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SODIUM EFFLUX FROM VOLTAGE CLAMPED SQUID GIANT AXONS

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SUMMARY

1. The efflux of radioactive sodium was measured from squid axons during simultaneous voltage clamp experiments such that it was possible to determine the efflux of sodium associated with a measured voltage clamp current.

2. The extra efflux of sodium associated with voltage clamp pulses increased linearly with the magnitude of the depolarization above 40 mV. A 100 mV pulse of sufficient duration to produce all of the sodium current increased the rate constant of efflux by about 10^{-6}.

3. Application of 100 nM tetrodotoxin eliminated the sodium current and the extra efflux of radioactive sodium.

4. Cooling the axon increased the extra efflux/voltage clamp pulse slightly with a Q_{10} of 1/1·1. On the same axons cooling increased the integral of the sodium current with a Q_{10} of 1/1·4.

5. Replacing external sodium with Tris, dextrose or Mg-mannitol reduced the extra efflux of sodium by about 50%. The inward sodium current was replaced with an outward current as expected.

6. Replacing external sodium with lithium also reduced the extra efflux by about 50% but the currents seen in lithium were slightly larger than those in sodium.

7. The effect of replacing external sodium was not voltage dependent. Cooling reduced the effect so that there was less reduction of efflux on switching to Tris ASW in the cold than in the warm.

8. The extra efflux of sodium into sodium-free ASW is approximately the same as the integral of the sodium current. Adding external sodium produces a deviation from the independence principle such that there is more exchange of sodium than predicted. Such a deviation from prediction was noted by Hodgkin & Huxley (1952*c*).

9. Using the equations of Hodgkin & Huxley (1952*c*) modified to

include the deviation from independence reported in this paper and its temperature dependence, one can predict the temperature dependence of the sodium efflux associated with action potentials and obtain much better agreement than is possible without these phenomena.

10. This deviation from independence in the sodium fluxes is the type expected from some kind of mixing and binding of sodium within the membrane phase.

INTRODUCTION

During the rising phase of the nerve impulse there is an increase in the electrical conductivity of the nerve membrane which has been associated with the movement of sodium ions. The quantitative description of this sodium conductance has united many electrical phenomena of excitable cells (Hodgkin & Huxley, 1952*c*). The early measurements of sodium fluxes associated with action potentials (Keynes, 1951) agreed with the calculations of Hodgkin & Huxley (1952*c*) only with respect to the net fluxes. The experimentally observed unidirectional fluxes of sodium were higher than predicted whereas those of potassium were lower. The discrepancy in the potassium fluxes was studied by Hodgkin & Keynes (1955) who suggested that if it is assumed that potassium ions move in single file, then it is possible to explain why potassium influx was smaller than predicted. Little attention has been given to the discrepancy in the sodium fluxes.

Recent experiments on the temperature dependence of sodium and potassium fluxes associated with nerve impulses have underlined these contradictions between observed and predicted tracer fluxes (Cohen & Landowne, 1974; Landowne & Scruggs, 1976) and have shown again the need for a more direct comparison between currents and fluxes. It is possible to measure the efflux of radioactive sodium from relatively intact squid axons while voltage clamping the membrane and measuring the associated currents (Mullins, Adelman & Sjodin, 1962). In this paper the work of Mullins *et al.* (1962) is extended to include a range of voltages, two temperatures, and the effects of replacing external sodium. Preliminary reports have already appeared (Landowne, 1975*a*, 1976).

METHODS

Axons from *Loligo pealii* were dissected and cleaned and mounted horizontally in the chamber shown schematically in Fig. 1. A 1–200 μm diameter glass tube loaded with ^{22}Na and mounted on a Hamilton 10 μl. syringe was inserted into the axon through a cut on the right hand end and then advanced through the length of the chamber inside the axon while observing the tip with the aid of a dissecting microscope and a mirror held at 45° so that both a top and side view of the axon could be seen. Upon reaching the left end of the axon a second cut was made

through the membrane and the injector tip was advanced out of the axon. Any axoplasm inside the injector was expelled and the extended end of the current wire of the voltage clamp electrode was inserted inside the injector. Any air remaining in the injector was expelled and the injector and electrode were then moved to the right together, each on its own micromanipulator until the tip of the injector was at the edge of the central chamber. Movement to the right from this point was made while injecting the ^{22}Na into the central region of the axon. When the tip of the voltage sensing portion of the electrode reached the centre of the centre chamber the movement of the electrode was stopped and the injector withdrawn while still injecting ^{22}Na. At the right hand edge of the central chamber the injection was stopped and the injector withdrawn leaving the electrode as shown in Fig. 1.

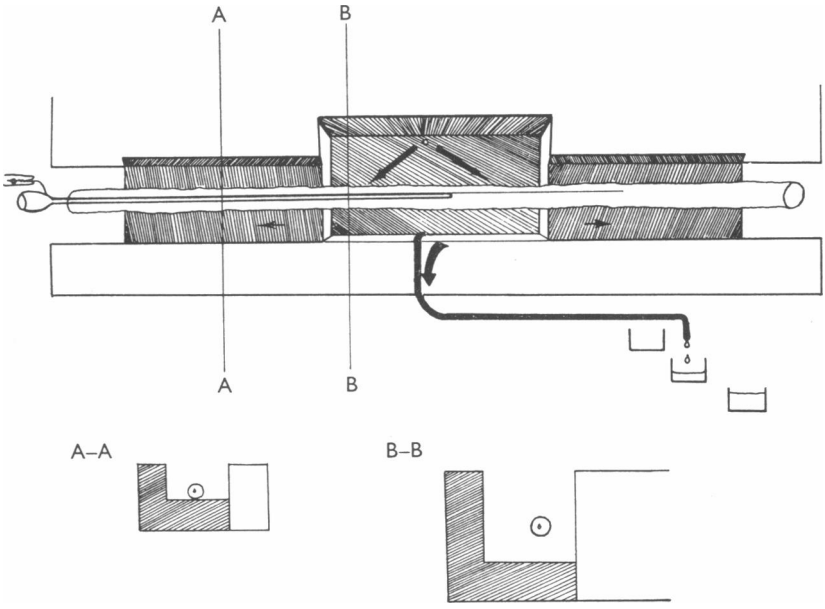

Fig. 1. Diagram of chamber used for simultaneous voltage clamp and efflux measurements on squid giant axons.

The electrode was the same as that described by Chandler & Meves (1965) except that a 50 μm platinum wire platinized for 10 mm on either side of the tip of the voltage sensing electrode was used to pass current. The central chamber was $12 \times 3 \times 3$ mm and the side chambers were $10 \times 2 \times 1$ mm. The bottom and rear wall of these chambers was a sheet of platinized platinum used as the external current electrodes for voltage clamping of the axon. The front wall was removable to facilitate insertion of the axon, injector and electrode assembly.

The voltage across the membrane was measured between the internal potassium chloride-filled capillary and one inserted in the central chamber just outside the axon. This voltage was compared to a command pulse and the difference between the actual and command voltages caused current to flow between the internal current electrode and the platinum sheets in the chamber. The two outer chamber platinum sheets were directly grounded and the centre sheet was held at ground by a

feedback control system. The current required to maintain this virtual ground was considered the current through the central guarded region of the axon. The axon diameter was measured at the middle and both ends of the central chamber and the average value used to adjust the gain of the virtual ground amplifier so that 1 mA/cm^2 gave a 100 mV signal. The voltage clamp circuit included 'compensation' for the effects of the resistance in series with the membrane (Hodgkin, Huxley & Katz, 1952).

