**MEMBRANE CURRENT AND INTRACELLULAR SODIUM CHANGES IN A SNAIL NEURONE DURING EXTRUSION OF INJECTED SODIUM**

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**SUMMARY**

1. Sodium was injected into an identified snail neurone by passing current between two intracellular micro-electrodes, the membrane potential being recorded with a third micro-electrode.

2. The injection of about 25 p-equiv Na, but not the injection of similar quantities of K or Li, caused a hyperpolarization of up to 20 mV. This response to Na injection was blocked by application of ouabain or removal of external K, indicating that it was due to the stimulation of an electrogenic pump.

3. To measure the current produced by the sodium pump the output of a feed-back amplifier was fed into the cell via a fourth intracellular micro-electrode so as to keep the average membrane potential constant. The pump current, measured in this way, rose at a constant rate during, and declined exponentially after, an injection of Na, the decline having an average time constant of 4.4 min. The total charge transferred by the pump was between a third and a quarter of the charge passed to inject sodium.

4. An intracellular Na-sensitive glass micro-electrode was used to follow changes in the concentration of intracellular Na ions. The results showed that both the pump current and the rate of Na extrusion were proportional to the concentration of intracellular Na ions above the normal level.

5. It was concluded that about two thirds of the Na extruded was coupled to the active transport of other ions, probably to the uptake of K, the uncoupled third producing the electrogenic effect.
INTRODUCTION

It has already been shown in a variety of nerve and muscle preparations that the sodium pump can be 'electrogenic', that is, it can act as a current source in the cell membrane. To show this it has usually been necessary to stimulate the Na pump by increasing intracellular Na concentrations above normal. Thus with snail neurones, Kerkut & Thomas (1965) showed that the injection of Na salts (by allowing them to leak into the cell from low resistance micro-electrodes) caused an increase in the membrane potential (i.e. an increase in internal negativity), which was blocked by removal of external K and by the application of ouabain. They concluded that the hyperpolarization caused by Na injection into snail neurones, which has also been reported by Chiarandini & Stefani (1967), was due to the stimulation of an electrogenic Na pump.

The purpose of the present experiments was, first, to confirm the earlier observations on snail neurones, using a different method of Na injection; secondly to measure the size and time course of the current generated by the pump; and finally to measure the changes in intracellular Na activity, [Na+]i, with a Na+-sensitive glass micro-electrode. The results suggest that approximately one third of the Na+ ions are extruded without coupling to K uptake, and that both 'pump current' and rate of Na efflux are proportional to the excess of [Na+]i above the normal level. A preliminary account of some of this work has been published (Thomas, 1968).

METHODS

Experiments were done on neurones of the common snail, *Helix aspersa*, and were generally confined to the largest cell in the right pallial ganglion. This cell, located near the entry of the right pallial nerve, is of the type classified as 'DInhi' by Gerschenfeld (1964). It is about 200 μ in diameter and readily identifiable from snail to snail. The 'brain' was removed from dormant snails and mounted, dorsal surface up, on a square block of Perspex. The thick outer connective tissue was then cut away over the right pallial ganglion, the preparation transferred to the experimental bath, and covered with snail Ringer (Kerkut & Thomas, 1965). Finally, the thin inner connective tissue was torn to expose the cells at the rear of the ganglion. All experiments were done at room temperature, 20–23° C.

*Measurement of membrane potential*. The cell was penetrated with conventional Pyrex micro-electrodes filled with KAc or KCl and having resistances of about 25 MΩ. The potential difference between the intracellular electrode and the bath electrode was amplified and displayed in the usual manner, with permanent records being made on a potentiometric pen recorder.

