J. Physiol. (1972), **220**, *pp.* 55–71
With 13 *text-figures*
Printed in Great Britain

INTRACELLULAR SODIUM ACTIVITY AND THE SODIUM PUMP IN SNAIL NEURONES

By R. C. THOMAS

From the Department of Physiology, University of Bristol, BS8 1TD

(*Received 11 June 1971*)

SUMMARY

1. Recessed-tip Na^+-sensitive micro-electrodes were used to measure $[Na^+]_i$ continuously in snail neurones for experiments lasting up to several hours. The average resting $[Na^+]_i$ in twenty-two cells was 3·6 mM.

2. Inhibition of the Na pump by ouabain caused $[Na^+]_i$ to increase at an average rate of 0·54 m-mole/min. This corresponds to a passive influx of Na quantitatively similar to that observed in squid axons.

3. Changing external K over the range 1–8 mM had little effect on $[Na^+]_i$, but K-free or 0·25 mM-K Ringer caused a rise in $[Na^+]_i$.

4. Increasing membrane potential by up to 90 mV caused an increased influx of Na, but did not inhibit the pump.

5. Reducing external Na caused a decrease in $[Na^+]_i$ but did not affect the pump rate at a given $[Na^+]_i$. The pump rate at low $[Na^+]_i$ was proportional to $[Na^+]_i$ minus a threshold value of about 1 mM.

6. The Na pump appeared still to be electrogenic at subnormal rates of activity.

7. It is concluded that, given sufficient external K, the rate of the Na pump depends principally on $[Na^+]_i$. Changes in external Na or membrane potential appear to affect the pump only indirectly, by changing the Na influx and thus $[Na^+]_i$.

INTRODUCTION

The low intracellular Na ion concentration in nerve cells is of great importance for their electrical activity. This low $[Na^+]_i$ is maintained by the Na pump, which uses metabolic energy to extrude Na and take up K. The properties of the Na pump have been widely investigated in nerve and muscle cells, usually by following the efflux of radioactively labelled Na from Na-loaded cells, but rather less is known about the dependence of pumping on $[Na^+]_i$.

The only technique for directly measuring $[Na^+]_i$ is the Na^+-sensitive glass micro-electrode, first used by Hinke (1959, 1961) to measure $[Na^+]_i$

in crustacean muscle and squid axon. Technical difficulties in constructing and recording from suitably fine Na^+-sensitive micro-electrodes have so far, however, largely prevented their use for any but brief measurements. This paper describes experiments performed with a 'recessed-tip' Na^+-sensitive microelectrode (Thomas, 1970), the 'effective' tip (the part that has to penetrate the cell) being only 1 or 2 μ in diameter. With this design of electrode it is possible to undertake experiments lasting several hours, during which the $[Na^+]_i$ in relatively normal-sized neurones can be continuously measured.

The aim of the experiment in this paper was firstly to determine the normal $[Na^+]_i$ in snail neurones, and then to determine the effect on $[Na^+]_i$ of inhibiting the Na pump, changing external K, increasing membrane potential and decreasing external Na.

The results show that inhibition of the Na pump causes a rapid rise in $[Na^+]_i$. While reduced external Na or increased membrane potentials do change the passive Na influx, they seem to have little direct effect on the Na pump. For conditions near the physiological range the pump rate appears to depend principally on $[Na^+]_i$.

METHODS

Experiments were done on large neurones (70–200 μ in diameter) located on the dorsal surface of the abdominal and right pallial ganglia of the snail *Helix aspersa*. After preliminary experiments successful penetrations were made of about half the cells attempted. A successful penetration was considered to be one in which the $[Na^+]_i$ did not rise after penetration by more than a few mM, as seen in Fig. 3. The circumoesophageal ring of ganglia was removed from dormant snails and mounted, dorsal surface up, on a shaped P.T.F.E. block. The outer connective tissue over the two ganglia was then removed using fine scissors, and the block placed in the experimental chamber. The P.T.F.E. block formed the floor and back of the experimental chamber. The ganglia were then covered with continuously flowing snail Ringer, and the thin inner connective tissue still covering the nerve cells was carefully torn with a fine hook.

The chamber had a volume of about 0·3 ml., and was perfused at a rate of about 1·5 ml./min. Solutions could be changed without interrupting the flow by means of a nine-way tap mounted near the chamber. The normal snail Ringer used had the following composition: KCl 4 mM, NaCl 80 mM, $CaCl_2$ 7 mM, $MgCl_2$ 5 mM, Tris chloride pH 8, 5 mM. Low Na solutions were made by substituting equivalent concentrations of Tris chloride, and when K was raised or lowered compensating changes were made in Na. All experiments were done at room temperature, 20–23° C.

Na^+-sensitive micro-electrodes. Electrodes made of the glass NAS 11–18 measure Na activity, that is the concentration of Na ions. This will be abbreviated to $[Na^+]$ to avoid confusion with total Na concentration [Na]. The electrodes used were of a recessed-tip design (Thomas, 1970, 1971) which permits a relatively long length of Na^+-sensitive glass to be exposed to the cell interior while the cell membrane is penetrated only by the 1–2 μ diameter tip of the outer, insulating glass. The design of the electrodes used is illustrated in Fig. 1. The relatively long exposure of Na^+-sensitive glass is desirable to minimize electrode resistance. With a 100 μ length the

resistance is about 10^{10} Ω. A detailed account of the construction procedure has been given elsewhere (Thomas, 1971), so only a brief account will be given here. First, a batch of micropipettes are made on a vertical electrode puller from the Na^+-sensitive glass NAS 11–18 (Eisenman, Rudin & Casby, 1957). The NAS 11–18 was obtained as 1 mm tubing, from Corning Glass Works, Corning, New York, and was cut into 3 cm lengths before pulling. Secondly, a batch of Pyrex micropipettes are made, with internal dimensions near the tip matching as closely as possible the outer dimensions of the corresponding parts of the NAS 11–18 micropipettes. Thirdly, the NAS 11–18 micropipettes are sealed by touching their tips to a red-hot wire in a microforge. The microforge consists of a horizontally mounted student type compound microscope with a Prior micromanipulator on each side. The microforge wire is Pt:Rh 80:20, approximately 25 μ in diameter. Fourthly, a selected Pyrex micropipette is mounted, vertically and tip down, in the microforge. The terminal 5 mm or so of a matching

