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SOME PROPERTIES OF THE EXTERNAL ACTIVATION SITE OF THE SODIUM PUMP IN CRAB NERVE

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SUMMARY

1. Methods are described for using the changes in respiration of intact *Libinia* nerve to follow the rate of energy utilization by the sodium pump in this tissue.

2. Short tetani in 10 K(Na)ASW (artificial sea water in which Na is the major cation and the potassium concentration is 10 mM) increased the oxygen uptake which then declined exponentially. From the net influx of Na during the tetanus and the associated oxygen uptake, values between 1·9 and 3·4 were calculated for the Na: ~ P ratio. After longer tetani, the recovery curve was S-shaped.

3. The pump was activated by potassium ions in the external medium and this activation was competitively inhibited by external sodium ions. The data are consistent with a Michaelis constant (K_m) for external potassium of 1 mM and an inhibitor constant (K_i) for external sodium of 60 mM.

4. In activating the pump, K could be replaced by Tl^+, Rb, NH_4 and Cs ions; but, of the monovalent ions tested, sodium seemed to be unique in its inhibitory action.

5. In sea waters containing 460 mM-Na, ouabain behaved like a mixed inhibitor of the pump, reducing both the maximum velocity and the apparent affinity for external potassium. At a given ouabain concentration, reducing the sodium content of the medium was without effect on the maximum rate of pumping; but the apparent affinity for potassium increased more steeply than in a ouabain-free solution.

6. The rate of energy utilization associated with pumping was unaffected by inclusion of quite high concentrations of sulphydryl-blocking agents in the external medium.

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INTRODUCTION

Sodium can be extruded from crab nerves against a steep electrochemical gradient. This process requires metabolic energy and appears to be associated with the operation of an ATP-utilizing system located in the cell membrane. In order to activate the enzyme system, both internal sodium and external potassium must be present. Inclusion of ouabain in the external medium causes inhibition. In intact nerve, changes in the rate of energy utilization by the pump can be followed either by assaying for orthophosphate (Baker, 1965) or by measuring the rate of oxygen consumption (Brink, Bronk, Carlson & Connelly, 1952; Connelly, 1959, 1962; Baker, 1965). The latter method is particularly useful because oxygen consumption can be monitored on the same nerve during exposure to a variety of conditions, whereas each measurement of orthophosphate necessitates sacrificing a fresh nerve.

The use of oxygen uptake as a measure of energy utilization by the pump rests on the assumption that all the ATP broken down during pumping is resynthesized by oxidative phosphorylation. This need not necessarily be the case; but in crab nerve there is good evidence that oxidative phosphorylation is very much more important than glycolysis (Baker, 1965). A drawback to using oxygen uptake as an index of pumping activity is that not all of the oxygen uptake is associated with pumping. For instance, in resting nerve the respiration is only reduced to about half following complete inhibition of the pump by immersion of the nerve in K-free sea water containing ouabain. Thus, in order to obtain quantitative results on the oxygen uptake associated with pumping, a correction must always be made for the residual respiration due to processes other than the sodium pump. Fortunately, this basal respiration is relatively easy to measure and it is possible to follow very small changes in the activity of the sodium pump. This approach has been directed, in particular, towards analysing the interactions between sodium, potassium and ouabain at the external (potassium) activation site of the sodium pump.

METHODS

Material

Nerves were normally obtained from the claws of the spider crab *Libinia emarginata*, although in a few experiments walking leg nerves were also used. The animals were collected at Woods Hole and kept in an aquarium at the Rockefeller University until required. Although the animals survived well in captivity, they were normally used within four weeks of arrival.

Experimental procedure

Dissection of the nerve. The nerve was withdrawn from a severed claw by the method of Furusawa (1929). Throughout the dissection the isolated limb was kept immersed in sea

water. Nerves obtained in this way were usually too large for immediate use in the respirometer; but, as the nerve is made up of a number of discrete fibre bundles, it was an easy matter to split the nerve longitudinally dissecting away groups of fibre bundles until a preparation of suitable size was obtained. In general, the wet weight of the nerve used was 2–4 mg/cm. Before insertion into the respirometer, the nerve was attached to a fine glass rod. This facilitated handling of the nerve and also ensured that the length of nerve in the

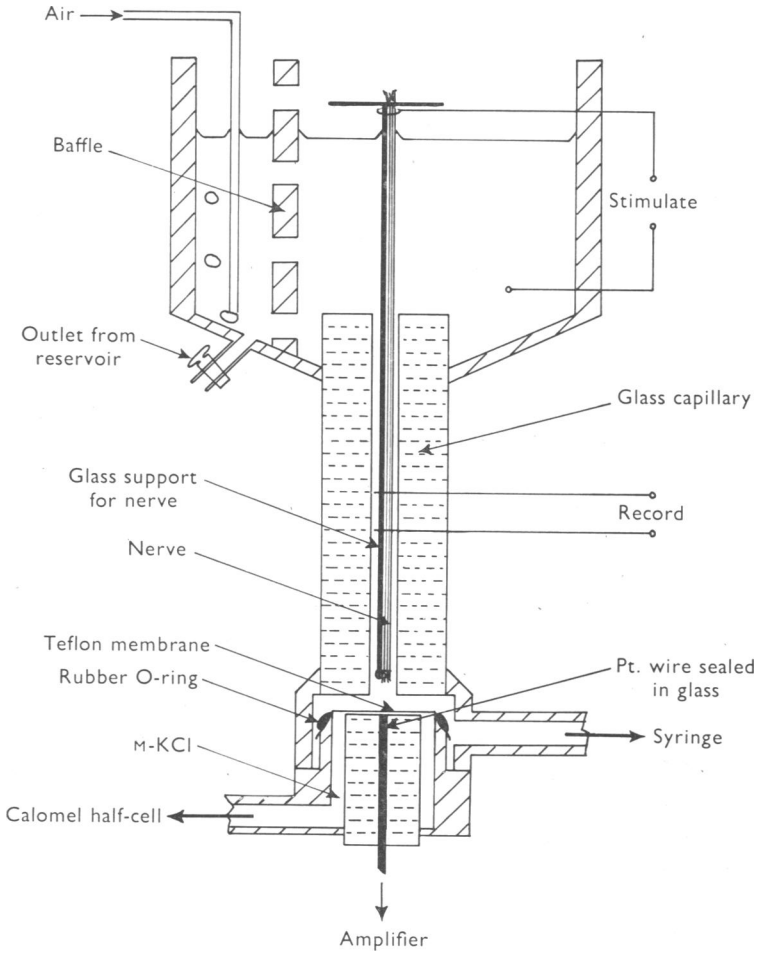

Fig. 1. Diagram showing the main features of the respirometer used in the present experiments. The drawing is not to scale.

capillary remained constant (Fig. 1). Once in position, the nerve was tested electrically and nerves which failed to give an action potential were discarded. At the end of the experiment, that part of the nerve which was within the capillary was removed from the support, carefully blotted on Whatman No. 1 filter paper and rapidly weighed.

The respirometer. This is illustrated in Fig. 1. It is the same apparatus as was used by Connelly in earlier work. The nerve is inserted into a fine capillary through which aerated fluid is drawn at a constant rate (281 mm^3/hr) by means of a motor-driven syringe. The

oxygen content of the fluid leaving the capillary is monitored by a Teflon-covered oxygen electrode (Clark, 1956). Provided the electrode is functioning properly, the electrode current is proportional to the concentration of oxygen in the flowing solution. Used in this way, the respirometer measures the total oxygen uptake of that portion of the nerve within the capillary.

Once in position, the electrode may have a life of many weeks during which time its operating characteristics remain extremely constant. The great stability and high sensitivity of the electrode make it very suitable for measuring small changes in respiration, provided the following conditions are fulfilled: (1) the syringe must be precisely bored; (2) the length of nerve in the capillary (in practice 22 mm) must be constant; (3) the nerve must not move about within the capillary. Movement of the nerve, owing to aeration of the reservoir, causes mixing of the fluid in the capillary with that in the reservoir, thus altering the effective length of nerve in the capillary. Movement can be much reduced by inserting a baffle into the reservoir (Fig. 1).

In the form used, the respirometer has three main drawbacks. (1) The time for fluid to pass through the capillary is between 5 and 7 min (depending on the size of nerve) and this makes it impossible to switch rapidly from one solution to another. A complete change of solution takes about 20 min. (2) As the lower end of the nerve receives fluid which has been in contact for some minutes with the upper end it is impossible to expose all the nerve to a completely K-free solution. (3) One end of the nerve is in the capillary. This means that the respiration of the damaged and depolarized ends of the axons is measured with that of the intact portion of nerve. Although this might represent a major source of error, there was no experimental evidence to suggest that it did.

Perhaps a better design for a respirometer of this type would be a T-shape with the nerve threaded through the stroke of the T and the oxygen electrode in the upright. In such a design, flow time and potassium accumulation would be reduced and both ends of the nerve would be outside the region under investigation.

Nerves were often kept in the respirometer at 16° C for more than 12 hr. Throughout these long experiments, the resting respiration and action potential did not alter appreciably. Between experiments, in an attempt to remove bacteria and residual pieces of nerve, the respirometer was washed with N-HCl. Every few days, the oxygen-sensitivity of the electrode was tested by bubbling nitrogen through the reservoir. Electrodes were discarded when the current in nitrogen failed to fall rapidly to less than 1 % of that in air.