*Injection of salts*. Controlled injections were made by the technique of 'interbarrel iontophoresis' (Eccles, Eccles & Ito, 1964; Frank & Tauc, 1964), two separate current-carrying electrodes (2 and 3 in Fig. 1) being used. These electrodes were filled with 3 M salt solutions and selected for resistances between 25 and 40 MΩ. Too low a resistance allows excessive leakage into the neurone, while too high a resistance prevents the passage of adequate injection currents. Current from a radio-frequency isolation unit (R.F. isol.
unit) was led via a 500 MΩ resistor to one electrode, into the cell, up the second injection electrode, and back to the isolation unit via a second 500 MΩ resistor, as shown on the left of Fig. 1. With care in insulation of the injection circuit, this method allows iontophoretic injections to be made without passing any current across the cell membrane. The injection current was monitored by measuring the voltage drop across a 1.0 MΩ resistor in the current circuit. A switch, not shown in Fig. 1, permitted current to be passed between one intracellular electrode and the bath electrode for measurements of membrane resistance.

![Diagram](image)

Fig. 1. Diagram illustrating experimental arrangement. The electrodes are numbered as follows: 1, membrane potential; 2 and 3, injection electrodes; 4, clamping electrode, and 5, Na⁺-sensitive glass micro-electrode. C.F., cathode follower; C.R.O., cathode ray oscilloscope; CAL, calibrator; TRIG, Schmitt trigger circuit; M.M., monostable multivibrator. Experiments described in the first section of Results used electrodes numbered 1, 2 and 3; in the second, electrode 4 was added, and in the final section all five intracellular electrodes were used.

**Feed-back circuit.** For measurements of the current generated by the Na pump the average membrane potential was kept constant by a ‘voltage clamp’ type of circuit, as shown on the right of Fig. 1. An FET-input operational amplifier, with its gain set to about 50,000, was used to amplify any difference between the membrane potential and a value (usually the normal resting potential) preset by a calibration unit in the lead from the bath electrode. The output from the amplifier was then led back to the cell via a 500 MΩ resistor and a fourth intracellular micro-electrode. As shown in Fig. 1, the clamping electrode was normally one of a double-barrelled pair with the membrane potential electrode, the double-barrelled electrodes being selected for coupling resistances less than 100 kΩ, which is two orders of magnitude less than the membrane resistance. To avoid disturbing the normal regular spontaneous activity of the cell, a 0.2 μF condenser was used to give the clamp circuit a long time constant, so that only maintained changes in the membrane potential were prevented. It was, however, difficult to eliminate all responses of the clamp amplifier to action potentials, so in later experiments the input to the amplifier was cut off for a 100 msec period after the beginning of each spike. The amplified spikes were taken from the oscilloscope output and fed into a Schmitt trigger circuit. This in turn triggered a monostable multivibrator which opened a reed relay in the input to the clamp amplifier, so that during the spikes the clamp output was held constant. The clamping current was measured between the bath electrode and earth, using a second FET operational amplifier as a current-to-voltage transducer.
Measurement of changes in intracellular $\text{Na}^+$. Potentials from $\text{Na}^+$-sensitive glass micro-electrodes were amplified by a vibrating reed electrometer and recorded on a potentiometric pen recorder. The input capacitance of the electrometer made its response time too long to follow the expected changes in $[\text{Na}^+]_i$ if the electrode resistance was higher than $10^{11}$ $\Omega$. The manufacture of suitable electrodes proved extremely difficult. The glass used was NAS 11–18 (Eisenman, Rudin & Casby, 1957), and was generously supplied by Dr N. C. Hebert of the Corning Glass Works, Corning, New York. This glass can be made into micropipettes relatively easily, using a conventional electrode puller at a high heat setting;

![Diagram showing construction of Na$^+$-sensitive micro-electrodes.](image)

Fig. 2. Diagram showing construction of Na$^+$-sensitive micro-electrodes. (a) Design used by Hinke (1959). (b) Inverted design used in the present experiments.

the great difficulty lies in providing a suitable insulation. The insulation must not only restrict the Na$^+$ sensitivity to that portion of the electrode that can be introduced into the cell, but must also electrically insulate the rest of the electrode so that the potential changes occurring at the sensitive tip are not shunted. The first method tried was the glaze insulation technique developed for pH-sensitive glass by Carter, Rector, Campion & Seldin (1967). It proved quite possible to make glazed electrodes of the required appearance and dimensions, but they gave no response to Na$^+$, probably because the glaze either did not provide adequate electrical insulation or in some way poisoned the Na$^+$-sensitive glass at the tip. A variety of other insulating materials applied to the electrode were also tried without success.