After the electrode was inserted the voltage clamp was adjusted so no current flowed at the resting potential. Resting potentials were in the range -49 to -65 mV. The command voltage consisted of a hyperpolarizing prepulse of 7–14 msec duration followed by a depolarizing pulse. The amplitude and duration of the prepulse were adjusted to remove any resting sodium inactivation (Hodgkin & Huxley, 1952*b*). Axons with less than 1 mA/cm^2 peak inward current at this point were discarded.

The experimental procedures involve several minutes of continuous voltage clamping with 20–30 depolarizing pulses per second. Photographs of the oscilloscope screen were made at least once a minute during the train of pulses. These photographs were projected and traced for analysis of the currents. At room temperature the capacity transient was not well resolved from the onset of the sodium current, it was obscured by ringing produced by the compensation for series resistance. The ringing was not traced and does not appear in the insets in Figs. 2, 3, and 5. Quantitative measurements of the sodium current were made by taking the difference between records for the same pulse taken in the absence and presence of tetrodotoxin (Fig. 2) and thus subtracting out the capacity transient, the leak current and the delayed potassium current for that record. The tracings shown in the Figures have not been so corrected. The leak conductance ($\bar{g}_l$) was measured either by dividing the minimum current seen in tetrodotoxin by the amplitude of the voltage clamp pulse or from the inward current which was seen during the hyperpolarizing prepulse. The range of $\bar{g}_l$ was 0·2 to $4{\cdot}1 \times 10^{-3}$ Ω^{-1} cm^{-2}.

The temperature was controlled by a water jacket below the chambers. The temperature was measured with a thermistor in the central chamber. 1–2 ml. artificial sea water were pumped each minute through a tube which passed through the water bath into a small hole shown in the rear of the central chamber. Three pumps removed the sea water, one for each of the three chambers. The central pump removed 80–90 % of the inflowing sea water and the pumps emptying the lateral guard chambers removed 5–10 % each. The pumping rate in the lateral pumps was adjusted so the level of sea water remained constant.

Experimental changes in solutions surrounding the axon were made with appropriate lead time to compensate for delays in the tubes running to or from the central chamber. In some experiments the axon was repetitively voltage clamped as the solution was changed. When sodium was replaced with Tris the early inward currents changed in the expected manner during one minute indicating the washout time of the chamber. The effectiveness of the guard chambers was demonstrated by adding phenol red to various locations in the chamber and watching the movement of the dye. The solution collected from the central chamber was dried in planchets and counted in a low background gas flow beta counter. When different compositions of sea water were used in an experiment the data from the counter were corrected for the different self absorption of the different sea waters by making appropriate standards with equal amounts of ^{22}Na and counting them. At the end of the experimental run the electrode was withdrawn and the central portion of the axon was cut out and counted. The data are expressed as the fraction of the radioactive sodium leaving the axon per minute. This was calculated by dividing the amount collected during the minute by the amount in the axon at the middle of that minute back

calculated from the amount which remained at the end of the run. Correction was made for the 10–20 % of the solution which bathed the middle section of the axon but was not collected. When not otherwise noted, results are given as mean ± S.E.

The standard artificial sea water (ASW) contained (mM) Na, 460; K, 10; Ca, 10; Mg, 55: Cl, 600 and HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethane sulphonic acid) buffer, pH 7·4, 2. In the sodium-substituted sea waters, sodium was replaced with 460 mM Tris (hydroxymethyl) aminomethane hydrochloride (Tris ASW), 460 mM-Li (Li ASW) or NaCl was replaced with 750 mM dextrose (dextrose ASW) or 460 mM mannitol plus 153 mM-$MgCl_2$ (Mg-Mannitol ASW) (DeWeer, 1970). All sea waters contained 10 μM ouabain to reduce the resting sodium efflux. The injection medium contained 570 mM-KCl, 5 mM HEPES, 1 mM phenol red and about 1 mc ^{22}Na/ml.

RESULTS

Efflux into normal artificial sea water

Efflux associated with voltage clamp pulses

When giant cephalopod axons which have been loaded with radioactive sodium are stimulated to produce nerve impulses they lose 10^{-6}–10^{-5} of their tracer per impulse. It is not surprising therefore that impressing across the membrane a potential of roughly the same amplitude and duration should also produce an efflux of the same order of magnitude. Such an experiment is shown in Fig. 2. During the time signified by the filled symbols the axon was repetitively depolarized 30 times/sec. The currents across the central guarded portion of the axon are shown in the insets. The extra efflux per pulse or the difference between the efflux during stimulation and the interpolated efflux expected of this axon at rest divided by the number of pulses given was 1·5 and $1{\cdot}9 \times 10^{-6}$ at room temperature and $2{\cdot}5 \times 10^{-6}$ in the cold. When TTX was added to the bathing media both the extra flux and the inward sodium current disappeared. Three estimates of the extra efflux of sodium in the presence of tetrodotoxin (TTX) gave values of less than 10^{-7} pulse^{-1}. The last measurement was made with a relatively long pulse of 1·5 msec demonstrating that there is very little extra sodium efflux associated with potassium currents.

Dependence on the amplitude of the voltage clamp pulse. The extra efflux per voltage clamp pulse increases roughly linearly as the amplitude of the pulse increases with no dramatic effect when the sodium current changes from inward to outward (Fig. 3). This linear relationship is to be expected if one assumes as Hodgkin & Huxley (1952*a*) assumed that the permeability of the membrane may be measured by the sodium conductance g_{Na}. The only additional requirement for a linear efflux *vs.* voltage relationship is that for depolarizations of more than 40 mV *the time integral of the sodium conductance is independent of voltage* when the duration of the current is long enough to allow inactivation of the conductance. There must, of course, be some zone of transition between a very small pulse which fails

to activate any sodium conductance increase and larger depolarization. This constancy of $\int g_{Na}\, dt$ can be seen in the g_{Na} *vs.* t curves from Hodgkin & Huxley (1952*c*) and from the finding that they can be superimposed by an axes transformation which preserves areas (Landowne, 1972). Analysis of the curves of current *vs.* time in Fig. 3 also shows this constancy.

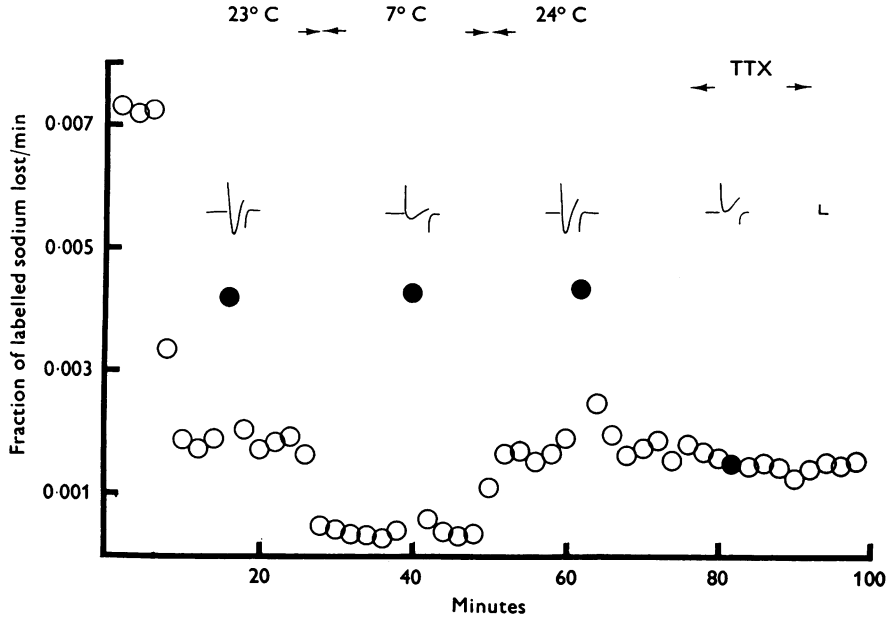

Fig. 2. The efflux of sodium from a squid axon during voltage clamp pulses. The efflux at rest is shown by the open symbols. After the third sample was taken 10 μM ouabain was added to the bathing medium for the rest of the experiment. During the periods indicated by the filled symbols the membrane was depolarized by 60 mV, 30 times/sec for 0·5 msec at room temperature and for 3 msec in the cold. The fourth voltage clamp run was done with 100 nM tetrodotoxin (TTX) added to the bathing medium. The insets show the currents flowing through the central portion of the axon. Vertical calibration, 0·5 mA/cm^2; horizontal calibration, 0·25 msec at room temperature; 1 msec at 7° C. Axon 11J5. Diameter 505 μm; resting potential -62 mV, $\bar{g}_l$, $0{\cdot}32 \times 10^{-3}\,\Omega^{-1}\,cm^{-2}$.