The respirometer was kept in a constant temperature enclosure which was normally maintained at $16 \pm 0{\cdot}1°$ C.

The electrode current was displayed on a chart recorder. Suitable portions of these records have been traced for inclusion in the present paper. Artifacts in the record were often produced immediately following a change of solution. These resulted from small changes in temperature and also from differences in density of the solutions. A solution of density greater than that of the fluid in the capillary tended to displace fluid from the capillary: thus, with very dense solutions, the electrode was suddenly exposed to an almost fully oxygenated solution. This made experiments with the very dense sucrose artificial sea waters rather difficult. Transfer to a solution of density less than that of the fluid in the capillary produced a small artifact in the opposite direction (see Figs. 5, 6 and 11).

Estimation of Na, K and orthophosphate (P_i). These were estimated as described by Baker (1965).

Solutions

The solutions and shorthand used to describe them were similar to those described by Baker (1965), except that the magnesium content was 35 mM instead of 55 mM and the solutions were buffered by 3 mM Tris-HCl, pH 7·7 and not by HCO_3^--CO_2, pH 8·0. Tris was used to minimize the possibility of pH changes which might result from the use of gas mixtures other than air. The notation is readily explained by way of an example: thus

10 K(Na)ASW refers to an artificial sea water in which the major cation is Na and the major anion is Cl and the potassium concentration is 10 mM.

Design of experiments

The two main problems were: (1) to distinguish between respiration associated with ion pumping and respiration due to other processes; (2) to find some way of loading nerves with a reproducible amount of sodium.

One way of avoiding both difficulties is to measure the time constant of recovery from a train of impulses. If, at the start, the respiration of the nerve is in a steady state, then following a train of action potentials the steady state will be displaced, because of the increased sodium content of the axons, and a burst of respiration should ensue. As the sodium is pumped out of the nerve, the respiration rate falls and, when determined over the final stages of recovery to the steady state, this fall is exponential. The time constant determined over this exponential phase of recovery is a function of the rate coefficient of pumping (Brink *et al.* 1952). This avoids absolute measurements of basal respiration and respiration rate associated with pumping; but the method has serious limitations. For instance, it can only be applied to solutions in which the nerves are excitable. This excludes solutions either with a high potassium content or low sodium content and also solutions (such as K-free sea water or ouabain) application of which results in an accumulation of sodium inside the axons. Even when stimulation is possible, use of the time constant of recovery is still not free from difficulties. In some instances, stimulation of the same nerve in the same solution—but for different lengths of time—was observed to give different recovery time constants; and in other instances, repeated applications of identical tetani gave a progressive reduction in the time constant throughout the experiment. These observations underline the necessity for very careful controls in experiments designed to detect changes in the time constant of recovery; but they also suggest that an observed difference in recovery time constant between two solutions might not necessarily reflect a change in pumping rate. Factors which might affect the time constant of recovery are a difference in sodium or calcium influxes during stimulation or changes in the initial steady-state levels of Na and K within the axon. Experiments on the isolated (Na+K)-activated ATPase (Skou, 1957) have provided evidence for a competition between Na and K at the sodium-activation site and if this occurs in the living nerve—for which there is some evidence (Baker, 1965)—changes in potassium content will alter the affinity of the pump for internal sodium and so change the time constant of recovery. A slow net loss of potassium, which might occur during a long experiment, could progressively decrease the time constant for recovery from a standard tetanus. A further factor which might be relevant is that if the internal Na content is very low (and K high) the pump might not respond linearly to internal sodium.

A more satisfactory method for comparing different solutions is to load the nerve with sodium by immersing it in K-free sea water. This seemed possible despite the unfavourable design of the respirometer. After soaking for 1 hr in 0 K(Na)ASW, the Na content of the nerve was about doubled (Table 1). On return to a medium in which pumping could occur, the extrusion of Na was associated with a burst of respiration. Measurement of the extra oxygen consumed (above the 0 K(Na)ASW base line) during the 20 min period immediately following application of the test solution gave a measure of the extent to which the pump had been activated. The nerve was then returned to 0 K(Na)ASW for a further hour before applying another test solution. A test solution was always bracketed between applications of either 5 K(Na)ASW or 10 K(Na)ASW. When the oxygen consumed in the test solution was expressed as a percentage of the average oxygen consumption in the two applications of 10 K(Na)ASW, the value was found to be very constant from one experiment to another; but, for a given test solution, the absolute amount of oxygen consumed (μl./g wet nerve) varied from nerve to nerve.

This method of measuring the oxygen consumption associated with the sodium pump

makes three assumptions. They are that respiration in 0 K(Na)ASW is not associated with pumping, that the respiration associated with processes other than the sodium pump is unaffected by changes in the external medium and that during the 20 min test period the sodium content of the cells does not fall appreciably. The first assumption is probably not entirely true because there will be enough residual potassium to allow some pumping to continue. Evidence for this comes from the further fall in respiration following either addition of ouabain to the 0 K(Na)ASW or replacement of all the external sodium by choline (Table 2). There is no evidence available for crab nerve which is relevant to the second assumption; but in perfused squid axons Baker & Shaw (1965) have shown that the axoplasmic ATPase is unaffected by changes in the Na, K and ouabain contents of the perfusion fluid. If the residual respiration of crab nerve can be equated with the axoplasmic ATPase of squid axons, the experiments on squid nerve suggest that the respiration not associated with pumping will be insensitive to changes in the external medium. The third assumption does not hold at high rates of pumping (see p. 285). This problem can be overcome by lowering the temperature to 8° C and so slowing down the pump. In the presence of ouabain, the rate of pumping is slowed down enough to enable accurate measurements to be made at 16° C.

TABLE 1. Sodium contents (m-moles/kg nerve) of *Libinia* nerves immersed in different media. Where the nerve was transferred from one solution to another, the second solution is termed solution 2. The figures have been calculated on the assumption that the fraction of the wet weight occupied by extracellular fluid is 0·3 (Baker, 1965). Only two experiments were made in each case and the results of both are included. The nerves were unstimulated, unless stated otherwise. Temperature 16° C

Solution 1	Solution 2	Sodium content
10 K(Na)ASW, 1 hr	—	18·1; 25·1
10 K(Na)ASW, 6 hr	—	22·1; 39·3
0 K(Na)ASW, 1 hr + stimulation at 30 impulses/sec for 5 min	—	76·4; 95·7
0 K(Na)ASW, 1 hr	—	47·9; 48·6
0 K(Na)ASW, 6 hr	—	96·4; 97·9
10 K(Na)ASW + 1 mM ouabain, 6 hr	—	185·7; 157·1
0 K(Na)ASW, 1 hr	10 K(Na)ASW, 20 min	31·4; 25·3
0 K(Na)ASW, 1 hr	10 K(choline)ASW, 20 min	13·4; 13·0
0 K(Na)ASW, 1 hr	0 K(choline)ASW, 20 min	20·9; 32·0
0 K(Na)ASW, 1 hr	10 K(choline)ASW + 1 mM ouabain, 20 min	22·3; 20·7

In all experiments in which a comparison was made between the oxygen uptake of the same nerve immersed in a series of test solutions, corrections were always applied for any differences in the solubility of oxygen in the various test solutions.

There may be an important difference between nerves which have been loaded with Na by stimulation and those which have been loaded by immersion in 0 K(Na)ASW. In stimulated nerves, almost all the increase may be expected to occur in the axons; whereas the increase in Na which occurs on soaking in 0 K(Na)ASW is probably distributed between both axons and Schwann cells. Despite this, no obvious differences in pumping were seen between nerves loaded with Na by these two methods.

RESULTS

Recovery from electrical stimulation

Evidence for saturation at the sodium activation site of the pump. At 16° C, the resting rate of oxygen consumption of nerves immersed in 10 K(Na)-ASW was $1{\cdot}19 \pm 0{\cdot}035$ μl./g wet nerve/min (Table 2). This figure is in close

agreement with earlier values (for discussion see Baker, 1965). Stimulation at a steady rate of 5 impulses/sec about doubled the rate of oxygen uptake. Stimulation for short periods resulted in a transient rise in respiration followed by a fall to the resting level. After stimulation at 2 impulses/sec for 10 min or less, the respiration declined exponentially; but after longer tetani the recovery curve was often more complex and consisted of a small, but rapid, initial fall followed by a return to the base line along a distinctly S-shaped curve of which only the final portion was exponential (Fig. 2, trace *C* and Fig. 3, trace *D*).

TABLE 2. Steady-state rates of oxygen uptake (μl. O_2/g wet nerve/min) of *Libinia* nerves immersed in various solutions. Temperature 16° C, unless otherwise stated. All nerves were unstimulated. The results are given as the mean ± S.E. of the mean. The number of determinations is given in brackets

Test solution	Oxygen uptake
10 K(Na)ASW	1·19 ± 0·035 (40)
5 K(Na)ASW	0·93 ± 0·1 (3)
0 K(Na)ASW	0·73 ± 0·034 (26)
0 K(Na)ASW, 8° C	0·26 ± 0·018 (19)
0 K(Na)ASW + 0·1 mM ouabain	0·504 ± 0·026 (20)
0 K(Na)ASW + 1 mM ouabain	0·49 ± 0·055 (6)
10 K(choline)ASW	0·48 (1)
0 K(Na)ASW + 100 μM dinitrophenol, pH 7·0*	2·3 ± 0·09 (5)

* In three of the experiments, 1 mM ouabain was also present.