The first potentially usable electrodes were eventually made by the method of Hinke (1959), the insulation and main body of the electrode being lead glass. Such electrodes gave excellent responses to Na$^+$ changes extracellularly, but for resistances less than $10^9$ $\Omega$, at least 60 $\mu$m of NAS 11–18 had to be exposed, as shown in Fig. 2a (Hinke (1959) quotes resistances of between $10^6$ and $10^8$ $\Omega$ for his electrodes, which had 150 $\mu$m exposed lengths of NAS 11–18). Such electrodes proved very difficult to insert fully into the neurone, because not only all the exposed NAS 11–18 but also the beginning of the insulation must be inside the cell. To overcome this problem, electrodes were constructed to a new design, which was basically an ‘inverted’ form of the Hinke (1959) electrode, as shown in Fig. 2b. With this ‘outside-in’ electrode it is possible to expose over 100 $\mu$m of Na$^+$-sensitive glass to the cell interior, without having an electrode tip diameter greater than 10 $\mu$m. (The electrodes used had tip diameters of 8–10 $\mu$m, and were tolerated well by the snail neurones studied. It is possible to make electrodes to this design with tip diameters as small as 4 $\mu$m, but their resistances are too high for the present purpose.) Since the insulation extends to the tip of the electrode, there is no uncertainty as to the insertion of both the sensitive
glass and the beginning of the insulation. Construction was as follows. First, lead glass micropipettes were made on the electrode puller and their tips broken to an outside diameter of 8–10 μ. Then NAS 11–18 micropipettes were made, using a strong pull and high temperature so that they were as long and as thin as possible, and their tips sealed by touching them to a red-hot platinum wire under microscopic control (Janáček, Morel & Bourguet, 1968). Then, with the lead glass and NAS 11–18 micropipettes held vertically tip to tip, the NAS 11–18 pipette was inserted into the tip of the lead glass pipette, and a glass-to-glass seal made with a microforge. The contraction of the glass occurring when the microforge was switched off usually broke off the NAS 11–18 cleanly at the seal. Some electrodes were made in this way using Pyrex for the outer micropipette instead of lead glass, and with the same exposed length of NAS 11–18 had somewhat lower resistances, suggesting that the lead glass might be in some way poisoning the NAS 11–18. The electrodes were filled in the usual way with methanol under reduced pressure, followed by two changes of distilled water and finally 0.1 M-NaCl.

RESULTS

Effects of salt injections on the membrane potential. When Na salts are injected into the snail neurone there is a considerable increase in the membrane potential, which is not seen with the injection of similar quantities of K or Li salts. This is illustrated in Fig. 3, which is taken from two experiments. In the first, shown in the top half of the figure, one injection electrode was filled with Na acetate (NaAc) and the other with KAc. Thus the passage of current in one direction causes the injection of Na ions from the first electrode and Ac ions from the second, while current passed in the opposite direction causes the injection of K and Ac ions. The recording shows that the injection of KAc, by a current of 38nA for one min, caused a depolarization of about one mV. The charge used in this injection would be expected to inject about 23 p-equiv KAc, and should raise the intracellular concentrations of K and Ac ions by about 6 mM. Increasing [K⁺]i would be expected to increase internal negativity, so the observed small decrease is presumably due to the Ac ions. The injection of a similar quantity of NaAc into the same cell caused a hyperpolarization of 14 mV, the increase in the membrane potential beginning within a few seconds of the start of the injection. After the injection, the membrane potential returned to the pre-injection level over a period of several minutes, with the spontaneous action potentials returning as the potential decreased.

The second experiment illustrated in Fig. 3 again shows the dramatic effects of Na injection on the membrane potential, in comparison this time with Li. The injection electrodes were filled with NaAc and LiAc. The injection of NaAc by a current of 34 nA for 1 min caused a hyperpolarization of again 14 mV, while the later injection of LiAc by the same charge caused a hyperpolarization of only about 0.5 mV. The results illustrated in Fig. 3, then, suggest that the hyperpolarization seen with
Na injection is caused by the Na pump, since the pump would be stimulated by Na injection, but not by Li or K.

