaneously of internal Na and external K and both were inhibited by external application of ouabain.

5. Measurement of the initial rate of P_i release following application of cyanide showed that, for nerves immersed in Na-artificial sea waters, changes in the P_i level reflected changes in the rate of energy-rich phosphate break-down in the tissue. In a limited number of experiments, the rate of phosphate break-down increased in proportion to the square of the P_i level.

6. In both resting and stimulated nerves, the measured rate of energy-rich phosphate break-down was almost identical with the rate of resynthesis calculated from measurements of oxygen uptake.

7. The K-sensitivity of the changes in P_i was little affected by a wide range of external pH, by the nature of the external anion or by variation in the concentration of external Ca or Mg.

8. In Na-artificial sea water the ouabain-sensitive phosphatase was half-maximally activated by 9·3 mM-K. Addition of ouabain increased the apparent Michaelis constant for external K and also decreased the maximum velocity of the phosphatase.

9. External K could be replaced by thallous, Rb and to some extent Cs and NH_4 ions and the order of effectiveness of these ions is activating the pump was the same as their action in depolarizing *Maia* nerve.

10. Between 2·7 and 4 Na ions were extruded for each energy-rich phosphate bond split.

11. The loss of P_i into the external medium was very small, as was the break-down of externally applied ATP.

12. The present experiments show that the axolemma and possibly the Schwann cell membranes of intact crab nerve contain an energy-rich phosphatase, the properties of which are closely similar to those of the ouabain-sensitive (Na + K)-activated ATPase isolated by Skou from crab nerve. This similarity is examined in detail and it is concluded that Skou's enzyme forms part of the sodium pump mechanisn in intact nerve.

I wish to thank the Director and staff of the Laboratory of the Marine Biological Association, Plymouth for their helpfulness throughout the course of this work.

REFERENCES

ABBOTT, B. C., HILL, A. V. & HOWARTH, J. V. (1958). The positive and negative heat production associated with a nerve impulse. *Proc. Roy. Soc.* B, **148**, 149–187.

ADRIAN, R. H. & SLAYMAN, C. L. (1964). Pumped movements of K and Rb in frog muscle. *J. Physiol.* **175**, 49*P*.

BAKER, P. F. (1963). The relationship between phosphorus metabolism and the sodium pump in intact crab nerve. *Biochim. biophys. Acta*, **75**, 287–289.

BAKER, P. F. (1964). An efflux of ninhydrin-positive material associated with the operation of the Na^+ pump in intact crab nerve immersed in Na^+-free solutions. *Biochim. biophys. Acta*, **88**, 458–460.

BAKER, P. F. (1965). A method for the location of extracellular space in crab nerve. *J Physiol.* **180**, 439–447.

BAKER, P. F., HODGKIN, A. L. & SHAW, T. I. (1962). Replacement of the axoplasm of giant nerve fibres with artificial solutions. *J. Physiol.* **164**, 330–354.

BAKER, P. F. & SHAW, T. I. (1965). A comparison of the phosphorus metabolism of intact squid nerve with that of the isolated axoplasm and sheath. *J. Physiol.* **180**, 424–438.

BERENBLUM, I. & CHAIN, E. (1938). An improved method for the colorimetric determination of phosphate. *Biochem. J.* **32**, 295–298.

BERESINA, M. & FENG, T. P. (1933). The heat production of crustacean nerve. *J. Physiol.* **77**, 111–138.

BONTING, S. L. & CARAVAGGIO, L. L. (1963). Studies on sodium-potassium-activated adenosinetriphosphatase V. Correlation of enzyme activity with cation flux in six tissues. *Arch. Biochem. Biophys.* **101**, 37–46.

BONTING, S. L., CARAVAGGIO, L. L. & HAWKINS, N. M. (1963). Studies on sodium-potassium-activated adenosinetriphosphatase VI. Its role in cation transport in the lens of cat, calf and rabbit. *Arch. Biochem. Biophys.* **101**, 47–55.

BRINK, F., BRONK, D. W., CARLSON, F. D. & CONNELLY, C. M. (1952). The oxygen uptake of active axons. In *Cold Spr. Harb. Symp. on Quant. Biol. XVII. The Neuron*, pp. 53–67.

BRINLEY, F. J. (1964). Ionic contents and resting fluxes in isolated lobster giant axons. *Abstracts Amer. Biophys. Soc.* Feb. 1964.