During stimulation, potassium tends to accumulate in the space immediately external to the axolemma and this raised potassium concentration should favour an increased rate of pumping. At the end of a tetanus the extra potassium which has accumulated should diffuse rapidly into the bathing solution and the small fall in respiration which is observed at this time probably reflects this loss. This effect can be seen most clearly after a tetanus in 0 K(Na)ASW. Under these conditions, the time course of any changes in respiration represents the time course of changes in potassium concentration in the extracellular space. In the experiment shown in Fig. 5, there was a rise in respiration during the tetanus; but once stimulation ceased, the respiration declined swiftly and returned to the base line with a time constant of 4 min.

The slower S-shaped phase of recovery seems likely to have resulted from partial saturation of the sodium-activation site inside the axon. This is borne out by the observation that the plateau was most pronounced after long tetani (Fig. 2). The time constant of the final phase of recovery was longer following massive tetani than after short ones. Thus, in Fig. 2, the time constant for curve *A* is about 13 min, whereas that for curve *C* is about 18 min. The reason for this difference is not known. The rather small respiration rate observed in the presence of DNP (Table 2) suggests

that an alternative explanation of the S-shaped response to massive stimulation might be that respiration was rate limiting. This possibility has not been excluded.

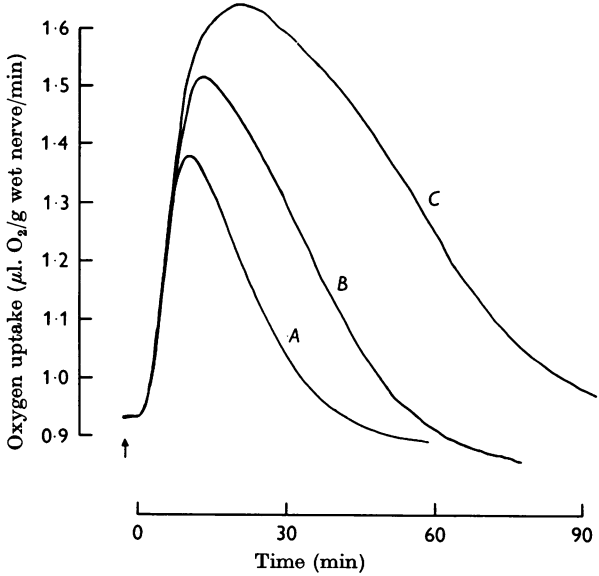

Fig. 2. Changes in the rate of respiration during and after a tetanus. All the responses were obtained from the same nerve immersed in 10 K(Na)ASW. Stimulation, which commenced at the arrow, was at 2 impulses/sec for 6 min (trace *A*); 4 impulses/sec for 10 min (trace *B*) and 4 impulses/sec for 10 min followed by 10 impulses/sec for 8 min (trace *C*). The records were obtained in the order *A*, *B*, *C*. Temperature 16° C.

Changes in the time constant of recovery from stimulation as a function of the external potassium concentration. For comparison of time constants of recovery from stimulation, nerves were given a standard tetanus of 2 impulses/sec for either 6 or 10 min. The results of these experiments are summarized in Table 3. The range of potassium concentrations which could be examined by this method was small, because at low potassium concentrations the nerves gained sodium and a steady state was not achieved and at high potassium concentrations excitability was impaired. Within these limits, the time constant of recovery increased as the potassium content of the external medium decreased. This was examined most carefully for the pair of solutions 5 K(Na)ASW and 10 K(Na)ASW. A longer time constant was always seen in 5 K(Na)ASW, irrespective of the order in which the tests were made. The most likely reason for this difference in time constant is that the rate coefficient of pumping was lower in 5 K(Na)-ASW than in 10 K(Na)ASW.

Despite the difference in time constant, for identical tetani the total extra oxygen consumption in the two solutions was very similar (Table 4). As the sodium content of both solutions was the same and, in both cases, the potassium content fairly low, it seems likely that the sodium entry per impulse would be similar. If this was the case, the similarity in extra oxygen uptake following identical tetani in 5 K(Na)ASW and 10 K(Na)ASW suggests that the number of sodium ions extruded for each energy-rich

TABLE 3. Time constants of recovery from stimulation in various media. The time constant was determined over the final phase of recovery. Data are included only from nerves stimulated at 2 impulses/sec for 6–10 min. The time constant is given in min and is expressed as the mean ± S.E. of the mean. The number of experiments is given in brackets. Temperature 16° C

Test solution	Time constant	
5 K(460 Na)ASW	24·8 ± 4·0	(7)
5 K(230 Na + 230 choline)ASW	19·0	(1)
5 K(115 Na + 345 choline)ASW	15·3 ± 2·5	(8)
5 K(115 Na + 345 Li)ASW	13·0	(1)
5 K(115 Na + sucrose)ASW*	10·0	(1)
10 K(700 Na)ASW	29 ± 1·4	(3)
10 K(460 Na + 240 choline)ASW	15·6 ± 0·2	(4)
10 K(460 Na + 240 Li)ASW	15·5	(1)
10 K(460 Na + sucrose)ASW†	15·5	(1)
10 K(460 Na)ASW	12·2 ± 0·64	(11)
10 K(180 Na + 280 choline)ASW	8·0	(1)
10 K(115 Na + 345 choline)ASW	9·25	(2)
10 K(180 Na)ferrocyanide-ASW + sucrose*	38·0	(1)
10 K(460 Na)ASW containing 45 mM ferrocyanide‡	25·5 ± 0·5	(3)

* Sucrose added to make the solution isotonic with sea water.

† Sucrose added to make the solution isotonic with 10 K(700 Na)ASW.

‡ Choline chloride added to make the solution isotonic with sea water.

TABLE 4. Calculation of Na:~P ratios for intact *Libinia* nerves immersed in sodium-ASWs

Solution	Number of impulses	Extra oxygen consumed (μl. O_2/g wet nerve)	Na:~P
5 K(Na)ASW	1200	39·4	1·9
5 K(Na)ASW	720	13·3	3·4
5 K(Na)ASW	300 (1/sec)	6·5	2·9
5 K(Na)ASW	720	18·6	2·4
10 K(Na)ASW	720	14·8	3·0
10 K(Na)ASW	600	13·8	2·7
10 K(Na)ASW	1200	34·4	2·2
10 K(Na)ASW	720	19·2	2·3
10 K(Na)ASW	720	15·5	2·9
		Mean ± S.E. of mean	2·6 ± 0·16

Only records taken early in an experiment are included. All nerves, except one, were stimulated at 2 impulses/sec. Oxygen consumed is estimated from the area under the recovery curve. The Na:~P ratio is calculated on the assumption that the net increase in internal sodium is 20 μmoles/g wet nerve/1200 impulses (Baker, 1965) and the P/O ratio is 3. Temperature 16° C.

phosphate bond split was also similar for nerves immersed in these two solutions. A numerical value for the Na: ~P ratio can be obtained if it is assumed that the P/O ratio is 3 and that the sodium entry/impulse is the same as that in *Maia* nerve (Baker, 1965). Na: ~P ratios calculated in this way are given in Table 4. The values obtained are in close agreement with earlier estimates of the Na: ~P ratio on crab nerve and other tissues (see Baker, 1965).

The present estimate of the Na: ~P ratio relies on two assumptions and is more open to error than the earlier values of Baker (1965). Lynn & Brown (1965) have recently presented evidence that in isolated rat liver mitochondria the P/O ratio might be as high as 7; but it is not known whether this applies *in vivo*. In *Maia* nerve, Baker (1965) has shown that the rate of ~P utilization can be equated with the rate of oxygen uptake if a P/O ratio of 3 is assumed. These calculations would not exclude a P/O ratio of 4 or 5; but they make a P/O ratio of 7 rather unlikely. If the P/O ratio is greater than 3, the calculated value for the Na: ~P ratio will be lower; but the Na entry/impulse as estimated by Baker (1965) may well be too low and so these errors will tend to cancel each other.

Changes in the time constant of recovery from stimulation as a function of external sodium concentration. In general, for solutions of similar potassium content, the time constant of recovery from identical tetani became shorter as the sodium content of the bathing fluid was decreased (Table 3). This was true when external sodium was replaced isosmotically by choline, lithium or sucrose and the effect was seen irrespective of the order in which the solutions were applied.

One problem in comparing solutions of different sodium contents is that the sodium entry/impulse might not be the same. If the sodium entry from Na-poor solutions is smaller, this might tend to shorten the time constant of recovery (see p. 274). In order to overcome this objection, in a few experiments the nerve was tetanized for a longer period in the Na-poor solution than in the Na-rich one. A shorter time constant was still observed in the Na-poor solution.

The most pronounced effect of external sodium was seen in sea water to which extra sodium had been added to give a final sodium content of 700 mM. In this solution, the time constant of recovery was greatly prolonged (Fig. 3). This effect was not due solely to the increased tonicity of the medium nor to its higher ionic strength, because only a small increase in time constant was seen in sea waters to which choline, lithium or sucrose had been added to give the same tonicity and, in the case of choline and lithium, the same ionic strength (Table 3). A noticeable feature of the respiration following stimulation in 10 K(700 Na)ASW was that both the rate at which respiration increased during the tetanus and the maximum rate of respiration reached were lower than following an identical tetanus in 10 K(460 Na + 240 choline)ASW or in a solution entirely comparable but of even lower Na content. A reduction of this

kind is to be expected if one effect of increasing the external sodium concentration is to reduce the rate of pumping. These experiments suggest that sodium antagonizes the action of potassium at the external activation site of the sodium pump. Sodium cannot be replaced in this action by choline, lithium or sucrose.