This suggestion is confirmed by the effect of the specific Na pump inhibitor, ouabain, on the membrane potential response to NaAc injection before and after ouabain was applied to the preparation. The first injection caused a hyperpolarization of 15 mV in the usual way. When the membrane potential had returned to the pre-injection level ouabain was applied, causing a further membrane potential decrease of about 2 mV, without, as was tested in other experiments, any detectable change in membrane resistance. The injection of the same quantity of NaAc as before then caused a hyperpolarization of only 3 mV. Thus ouabain greatly inhibits the membrane potential increase caused by Na injection, giving further evidence that the Na pump is involved.

There are two possible mechanisms by which the Na pump could cause an increase in the membrane potential. First, the pump may be electrically neutral and cause the hyperpolarization by the removal of K from a restricted area outside the cell membrane; or secondly, the pump may be electrogenic, itself generating a current across the cell membrane.

Fig. 3. Pen recordings of the membrane potential, from two experiments, showing the effects of injections of KAc, NaAc and LiAc. In this and other recordings of the membrane potential, the long time constant of the pen recorder has reduced the action potentials to only a few mV. Solid bars indicate periods of injection; injection current in top half of figure was 38 nA, and in bottom half 34 nA.
which leads to the observed potential change (Ritchie & Straub, 1957). The first possibility is ruled out by the results of experiments such as that shown in Fig. 5, in which the normal snail Ringer, containing 4 mM-K, is replaced by a K-free solution. The first injection of NaAc caused a hyperpolarization of 12 mV. When the membrane potential had returned to the normal level, the Ringer in the bath was replaced by the K-free solution, which caused a decrease in the membrane potential of about 1.5 mV. A second injection of NaAc now caused a hyperpolarization of 2 mV, but when the K outside the cell was replaced 2 min later there was an immediate further hyperpolarization of about 15 mV, the membrane potential then declining in the same way as after a normal Na injection. (Similar effects of K removal and replacement on the post-tetanic hyperpolarization in mammalian non-myelinated nerve fibres have been shown by Rang & Ritchie, 1968.) The similarity of this response to K replacement to that seen with the previous Na injection suggests that the Na injected during the period in K-free solution was not extruded until the external

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**Fig. 4.** Pen recording of the membrane potential response to NaAc injection before and after the application of $2 \times 10^{-5}$ (w/v) ouabain. Solid bars indicate periods during which NaAc was injected by a current of 35 nA.

**Fig. 5.** Pen recording of the effect of the removal of external K on the membrane potential response to Na injection. Between the arrows the normal snail Ringer was replaced with a K-free solution. Solid bars indicate the periods during which NaAc was injected by a current of 34 nA.
K was replaced, and thus that K removal inhibits the Na pump. It has been shown that this occurs in squid axons by Hodgkin & Keynes (1956) and in frog muscle by Keynes & Swan (1959), as well as in other preparations. Thus the experiment shown in Fig. 5 again implicates the Na pump as the cause of the increase in membrane potential. The main point of this result, however, is that as well as blocking the hyperpolarization normally caused by Na injection, K removal does not itself cause any increase in the membrane potential. Thus the Na pump in these experiments is electrogenic, generating a current across the cell membrane.

The finding that both ouabain application and K removal cause a decrease in the membrane potential in these experiments is interesting because of the possibility that the electrogenic Na pump may normally make a significant contribution to the membrane potential. In the present experiments, however, one of the intracellular electrodes was always filled with a Na salt, so there would always have been a continuous small leakage of Na into the cell. The experiments, nevertheless, do place an upper limit on the extent of the pump’s normal contribution to the membrane potential in this cell, showing that it is not more than 2 mV.

Measurement of the current generated by the Na pump. Increasing the membrane potential of the cell leads to a decrease in the membrane resistance, and the longer the hyperpolarization the greater this effect (similar phenomena have been described by other investigators in snail neurones; see Kandel & Tauc, 1966). It is therefore not easy to determine the pump current simply by measuring membrane potential changes. Furthermore, the hyperpolarization following Na injection changes the electrical gradient against which Na is extruded, and by altering Na influx, passively and during each action potential, may also change the pump rate. For these reasons it was decided to use a ‘voltage clamp’ arrangement to keep the average membrane potential constant. Special precautions were taken, as described in Methods, to avoid clamping the action potentials.