CALDWELL, P. C. (1958). Studies on the internal pH of large muscle and nerve fibres. *J. Physiol.* **142**, 22–62.

CALDWELL, P. C. (1960). The phosphorus metabolism of squid axons and its relationship to the active transport of sodium. *J. Physiol.* **152**, 545–560.

CALDWELL, P. C., HODGKIN, A. L., KEYNES, R. D. & SHAW, T. I. (1960). The effects of injecting 'energy-rich' phosphate compounds on the active transport of ions in the giant axons of *Loligo*. *J. Physiol.* **152**, 561–590.

CALDWELL, P. C., HODGKIN, A. L., KEYNES, R. D. & SHAW, T. I. (1964). The rate of formation and turnover of phosphorus compounds in squid giant axons. *J. Physiol.* **171**, 119–131.

CALDWELL, P. C. & KEYNES, R. D. (1959). The effect of ouabain on the efflux of sodium from a squid giant axon. *J. Physiol.* **148**, 8*P*.

CALDWELL, P. C. & WALSTER, G. E. (1962). The turnover of injected ^{32}P-labelled ATP in crab muscle. *J. Physiol.* **163**, 15*P*.

CHANCE, B. & CONNELLY, C. M. (1957). A method for the estimation of the increase in concentration of adenosinediphosphate in muscle sarcosomes following acontraction. *Nature, Lond.*, **179**, 1235–1237.

CHANCE, B. & WEBER, A. (1963). The steady state of cytochrome *b* during rest and after contraction in frog sartorius. *J. Physiol.* **169**, 263–277.

CHANCE, B. & WILLIAMS, G. R. (1955*a*). Respiratory enzymes in oxidative phosphorylation. I. Kinetics of oxygen utilization. *J. biol. Chem.* **217**, 383–393.

CHANCE, B. & WILLIAMS, G. R. (1955*b*). Respiratory enzymes in oxidative phosphorylation. II. Difference spectra. *J. biol. Chem.* **217**, 395–407.

CHANG, T. H. (1931). Metabolism of peripheral leg nerves and the ganglionated nerve cord of the lobster. *Proc. Soc. exp. Biol., N.Y.* **28**, 954–955.

CONNELLY, C. M. (1959). Recovery processes and metabolism of nerve. *Rev. mod. Physiol.* **31**, 475–484.

CONNELLY, C. M. (1962). Metabolic and electrochemical events associated with recovery from activity. *XXII Int. Cong. Physiol. Leiden*, vol. 1, 600–602.

CONWAY, E. J. (1963). The significance of various factors including lactic dehydrogenase on the active transport of sodium ions in skeletal muscle. *Nature, Lond.*, **198**, 760–763.

CUMMINS, J. & HYDÉN, H. (1962). Adenosinetriphosphate levels and adenosinetriphosphatases in neurons, glia and neuronal membranes of the vestibular nucleus. *Biochim. biophys. Acta*, **60**, 271–283.

DAWSON, R. M. C. & RICHTER, D. (1950). Effect of stimulation on the phosphate esters of the brain. *Amer. J. Physiol.* **160**, 203–211.

DIXON, M. & WEBB, E. (1964). *Enzymes*. London: Longmans, Green and Co.

ENNOR, A. H. & STOCKEN, L. A. (1950). The determination of inorganic phosphate in the presence of labile organo-phosphorus compounds. *Austr. J. exp. Biol. med. Sci.* **28**, 647–655.

FURUSAWA, K. (1929). The depolarization of crustacean nerve by stimulation or oxygen want. *J. Physiol.* **67**, 325–342.

GERARD, R. W. (1932). Nerve metabolism. *Physiol. Rev.* **12**, 469–592.

GERARD, R. W. & TUPIKOVA, N. (1938). Creatine in nerve, muscle and brain. *J. cell. comp. Physiol.* **12**, 325–360.

GERARD, R. W. & TUPIKOVA, N. (1939). Nerve and muscle phosphates. *J. cell. comp. Physiol.* **13**, 1–13.

GEREN, B. B. & SCHMITT, F. O. (1954). The structure of the Schwann cell and its relation to the axon in certain invertebrate nerve fibres. *Proc. nat. Acad. Sci., Wash.*, **40**, 863–870.