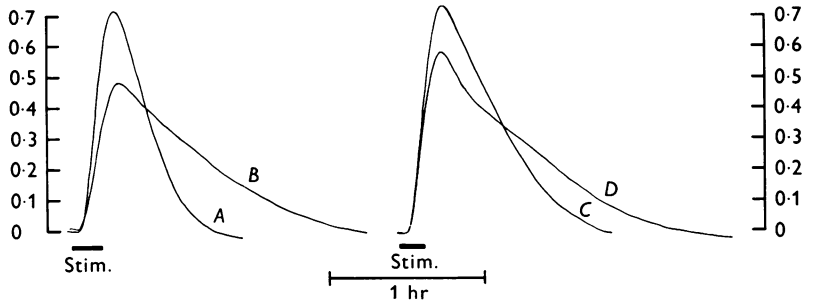

Fig. 3. Changes in the rate of respiration during and after identical tetani in media of different sodium content. Ordinate: increase in the rate of oxygen consumption (μl. O_2/g wet nerve/min) above the resting level. Stimulation was at 2 impulses/sec for the period shown. Traces *A* and *B* are from one nerve and *C* and *D* from another. In all cases, the nerve was left in the test solution for about 1 hr before applying the tetanus. The bathing solutions were: trace *A*, 10 K(115 Na + 345 choline)ASW; trace *B*, 10 K(700 Na)ASW; trace *C*, 10 K(460 Na + 240 choline)ASW; trace *D*, 10 K(700 Na)ASW. The records were obtained in the order *A*, *B* and *C*, *D*. Temperature 16° C.

Effect of ferrocyanide ions on recovery. While investigating the effects of ionic strength on the time constant of recovery, a few experiments were made with solutions the ionic strength of which had been increased by addition of sulphate or ferrocyanide ions. Inclusion of sulphate in the external medium often made the nerves fire spontaneously; but addition of ferrocyanide to the bathing medium while maintaining excitability did not produce repetitive firing and a few stimulation experiments were performed in artificial sea waters containing this ion. The repetitive firing observed in sulphate sea waters probably resulted from a reduction in the level of ionized calcium in the medium. When compared with a solution of similar tonicity and Na and K content, the presence of ferrocyanide in the external medium had little effect on the basal level of respiration, but greatly lengthened the time constant of recovery from stimulation and much reduced the maximum rate of respiration during recovery (Table 3 and Fig. 4). These effects were completely reversed following replacement of ferrocyanide by chloride. In the presence of ferrocyanide, an increase in the potassium or chloride content of the medium increased the rate of respiration, which suggests that respiration was not rate-limiting under these conditions. Further experiments showed that the action of ferro-

cyanide did not result from changes in ionic strength because a similar reduction in the rate of respiration during recovery from stimulation was seen in nerves immersed in ferrocyanide solutions the ionic strength of which was the same as that of sea water (Fig. 4 and Table 3). In fact, in the presence of 45 mM ferrocyanide, solutions made isotonic by addition of choline chloride or sodium chloride supported a higher respiration rate following stimulation than solutions to which an osmotically equivalent quantity of sucrose had been added. These experiments suggest that, in *Libinia* nerve, the ferrocyanide ion has an inhibitory effect on the sodium pump; but more experiments are required to establish this point and to determine its mode of action. It would also be of interest to know whether ferrocyanide ions inhibit the (Na+K)-activated ATPase which can be isolated from crab nerve.

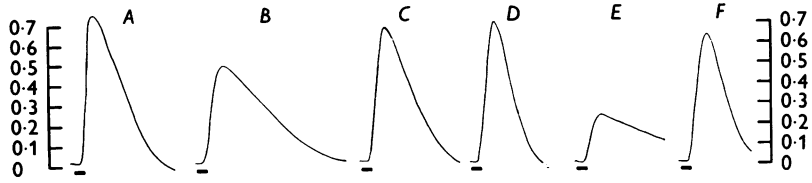

Fig. 4. The effect of ferrocyanide ions and N-ethyl maleimide on the rate of respiration during and after a tetanus. Ordinate: increase in the rate of oxygen consumption (μl. O_2/g wet nerve/min) above the resting level. The period of stimulation is shown by a black bar: in all cases it was at 2 impulses/sec for 6 min. The traces are all from the same nerve and were obtained in the order *A–F*. Before each test, the nerve was immersed in the test solution for 30–60 min. Trace *A*, 10 K(460 Na)ASW; trace *B*, 10 K(460 Na)ASW containing 45 mM sodium ferrocyanide—tonicity maintained by addition of choline chloride; trace *C*, 10 K(460 Na)ASW; trace *D*, 10 K(180 Na+280 choline)ASW; trace *E*, 10 K(180 Na)ferrocyanide-ASW in which tonicity was maintained by addition of sucrose; trace *F*, 10 K(460 Na)ASW containing 1 mM N-ethyl maleimide. The ionic strength of the solutions was 0·6, except for solution *B* which was 0·95. Temperature 16° C.

Stimulation in the presence of ouabain. Ouabain inhibits the sodium pump in squid nerve (Caldwell & Keynes, 1959), frog nerve (Connelly, 1962) and *Maia* nerve (Baker, 1965). The same is true for *Libinia* nerve where inclusion of 0·1 or 1 mM ouabain in 10 K(Na)ASW reduced the resting respiration (Table 2) and largely abolished the burst of oxygen consumption associated with stimulation.

Recovery from the effects of soaking in K-free sea water

Changes in respiration and pumping during immersion in K-free sea water. Immersion of nerves in K-free sea water provides a convenient and fully reversible method for inhibiting the sodium pump. In the absence of

external potassium, the rate of sodium extrusion is much reduced and the nerves gain Na and lose K (Baker, 1965). This marked reduction in pumping rate was associated with a sharp fall in respiration (Table 2 and Fig. 5). The time constant for the reduction in respiration following transfer from 10 K(Na)ASW to 0 K(Na)ASW averaged 5 min.

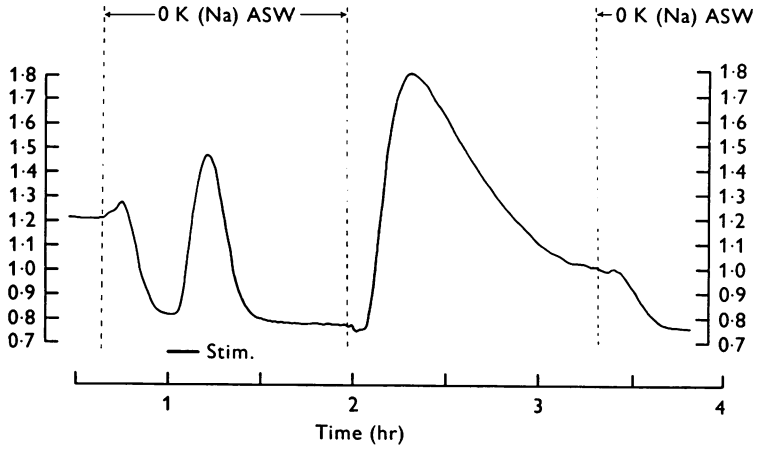

Fig. 5. Effect of removing external potassium on the respiration of *Libinia* nerve immersed in 10 K(Na)ASW. Ordinate: rate of oxygen uptake (μl. O_2/g wet nerve/min). Stimulation was at 5 impulses/sec for 10 min. Temperature 16° C.

Effect of restoring potassium or K-like ions to the bathing medium. The low rate of oxygen uptake of nerves immersed in K-free sea water was maintained for many hours; but addition of potassium to the sea water produced a rapid and prolonged burst of respiration (Fig. 5). When 10 K(Na)-ASW was applied, the burst of respiration declined to a new steady state with an average time constant of 11 min and after 20 min of recovery the Na and K contents of the nerve had returned nearly to their resting values (Table 1).

With artificial sea waters containing 20 or 30 mM potassium, the burst of respiration occurred in two phases (Fig. 6*A*). The second phase was not seen in solutions of raised calcium content (Fig. 6*B*). The first phase, which lasted for 20–30 min, probably reflected the extent to which, at the initial internal Na content, the pump could be activated by the applied potassium. The origin of the second phase is not clear.

When periods of sodium-loading in K-free sea water were alternated with applications, for 20 min, of various test solutions, the effectiveness of different media in reactivating the pump could be compared (see p. 274). The results of this approach are given in Fig. 7. From a Lineweaver & Burk plot of these values, the apparent Michaelis constant for external

potassium in sea water containing 460 mM sodium was found to be between 8·7 and 10 mM.

In a few experiments the effectiveness of some other monovalent cations in re-activating the pump was examined (Fig. 8). Comparison of Cs, Rb, NH_4 and K was made in Cl-ASW and of Tl^+ and K in NO_3-ASW. Nitrate did not have any detectable effect of its own either on the basal respiration or on the pump. The order of effectiveness of equimolar concentrations of these cations in stimulating respiration of nerves immersed in K-free sea water was $Tl^+ > K \simeq Rb > NH_4 > Cs > Na$. If it is assumed that the maximum rate of oxygen uptake associated with pumping is the same for each ion, as it is for ATP utilization in red cell ghosts (Whittam & Ager, 1964), values for the apparent Michaelis constant (K_p) for the different cations can be calculated. Taking K_p for K as 10 mM, K_p for the other ions is 91 mM for Cs, 33 mM for NH_4, 11 mM for Rb and 7 mM for Tl^+. These values are similar to those obtained from direct measurements of the rate of energy-rich phosphate utilization by the sodium pump in *Maia* nerve (Baker, 1965); but, because of the greater sensitivity of the present method, they are probably much more accurate.