The operation of this ‘slow clamp circuit’ is illustrated in Fig. 6. On the left is shown the typical response of the membrane potential to an injection of Na, a hyperpolarization of 19 mV occurring as the Na is injected. When the potential had returned to the pre-injection level the clamp circuit was switched on, and set to maintain the normal average membrane potential. The potential trace (b) now shows only a small, transient response to a second injection of NaAc, but the clamp current (c) rises linearly during the injection to a peak of 1.7 nA, and then declines exponentially. Careful checks in other experiments have established that for Na injections with charges of less than about 3 μC there is no change in membrane resistance when the membrane potential is kept constant.
in this way. Thus the clamp current should be equal and opposite to that generated by the pump.

Figure 7 illustrates the clamp currents for four injections of different amounts of Na into one neurone, with semi-logarithmic plots of the

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Fig. 6. Response to Na injection before and after clamping. (a) and (b) are pen recordings of the membrane potential (M.p.) before and after the average membrane potential was held constant; record (c) is the clamp current recorded simultaneously with trace (b). Solid bars indicate periods during which Na was injected by a current of 39 nA.

Fig. 7. Recordings of clamp current for four different injections of Na into one cell. On the right are graphs of the clamp current on a logarithmic scale against time. The charges passed to inject Na were for (a) 0.29 μC, (b) 0.77 μC, (c) 1.12 μC and (d) 2.28 μC.
clamp currents against time from the end of the injections. The graphs clearly show the exponential decline of the clamp current from the peak at the end of each injection, at least for the time that the currents are large enough to be measured accurately. The time constants for these four injections ranged from 3·76 to 4·1 min, but this variation was probably due to experimental errors. The average time constant for a total of thirty-three similar injections in thirteen different experiments was 4·4 ± 0·17 min (s.e. of mean). A similar exponential decline of current generated by the Na pump has been deduced by Rang & Ritchie (1968) from measurements of the decline in post-tetanic hyperpolarization in mammalian C fibres, by assuming that the membrane resistance was constant. These authors, however, found that the duration of stimulation, and thus presumably the quantity of Na entering the fibres, affected the time constant of the decline of the hyperpolarization, the longer the period of tetanus the slower being the recovery. This is in contrast with the present results, where the time constants for different injections into the same cell were not affected by the quantity of Na injected.

In all experiments where Na was injected rapidly compared with the rate of decline of the clamp current, the clamp current rose at a relatively constant rate during the period of injection. Since the injection current was constant during the injection period, [Na+]i should be increasing at a constant rate as long as the injection rate is considerably higher than the rate of extrusion. The clamp current, then, rises in parallel with the expected rise in [Na+]i, suggesting that the current generated by the Na pump is proportional to the level of [Na+]i above the normal level.

The total charge generated by the Na pump for each Na injection can be estimated by measuring the area under the current record, and this may be compared with the charge used to inject the Na. This comparison should then give some measure of the fraction of Na pumped out which is not directly coupled to either the uptake of other cations, probably K, or the extrusion of anions. Figure 8 shows a comparison of clamp and injection charges for thirty-one experiments in which NaAc was injected and two in which NaCl was injected. There is clearly a good correlation between injection and clamp charges, showing that the fraction of uncoupled Na extrusion is reasonably constant for different quantities of injected Na, as well as for different experiments. The average ratio of clamp to injection charge was 0·21 ± 0·006 (s.e. of mean).

An exact comparison between the number of Na atoms injected and the number of ions recorded as the clamp charge is only possible if all the injection current is carried by Na ions leaving the injection electrode, if there is no significant electro-osmosis, and if no intracellular Na enters the negative injection electrode. (The absence of any displacement of
the clamp current recording during the injection shows that none of the injection current is crossing the cell membrane.) The high salt concentrations inside the injection electrodes should prevent any significant electro-osmosis (Krnjević, Mitchell & Szerb, 1963), and should also largely prevent intracellular ions entering the injection electrodes (Coombs, Eccles & Fatt, 1955). Any errors from these two sources would also tend to cancel each other. Thus the injection charge should give a reasonably accurate measure of the number of Na atoms injected.