GERLACH, E. & DÖRING, H. (1955). Schnell-Identifizierung von Adenin, Adenosin, Adenosinphosphosäuren und Pyridinnukleotiden in Papierchromatogrammen. *Naturwissenschaften*, **42**, 344.

GLYNN, I. M. (1957). The action of cardiac glycosides on sodium and potassium movement in human red cells. *J. Physiol.* **136**, 148–173.

GLYNN, I. M. (1962). Activation of adenosinetriphosphatase activity in a cell membrane by external potassium and internal sodium. *J. Physiol.* **160**, 18*P*.

GLYNN, I. M. (1963). 'Transport adenosinetriphosphatase' in electric organ. The relation between ion transport and oxidative phosphorylation. *J. Physiol.* **169**, 452–465.

GORE, M. B. R. & MCILWAIN, H. (1952). Effects of some inorganic salts on the metabolic response of sections of mammalian cerebral cortex to electrical stimulation. *J. Physiol.* **117**, 471–483.

GREEN, A. L. & TAYLOR, C. B. (1964). Kinetics of (Na+K)-stimulated ATPase of rabbit kidney microsomes. *Biochem. biophys. res. Comm.* **14**, 118–123.

GREENGARD, P. & STRAUB, R. W. (1959). Effect of frequency of stimulation on the concentration of intermediary metabolites in mammalian non-myelinated fibres. *J. Physiol.* **148**, 353–361.

HASHIMOTO, T. & YOSHIKAWA, H. (1963). Adenosinetriphosphate-dependent transport of rubidium into erythrocyte ghost. *Biochim. biophys. Acta*, **75**, 135–136.

HOBSON, G. E. & REES, K. R. (1955). The annelid phosphagens. *Biochem. J.* **61**, 549–552.

HODGKIN, A. L. (1947). The membrane resistance of a non-medullated nerve fibre. *J. Physiol.* **106**, 305–318.

HODGKIN, A. L. (1957). Ionic movements and electrical activity in giant nerve fibres. *Proc. Roy. Soc.* B, **148**, 1–37.

HODGKIN, A. L. & HUXLEY, A. F. (1947). Potassium leakage from an active nerve fibre. *J. Physiol.* **106**, 341–367.

HODGKIN, A. L. & KEYNES, R. D. (1954). Movements of cations during recovery in nerve. *Symp. Soc. exp. Biol.* **8**, 423–437.

HODGKIN, A. L. & KEYNES, R. D. (1955). Active transport of cations in giant axons from *Sepia* and *Loligo*. *J. Physiol.* **128**, 28–60.

HOSIE, R. J. A. (1964). The adenosinetriphosphatase activity of cerebral membranes of known morphological origin. *Biochem. J.* **93**, 13*P*.

KEYNES, R. D. (1951). The ionic movements during nervous activity. *J. Physiol.* **114**, 119–150.

KEYNES, R. D. & LEWIS, P. R. (1951). The resting exchange of radioactive potassium in crab nerve. *J. Physiol.* **113**, 73–98.

KEYNES, R. D. & SWAN, R. C. (1959). The permeability of frog muscle fibres to lithium ions. *J. Physiol.* **147**, 626–638.

LARIS, P. C. & LETCHWORTH, P. E. (1962). Cation influence on inorganic phosphate production in human erythrocytes. *J. cell. comp. Physiol.* **60**, 229–234.

LEAF, A., PAGE, L. B. & ANDERSON, J. (1959). Respiration and active sodium transport in isolated toad bladder. *J. biol. Chem.* **234**, 1625–1629.

LÜTTGAU, H. C. & NIEDERGERKE, R. (1958). The antagonism between Ca and Na ions on the frog's heart. *J. Physiol.* **143**, 486–505.

MCCLURKIN, I. T. (1964). A method for the cytochemical demonstration of sodium-activated adenosine triphosphatase. *J. Histochem. Cytochem.* **12**, 654–658.

MAIZELS, M. (1951). Factors in the active transport of cations. *J. Physiol.* **112**, 59–83.

MEYERHOF, O. & SCHULZ, W. (1929). Über die Atmung des marklosen Nerven. *Biochem. Z.* **206**, 158–170.

MULLINS, L. J. & MOORE, R. D. (1959). The movement of thallium ions in muscle. *J. gen. Physiol.* **43**, 759–773.