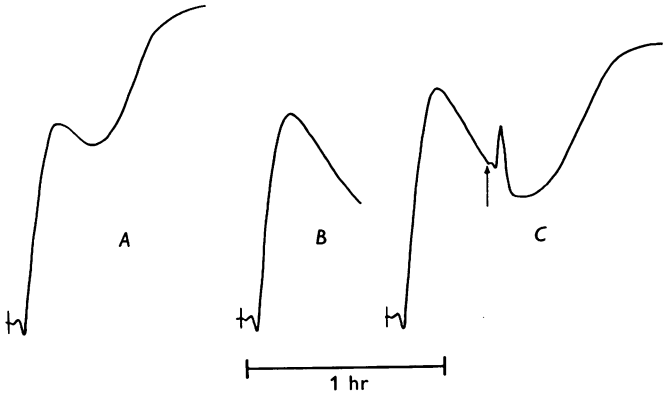

Fig. 6. Increase in respiration on adding sea waters containing 20 mM potassium to a nerve presoaked in 0 K(Na)ASW. In each case the nerve was presoaked in 0 K(Na)ASW for 1–2 hr. The small vertical line marks the time at which the test solution was applied. The vertical scale is the same in each record and an increase in oxygen consumption is shown as an upward deflexion. The small downward deflexion in each trace which occurs shortly after adding the test solution is a solution-change artifact, as is the larger upward deflexion after the arrow in trace *C*. Trace *A*, 20 K(Na)ASW; trace *B*, 20 K(Na)ASW containing an extra 30 mM calcium; trace *C*, the same solution as for trace *B*; but at the arrow the extra calcium was removed, the nerve being immersed in 20 K(Na)ASW. Before applying the solution of raised calcium content at the beginning of trace *C*, the nerve was soaked for 30 min in 0 K(Na)ASW of this calcium content. The traces were obtained in the order *A*, *B*, *C*. Temperature 16° C.

In making these measurements, the main source of error was the assumption that the respiration in 0 K(Na)ASW did not include any oxygen uptake associated with pumping. As an estimate of the ouabain-sensitive respiration in 0 K(Na)ASW could not be made until the end of the experiment, when potassium permeability and other factors may have changed, no correction was attempted.

In a single test, the activation produced by 20 mM-Cs + 2 mM-K was that expected from

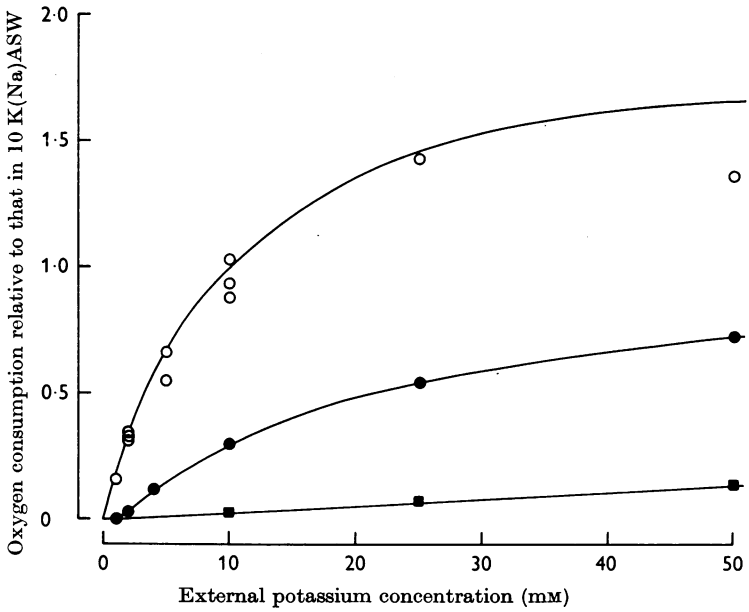

Fig. 7. Oxygen consumption of *Libinia* nerve as a function of the potassium content of the bathing medium. Oxygen uptake in a test solution was measured after soaking the nerves for 1 hr in 0 K(Na)ASW (see text, p. 274). ○ (460 Na)-ASW; ● (460 Na)-ASW containing 0·1 mM ouabain; ■ (460 Na)-ASW containing 1 mM ouabain. The curve drawn through the points in (Na)-ASW is a rectangular hyperbola (apparent Michaelis constant for K of 8·5 mM). The points in ouabain are included as described in the text and the curves have been drawn by eye. Temperature 16° C.

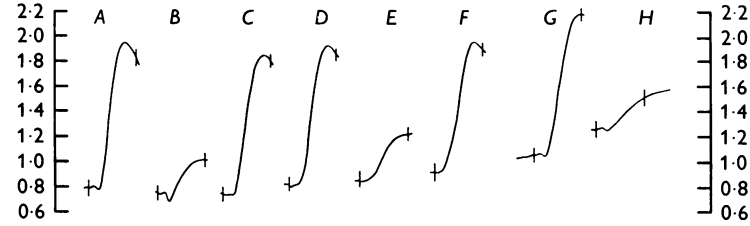

Fig. 8. Increase in respiration following addition of sea waters containing potassium or potassium-like ions to a nerve presoaked in 0 K(Na)ASW. Ordinate: rate of oxygen uptake (μl. O_2/g wet nerve/min). The records were obtained on the same nerve in the order shown. There was a small increase in the basal respiration throughout the experiment. The nerve was soaked for 1 hr in 0 K(Na)ASW before each application of a test solution, except between traces *G* and *H* where the nerve was soaked for 1 hr in 0 K(Na)ASW containing 0·1 mM ouabain. The time at which the test solution was applied is marked by a short vertical line. The second vertical line is drawn 20 min after the first. *A*, 10 K(Na)ASW; *B*, 10 Cs(Na)-ASW; *C*, 10 Rb(Na)ASW; *D*, 10 K(Na)ASW; *E*, 2 K(Na)ASW; *F*, 30 NH_4(Na)-ASW; *G*, 10 K(Na)ASW; *H*, 10 K(Na)ASW containing 0·1 mM ouabain. Temperature 16° C.

an equivalent concentration of K (20 Cs ≡ 2·2 mM-K), indicating that there is no synergistic action between these two ions at the external activation site. Other pairs of ions were not tested.

Re-activation of the pump by potassium in sea waters of reduced sodium content. At 16° C, application of 10 K-ASW in which sodium had been replaced by an osmotically equivalent quantity of choline, lithium or sucrose, gave a burst of respiration which reached a peak between 10 and 15 min and then declined rapidly. In comparable tests on 10 K(Na)ASW, the respiration only reached a peak between 15 and 25 min. This difference, which resulted from the rapid loss of internal Na into the Na-free medium, made it impossible to compare accurately the effectiveness of 10 K(Na)-ASW in reactivating the pump with that of Na-free solutions. When the results of the few experiments of this kind which were made are expressed as a fraction of the respiration over a comparable 20 min period in 10 K-(Na)ASW, the values obtained are 0·4 for 0 K(choline)ASW, 0·8 for 10 K(choline)ASW and 0·95 for 10 K(Li)ASW.

When only part of the sodium in the sea water was replaced by choline it was possible to compare solutions of low potassium content without the problem of early peaking in the Na-deficient solution. This was examined for the pair of solutions 2 K(460 Na)ASW and 2 K(230 Na + 230 choline)-ASW. When bracketed between tests in 460 mM-Na, the respiration in 230 mM-Na averaged 140 % of that in 460 mM-Na.

A much more convenient way of comparing the respiration in Na-poor and Na-rich solutions was to work at a temperature lower than 16° C. At 8° C the pump was considerably slowed and application of a Na-poor sea water to a Na-loaded nerve gave a prolonged burst of respiration. As the respiration reached what might be described as a 'pseudo steady state', it was possible to compare the effectiveness of different solutions in re-activating the pump by measurement of the 'pseudo steady state' level of respiration achieved. Nerves were loaded with Na by soaking in 0 K(Na)-ASW at 16° C for 1–3 hr and test solutions were routinely compared with 2 K(Na)ASW (Fig. 9). The results of those experiments which did show a sharp peak in respiration were rejected on the grounds that the nerves were probably not loaded adequately with sodium. One problem which was encountered during these experiments was that the response of Na-loaded nerves to Na-deficient solutions tended to decline over a series of tests while that to 10 K(Na)ASW was unaltered. The explanation for this is not clear; but, in order to avoid this source of error, a separate nerve was used for each measurement of respiration in Na-poor solutions.