![Diagram](http://example.com/diagram.png)

**Fig. 8.** The relation between clamp and injection charges.

Although the clamp current is clearly closely related to the current developed by the Na pump, there is some reason for doubting that the two currents are in fact equal. In measuring the clamp charge a straight base line was drawn from the pre-injection current level to that towards which the current declined exponentially after the injection. Due to instability, these two levels were not always the same. This assumption of a straight base line takes no account of the effect of the anion injection, which was shown in Fig. 3 to cause a small decrease in the membrane potential. The effect of a KAc injection on the clamp current is shown in Fig. 9, and this recording confirms the depolarizing effects of anion injection. Since equal injections of anions were made during the Na injections, a better estimate of the charge generated by the pump will be obtained if the clamp charge is corrected for the effect of the anions.
on the current base line. This was done by using the record of a KAc injection as the baseline for calculation of the clamp charge for a similar injection of NaAc. The effect of this on the clamp current is shown in Fig. 10, the filled circles being the current corrected for acetate. The total clamp charge corrected for Ac is 0.71 9C as against 0.56 9C uncorrected. The corrected clamp charge is then 28 % of the injection charge, compared with 22 % if no allowance is made for Ac. For some reason the iontophoretic injection of K often led to blockage of the injection electrodes, so it was only possible to make this correction for eight different injections of Na. The effect of the correction procedure was, however, very similar in all cases, and gave an increase of about 30 % in the calculated clamp charge for Na. Thus the ratio of the charge generated by the Na pump in the cell body is probably closer to 0.27 % than the average of 0.21 % for the results shown in Fig. 8.

To relate this figure to the quantity of Na actually extruded by the pump two further points must be considered. One is that not all the injected Na will be pumped out across the membrane of the cell body, which is the only part of the cell which is completely controlled by the clamp. Some of the injected Na will diffuse down the axon. However, the large diameter of the cell body relative to the axon, and the high rate of Na extrusion, should reduce such a loss to the clamp of injected Na to a minimum. Any significant loss would of course tend to cause an underestimate of the true pump to injection charge ratio. A second question is whether all the injected Na is in fact extruded by the pump, that is, does the [Na+]i return to the pre-injection level when the pump current stops? That it does so return is shown by the experiments described in the next section.

Measurement of changes in intracellular Na+. The finding that the current produced by the Na pump rises linearly during, and declines exponentially
after, an injection of Na into the snail neurone suggests that the current is proportional to the pump rate. To measure this rate, or more strictly the rate of change of \([\text{Na}^+]_i\), an intracellular \(\text{Na}^+\)-sensitive glass electrode (\(\text{Na}^+\) electrode) was used. The general experimental procedure was the same as for the experiments described in the previous section, with two separate injection electrodes, a double-barrelled pair for recording and

![Graph](https://via.placeholder.com/150)

**Fig. 10.** Clamp current, plotted on a logarithmic scale, versus time from the end of an injection of NaAc. Open circles: no allowance made for effects of acetate; filled circles: acetate allowed for. Results taken from the experiment illustrated in Fig. 9.

clamping the average membrane potential, and the \(\text{Na}^+\) electrode. The holding of the average membrane potential at a fixed level which was necessary to measure the pump current had the advantage that changes in the potential recorded by the \(\text{Na}^+\) electrode would be solely due to changes in \([\text{Na}^+]_i\). Figure 11 illustrates an experiment in which the effects of two injections of NaAc on the clamp current and \([\text{Na}^+]_i\) were recorded. In this experiment the resistance of the \(\text{Na}^+\) electrode was very high, so that the amplifier responded rather slowly, as is shown by the large artefacts occurring when the injection current was switched on and off. The records clearly show, however, that the \([\text{Na}^+]_i\) rises during the
injection, and then declines towards the pre-injection level at a rate similar to that for the decline of the clamp current.