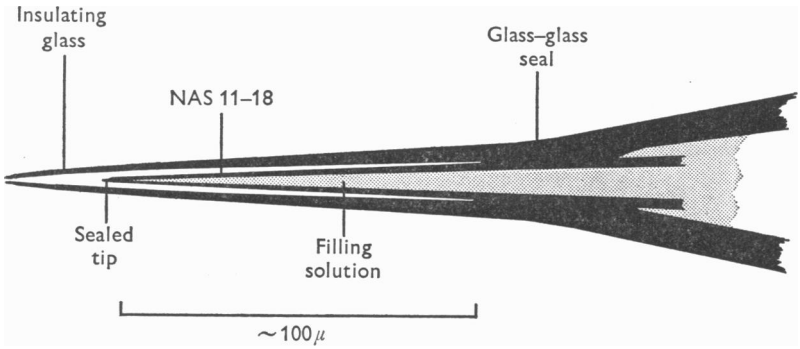

Fig. 1. Diagram showing construction of recessed-tip Na^+-sensitive micro-electrode.

NAS 11–18 micropipette is then broken off after scratching with a diamond, and dropped, tip down, into the top of the Pyrex micropipette. With care the NAS 11–18 will lodge with its tip intact and 10–30 μ behind the Pyrex tip (see Fig. 1). A glass to glass seal is then made 100–150 μ behind the electrode tip by applying heat at that point with the microforge. Electrodes are made in this way and stored dry in batches. They are filled individually as required with 0·1 M-NaCl, buffered to pH 8 with Tris-Cl, using a fine-needled syringe to fill the electrode as completely as possible, and expelling remaining air bubbles using the microforge.

On immersion of the electrode tip in Ringer solution, the dead space between the outside of the NAS 11–18 and the inside of the Pyrex tip is full of air, but within a few minutes this air dissolves and the space fills with Ringer. The electrodes become electrically stable within 1 or 2 hr of filling, and are then at their best, with least noise, lowest electrical resistance and greatest sensitivity to Na^+. They are usable for several days. The responses of the electrodes to Na^+ were calibrated, always after and usually before an experiment, by using Ringer solutions with K substituted for Na, but with the same Mg, Ca, Tris and Cl as normal snail Ringer. Three calibration solutions were normally used; one being normal Ringer (80 Na, 4K) the others being 8 Na, 76 K and 2 Na, 82 K. The latter two solutions had K and Na concentrations of the same order as expected intracellularly; having high K in these low Na calibration solutions should automatically correct the Na^+-sensitive electrode response for the small error caused by the slight response of NAS 11–18 glass to the high $[K^+]_i$. No correction was made for the difference between the Na activity and con-

centration in the calibration solutions. That is, the levels of $[Na^+]_i$ presented in the results will be higher than the true values by perhaps some 25 % (Hinke, 1961).

The volume of the dead space in the electrode tips was less than 10^{-9} ml., but because of the small tip diameter the electrodes responded rather slowly, with time constants of several seconds. This should cause errors only when $[Na^+]_i$ is changing rapidly.

Reference and current-passing micro-electrodes. Conventional micro-electrodes were made on a vertical electrode puller from 2 mm diameter Pyrex tubing with a Pyrex filament of about 0·1 mm diameter glued inside. This modification of the technique of Tasaki, Tsukahara, Ito, Wayner & Yu (1968) permitted the filling of the micro-electrodes just before use by means of a syringe. Electrodes were filled with 2 M-KCl (except for Na injections), buffered to pH 8 with 1 mM-Tris chloride. Electrodes made and filled in this way had tip potentials less than 3 mV, and appeared to penetrate cells more easily than electrodes filled by boiling. The resistances of electrodes used ranged from 15 to 30 MΩ. The indifferent electrode was a Ag-AgCl wire in an agar-Ringer bridge, and electrical connexions to the micro-electrodes were made directly by Ag-AgCl wired dipped into the filling solution.

Electrical arrangements. These were basically the same as previously described (Thomas, 1969) except that the membrane potential was electrically subtracted from the Na^+-sensitive electrode potential by using the intracellular membrane potential electrode, after current amplification, as reference for the electrometer. The electrometer used had a higher input resistance (10^{16} Ω) than before. For Na injections a photon-coupled floating current clamp was used rather than a radio-frequency isolation unit. The very high electrical resistances (10^{10}–10^{11} Ω) of the Na^+-sensitive micro-electrodes made them very sensitive to electrical interference. To minimize this the preparation had to be very carefully shielded.

RESULTS

Normal value of $[Na^+]_i$ in snail neurones

Fig. 2 illustrates an experiment in which $[Na^+]_i$ and membrane potential were measured in a large spontaneously active neurone over a period of about 15 min. The Na^+-sensitive and conventional micro-electrodes were first kept outside the cell for some time to equilibrate. Then the cell was penetrated with the Na^+-sensitive micro-electrode, and, about 30 sec later, with the conventional KCl electrode. As the cell membrane was penetrated by the KCl electrode, the membrane potential was automatically subtracted from the potential recorded by the Na^+-sensitive micro-electrode, causing an upwards jump of the $[Na^+]_i$ record. The membrane potential was close to 50 mV throughout the experiment, with some spontaneous action potentials. The recorded action potentials are greatly reduced in size by the slow time constant of the pen recorder, and are seen on the $[Na^+]_i$ record because of imperfect cancellation of such rapid changes in membrane potential. The value for $[Na^+]_i$ in this cell was about 3·5 mM a few minutes after penetration, falling to about 3 mM over the next 10 min. Such a decrease in $[Na^+]_i$ from the value observed in the first few minutes after penetration was found in all experiments, and was pre-

sumably due to recovery from damage. In the experiments shown in Figs. 3, 4 and 10 there was more damage, as judged by lower initial membrane potentials, than seen in Fig. 2, and in these experiments a distinct but transient rise in $[Na^+]_i$ is seen at the beginning. Fig. 3 shows that this rise