Sodium was normally replaced by choline and the results of these experiments are collected in Fig. 10. Essentially similar results were obtained in the few experiments in which Na was replaced by an osmotically

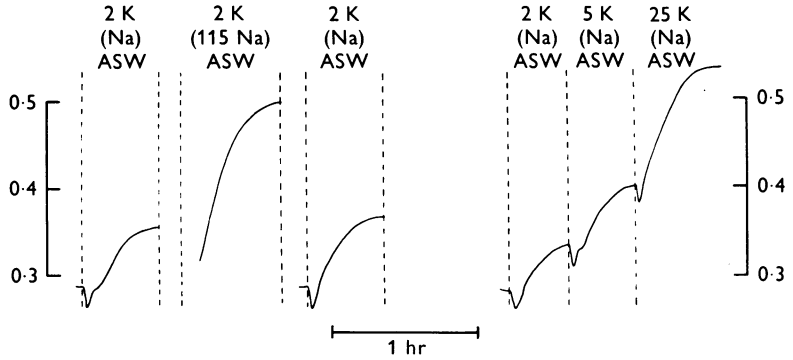

Fig. 9. Changes in respiration of a Na-loaded nerve in solutions of different Na and K contents. Sodium was replaced by an equivalent quantity of choline. Ordinate: oxygen uptake (μl. O_2/g wet nerve/min). The traces were all obtained on the same nerve. Each of the traces in the first sequence of three was obtained after soaking for 1 hr in 0 K(Na)ASW. Between the first set of traces and the second, the nerve was immersed in 0 K(Na)ASW for 3 hr. The downward deflexions which occur shortly after a solution change are artifacts. 2 K(115 Na)ASW gave a large artifact and this has been excluded from the trace. The position of the trace in 2 K(115 Na)ASW has been corrected for the difference in oxygen solubility between this solution and 2 K(Na)ASW. Temperature 8° C.

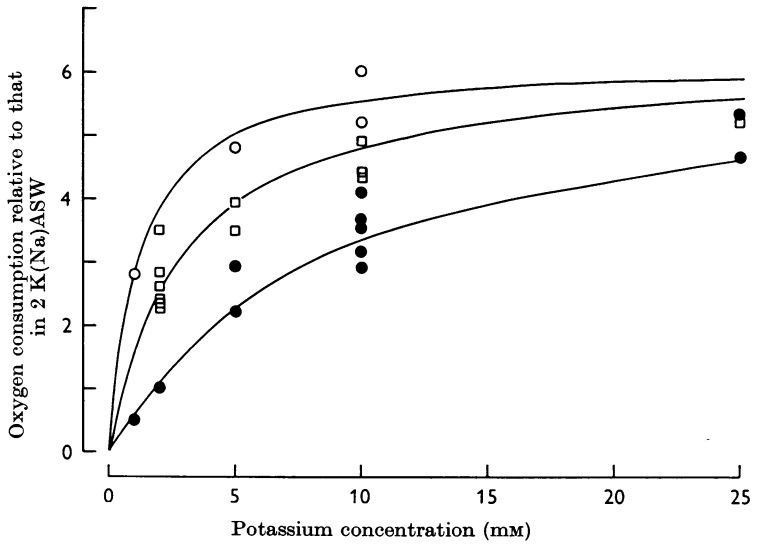

Fig. 10. Collected results of experiments at 8° C. The solutions used were all (Na)ASWs in which Na was replaced by choline. ● (460 Na)ASW; □ (115 Na)-ASW; ○ (10 Na)ASW. The curves drawn through the experimental points are all rectangular hyperbolae calculated on the assumption that Na and K compete in a 1:1 manner at the external activation site of the sodium pump. The maximum rate to which the curves are tending is 6·2. In absolute terms, the mean increase in respiration at 8° C on adding 2 K(Na)ASW to a nerve presoaked in 0 K(Na)ASW was $0{\cdot}095 \pm 0{\cdot}007$ μl. O_2/g wet nerve/min.

equivalent amount of lithium or sucrose. At low potassium concentrations, the respiration rate increased as the Na content of the bathing medium decreased. The experimental points can be fitted by a series of rectangular hyperbolae in which the maximum rate of respiration associated with pumping is the same at different external sodium concentrations; but the apparent Michaelis constant (K_p) for potassium falls as the external sodium concentration is reduced. From a Lineweaver & Burk plot of the points in Fig. 10, K_p for external potassium was found to be 8·5 mM at 460 mM-Na, 2·9 mM at 115 mM-Na and 1·2 mM at 10 mM-Na. These values are consistent with a 1:1 competition between external sodium and potassium. For simple competitive inhibition, the relation between K_p, K_m and K_i is $K_p = K_m(1+i/K_i)$ (Dixon & Webb, 1964). From the above data, the calculated value for K_m for external potassium is close to 1 mM and that for K_i for external sodium is close to 60 mM.

With this method it was not possible to examine the respiration in a completely Na and K-free medium; but from the observed reduction in respiration following addition of ouabain to 0 K(Na)ASW (Table 2) the residual potassium concentration was probably not greater than 2 mM. The amount of residual Na is not known.

Re-activation of the pump by potassium in the presence of sulphydryl-blocking reagents. There have been a number of reports that rather high concentrations of sulphydryl-blocking reagents inhibit the ouabain-sensitive break-down of ATP in tissue homogenates (Skou, 1963; Glynn, 1963; Wheeler & Whittam, 1964; Gibbs, Roddy & Titus, 1965). A few experiments were made to see whether external application of these compounds affected the sodium pump in *Libinia* nerve. The nerve was soaked in 0 K(Na)ASW containing the reagent for 1 hr and then 10 mM potassium was added to the medium. *p*-Chloromercuribenzoate (10^{-5} M), N-ethyl maleimide (1 mM) and iodoacetamide (1 mM) were without effect on either the basal respiration in 0 K(Na)ASW or the increase in 10 K(Na)-ASW, indicating that at the concentrations used these reagents were without action on the sodium pump (see also Fig. 4). A ouabain-sensitive increase in respiration was seen in 0 K(Na)ASW containing 10^{-4} M *p*-chloromercuribenzoate. The most likely explanation of this effect is an increase in the passive permeability of the nerve membrane to Na and K. This was supported by the observation that nerves became inexcitable in 10^{-4} M; but not in 10^{-5} M *p*-chloromercuribenzoate. The ouabain-sensitive increase in respiration indicates that, although the passive permeability was increased, the pump was not inhibited under these conditions. It is not known to what extent these reagents penetrated the cells; but this series of experiments makes it unlikely that sulphydryl groups play an

important role at the external activation site of the sodium pump in crab nerve.

These results do not conform with those of Kernan (1963) who stated that 7×10^{-4} M *p*-chloromercuribenzoate completely inhibited the sodium pump in frog muscle; but, as he was measuring net movements of sodium, his results are also compatible with little change in the rate of pumping but an increase in the sodium influx. Kerkut & Thomas (1965) found that the electrogenic sodium pump in snail ganglia was inhibited by 2×10^{-5} M *p*-chloromercuribenzoate; but this also might have resulted from an increased permeability to Na and K which would reduce the membrane resistance and short circuit the pump. However, it is possible that there is considerable variation from tissue to tissue in the sensitivity to sulphydryl blocking reagents—just as there is to ouabain.

Recovery from the effects of soaking in K-free sea water containing ouabain

General features of the kinetics. Inclusion of 0·1 mM ouabain in 0 K(Na)-ASW depressed the resting respiration below that in 0 K(Na)ASW alone (Table 2). The most likely explanation of this reduction is that the lower portion of the nerve in the respirometer was never bathed in a completely K-free solution and so some pumping could continue. But, on the evidence of these results alone, it is not possible to exclude some other explanation such as the occurrence of energy-dependent exchange diffusion of sodium in 0 K(Na)ASW. This latter explanation seems unlikely because, with *Maia* nerves immersed in a large volume of well-stirred sea water, Baker (1965) was unable to demonstrate any significant difference in the rates of energy-rich phosphate utilization in 0 K(Na)ASW with and without ouabain.

At 16° C, addition of 10 mM potassium to 0 K(Na)ASW containing 0·1 mM ouabain initiated a slow increase in the rate of oxygen uptake which usually reached a steady level after about 30 min; but in some experiments continued to rise for as long as 1 hr. Addition of ouabain-free 10 K(Na)ASW produced a much longer rise in respiration. For a particular potassium concentration, the changes in respiration in the presence of ouabain were consistently somewhat slower than the rate of attainment of the 'pseudo steady state' level of respiration in a ouabain-free system at 8° C. This suggests that, in the presence of ouabain, some factor other than the time taken for potassium to penetrate into the extracellular space was limiting the rate of increase of oxygen consumption. A slow displacement of ouabain in the presence of potassium might explain the results; but the displacement must only be partial because, even at high potassium concentrations, the respiration seems to level off at a value

well below that obtained in the absence of the drug. This might occur if the affinity for ouabain of the ouabain-binding site is reduced, but not abolished, by the presence of external K. Ouabain also seems to reach its site of action rather more slowly than potassium. For instance, application of 10 K(Na)ASW containing 0·1 mM ouabain to a nerve immersed in 0 K(Na)ASW gave a burst of respiration followed by inhibition. Also, the respiration of a Na-loaded nerve immersed in 10 K(Na)ASW was cut off with a time constant of 12 min following addition of 1 mM ouabain, whereas transfer to 0 K(Na)ASW reduced respiration with a time constant of 5 min.