Since the bath electrode was used as a reference for the Na\(^+\) electrode, the recorded potential included both the membrane potential and the \('[Na^+] gradient potential' across the membrane, and the membrane potential had to be subtracted from the Na\(^+\) electrode potential to calculate \([Na^+]_i\). In the present experiments no special precautions were taken to eliminate tip potentials in the micro-electrodes, so the precise value of the membrane potential is uncertain, and the calculated \([Na^+]_i\) values obtained in these experiments will not be very accurate. The error is unlikely to be large, however, because the recorded changes in \([Na^+]_i\) are close to those calculated from the cell diameter and the injection charges. For example, for the first NaAc injection of Fig. 11, if all the NaAc injected remains ionized, it should cause an increase in \([Na^+]_i\) of about 5 mm. A rough estimate for the peak level of \([Na^+]_i\) from the recording

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**Fig. 11.** Pen recordings of the response of the membrane potential, \([Na^+]_i\) and clamp current, recorded simultaneously, to two injections of NaAc. The membrane potential was held constant by the feed-back circuit. Solid bars indicate periods during which Na was injected: on the left by a current of 31.3 nA for 1 min, and on the right by a current of 30.1 nA for 2 min.
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is 11.5 mm, compared with a pre-injection level of 7.5 mm. There is thus quite reasonable agreement between the recorded and expected rises in [Na\(^+\)]\(_i\), although in fact the accuracy of the measurement of this does not affect the conclusions about the rate of relative changes of [Na\(^+\)]\(_i\) which were the main object of these experiments.

Fig. 12. Pen recordings of the response of the membrane potential, [Na\(^+\)]\(_i\), and clamp current, recorded simultaneously, to an injection of NaAc by a current of 41 nA for 1 min.

The average value for [Na\(^+\)]\(_i\) obtained in the present experiments was 6.3 mm, which is lower than the 13 mm reported for Helix pomatia neurones by Sorokina (1966). This may be due to variations between different cells, since the latter figure is based on an average of all the giant cells.

Figure 12 is a recording from an experiment with a different Na\(^+\) electrode, which had a lower resistance but higher noise level than that used for the experiment of Fig. 11. Again, the recording clearly shows that [Na\(^+\)]\(_i\) rises and falls at a similar rate to the clamp current. The Na\(^+\) electrode in this case recorded a 4 mm increase in [Na\(^+\)]\(_i\), as against
a calculated increase from the injection of about 6 mM. The results from this experiment are plotted on semi-logarithmic scales in Fig. 13, with the \( [Na^+]_i \) given as the value above the pre-injection level of 3·9 mM. This shows that both the clamp current and excess \( [Na^+]_i \) decline exponentially, with time constants for the current of 3·55 min and for the \( [Na^+]_i \) of 3·65 min.

![Fig. 13. Relation between clamp current (open circles) and excess \( [Na^+]_i \) (filled circles), both plotted on logarithmic scales, and time from the end of the injection of NaAc, the recordings of which are shown in Fig. 12.](image)

The average time constant for loss of Na was 4·8 min for six injections into four different cells, and since in this situation the major cause of Na movement will be the Na pump, the exponential decrease in excess \( [Na^+]_i \) shows that the rate of the Na extrusion is proportional to the \( [Na^+]_i \) above some threshold value, which is probably close to the normal intracellular \( [Na^+]_i \). Exponential declines in the rate of Na extrusion have been shown in other preparations, at least for levels of \( [Na^+]_i \) above normal. Thus Hodgkin & Keynes (1956) showed that Na efflux from squid axon declined exponentially with a time constant of about 5 h, and Hodgkin & Horowicz (1959) showed that single frog muscle fibres,
loaded with radioactive Na, lost it exponentially with a time constant of about 90 min.

By taking the values of $[\text{Na}^+]_i$ and clamp current at a series of time intervals after the injections, it is possible to show the correlation between the two in a different way, as is illustrated in Fig. 14 for experiments on three different preparations. The linear relationship shows again that the current is directly proportional to the $[\text{Na}^+]_i$ above the resting level.