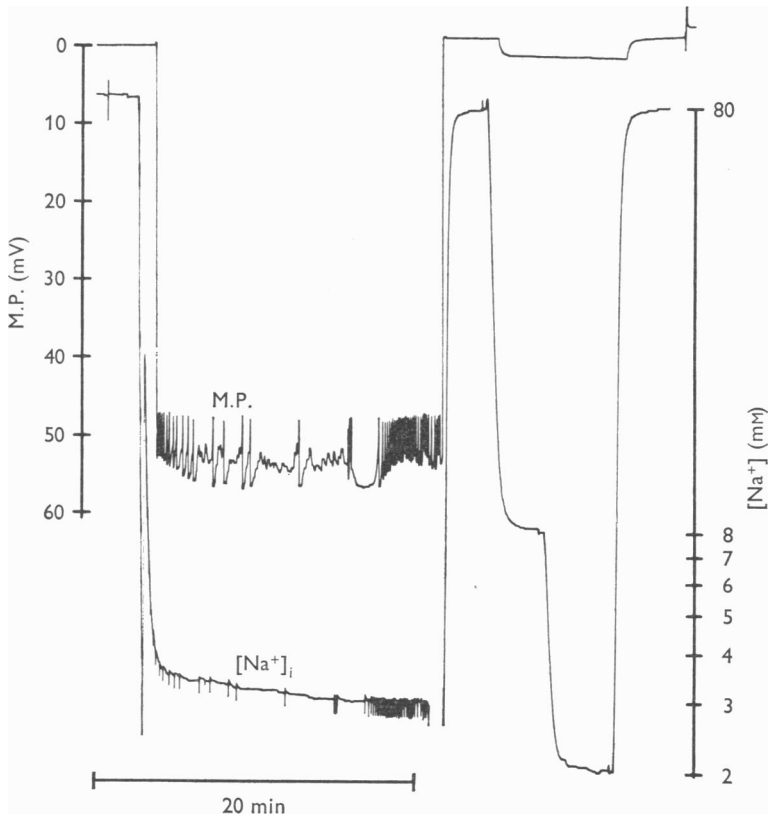

Fig. 2. Pen-recording of experiment to determine $[Na^+]_i$ and membrane potential of a large snail neurone. The Na^+-sensitive electrode was inserted first, then the membrane potential electrode. The electrodes were withdrawn in reverse order, and then the Na^+-sensitive electrode was calibrated by perfusing the experimental chamber with 8 mM-Na and 2 mM-Na Ringer solutions. The slow time constant of the recorder has reduced the recorded size of the action potentials. The membrane potential pen of the recorder was displaced in time some 50 sec behind the $[Na^+]$ pen in all experiments.

is not due to some special property of the Na^+-sensitive micro-electrode. In this experiment on a silent cell penetration of the cell membrane by a third micro-electrode caused similar changes in the membrane potential and $[Na^+]_i$ to those seen at the beginning of the experiment. All deter-

minations of normal resting $[Na^+]_i$ were therefore made only when the $[Na^+]_i$ had recovered from the initial rise caused by damage. While recovery always seemed complete, it is impossible to rule out the possibility that some permanent damage had occurred. The finding that $[Na^+]_i$ was maintained at essentially constant resting levels during experiments lasting several hours, however, suggests that any permanent damage was minor.

The value for $[Na^+]_i$ found in twenty-two experiments on a variety of neurones was $3{\cdot}6 \pm 0{\cdot}15$ mM (mean $\pm$ S.E. of mean). The corresponding figures for membrane potential were $47{\cdot}8 \pm 1{\cdot}0$ mV.

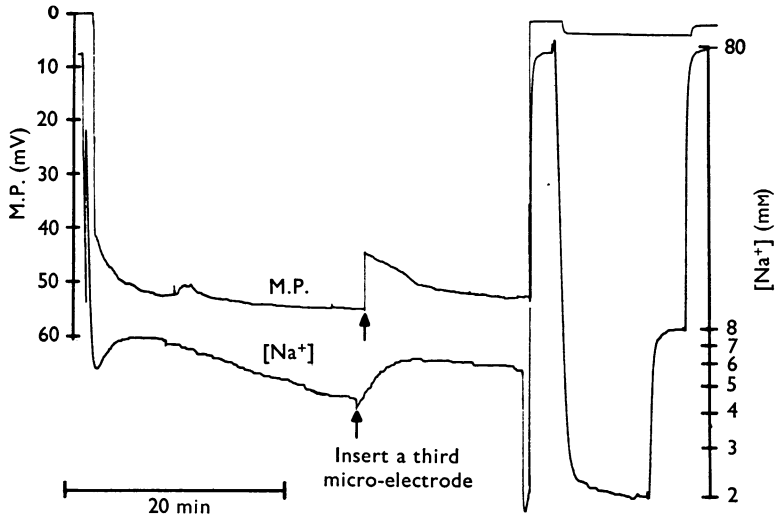

Fig. 3. Pen-recording of experiment showing the effect of inserting a third micro-electrode on the $[Na^+]_i$ of a 70 μ silent neurone. Calibration of the Na^+-sensitive micro-electrode is also shown.

Apart from some brief observations with a reversed-tip electrode (Thomas, 1969) the only other measurements of $[Na^+]_i$ values in snail neurones are those reported by Sorokina (1966) and Kostyuk, Sorokina & Kholodova (1969). The membrane potentials they report are not greatly different from those found in the present experiments, but their value of 12–13 mM for $[Na^+]_i$ is over three times higher than that found in the present experiments. It is difficult to ascribe such a difference simply to a different species (*Helix pomatia*) or different physiological salines, and it seems more likely that the relatively large electrode tips used by these workers did not completely penetrate the cell membrane. To accurately measure $[Na^+]_i$ the Na^+-sensitive micro-electrode must record both the membrane potential and $[Na^+]_i$ and any failure to record the full membrane potential, caused by incomplete penetration, would lead to over-

estimation of $[Na^+]_i$, assuming accurate measurement of membrane potential alone with a conventional micro-electrode. In the present series of experiments the lack of direct effects of membrane potential changes on recorded $[Na^+]_i$ seen, for example, in Figs. 4, 7, 10 and 12, confirms that the recessed-tip design of Na^+ sensitive microelectrode records the full membrane potential.