Changes in respiration resulting from alterations in the concentration of sodium and potassium in the external medium. At 16° C, addition of potassium to a Na-loaded nerve immersed in 0 K(Na)ASW containing 0·1 mM ouabain ultimately produced a new 'pseudo steady state' level of respiration (Fig. 11) comparable to that seen in the absence of ouabain at 8° C (Fig. 9). Nerves were routinely loaded with Na by soaking for 1 hr in 0 K(Na)ASW containing ouabain and test solutions were compared with 10 K(Na)ASW containing ouabain. Figure 12 shows the main results of this series of experiments. Superficially, the results are similar to those obtained in the absence of ouabain; but there are two main differences. In the presence of 460 mM-Na and 0·1 mM ouabain, the relation between external K concentration and respiration was S-shaped. Also, with potassium concentrations less than 20 mM, progressive replacement of external Na by an osmotically equivalent amount of choline, lithium or sucrose gave an increase in respiration; but the respiration increased much more steeply than would be expected from a 1:1 competition between external Na and K. The curves for 0·1 mM ouabain would be fitted quite closely if two K ions competed with 3 Na ions at the external activation site; but this relation does not fit the results in 1 mM ouabain so well and is probably fortuitous. However, the results do suggest that external Na is more effective in inhibiting the pump in the presence of ouabain than in its absence. Essentially similar results have been obtained by Schatzmann (1965) for the action of external Na and K on the rate of ATP hydrolysis in ouabain-treated red cell ghosts.

Provided, as seems to be the case, that, at a particular ouabain concentration, all the curves have the same maximum value and assuming that all the extra oxygen uptake results from activation of the pump by potassium, it is possible to calculate K_p for external potassium at different external sodium concentrations. From experiments in which Na was replaced by choline, in the presence of 0·1 mM ouabain, K_p was 25 mM at 460 mM-Na, 9 mM at 230 mM-Na, 1·5 mM at 115 mM-Na and 0·05 mM at 10 mM-Na and in the presence of 1 mM ouabain, K_p was 85 mM at 460 mM-Na,

30 mM at 230 mM-Na, 5 mM at 115 mM-Na and 0·6 mM at 10 mM-Na. From these figures it appears that K_p at low Na concentrations is lower in the presence of ouabain than in its absence. This might be true; but if some of the respiration in low sodium solutions was not dependent on potassium—as might be the case if sodium was lost in association with an anion (Baker, 1964)—the calculated values of K_p would be much larger.

In Na-deficient sea waters, the respiration tended to fall at high potassium concentrations (Fig. 12*A*). This effect was reduced in Ca-free media. It is possible that the fall in respiration resulted from a high calcium influx into depolarized nerves immersed in Na-deficient

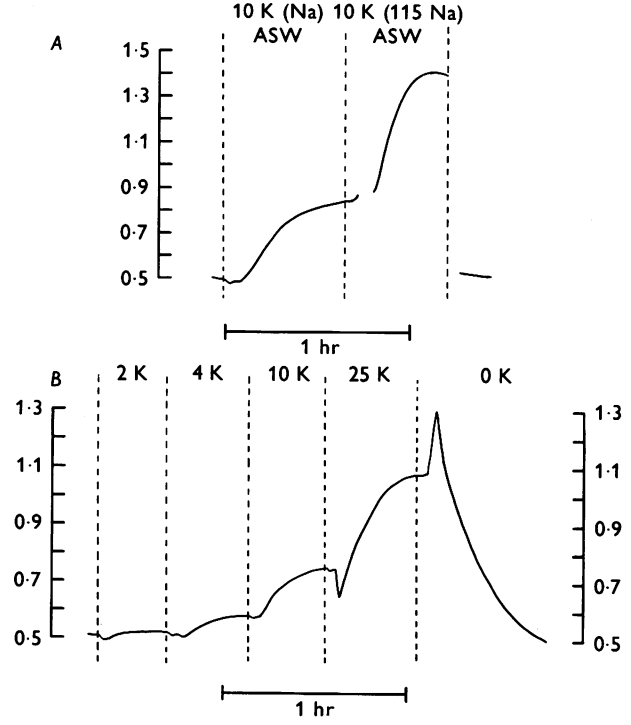

Fig. 11. The effect of 0·1 mM ouabain on the respiration of a Na-loaded nerve immersed in solutions of different Na and K contents. Sodium was replaced by choline; but extra potassium was added without reduction in the Na content. Trace *A* was obtained on one nerve and trace *B* on another. Ordinate: oxygen uptake (μl. O_2/g wet nerve/min). In both cases, the nerve was soaked for 1 hr in 0 K(Na)ASW containing 0·1 mM ouabain before application of the test solution. All the solutions used in trace *B* were sea waters containing 460 mM-Na. Solution-change artifacts are particularly conspicuous in trace *B* following transfer from 10 to 25 K and from 25 to 0 K. A very large artifact was present in trace *A* during the break in the record. Trace *A* has been corrected for the difference between oxygen solubility in (460 Na)ASW and in (115 Na)ASW. The final column in trace *A* shows the level to which respiration returned after 1 hr in 0 K(Na)ASW. Temperature 16° C.

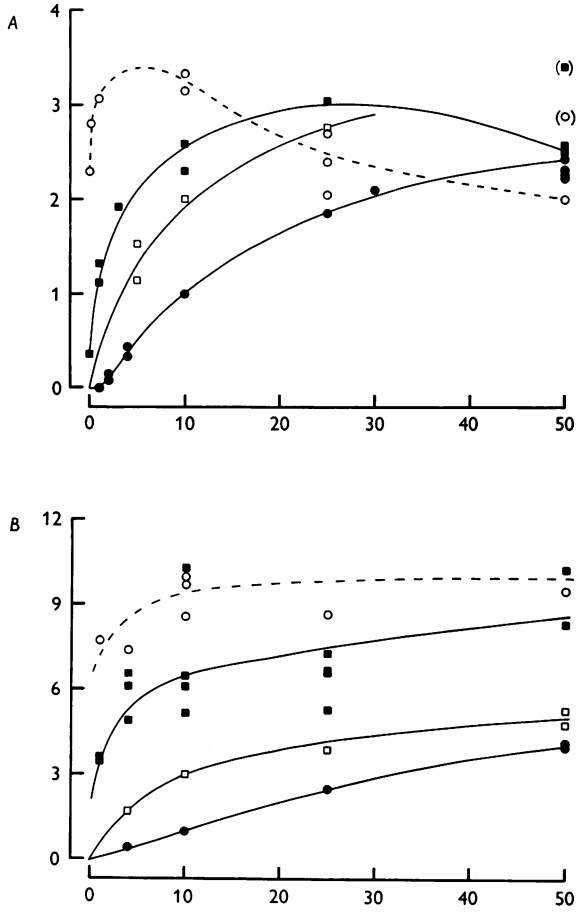

Fig. 12. Collected results of experiments showing the effect of ouabain on the changes in respiration of Na-loaded nerves immersed in solutions of different Na and K contents. Na was replaced by choline. Experiments in the presence of 0·1 mM ouabain are given in Fig. 12*A* and those in the presence of 1 mM ouabain in Fig. 12*B*. For both *A* and *B*, ordinate: oxygen consumed in the test solution relative to that in 10 K(460 Na)ASW containing the same concentration of ouabain; abscissa: external potassium concentration (mM). The same symbols are used in both graphs. ● (460 Na)ASW; □ (230 Na)ASW; ■ (115 Na)ASW; ○ (10 Na)ASW. Symbols surrounded by brackets refer to experiments in calcium-free media. The curves have been drawn through the points by eye. Temperature 16° C. In absolute terms, the mean increase in respiration in the presence of 0·1 mM ouabain on adding 10 K(Na)ASW to a nerve presoaked in 0 K(Na)ASW was $0{\cdot}32 \pm 0{\cdot}02$ μl. O_2/g wet nerve/min.

solutions (by analogy with the work of Lüttgau & Niedergerke, 1958). If such an influx was occurring, it would be reduced in Ca-free media; but the high respiration in Ca-free media might have been fortuitous because in 10 K(Na)ASW containing ouabain removal of either Ca or Mg also increased the respiration. It is possible that these ions exert an inhibitory action at the external activation site of the pump somewhat similar to that shown by external sodium and this effect may be particularly pronounced in the presence of ouabain; but the action of Ca and Mg in this system requires further study.

In a few experiments, an examination was made of the ability of some K-like ions to increase the respiration of nerves immersed in 0 K(Na)ASW containing ouabain. Equimolar concentrations of the ions were compared in 460 mM-Na-ASW containing 0·1 mM ouabain. No experiments were made at lower Na concentrations. Of the cations examined, the order of effectiveness in increasing respiration was $K \simeq Tl^+ \simeq Rb > NH_4 > Cs > Na$. This is similar to the action of these cations on respiration in the absence of ouabain. The average test ion concentration required to produce the same degree of activation as 10 mM-K was 10 mM-Tl^+, 11·6 mM-Rb, 18·6 mM-NH_4, and 61 mM-Cs.

Comparison of the absolute rates of pumping in the presence and absence of ouabain. Satisfactory comparison of the rates of pumping of nerves immersed in 10 K(Na)ASW in the presence and absence of ouabain was only possible at 8° C where, in both instances, a 'pseudo steady state' level of respiration was reached. Only two experiments of this kind were made; in both cases, when the 'pseudo steady state' levels of respiration in the presence and absence of ouabain were referred to a base line in 0 K(Na)ASW containing 0·1 mM ouabain, the rate of respiration was 3–4 times higher in 10 K(Na)ASW than in the same solution containing 0·1 mM ouabain. Comparison of the respiration rate in 10 K(Na)ASW containing 0·1 and 1 mM ouabain was made at 16° C. The curves for 0·1 and 1 mM ouabain have been included in Fig. 7 on the assumption that the rate of K-sensitive respiration in 10 K(Na)ASW containing 0·1 mM ouabain is 30 % of that in the absence of the drug.