The sodium current was separated from the total currents shown in Fig. 3 by subtracting off the current seen for the same potential step in the presence of TTX. The area under the sodium current *vs.* time curves was then measured and found to be 953, 365 and 385 nC/cm^2 for the three records in the order shown in Fig. 3. The third charge transfer is outward and the first two inward. Between each stimulation to produce the efflux a series of voltage clamp currents were recorded for pulses of varying

amplitude in order to estimate V_{Na}. In this axon $V-V_{Na}$ for the three traces was 31, 11 and 11 mV, the third being in the opposite direction of the first two. Therefore the values for $C_{Na} = \int g_{Na}\, dt$ are 31, 33 and 35 nC/mV cm^2. Within experimental error these are all the same. A 1 mV change in the estimation of V_{Na} produces a 10 % change in the second and third estimate of C_{Na}.

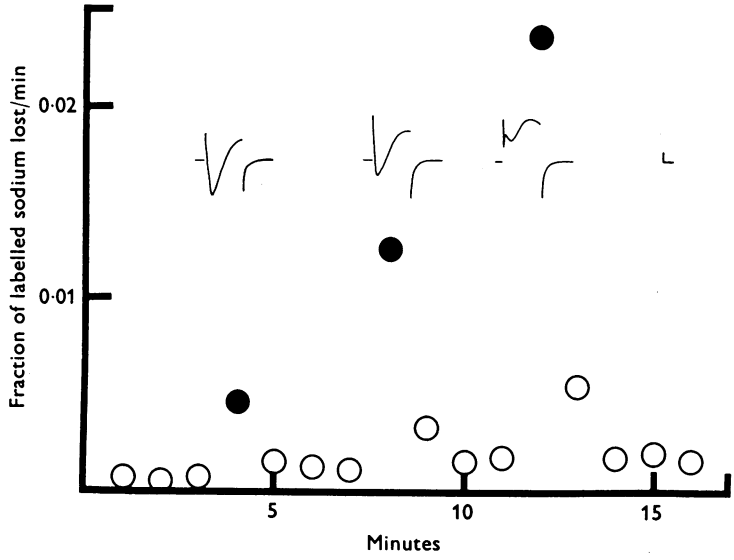

Fig. 3. The effect of potential on the efflux of sodium during voltage clamp pulses. During the three periods indicated by the filled symbols the axon was repetitively depolarized 30 times/sec for 0·8 msec. The magnitudes of the depolarizations used were 62, 82, and 104 mV respectively. Axon 13J5. Diameter, 660 μm. Temperature 22° C. Horizontal calibration 0·25 msec; vertical, 1 mA/cm^2. Resting potential -55 mV, $\bar{g}_l$, $1{\cdot}7 \times 10^{-3}\,\Omega^{-1}$ cm^{-2}.

Fig. 4 shows the extra efflux per pulse as a function of pulse voltage. Instead of the fractional increase in the rate constant the extra efflux is shown as the *equivalent efflux* or the efflux divided by the internal concentration (Landowne, 1975*b*). The equivalent efflux is formally the same as the proportionality constant between efflux and internal concentration, k_2, used by Hodgkin & Huxley (1952*a*) in the description of their independence principle. k_2 was described as a constant which depends on the condition of the membrane and on the potential difference across it. The equivalent efflux is obtained from the rate constant, k_s, by multiplying by the volume to surface ratio or one fourth the diameter of the axon.

The line, which is a least-squares fit of the room temperature data, has a slope of $2{\cdot}7 \pm 0{\cdot}7 \times 10^{-9}$ cm/pulse mV and intercepts the voltage axis at

40 mV. Thus for a 140 mV depolarization the equivalent efflux is about $2{\cdot}7 \times 10^{-7}$ cm/pulse. This may be compared to the data of Mullins *et al.* (1962) by using their value of 147 mM for the internal sodium concentration in their axons. $2{\cdot}7 \times 10^{-7}$ cm/pulse then corresponds to an extra efflux of $2{\cdot}7 \times 10^{-7} \times 147 \times 10^{-6} = 40 \times 10^{-12}$ mole/cm^2 pulse or $4{\cdot}1 \times 10^{-6}$ C/cm^2 pulse by Faraday's law. This is slightly larger than the average

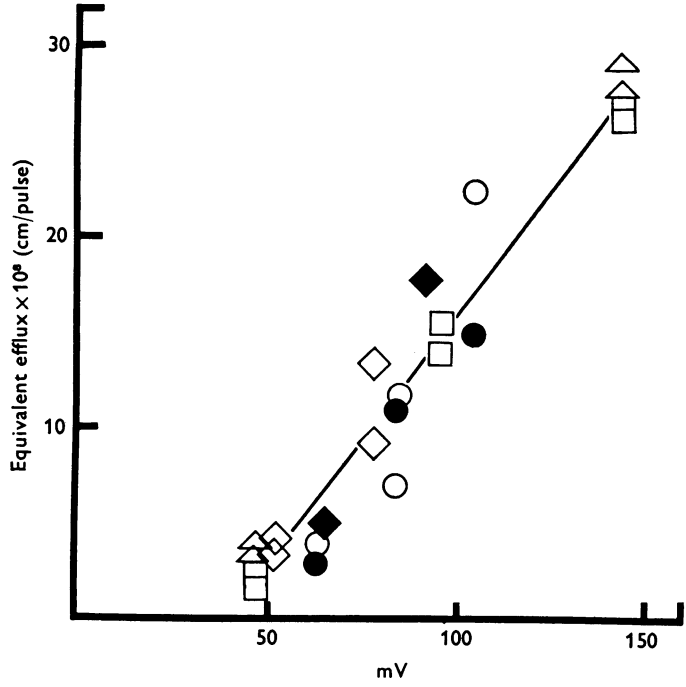

Fig. 4. The voltage dependence of the extra sodium efflux associated with voltage clamp pulses. Horizontal axis, amplitude of voltage clamp pulse. Vertical axis, extra equivalent efflux. Open symbols indicate measurements made at room temperature, filled symbols indicate measurements made at 5–8° C. Different symbols represent different axons.

of the values reported by Mullins *et al.* (1962) ($2{\cdot}4 \times 10^{-6}$ C/cm^2 pulse) but the difference is within experimental error. There was a tendency for the voltage sensitivity to decrease as the axon deteriorated along with the sodium current and efflux at each potential. However this was not studied systematically because as will be shown below, the slope changed dramatically when the external sodium concentration was altered suggesting that the condition of the axon membrane was not the only variable involved.