The effect of Na pump inhibition on $[Na^+]_i$

It is well established that the low $[Na^+]_i$ of excitable cells is maintained by the Na pump. The passive influx of Na from outside the cell is normally balanced by the active extrusion of Na by the pump. Inhibition of the

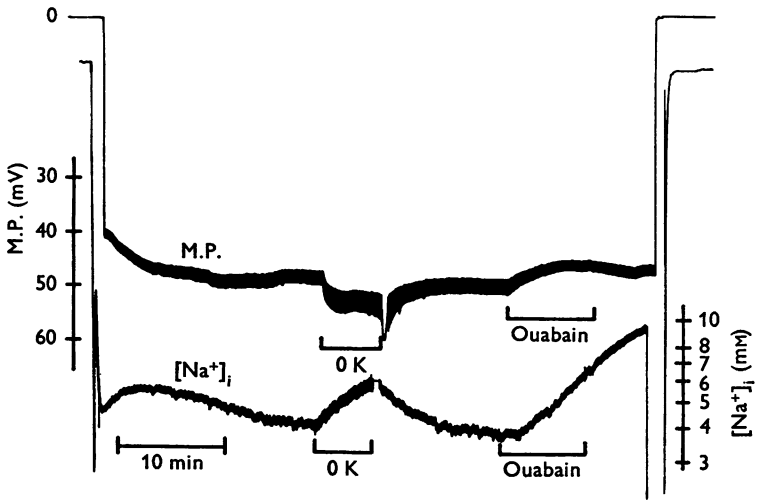

Fig. 4. Pen-recording of response of $[Na^+]_i$ and membrane potential to K-free Ringer and application of 10^{-4} M ouabain.

pump should then reveal the size of the passive influx of Na. In the experiment illustrated in Figs. 4 and 5 the Na pump was inhibited first reversibly by removal of external K, and, secondly, irreversibly by application of the cardiac glycoside ouabain. It can be seen that pump inhibition led to a continuous rise in $[Na^+]_i$ at a rate of about 0·37 m-mole/min for K removal and 0·45 m-mole/min for ouabain. The lower rate of rise seen with K removal is probably due to incomplete removal of K, and thus incomplete inhibition of the pump.

If the cell is regarded as a sphere of, in this case, a radius of 100 μ, the rate of rise of $[Na^+]_i$ seen with ouabain is equivalent to a net Na^+ influx of approximately 25 p-equiv/cm^2.sec, a value close to that found for squid axon by Hodgkin & Keynes (1955). It should be noted perhaps that

Hodgkin & Keynes in fact measured influx of radioactive Na, and did not exclude exchange diffusion, which would cause no increase in $[Na^+]_i$. The linearity of the rise in $[Na^+]_i$ with ouabain (see also Fig. 11) suggests that if there is any binding of the Na^+ ions entering the cell, then this binding is proportional to $[Na^+]_i$.

Similar experiments have been done on seven different snail neurones, both silent and spontaneously active, and in all cases pump inhibition led to a relatively rapid increase in $[Na^+]_i$, the average rate of increase being

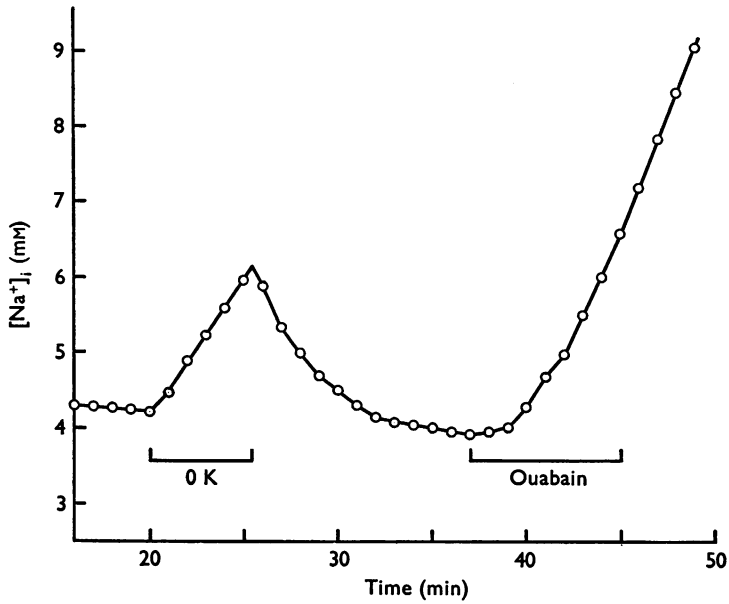

Fig. 5. Plot on a linear scale against time of measurements of $[Na^+]_i$ taken from the experiment shown in Fig. 4.

0·54 m-mole/min. These experiments reveal that the net influx of Na is at such a rate that it would rapidly increase $[Na^+]_i$ were it not for the continuous activity of the Na pump, assuming ouabain has no effect on the cell other than on the Na pump.

The effect of external K on $[Na^+]_i$

Because of the coupling between K uptake and Na extrusion by the Na pump it seems possible that relatively small changes in external K, perhaps by altering the K equilibrium potential, might affect $[Na^+]_i$. The experiment illustrated in Fig. 6, however, shows that only very low external K has much effect. Doubling external K, although causing about 5 mV depolarization of the membrane potential, had no effect on $[Na^+]_i$, and

1 mM-K causes at most a small rise in $[Na^+]_i$. Lower concentrations of K, 0·25 mM or K-free Ringer, however, do cause a rise in $[Na^+]_i$, presumably by inhibiting the pump rather than increasing the Na^+ influx. Over the range of 1–8 mM external K, it is clear that $[Na^+]_i$ is relatively unaffected. Because of unknown effects of external K on the Na influx it is not possible to conclude that the Na pump is itself unaffected by external K over this range, but it is clear that the K equilibrium potential does not in any way re-set $[Na^+]_i$.

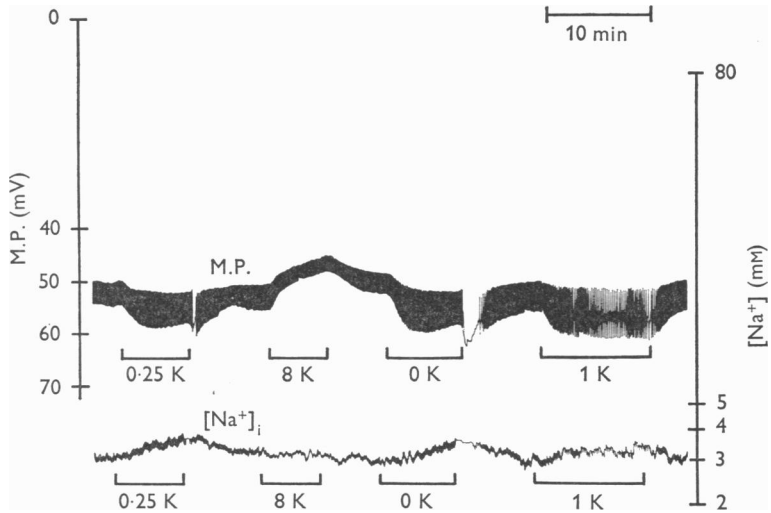

Fig. 6. Pen recording of experiment to show the effects on membrane potential and $[Na^+]_i$ of different external K concentrations. K concentrations given in mM.