![Graph showing relation between clamp current and $[\text{Na}^+]_i$](image)

Fig. 14. Relation between clamp current and $[\text{Na}^+]_i$ for injections of NaAc into three different neurones.

The differences in slope may partly be due to errors in the calibration of the Na$^+$ electrode, but are more likely to be due to real variations from snail to snail, since the time constants for the decline of the current also varied in these cells, being (from left to right) respectively 3.65, 4.35 and 5.5 min.

**DISCUSSION**

The results of the present experiments show that when the Na pump is stimulated by Na injection it acts as a current source across the membrane. The intensity of the current generated, and the rate of the Na extrusion are both proportional to the excess of $[\text{Na}^+]_i$ above the resting level, and the total charge transferred is equivalent to the charge carried by between one third and a quarter of the Na ions extruded.

If the pump extruded only Na ions, uncoupled with any other ion movements, then it would transfer a charge equivalent to the whole of
the Na transported, and the ratio of pump to injection charge would be unity. Any active uptake of K ions would reduce the net charge moved by the pump; in the extreme case of equal Na extrusion and K uptake the pump would be electrically neutral.

The stoichiometry of the Na pump has been extensively investigated only in red blood cells. Post & Jolly (1957) estimated that two K ions were actively transported into the cell for every three Na ions extruded, and this ratio has been confirmed by many later investigators (for references see Post, Albright & Dayani, 1967). Estimates of the number of ions transported for each molecule of ATP consumed by the pump have been made by Sen & Post (1964), Whittam & Ager (1965) and Garrahan & Glynn (1967), and there is general agreement that the ratio of ATP:Na:K for red blood cells is 1:3:2.

Apart from an estimate by Nakajima & Takahashi (1966) that during post-tetanic hyperpolarization in crayfish stretch receptor neurones 20–30% of the accumulated Na was extruded uncoupled to presumed K uptake, there is little information on the Na:K ratio for the Na pump in nerve or muscle cells. (One reason for this is that it is difficult to distinguish between K actively transported by the pump and K entering passively in response to any increase in membrane potential generated by an electrogenic pump.) However, measurements of the ATP:Na ratio suggest it might be the same as in red blood cells. Thus Baker (1965) calculated that for the Na pump in crab axons the ATP:Na ratio was close to 1:3, and Baker & Shaw (1965) concluded that this ratio probably also applied to squid giant axons. For frog muscle, Dydynska & Harris (1966) and Harris (1967) showed that the ATP:Na ratio was also close to 1:3. Since this ratio is the same as in red blood cells, it seems reasonable to suggest that the Na:K ratio is also the same, and that the ratio for the Na pump in snail neurones might well be 3Na:2K. There is, of course, no direct evidence in the present experiments that the pump actively transports K ions, but removal of K from outside the cell certainly blocks the electrogenic effect, and judging from the response to readmission of external K, it also blocks Na extrusion. The inhibitory effect of ouabain also suggests that the pump in snail neurones is very similar to that in other cells, where it has been clearly shown that the Na pump does actively transport K. If, then, the Na pump in snail neurones is the same as in red blood cells, in that it takes up two K ions for every three Na ions extruded, then the charge transferred by the pump should be one third of the charge on the Na ions. The ratio found in the present experiments, corrected for the effects of anion injection, is rather less than this, but the discrepancy may be due to escape of injected Na down the axon, or to some systematic error in the measurements.
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It has been suggested that the Na:K coupling ratio for the Na pump in nerve and muscle cells may vary with different conditions (Keynes, 1965; Adrian & Slayman, 1966; Rang & Ritchie, 1968), but the results of the present experiments do not support this idea. The finding that the pump current is directly proportional to the rate of Na extrusion is evidence that there is a fixed linkage between Na extrusion and presumed K uptake, at least for levels of [Na⁺], above normal. Some preliminary experiments have also been done on the effects of different external K levels, and for concentrations from 1 to 16 mm there was no detectable change in the ratio of pump to injection charge. The present results, then, suggest that the stoichiometry of the pump in snail neurones is the same as in red blood cells, and that the reason for the electrogenic effect of the pump is that the extrusion of every three Na ions is directly coupled to the uptake of only two K ions.

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