Temperature dependence. Reducing the temperature of the axon from room temperature to 6° increases the extra efflux of sodium associated with

action potentials only slightly (Cohen & Landowne, 1974). It was of interest to see if this low temperature dependence would also be seen in voltage clamp experiments where the potential across the membrane is better defined than with action potentials. In Fig. 2 it can be seen that the extra efflux associated with voltage clamp pulses also has a low temperature coefficient. In this experiment the extra efflux (k_s) at room temperature was increased by 47 % when the temperature was reduced 16–17° C. This corresponds to a Q_{10} of 1/1·26. Seven such bracketed measurements on four axons gave a value of 1/1·13 ± 0·07 (Table 1) which is essentially the same as the value 1/1·2 found for action potentials (Cohen & Landowne, 1974). Changing the temperature did not dramatically affect the voltage sensitivity of the efflux. Included in Fig. 4 are two experiments in which the voltage dependence was measured at high and low temperatures. Five measurements of the voltage dependence of the equivalent efflux in the cold gave a value of $2{\cdot}7 \pm 0{\cdot}9 \times 10^{-9}$ cm/pulse mV, the same as at room temperature and in two bracketed experiments the voltage dependence had Q_{10}s of 1·11 and 0·74.

The effect of cooling on the currents measured during a voltage clamp pulse can be seen in Fig. 2. The peak amplitude of the sodium current was 1·4 and 1·2 mA/cm² at room temperature and 0·34 mA/cm² in the cold. This change seems larger than the finding of Moore (1958) but the effect was not studied systematically. The net transfer of charge associated with the sodium current for the three records was 295 and 268 nC/cm² in the warm and 405 nC/cm² in the cold. This gives a Q_{10} of 1/1·36 for the net currents, slightly larger than that found for the efflux. The seven bracketed measurements of the temperature dependence of the integral of the sodium current gave a Q_{10} of 1/1·36 ± 0·16 (Table 1). A paired t test suggested that the temperature coefficient of the flux measurements was less than that of the integrated currents ($P < 0{\cdot}1$).

Resting efflux

In four experiments ouabain was added to the bathing media after the first three efflux samples were taken. One of these experiments is shown in Fig. 2. As expected ouabain dramatically reduced the resting efflux. These four axons at 22–25° C lost ^{22}Na with a rate constant of 0·006 ± 0·002 min^{-1} (mean ± S.D.) in the absence of ouabain and 0·0011 ± 0·0005 min^{-1} in its presence. Ouabain was normally used to lower the resting efflux in order to facilitate measuring the extra efflux associated with voltage clamp pulses.

The resting efflux in the presence of ouabain was somewhat variable and seemed to be related to the leakage current measured in voltage clamp experiments. The resting efflux rate constant was determined by

TABLE 1. The effect of temperature changes on the extra efflux of sodium associated with voltage clamp pulses. The same magnitude depolarization was applied successively at high, low and high temperatures to measure k_s and $\int I_{Na}\,dt$

Expt.	Diam. (μm)	Depol. (mV)	Temp. (°)			$k_s \times 10^6$ (pulse^{-1})				$\int I_{Na}\,dt$ (μC/cm^2)			
			High	Low	High	High	Low	High	Q_{10}	High	Low	High	Q_{10}
11J	505	63	23	7	24	1·5	2·5	1·9	1/1·26	0·30	0·41	0·27	1/1·36
13J	650	62	22	6	22	2·3	1·6	1·8	1/0·84	1·27	0·48	0·32	1/0·72
		82	22	6	22	7·3	6·7	4·3	1/1·09	0·34	0·24	0·14	1/0·99
		104	22	6	22	13·8	9·1	2·1	1/1·09	0·23	0·73	0·98	1/1·64
24Jy A	440	53	23	9	23	6·2	5·1	3·0	1/1·14	1·23	1·37	0·40	1/1·41
		53	23	9	23	4·1	3·9	1·9	1/1·41	1·15	1·15	0·24	1/1·40
24Jy B	435	53	29	9	23	2·7	2·1	0·5	1/1·10	0·69	1·28	0·08	1/2·03
Mean ± S.E.									1/1·13 ± 0·07				1/1·36 ± 0·16

least-squares fitting at least five points such as shown in Fig. 2 and then interpolating the efflux at the time of the voltage clamp pulses. Sixty-four estimations of the resting efflux in twelve axons at 21–27° C had a mean ± S.E. of 0·0020 ± 0·0009 min^{-1} in the presence of ouabain. There was much less variation within any single axon as can be seen in Figs. 2, 3 and 6. This variability of the ouabain-insensitive component of the sodium efflux was reported by Baker, Blaustein, Hodgkin & Steinhardt (1969). The mean value reported here is higher than Baker *et al.* (1969) obtained, which may be related to the trauma associated with inserting the electrode assembly. However their experiments were at slightly lower temperatures and with larger axons; both factors which are consistent with lower efflux rate constants.

The temperature sensitivity of the resting efflux into ouabain containing sea water is high. In four bracketed experiments where the efflux at 22–25° C was compared to that at 6–8° C a Q_{10} of 2·5 ± 0·1 was obtained. TTX which blocks the voltage dependent sodium conductance had little effect on the resting sodium efflux. In six bracketed experiments TTX reduced the efflux by 0·97 ± 0·11. Both the effect of temperature and TTX are similar to those reported by Baker *et al.* (1969).

Efflux into reduced sodium sea waters

The theory advanced by Hodgkin & Huxley (1952*c*) predicted too little exchange of sodium and too much exchange of potassium. That is, the observed extra influx and efflux of sodium associated with action potentials are greater than predicted although for the net flux of sodium, theory and observation are in agreement. For potassium, the condition is complementary, the observed extra influx and efflux is smaller than predicted. These predictions of unidirectional fluxes were made from the observed electrical currents and a very general assumption about the manner in which ions cross the membrane, the 'independence principle' (Hodgkin & Huxley, 1952*a*). This assumption is that the chance that any individual ion will cross the membrane in a specific interval of time is independent of the other ions which are present. In particular this assumption demands that the tracer efflux of sodium or potassium ions only depends on the internal concentration of these ions and is independent of the external concentrations. In the case of potassium fluxes, Hodgkin & Keynes (1955) found very large departures from independence; lowering external potassium concentration increased the efflux of potassium. Hodgkin & Keynes' (1955) experiments on potassium movements were done without internal electrodes, and the potential across the nerve membrane was changed for several minutes while the flux measurements were made. It is not convenient to study the sodium system this way because the sodium currents

inactivate when subjected to a sustained depolarization. Accordingly in these studies, the axon was repetitively depolarized with short pulses to allow sodium ion movements and, in between pulses, repolarized to remove sodium inactivation. During each experiment the amplitude, duration and frequency of the pulses was kept constant, only the external sodium concentration was changed. Since the discrepancy between observation and prediction of sodium fluxes associated with nerve impulses is complementary to that of potassium fluxes one might expect that there is a complementary deviation from independence and that lowering the external sodium concentration would decrease the sodium efflux. If, on the other hand, sodium ion movements followed the independence principle, altering external sodium should not alter the efflux.

Efflux associated with voltage clamp pulses

Replacing sodium with Tris. Fig. 5 shows a record of an experiment where all of the sodium in the external bathing solution was replaced with Tris. This had the expected effect on the voltage clamp currents recorded for the same 42 mV depolarization, the transient early inward current disappeared and was replaced by a small outward current. The effect on the delayed potassium current is much smaller although there was consistently a slight reduction in Tris ASW. Upon returning to sodium ASW the currents returned to normal. The extra efflux of sodium associated with the voltage clamp pulses was dramatically reduced in Tris ASW. Thus it appears as if sodium ion movements are not independent and that the deviation from independence is in the appropriate direction to explain the discrepancy between theory and experiments. In this particular case the extra efflux into Tris ASW was about 32 % of the average value of the extra efflux in the bracketing measurements in sodium ASW. Six such experiments in five different axons gave a value of $0{\cdot}40 \pm 0{\cdot}08$ (S.E.) for the ratio of extra efflux into Tris ASW/extra efflux into Na ASW (Table 2). Tris ions are generally accepted as being impermeant through sodium channels and not having adverse effects on the membrane (Armstrong & Bezanilla, 1974; Keynes & Rojas, 1974). Furthermore, the flux and current data showed that the effects of Tris were quickly reversible. Finally, as shown in Fig. 5, the resting efflux increased in Tris ASW indicating that bathing the axon in sodium-free ASW did not just reveal a generalized axonal process which was able to recapture half of the sodium leaving during the voltage clamp pulse.