The effect on $[Na^+]_i$ of increasing the membrane potential

Since the Na pump is extruding Na against both an electrical gradient and a concentration gradient, increasing the membrane potential might be expected to increase the energy required to operate the pump beyond that available from cellular metabolism. A number of experiments were therefore done to observe the effects on $[Na^+]_i$ of hyperpolarizations of up to 100 mV. The results completely failed to demonstrate any blockage of the pump. Such an experiment is shown in Fig. 7. The membrane potential was controlled throughout the experiment by the slow voltage clamp circuit described previously (Thomas, 1969). Fig. 7 shows that raising the membrane potential, firstly by 50 mV and then by 90 mV, does lead to a rise in $[Na^+]_i$, presumably because passive Na influx is increased, as expected from the increase in electrical gradient. The rate of increase, however, is less than that seen with external K removal at the normal membrane

potential. This suggests that, even if there is no extra Na influx, raising the membrane potential causes less inhibition of the pump than removing external K.

To see if the pump could cause a net efflux of Na from the cell even if it was hyperpolarized by 90 mV, the $[Na^+]_i$ was first raised by removing external K and then increasing the membrane potential. The pump inhibition, and presumably the increased passive Na influx caused by this

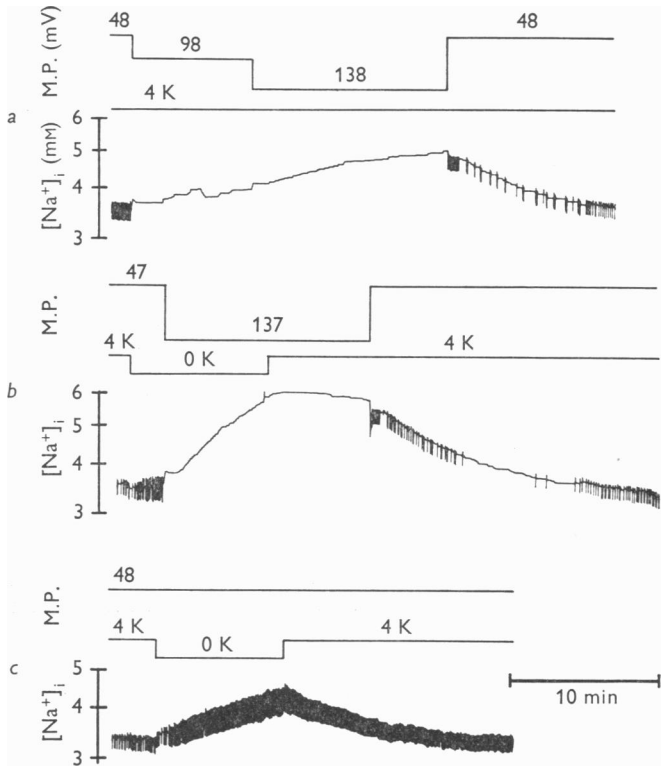

Fig. 7. Pen recordings of the effect of increasing membrane potential on $[Na^+]_i$. The levels of external K and membrane potential are indicated by the lines above the $[Na^+]_i$ recordings. *a*, The effects of increasing the membrane potential by 50 mV and then by 90 mV. *b*, The effects of both external K removal and increasing the membrane potential by 90 mV. *c*, The effects of K removal alone.

procedure, led to a rapid rise in $[Na^+]_i$. Then when external K was replaced $[Na^+]_i$ actually decreased slowly, until returning the membrane potential to normal allowed $[Na^+]_i$ to return also. Thus for the period with normal external K but increased membrane potential the Na pump was not only extruding the Na entering the cell; it was also causing a net reduction in $[Na^+]_i$. Unfortunately the pump rate under these conditions cannot be

easily compared with the pump rate at normal membrane potentials and the same high $[Na^+]_i$, but it is clear that the pump is not blocked by a 90 mV hyperpolarization.

This apparent lack of direct effect of hyperpolarization on the Na pump confirms the results obtained on squid axon, with rather smaller hyperpolarizations, by Hodgkin & Keynes (1955) and Brinley & Mullins (1971).

The effect of reducing external Na on $[Na^+]_i$

Decreasing external Na should decrease the passive Na influx into the cell and thus allow the Na pump to decrease $[Na^+]_i$. If influx is essentially abolished, and there is no significant passive efflux of Na, then the rate of

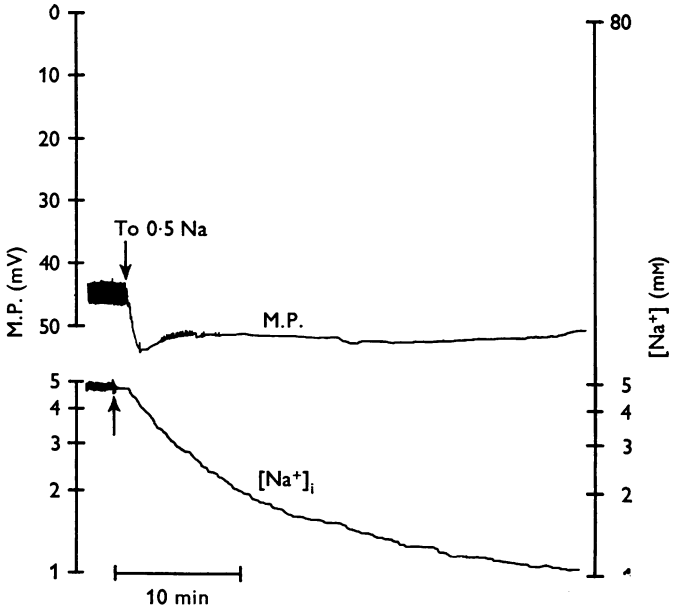

Fig. 8. Pen recording of membrane potential and $[Na^+]_i$ showing the effect of changing the external Na from 80 to 0·5 mM.

decrease in $[Na^+]_i$ should give the relationship between pump rate and $[Na^+]_i$. Fig. 8 illustrates an experiment in which the external Na was reduced from the normal 80 to 0·5 mM. This level was selected so that the Na equilibrium potential would be close to the membrane potential, minimizing net passive fluxes. In the experiment the membrane potential increased, as expected from the constant field equation, and $[Na^+]_i$ rapidly decreased. McLaughlin & Hinke (1966) describe a decrease in $[Na^+]_i$ from 10–14 mM to 6–7 mM in barnacle muscle fibres treated with sucrose Ringer for 30 min. In the present experiments, of course, continuous measurements were

made, and in Fig. 9*a* values of $[Na^+]_i$ from the experiment of Fig. 8 are plotted on a linear scale against time. It is clear that the decline in $[Na^+]_i$ is exponential. This shows that the rate of decrease in $[Na^+]_i$ is directly proportional to $[Na^+]_i$ minus a threshold value, as seen in Fig. 9*b* where the rate of decrease in $[Na^+]_i$ is plotted against $[Na^+]_i$. This suggests that the pump rate is directly proportional to the level of $[Na^+]_i$ above a threshold value of about 1 mM, assuming that the decrease in $[Na^+]_i$ is caused only by the pump.