These experiments show that inclusion of ouabain in sea water both reduces the maximum rate of oxygen consumption associated with pumping and also decreases the affinity of the pump mechanism for external potassium. Thus, in the terminology of Dixon & Webb (1964), ouabain is a mixed inhibitor of the sodium pump. These conclusions are in agreement with those of Baker (1965) from studies of *Maia* nerve and are also in accord with the results of Dunham & Glynn (1961) on red cell ghosts and of Schatzmann (1965) on intact red cells; but they seem to differ from those of Glynn (1957) from work on intact red cells. At high potassium concentrations Glynn was able to cause complete reversal of the inhibition produced by a low concentration of the related glycoside scillaren. This result may have been obtained simply because the low scillaren concentration used had little effect on the maximum velocity of pumping or it is possible that there is some difference between the actions of ouabain and scillaren.

DISCUSSION

The main aim of the present experiments was to use the changes in respiration of intact crab nerve as a means of following the interactions between Na, K and ouabain at the external activation site of the sodium pump. The interpretation of changes in respiration as changes in the rate of ~P breakdown associated with pumping is based on a number of assumptions which have been discussed in the methods section of this paper. The validity of the assumptions is supported by the finding that, where comparison is possible, the results of the present experiments are in agreement with direct measurements of the rate of ~P utilization (Baker, 1965; Schatzmann, 1965) and net movements of Na and K (Baker, 1965). Provided the assumptions are valid, the present experiments show quite clearly that activation of the pump by external potassium is competitively inhibited by external sodium and that this interaction is modified in the presence of ouabain. Of the monovalent ions tested, Na was unique in exerting an inhibitory action.

The kinetics of inhibition were consistent with a 1 : 1 competition between external Na and K. It is not clear whether the K ions required to activate the pump are also the K ions transported into the nerve; but if the K-activation and K-transport sites are the same, the simple relation obtained in the present experiments is rather surprising in view of the accumulating evidence that, for each ~P bond split, about three Na ions are pumped out of the cell in exchange for at least two K ions (for references see Baker, 1965; Whittam & Ager, 1965). If activation of the ~Pase and subsequent uptake of potassium involves binding of two or more K ions to the membrane, the curve relating pumping rate to external K concentration ought to be S-shaped. In the red cell, an S-shaped curve is obtained; but only at very low external potassium concentrations (Sachs & Welt, 1965), while in nerve the curve is only clearly S-shaped after treatment with low concentrations of ouabain. The absence, in both nerve and red cells, of a markedly S-shaped curve under normal conditions can be explained if one of the K-binding sites has a much lower affinity for potassium than the other sites. Competition with Na would probably occur at the binding site of lower affinity for potassium. The appearance, in both nerves and red cells immersed in low concentrations of ouabain, of an S-shaped relation between extra-oxygen consumption and external K concentration might be explained if ouabain reduces the affinity of those K-binding sites which normally have a very high affinity for external potassium. This might make Na–K competition possible at more than one site and explain both the S-shaped response to external K and also the steep increase in respiration associated with pumping, which is observed

after removal of external Na in the presence of ouabain. It is possible that any interaction with ouabain, which modifies the affinity of the pump for external K, might also be responsible for the observed reduction in the maximum velocity of pumping.

Sodium has been shown to inhibit competitively the activation by potassium of ouabain-sensitive ATPase preparations from many tissues; but experiments have not been published showing this effect in homogenates of crab nerve. However, Skou (1964) refers to unpublished experiments which demonstrate this in his *Carcinus* nerve preparation. He does not give values for the constants K_m for external potassium and K_i for external sodium; but the values obtained for these constants in the present experiments are close to those obtained for ATP break-down in red cells (Whittam & Ager, 1964). On the assumption that K_i for external Na is 60 mM, K_m for the other monovalent cations examined can be calculated. The values are: K, 1 mM; Tl^+, 0·7 mM; Rb, 1·1 mM; NH_4, 3·3 mM and Cs, 9·1 mM. These values are very close to the ion concentrations required for half-maximal inhibition of the anion efflux from *Maia* nerves immersed in Na and K-free solutions (Baker, 1964).

The sodium efflux from intact cephalopod nerve is increased following replacement of external Na by an osmotically equivalent amount of choline or dextrose (Hodgkin & Keynes, 1955) and, although other factors may be involved, it seems likely that much of this increase results from removal of an inhibitory effect of external Na on the Na efflux. More experiments are clearly required, in particular measurements of the ouabain-sensitive Na efflux from squid axons at different external Na and K concentrations.

In nominally Na and K-free solutions, the Na efflux from cephalopod nerves is quite high and, although some of this efflux might be caused by residual potassium, it seems unlikely that the whole flux can be explained in this way. Under comparable conditions, Na is extruded from crab nerve in association with anions (Baker, 1964) and the operation of a similar mechanism in squid axons might contribute to the high Na efflux.

Because of the design of the respirometer, it was not possible to make measurements of the respiration of Na-loaded nerves immersed in completely Na and K-free media, but measurements of the P_i level in *Maia* nerve indicate that there is a ouabain-sensitive energy requirement for the extrusion of Na into a large volume of well-stirred Na and K-free medium. Thus, the average P_i content of stimulated nerves immersed for 10 min at 16° C in 0 K(Li)ASW was 162 μg P/g nerve and in 0 K(choline)ASW was 193 μg P/g nerve; whereas in the same solutions containing 1 mM ouabain, it was 89·5 and 127 μg P/g nerve respectively. If this also applies to *Libinia* nerve, the respiration associated with pumping probably would not reach zero even in solutions devoid of Na and K ions.

The present results, together with those of earlier work, suggest that external Na is involved in at least three separate processes all of which

seem to be part of the sodium pump mechanism: (1) The coupled pumping of Na in exchange for K, where external K is essential and external Na inhibitory; (2) the extrusion of Na in exchange for Na, where external Na is obligatory and external K either inhibits or appears to have little effect (Keynes & Swan, 1959; Glynn, 1957; Caldwell, Hodgkin, Keynes & Shaw, 1960; Garrahan & Glynn, 1965); and (3) the loss of Na accompanied by an anion, where external Na and K are both inhibitory (Baker, 1964). In all three cases, where information is available, the affinity for external Na is low, K_m about 50 mM, and the affinity for external K is high, K_m about 1 mM. These values are very different from those obtained on broken cell preparations for the affinity of the presumed internal activation site, where K_m for Na is 1–3 mM and K_i for K is 8–10 mM (Skou, 1960). The apparent affinity of the internal activation site of the pump in intact nerve, muscle and red cells is consistent with the figures derived from broken cell preparations (Post, Merritt, Kinsolving & Albright, 1960; Baker, 1965; Keynes, 1965; Whittam & Ager, 1965).

The question naturally arises as to where in the pumping process the change in affinity occurs. The first possibility is that it might reflect a difference in the environment of the 'carrier molecule' at the inner and outer faces of the cell membrane. The most likely difference of this kind is the high concentration of divalent cations outside the cell as compared with inside. With isotonic sodium chloride containing 10 mM-K but no Ca or Mg, the operation of the pump in *Maia* nerve was essentially unchanged (Baker, 1965); whereas, if the external activation site was behaving under these conditions rather like the internal site—that is with a high affinity for Na and a low affinity for K—the pump should have been completely inhibited. Although there are, certainly, further environmental differences, these results suggest that other possibilities should also be examined. Perhaps the simplest explanation is to assume that the change in affinity is produced by reaction with ATP.

For instance, within the cell, ATP might convert a 'Na-carrier'—already loaded with three Na ions—into a 'potential K-carrier' which is then passed to a site within the membrane where it cannot exchange Na for internal potassium. According to this scheme, the 'Na-carrier' can only cross the membrane by reaction with ATP and exchange diffusion of external Na with internal Na can only occur through alternate break-down and resynthesis of ATP. At the outer face of the membrane, the only form of 'carrier' available to react with the ions present in the external medium is a 'potential K-carrier'. For exchange diffusion to occur, enough Na must be present in the external medium to keep at least one and probably all three Na sites on the 'carrier' full. For coupled pumping to occur, Na on the 'carrier' must be replaced by K ions from the external medium.

As mentioned earlier, if the 'carrier' combines with two K ions, it probably has a much higher affinity for one K ion that the other and it would be of interest to enquire whether the 'potential K-carrier' has a site of very high affinity for K or whether reaction with the first K ion creates such a high affinity site. If reaction at the high affinity site occurs first, it is

possible that both exchange diffusion and the anion efflux might be inhibited by an external K concentration which is too low to support coupled pumping; but this has not been observed (Glynn, 1957; Baker, 1964). In the case of exchange diffusion, Glynn (1957) has shown that under conditions suitable for exchange diffusion in red cells there is only a very small ouabain-sensitive influx of K from solutions of very low K content. This observation seems to rule out the possibility that reaction with a site of very high affinity for K might occur without blocking Na loss. The conclusion which can be drawn from these observations is that, if a site of high affinity for K is part of the pump mechanism, it is only made available after reaction of one K ion with a site of relatively low affinity (K_m about 1 mM).

A further feature of this model is that the 'potential K-carrier' might cross the membrane via a series of sites. If exchange of Na between 'carrier molecules' at these sites is impossible, when all the sites are filled, exchange diffusion of internal Na with external Na will be much reduced. This seems able to account for the reduction in the amount of exchange diffusion into K-free solutions at high ATP/ADP + P_i ratios and high levels of internal sodium—both of which would favour the formation of 'potential K-carriers' (Caldwell *et al.* 1960; Frumento & Mullins, 1964; Garrahan & Glynn, 1965).

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