Replacing sodium with dextrose or magnesium-mannitol. In order to test the hypothesis the decrease in extra sodium efflux in Tris ASW is associated with the absence of sodium rather than some previously unknown pharmacological action of Tris, two other sodium-deficient artificial sea

waters were prepared. Three bracketed measurements were made with 90% of the sodium chloride replaced with an isosmotic amount of dextrose. This also lowered the extra efflux of sodium to about one half of control values (Table 2). When the sodium was replaced with magnesium and mannitol, which mimics sodium ASW with regard to osmolality and ionic strength (DeWeer, 1970) the effect was the same as with Tris or dextrose. Seven bracketed measurements of the ratio of extra efflux in

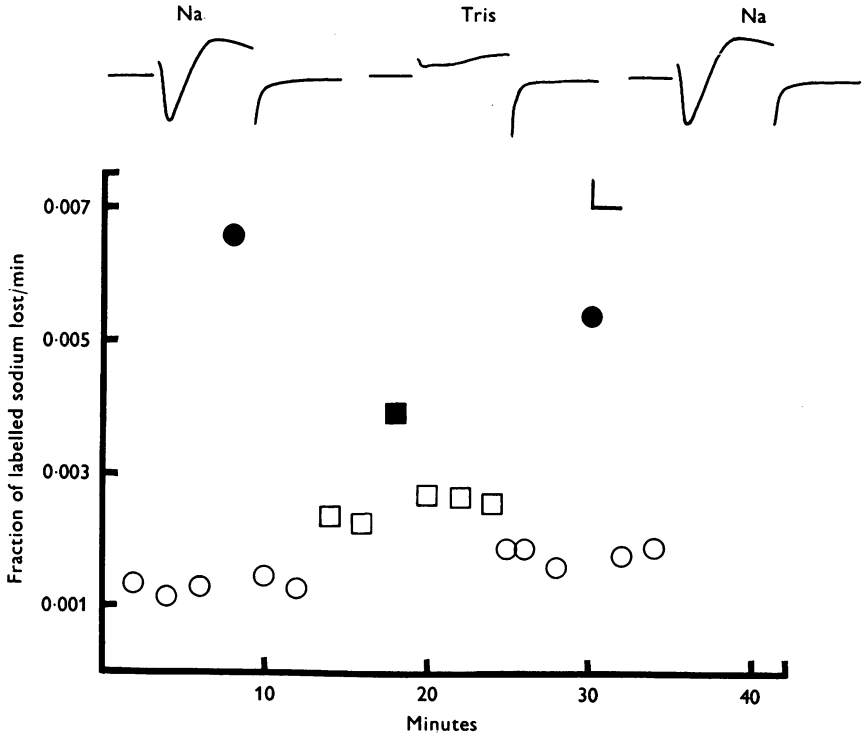

Fig. 5. Replacing external sodium with Tris reduces the extra efflux of sodium associated with voltage clamp pulses. Open symbols represent resting fluxes. During the time represented by filled symbols the axon was repetitively voltage clamped with a 42 mV depolarizing pulse for 0·8 msec 30 times/sec. The currents observed are shown in the insects above each period. Axon diameter 660 μm. Temperature, 26° C. Calibration: vertical 1 mA/cm^2; horizontal, 0·2 msec. Resting potential -51 mV, $\bar{g}_L$, $3{\cdot}2 \times 10^{-3}\ \Omega^{-1}\ \mathrm{cm}^{-2}$.

magnesium-mannitol ASW/the extra efflux in sodium ASW provided a value of $0{\cdot}58 \pm 0{\cdot}07$ (Table 2). Some of the experiments were performed with only a 70 or 90% replacement of the sodium but there was too much variability in the data to distinguish this from 100% replacement, so the

TABLE 2. The effect of sodium replacement on the extra efflux of sodium associated with voltage clamp pulses. Where the replacement was not complete the remainder of the solution was sodium ASW

Expt.	Diam. (μm)	Temp. (°)	Test soln.	Depol. (mV)	$k_s \times 10^6$ (pulse^{-1}) Na	Test	Na	Ratio of k_s
18 June	605	25	0·7 Tris	40	5·1	2·8	3·9	0·63
25 June	660	26	1·0 Tris	42	6·1	1·6	4·0	0·32
2 July	620	21	0·9 Tris	95	10·0	4·9	8·9	0·52
		21	0·9 Tris	142	16·9	8·9	17·2	0·52
24 July A	440	23	1·0 Tris	53	6·2	1·5	4·1	0·29
24 July B	435	29	1·0 Tris	53	3·1	0·3	2·7	0·10
Mean ± S.E.		25						0·40 ± 0·08
3 July	550	22	0·9 Dex	47	2·9	1·8	2·3	0·71
		22	0·9 Dex	142	20·1	6·6	21·1	0·32
9 July B	440	21	0·9 Dex	40	11·1	4·5	6·7	0·51
Mean ± S.E.		22						0·51 ± 0·11
10 July A	440	21	1·0 Man	88	12·2	6·2	7·6	0·31
10 July B	530	21	1·0 Man	52	4·7	2·5	3·7	0·60
		21	1·0 Man	97	7·1	5·0	7·2	0·70
11 July A	550	21	0·7 Man	38	6·5	2·1	4·4	0·39
		21	0·7 Man	106	8·0	3·8	7·2	0·50
		21	1·0 Man	38	4·4	3·0	2·9	0·82
		21	1·0 Man	106	7·2	4·2	3·8	0·76
Mean ± S.E.		21						0·58 ± 0·07

Table 2 (*cont.*)

9 July A	440	21	1·0 Li	52	6·9	1·8	5·2	0·31
9 July B	440	21	1·0 Li	40	6·7	3·1	5·9	0·50
10 July A	440	21	1·0 Li	88	7·6	6·9	6·4	0·99
24 July A	440	23	1·0 Li	53	3·0	0·9	1·9	0·37
Mean ± S.E.		21						0·54 ± 0·15
			Low temperature					
18 June	605	8	0·7 Tris	37	2·4	2·8	3·6	0·95
11 July B	475	11	1·0 Tris	44	10·2	2·3	5·8	0·29
		11	1·0 Tris	139	18·1	14·9	11·1	1·02
		11	0·7 Tris	44	5·8	4·7	8·8	0·64
		11	0·7 Tris	139	11·1	7·0	14·9	0·54
24 July A	440	9	1·0 Tris	53	5·1	1·1	3·9	0·24
24 July B	435	9	1·0 Tris	53	2·1	1·3	2·9	0·52
Mean ± S.E.		10						0·60 ± 0·11

results have been pooled. Thus, replacing external sodium with three chemically quite different species which are unable to substitute for sodium in producing inward current, reduced the extra efflux associated with voltage clamp pulses by about 50 %.