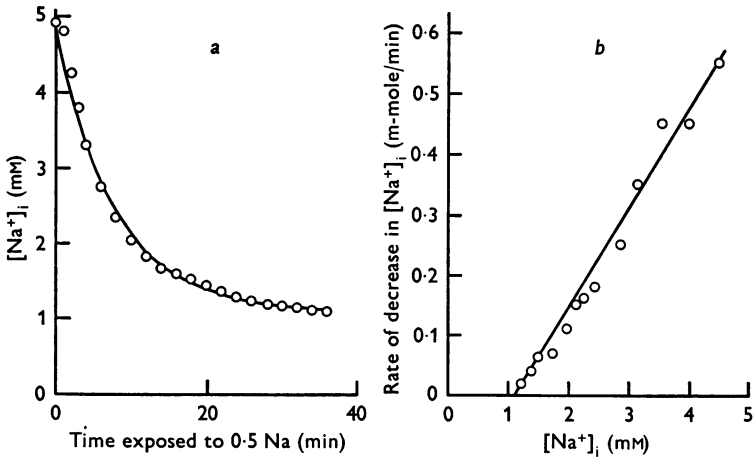

Fig. 9. *a*, Values of $[Na^+]_i$ plotted on a linear scale against time of exposure to 0·5 mM external Na. The line is a calculated exponential decline to 1·1 mM-$[Na^+]_i$ with a time constant of 7·7 min. *b*, Plot of the rate of decrease in $[Na^+]_i$ against $[Na^+]_i$. Data taken from the experiment illustrated in Fig. 8.

Reducing external Na will also reduce the energy required to operate the pump, and might therefore be expected to allow the pump to operate at a faster rate. For the experiment shown in Fig. 8, unfortunately, the pump rate in normal external Na was not determined, although the pump rate at 4 mM $[Na^+]_i$, taken from Fig. 9*b*, is close to the average value for the normal pump rate calculated from the experiments with ouabain.

The experiment illustrated in Figs. 10 and 11 does however allow a comparison of pump rates in normal and reduced external Na. The application of ouabain at the end of the experiment (shown in Fig. 11 only) indicates that the passive Na influx was at a rate which increased $[Na^+]_i$ at 0·48 m-mole/min. Since the equilibrium value for $[Na^+]_i$ in normal external Na was about 3·8 mM, the normal pump rate at $[Na^+]_i$ of 3·8 mM must have been 0·48 m-mole/min. The rate of decrease in $[Na^+]_i$ when external Na was reduced to 10 mM was about 0·4 m-mole/min at an $[Na^+]_i$ of 3·8 mM.

Since in 10 mM external Na there will be perhaps an eighth of the normal net Na influx, a $[Na^+]_i$ reduction rate of 0·4 mM indicates an actual pump rate of 0·46 mM, in good agreement with the calculated pump rate in normal external Na. Thus it appears that reduction of the external Na from 80 to 10 mM has had no direct effect on the Na pump.

The final experiment, illustrated in Fig. 12, shows that even complete removal of external Na does not apparently affect the pump rate. In this experiment the cell was penetrated with two additional micro-electrodes

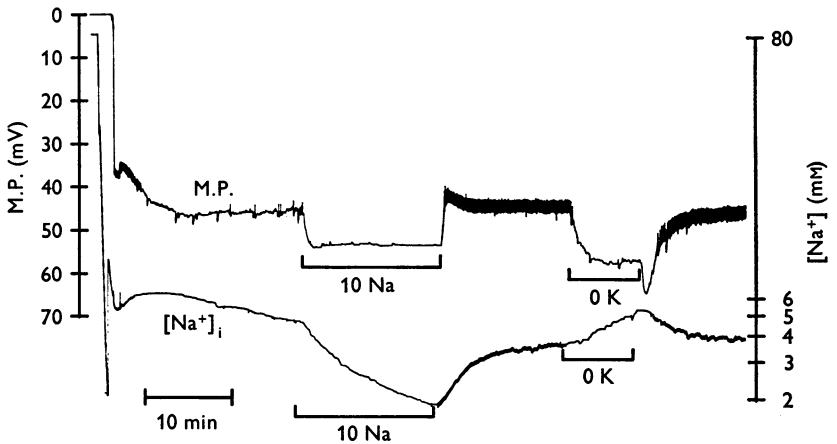

Fig. 10. Pen recording showing the effects on membrane potential and $[Na^+]_i$ of 10 mM-Na Ringer and K-free Ringer.

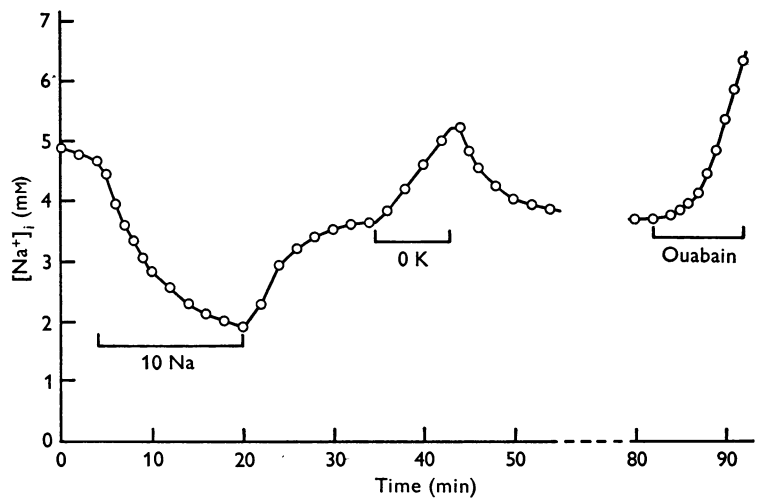

Fig. 11. Graph on a linear scale of values of $[Na^+]_i$ against time from the experiment partially illustrated in Fig. 10, showing the effects of 10 mM-Na Ringer, K-free Ringer and 10^{-4} M ouabain.