OBERHOLZER, R. I. H., BRINK, F. & BRONK, D. W. (1951). Effect of various potassium concentrations on resting respiration of nerve. *Amer. J. Physiol.* **167**, 813–814.

PAGE, E., GOERKE, R. J. & STORM, S. R. (1964). Cat heart muscles *in vitro* IV. Inhibition of transport in quiescent muscles. *J. gen. Physiol.* **47**, 531–543.

POST, R. L., MERRITT, C. R., KINSOLVING, C. R. & ALBRIGHT, C. D. (1960). Membrane adenosinetriphosphatase as a participant in the active transport of sodium and potassium in the human erythrocyte. *J. biol. Chem.* **235**, 1796–1802.

RITCHIE, J. M. & STRAUB, R. W. (1957). The hyperpolarization which follows activity in mammalian non-medullated fibres. *J. Physiol.* **136**, 80–97.

ROSENBERG, H., ENNOR, A. H. & MORRISON, J. F. (1956). The estimation of arginine. *Biochem. J.* **63**, 153–159.

ROTHSTEIN, A. (1946). Phosphorylation in living yeast cells. *J. cell. comp. Physiol.* **28**, 221–230.

SCHREIBER, S. S. (1956). Potassium and sodium exchange in the working frog heart. Effects of overwork, external concentrations of potassium and ouabain. *Amer. J. Physiol.* **185**, 337–347.

SEN, A. K. & POST, R. L. (1964). Stoichiometry and localization of adenosinetriphosphate-dependent sodium and potassium transport in the erythrocyte. *J. biol. Chem.* **239**, 345–352.

SHANES, A. M. & BERMAN, M. D. (1955). Kinetics of ion movement in the squid giant axon. *J. gen. Physiol.* **39**, 279–300.

SHANES, A. M. & HOPKINS, H. S. (1948). The effect of potassium on 'resting potential' and respiration of crab nerve. *J. Neurophysiol.* **11**, 331–342.

SHAPOT, V. S. (1957). Brain metabolism in relation to the functional state of the central nervous system. In *Metabolism of the nervous system*, ed. D. RICHTER, pp. 257–262. New York: Pergamon Press.

SKOU, J. C. (1957). The influence of some cations on an adenosine-triphosphatase from peripheral nerves. *Biochim. biophys. Acta*, **23**, 394–401.

SKOU, J. C. (1960). Further investigations on a Mg^{++}, Na^{+}-activated adenosinetriphosphatase, possibly related to the active transport of Na^{+} and K^{+} across the nerve cell membrane. *Biochim. biophys. Acta*, **42**, 6–23.

VILLEGAS, G. M. & VILLEGAS, R. (1964). Extracellular pathways in the peripheral nerve fibres: Schwann-cell-layer permeability to thorium dioxide. *Biochim. biophys. Acta*, **88**, 231–233.

VILLEGAS, R., GIMENEZ, M. & VILLEGAS, L. (1962). The Schwann-cell electrical potential in the squid nerve. *Biochim. biophys. Acta*, **62**, 610–612.

WHITTAM, R. (1962*a*). The dependence of the respiration of brain cortex on active cation transport. *Biochem. J.* **82**, 205–212.

WHITTAM, R. (1962*b*). The asymmetrical stimulation of a membrane adenosinetriphosphatase in relation to active cation transport. *Biochem. J.* **84**, 110–118.

WHITTAM, R. & AGER, M. E. (1962). Dual effects of sodium ions on an erythrocyte membrane adenosinetriphosphatase. *Biochim. biophys. Acta*, **65**, 383–385.

WHITTAM, R. & AGER, M. E. (1964). Vectorial aspects of adenosinetriphosphatase activity in erythrocyte membranes. *Biochem. J.* **93**, 337–348.

WHITTAM, R. & WHEELER, K. P. (1961). The sensitivity of a kidney ATPase to ouabain and to sodium and potassium. *Biochim. biophys. Acta*, **51**, 622–624.

WHITTAM, R. & WILLIS, J. S. (1963). Ion movements and oxygen consumption in kidney cortex slices. *J. Physiol.* **168**, 158–177.

WILBRANDT, W. (1937). The effect of organic ions on the membrane potential of nerves. *J. gen. Physiol.* **20**, 519–541.

ZERAHN, K. (1956). Oxygen uptake and active sodium transport in the isolated and short circuited frog skin. *Acta. physiol. Scand.* **36**, 300–318.