Replacing sodium with lithium. Lithium is able to substitute for sodium in supporting the action potential and in producing the transient inward

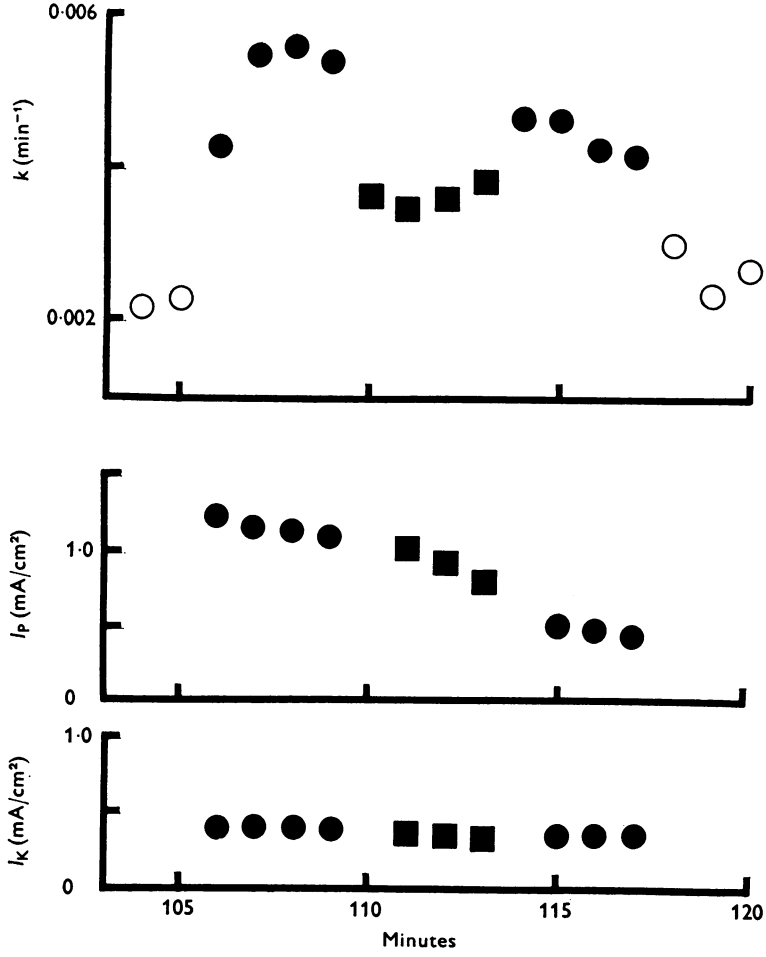

Fig. 6. Replacing external sodium with lithium reduces the extra efflux of sodium associated with voltage clamp pulses. The upper graph shows the fraction of labelled sodium leaving the axon at rest (open symbols) and when repetitively voltage clamped 20 times per second with a 53 mV pulse 0·7 msec long. During the period indicated by the squares the sodium ASW was replaced by lithium ASW. The lower graphs show the magnitude of the peak inward current and the delayed outward current through the membrane during the voltage clamp pulse. Axon diameter 440 μm. Temperature 23° C.

current (Overton, 1902; Moore, 1958; Chandler & Meves, 1965). In Fig. 6 it can be seen that Li is unable to substitute for sodium in the process that is responsible for producing one half of the extra sodium efflux. In this particular experiment a slightly different protocol was used. During the period indicated by the filled symbols repetitive voltage clamp pulses were applied and the bathing solution was changed during the voltage clamp run. In the upper graph, showing the fraction of labelled sodium leaving the axon per minute, it can be seen that substituting lithium for sodium externally reduced the extra efflux of sodium by about one half. In four bracketed experiments lithium substitution reduced the extra efflux of sodium to $0{\cdot}54 \pm 0{\cdot}15$ of control values in sodium ASW (Table 2). In the lower two graphs of Fig. 6 it can be seen that there was a general decline of the peak inward current and very little change in the delayed outward current throughout the experiment. On first switching to lithium the inward current actually increased slightly, this effect can be seen by extrapolating the graph in sodium and in lithium to the time of the switch. This is consistent with the finding of Chandler & Meves (1965) that the sodium channel permeability to lithium is greater than to sodium when estimated by the constant field equation. By way of contrast, in the frog myelinated nerve the permeability of the sodium channel is less for lithium than sodium and the currents seen in lithium are smaller than in sodium (Hille, 1972).

Voltage dependence of sodium efflux in reduced sodium sea water. The experiments reported in Table 2 show that the reduction of Na efflux that accompanies replacement of external sodium is not highly dependent on the potential at which it is measured. Thus the twelve measurements with depolarizations of less than 60 mV had a ratio of k_s of $0{\cdot}46 \pm 0{\cdot}06$ essentially the same as $0{\cdot}58 \pm 0{\cdot}08$ for larger depolarizations. A paired t test applied to the five experiments where bracketed measurements were made at two potentials indicate no significant difference between the two potentials ($P > 0{\cdot}5$). Thus replacement of external sodium reduces the extra efflux of sodium by about one half irrespective of the potential at which the extra efflux is observed.

The voltage independence of the reduction in extra efflux with removal of external sodium leads one to expect the relationship between the efflux and the voltage in the absence of external sodium to be similar to the relationship in its presence (Fig. 4) only the slopes of the lines would be different. The most complete experiment is shown in Fig. 7 which shows that roughly this is the case. Seven estimates of the slope of the equivalent efflux *vs.* potential relationship gave a value of $0{\cdot}74 \pm 0{\cdot}14 \times 10^{-9}$ cm/pulse mV which is about one third of the value found with normal external Na.

Low temperatures. Replacing external sodium with Tris was slightly less

effective at reducing the sodium efflux in the cold than at room temperature ($P < 0{\cdot}1$, Table 2). That is the deviation from independence was less pronounced in the cold. The magnitude and direction of this change with temperature are consistent with the idea that in normal external sodium the integral of the sodium current is more temperature dependent than the extra efflux. It is as if at low temperatures some of the increased current

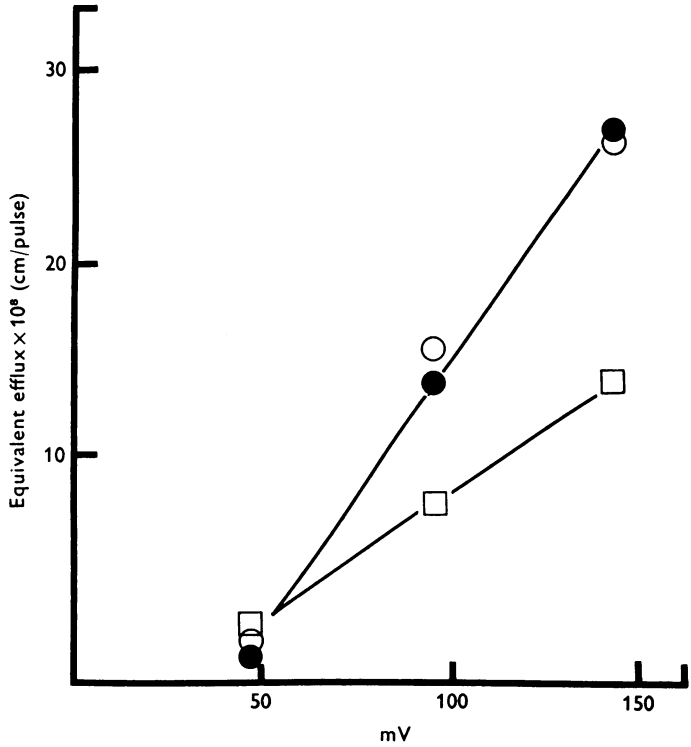

Fig. 7. The voltage dependence of the extra sodium efflux associated with voltage clamp pulses in the presence and absence of external sodium. Open circles indicate measurements in sodium ASW; squares, in 0·9 Tris ASW; and filled circles, return to sodium ASW. Axon diameter 620 μm. Temperature 21° C.

arises because the fluxes are more independent and some from a general increase of the fluxes. This approach towards independence of ion movements associated with cooling was also seen for extra potassium fluxes associated with nerve impulses (Landowne & Scruggs, 1976). As was the case in sodium ASW the voltage dependence of sodium efflux into low sodium ASW was not very temperature dependent. Two measurements made in the cold were not significantly different from those made at room temperature.