so that Na acetate could be injected by interbarrel iontophoresis. No attempt was made to prevent leakage of Na acetate from the Na injection electrode so there was a continuous injection of Na acetate throughout the experiment. Once the membrane potential and $[Na^+]_i$ had recovered from the microelectrode penetrations, external Na was removed and, 8 min

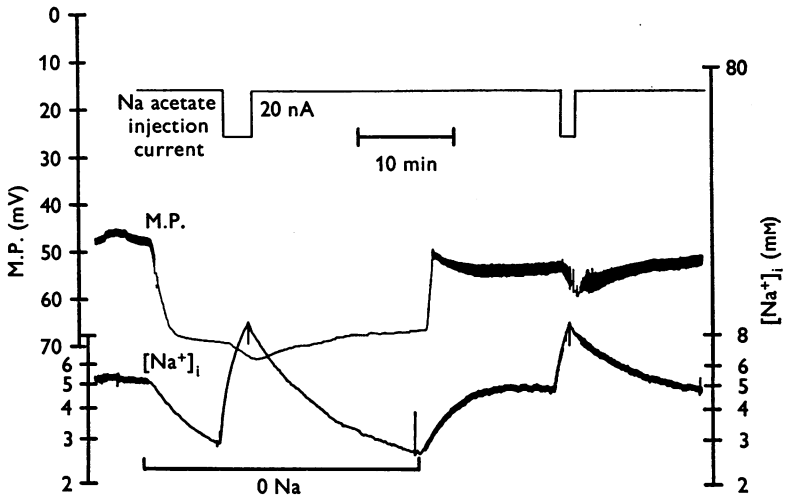

Fig. 12. Pen recording of the effect of the removal of external Na on the extrusion of injected Na. Na acetate was injected into the cell by interbarrel iontophoresis, as indicated at the top of the Figure, during exposure to Na-free Ringer and again on return to normal Ringer.

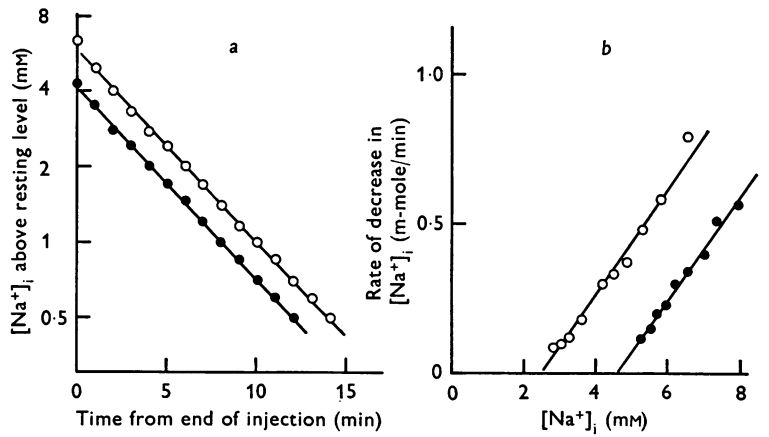

Fig. 13. Data taken from experiment shown in Fig. 12. Open circles: Na-free Ringer; filled circles: normal Ringer. *a*, $[Na^+]_i$ above resting level plotted on a linear scale against time from end of injection. Resting levels taken as 2·5 mM in Na-free Ringer and 4·5 mM in normal Ringer. (*b*) Rate of decrease in $[Na^+]_i$ plotted against $[Na^+]_i$.

later, Na acetate was injected by passing a 20 nA current between the two injection electrodes for 3 min. $[Na^+]_i$ rose to 8·8 mM during the injection, and then declined exponentially towards a resting level of about 2·5 mM. External Na was replaced, and a second injection of Na acetate made to raise $[Na^+]_i$ to a similar level as at the end of the first injection. After the second injection the $[Na^+]_i$ declined exponentially towards the resting level of about 4·5 mM (cf Thomas, 1969). Levels of $[Na^+]_i$ above the respective resting levels of 2·5 mM in Na-free Ringer and 4·5 mM in normal Ringer are plotted on a logarithmic scale against time in Fig. 13*a*. The points fall on two parallel straight lines, confirming both that the pump rate is directly proportional to excess $[Na^+]_i$ and, as also shown in Fig. 13*b*, that removal of external Na has no effect on the rate constant of the pump.

DISCUSSION

The results show that $[Na^+]_i$ in snail neurones is very low, but is stable for many hours. Inhibition of the Na pump reveals that there is a continuous large influx of Na, at a rate that would double $[Na^+]_i$ within 10 min. Increasing the membrane potential and decreasing external Na appear to have no direct effect on the pump. But by changing the rate of Na influx these procedures do cause an increase or decrease in $[Na^+]_i$. Apart from low concentrations, changes in external K do not affect $[Na^+]_i$. Given sufficient external K, then, the rate of the Na pump in these experiments is controlled principally by $[Na^+]$. Since snail blood has K concentrations around 4 mM, this suggests that in the snail the rate of the Na pump is controlled principally by $[Na^+]_i$.

Most previous measurements of $[Na^+]_i$ have been made with similar types of Na^+-sensitive micro-electrode to that used by Hinke (1959). This design can give difficulties when the electrode has to cross the cell membrane, as mentioned earlier, but Hinke used a cannulation technique which avoided this problem. He reported values of 12–15 mM for crustacean muscle (Hinke, 1959, see also McLaughlin & Hinke, 1966) and about 30 mM for squid axon (Hinke, 1961). These values are much higher than those reported here for the snail, but if one considers only the ratio of internal to external Na^+ then the present results give values quite similar to those reported by Hinke.

The conclusions reached about the lack of effects of external Na^+ removal on the Na pump are in agreement with some findings on squid axon, but not with others. For example Mullins & Brinley (1967) found no effect of external Na removal on the Na efflux from dialysed squid axons, while Hodgkin & Keynes (1955) and Baker, Blaustein, Keynes, Manil, Shaw & Steinhardt (1969) and others report that Na removal increases

Na efflux. According to Baker *et al.* (1969) removal of external Na increases the affinity of the pump mechanism for external K ions. A possible explanation for the lack of effect of low external Na in the present experiments is that the K-binding site may be saturated by the K concentration in the snail Ringer.