The relationship between current and efflux. In sodium ASW the relationship between sodium current and sodium efflux during a depolarizing voltage clamp pulse is complicated because the direction of the current changes from inward to outward as the amplitude of the pulse is increased whereas the flux increases approximately linearly with the amplitude of the pulse (Figs. 3, 4, 7). In the absence of external sodium, however, the sodium current is outward for all depolarizing pulses and one might expect the ratio of extra sodium efflux/$\int I_{Na} dt$ to be close to unity if sodium is the only ion carrying current. It is necessary to estimate the internal sodium concentration to make this calculation as this was not measured in these experiments. Using $[Na]_i$ = 46 mM (Steinbach & Spiegelman, 1943) one obtains from the data in Table 2 a value of $1{\cdot}3 \pm 0{\cdot}3$ for this ratio. This is in accord with Mullins *et al.* (1962) who obtained a mean value of 1·2. Furthermore, comparing the extra influx of sodium to the measured inward current in axons perfused with Na free solutions Atwater, Bezanilla & Rojas (1970) and Bezanilla, Rojas & Taylor (1970) found the ratio of flux/current to be near unity.

Raising external sodium from 0 to 460 mM *reduces* outward current (or produces inward current) while *increasing* the extra efflux of sodium (Fig. 5). This is consistent with the finding of Mullins *et al.* (1962) that, for large depolarizations, the extra sodium efflux into sodium ASW was larger than one might suppose from the current measurements, a ratio of $2{\cdot}37 \pm 0{\cdot}51$ can be calculated from their data. This is also consistent with the values reported here in Table 2, that is adding 460 mM sodium to the external medium roughly doubles the efflux.

Resting efflux

Replacing external sodium with Tris, dextrose or lithium generally increased the resting sodium efflux from these axons which had been treated with ouabain. The efflux relative to that seen into sodium ASW for these three solutions at room temperature was $1{\cdot}43 \pm 0{\cdot}13$ (5); $1{\cdot}43 \pm 0{\cdot}06$ (3) and $1{\cdot}16 \pm 0{\cdot}24$ (6) respectively where the numbers indicate the mean ± S.E. (number of bracketed determinations). These results are similar to those of Baker *et al.* (1969) where an increased resting efflux was reported for lithium, choline or dextrose replacement of the sodium. Magnesium-mannitol replacement of external sodium reduced the resting efflux to $0{\cdot}81 \pm 0{\cdot}14$ (4) normal. Three measurements of the resting efflux into Tris ASW in the cold were $1{\cdot}04 \pm 0{\cdot}23$ times the efflux into sodium ASW.

DISCUSSION

Hodgkin & Huxley (1952*c*) recognized that their equations predicted less extra efflux of sodium associated with nerve impulses than had been observed by Keynes (1951). Cohen & Landowne (1974) found that the extra efflux associated with nerve impulses had a lower temperature dependence than predicted by Hodgkin & Huxley (1952*c*). The findings reported here help to resolve these conflicts between theory and observation. Firstly, extra Na efflux is 1·5–2·5 times as large in the presence of external sodium as in its absence. Secondly, the temperature dependence of the extra sodium efflux is different from the temperature dependence of the integral of the sodium current. Correspondingly, the effect of external sodium on the extra sodium efflux appears to be larger at higher temperatures. These findings are sufficient to account for the temperature dependence of sodium efflux associated with action potentials (Fig. 8).

The points in Fig. 8 represent experimental measurements of the extra sodium efflux associated with nerve impulses at various temperatures. Curve *a* was calculated from the equations of Hodgkin & Huxley (1952*c*). Curve *b* was calculated from these same equations incorporating Moore's (1958) finding that the conductance increases 4% per degree as the temperature is increased. Curve *c* was fitted to the data by eye with the additional restriction that it fit the predictions made by dividing the values along curve *b* by the ratio of extra efflux into sodium-free ASW/sodium ASW (Table 2). Thus at 25° C the extra efflux is 2–3 times as large in sodium ASW as in sodium-free ASW so the theoretical curve *b*, which was generated assuming the independence principle held, must be increased correspondingly. As the effect of altering external sodium on the extra efflux was, in the first approximation, independent of the potential it seems appropriate to use the values obtained from voltage clamp experiments to describe the properties of the flux associated with the action potential.

The finding that the deviation from independence decreases as the temperature decreases can also be made by comparing flux ratio calculations between observations made of fluxes associated with nerve impulses and the same calculations made on the predictions of Hodgkin & Huxley (1952*c*). Landowne & Scruggs (1976) made such calculations for potassium fluxes and found the deviation from independence decreased with cooling. This was done by computing the potential $V = (kT/e) \ln (M_i/c_o)/(M_o/c_i)$ where k is Boltzmann's constant, T the absolute temperature, e the electronic charge, M_i and M_o the influx and efflux, and c_i and c_o the internal and external concentrations of the ions. Note that M_o/c_i is equal to k_s, the rate constant for extra loss of isotope, times the volume:surface area ratio.

Using Hodgkin & Huxley's (1952*c*) predictions, based on the assumptions of independent ion movements, one obtains values for V of -10 mV at room temperature and -17 mV in the cold. Using the data from Cohen & Landowne (1974) one obtains $V = -49$ mV at room temperature and

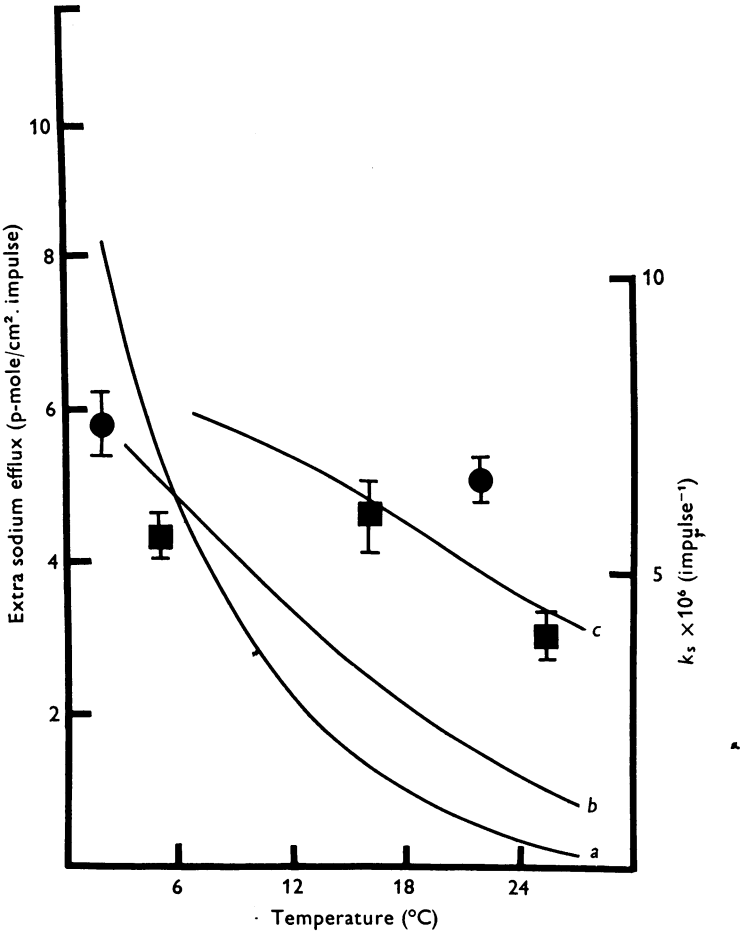

Fig. 8. The temperature dependence of sodium efflux associated with nerve impulses. Curve *a* represents the predictions of the unmodified equations of Hodgkin & Huxley (1952*c*). Curve *b* uses these equations but also incorporates the temperature dependence of the conductances, $\bar{g}_{Na}$ and $\bar{g}_K$, described by Moore (1958). Curve *c*, which is considered a better fit to the data than curves *a* or *b*, was obtained by multiplying curve *b* by the ratio of sodium efflux in sodium ASW/sodium efflux in Tris ASW as described in the text. The experimental data represent the mean values ± 1 S.E. from Table 3 of Cohen & Landowne (1974). The experimental data are plotted against the right hand vertical axis which was obtained from the left hand axis using a diameter of 476 μm and an internal sodium concentration of 65 mM.

−39 mV in the cold indicating there is a deviation from independence for the sodium fluxes during action potentials and this deviation is less in the cold.

The sodium fluxes during nerve impulses are not independent. The major new finding reported in this paper is that the efflux of sodium during voltage clamp pulses is not independent of the external sodium concentration but is reduced by about half when external sodium is removed. This deviation from independence is in the correct direction and of the correct magnitude to explain the differences between calculated and observed sodium effluxes associated with action potentials (Fig. 8), and also provides an explanation for the high transfer numbers reported by Mullins *et al.* (1962). This effect of external sodium is reminiscent of the exchange diffusion of sodium seen in the resting frog muscle (Ussing, 1949*a*; Keynes & Swan, 1959). There are also a few reports on sodium influx in perfused squid axons which also indicate the possibility of extensive exchange diffusion of sodium during nerve impulses.

Rojas & Canessa-Fischer (1968) reported that increasing internal sodium in perfused squid giant axons increased the influx of sodium associated with activity such that the net flux remained constant. The increase of the influx was equal to the increase in the efflux as internal sodium was increased. In perfused voltage clamped axons Bezanilla *et al.* (1970) reported two experiments in which they measured sodium influx and increased the internal sodium concentration from 0 to 100 mM. This increased the extra influx of sodium. From their data one can calculate the ratio of extra influx with $[Na]_i$ = 100 mM/extra influx with $[Na]_i$ = 0 and obtain 1·26 and 1·13 for the two bracketed experiments. These experiments were performed at 10° C and the changes in internal sodium are less than the changes in external sodium reported in this paper. Nevertheless the changes are in the appropriate direction and of the appropriate magnitude to support the sodium–sodium exchange hypothesis.

Lithium is not able to substitute for sodium in enhancing the extra efflux of sodium whereas it is capable of producing early inward current with kinetics which are thus far indistinguishable from the current produced by sodium ions (Fig. 5). This suggests that lithium's interaction with the membrane while passing through is different from that of sodium. A similar, but complementary difference, was seen when comparing thallium fluxes in squid axons with those of potassium (Landowne, 1975*b*). From flux ratio calculations it was shown that Tl fluxes appeared to be independent while those of potassium were consistent with the 'single file' model.

Modelling

Implicit in the finding that the sodium fluxes are not independent and do not satisfy Ussing's (1949*b*) flux ratio criterion, is that at least some of the sodium which flows across the membrane during the nerve impulse enters or binds to the membrane phase. While in the membrane phase, there is mixing such that the influx of sodium from the external solution into the membrane phase enhances the efflux of sodium from the membrane into the external solution. From the experiments of Rojas & Canessa-Fischer (1968) and Bezanilla *et al.* (1970) it seems very likely that this phenomenon is qualitatively symmetric across the membrane, some of the

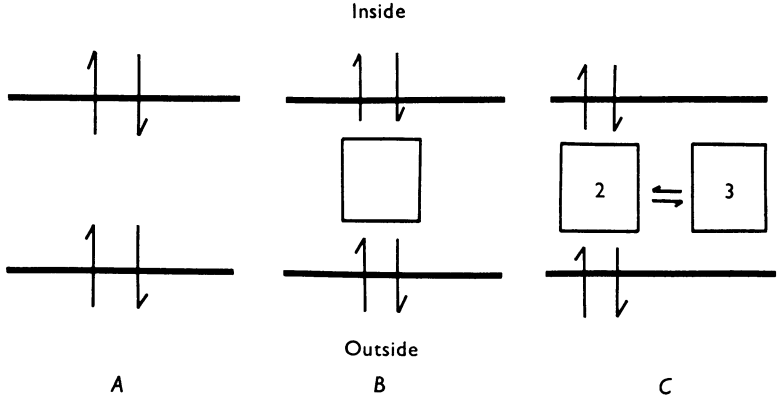

Fig. 9. Compartmental models for sodium fluxes through membranes.

sodium efflux enhancing the influx. The effect is not likely to be a simple allosteric effect of sodium at the interface between aqueous and membrane phases because these enhancements of the unidirectional fluxes are independent of the net flux and of the sodium conductance. The sodium conductance, g_{Na}, is not dramatically altered by lowering the external sodium concentration (Hodgkin & Huxley, 1952*a*), whereas the sodium efflux is reduced by half. This fraction is independent of potential in the first approximation but the magnitude and direction of the current both change with potential. The effect is independent of the current since it is seen equally well with impermeant and with the permeant ions, both Tris and lithium ion replacement reducing the efflux.

The amount of sodium from inside the axon which acts as if it were being exchanged for sodium outside the axon is proportional to the total amount which leaves. In addition, all of the extra sodium efflux is blocked by the external application of tetrodotoxin. These two observations make it very unlikely that sodium efflux occurs by two independent parallel

pathways such as a gate and a carrier mechanism as this would require that both pathways have the same voltage sensitivity and ability to bind tetrodotoxin.

Fig. 9 shows three different compartmental models which could describe the relationship of ions within the membrane phase. For the purposes of this discussion, no assumption need be made about the qualities of the membrane compartment other than that it lies in between the two aqueous compartments. Sodium in the membrane compartment could be within waterfilled pores, combined with a carrier or just free in the lipid environment. The only assumption is that the compartments be recognizable and that in models (*b*) and (*c*) the membrane compartments have a finite capacity.

Case (*a*) was considered by Ussing (1949*b*), and is independent in the sense that adding unlabelled sodium to the outside medium does not change the movement of labelled sodium from inside to outside. Case (*b*) with a limited membrane capacity for sodium has characteristics similar to those described by Hodgkin & Keynes (1955). Adding unlabelled sodium to the outside medium reduces the movement of labelled sodium from inside to outside. Case (*c*) has two compartments within the membrane both with limited capacity. In the steady-state case (*c*) reduces to case (*b*) and the system exhibits 'long pore' characteristics. However, with appropriate selection of rate constants, case (*c*) transiently will display characteristics similar to the sodium efflux from squid axons associated with transient voltage clamp pulses. That is adding sodium outside increases the amount of labelled sodium that moves from inside to outside. This occurs because the added sodium fills compartment 3 more rapidly thus allowing the labelled sodium in compartment 2 to flow to the outside compartment sooner.

The compartmental models considered by Hille (1975) are independent (case (*a*)) or long-pore (case (*b*)). In order to use Hille's type of model to explain the flux data reported here it would appear to be necessary to invoke some form of co-operativity whereby the sodium ions which enter the axon alter the property of the channel as seen by the tracer sodium ions which leave the axon. That is, not only does the channel alter the flow of sodium, the flow of sodium alters the channel. This corresponds to case (*c*).

These three models represent the simplest compartmental models which could be included within the membrane. Undoubtedly more complicated models could be constructed which also have these properties. Without considerably more experimental data it does not appear fruitful, at present, to assign values to the various rate constants and compartment sizes. Perhaps the most interesting quality of model (*c*) is that with different

rate constants, it can be used to describe both the sodium and the potassium fluxes, and it can deviate from independence in either direction.

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