The linear relationship between pump rate and $[Na^+]_i$ at subnormal levels agrees with results obtained on squid axons by Brinley & Mullins (1968), assuming that their $[Na]_i$ was proportional to $[Na^+]_i$. This relationship has long been established for higher levels of $[Na]_i$ (Hodgkin & Keynes, 1956). For muscle fibres, however, a highly non-linear relationship between efflux and $[Na]_i$ has been found. Perhaps this is due to a non-linear relationship between $[Na]_i$ and $[Na^+]_i$ in muscle fibres.

Contribution of the Na pump to the membrane potential

Previous studies of the electrogenic Na pump in snail neurones have been confined to cells with elevated $[Na^+]_i$. Indeed no studies of the electrogenic Na pump in tissues with subnormal $[Na^+]_i$ have been reported. The present results suggest that the pump is still electrogenic at normal and low values of $[Na^+]_i$. In the experiment illustrated in Fig. 4, for example, ouabain is seen to cause a decrease in membrane potential which reaches a peak after about 6 min, which is a similar period to that needed for maximal inhibition of the pump, as judged by the rate of increase in $[Na^+]_i$. The size of the depolarization is hard to determine because of the spontaneous action potentials but it appears to be about 3 mV. Such a contribution of the pump to the normal membrane potential is also seen in marine molluscan neurones (Carpenter & Alving, 1968; Gorman & Marmor, 1970). The increased pump activity occurring after the period of exposure to K-free Ringer is clearly also electrogenic, causing a hyperpolarization of about 10 mV in the experiment of Fig. 4; and see also Figs. 6 and 10. Again this effect is seen in certain marine mollusc neurones (Gorman & Marmor, 1970) but is less marked probably because of these cell's greater size and the lower temperature of the experiments. Of course the electrogenic property of the sodium pump working at above normal rates is well established for a variety of tissues.

Evidence that the pump is electrogenic at subnormal rates of activity can be seen in the results of the experiments in which external Na was reduced. For example, in Fig. 10, the return to normal snail Ringer after the exposure to 10 mM-Na caused the membrane potential to depolarize beyond the normal level. As $[Na^+]_i$ rose, however, causing a corresponding increase in pump activity, the membrane potential increased to its normal level. A similar response of the membrane potential is seen in Fig. 12. These results suggest that the electrogenic current is proportional to the pump

rate even at subnormal pump rates, as has previously been shown for above normal pump rates (Thomas, 1969).

I wish to thank my colleagues for their help and interest in my experiments, and for comments on drafts of this paper. I am particularly grateful to Mrs Barbara Moreau who constructed most of the Na^+ sensitive micro-electrodes used, and to the Medical Research Council for a grant to buy apparatus.

REFERENCES

BAKER, P. F., BLAUSTEIN, M. P., KEYNES, R. D., MANIL, J., SHAW, T. I. & STEINHARDT, R. A. (1969). The ouabain-sensitive fluxes of sodium and potassium in squid giant axons. *J. Physiol.* **200**, 459–496.

BRINLEY, F. J. & MULLINS, L. J. (1968). Sodium fluxes in internally dialysed squid axons. *J. gen. Physiol.* **52**, 181–211.

BRINLEY, F. J. & MULLINS, L. J. (1971). The fluxes of sodium and potassium across the squid axon membrane under conditions of altered membrane potential. *Fedn Proc.* **30**, 255.

CARPENTER, D. O. & ALVING, B. O. (1968). A contribution of an electrogenic Na^+ pump to membrane potential in Aplysia neurones. *J. gen. Physiol.* **52**, 1–21.

EISENMAN, G., RUDIN, D. O. & CASBY, J. U. (1957). Glass electrode for measuring sodium ion. *Science, N.Y.* **126**, 831–834.

GORMAN, A. L. F. & MARMOR, M. F. (1970). Contributions of the sodium pump and ionic gradients to the membrane potential of a molluscan neurone. *J. Physiol.* **210**, 897–917.

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

HODGKIN, A. L. & KEYNES, R. D. (1956). Experiments on the injection of substances into squid giant axons by means of a microsyringe. *J. Physiol.* **131**, 592–616.

HINKE, J. A. M. (1959). Glass micro-electrodes for measuring intracellular activities of sodium and potassium. *Nature, Lond.* **184**, 1257–1258.

HINKE, J. A. M. (1961). The measurement of sodium and potassium activities in the squid axon by means of cation-selective glass microelectrodes. *J. Physiol.* **156**, 314–335.

KOSTYUK, P. G., SOROKINA, Z. A. & KHOLODOVA, Y. D. (1969). Measurement of activity of hydrogen, potassium and sodium ions in striated muscle fibres and nerve cells. In *Glass Microelectrodes*, pp. 322–348, ed. LAVALLEE, M., SCHANNE, O. F. & HEBERT, N. C. New York: Wiley.

MCLAUGHLIN, S. G. A. & HINKE, J. A. M. (1966). Sodium and water binding in single striated muscle fibres of the giant barnacle. *Can. J. Physiol. Pharmac.* **44**, 837–848.

MULLINS, L. J. & BRINLEY, F. J. (1967). Some factors influencing sodium extrusion by internally dialyzed squid axons. *J. gen. Physiol.* **50**, 2333–2355.

SOROKINA, Z. O. (1966). Activity of sodium and potassium ions in giant neurones of molluscs. *Fiziol. Zh. ukrajin. R.S.R.* **12**, 776–779.

TASAKI, K., TSUKAHARA, Y., ITO, S., WAYNER, M. J. & YU, W. Y. (1968). A simple, direct and rapid method for filling microelectrodes. *Physiol. & Behav.* **3**, 1009–1010.

THOMAS, R. C. (1969). Membrane current and intracellular sodium changes in a snail neurone during extrusion of injected sodium. *J. Physiol.* **201**, 495–514.

THOMAS, R. C. (1970). New design for sodium-sensitive glass micro-electrode. *J. Physiol.* **210**, 82–83*P*.

THOMAS, R. C. (1971). pNa microelectrodes with sensitive glass inside the tip. In *Ion Selective Microelectrodes*, ed. HEBERT, N. C. & KHURI, R. New York: Dekker (in the Press).