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THE TEMPERATURE DEPENDENCE OF THE MOVEMENT OF SODIUM IONS ASSOCIATED WITH NERVE IMPULSES

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SUMMARY

1. The movement of sodium ions across the membrane of the squid giant axon was measured by the use of radioactive tracers. Unidirectional fluxes were measured at rest and when the nerve was stimulated. The difference was considered the extra flux association with nerve impulses.

2. The extra influx in intact axons at room temperature was 5·5 p-mole/cm^2. impulse. At 6° C the extra influx was 6·5 p-mole/cm^2. impulse giving a Q_{10} of 1/1·2.

3. In perfused axons a Q_{10} of 1/1·6 was obtained for the extra sodium influx in bracketed experiments on individual axons.

4. The Q_{10} of the extra sodium efflux associated with nerve impulses was found to be 1/1·2 in intact axons.

5. Hodgkin & Huxley had predicted a much larger temperature dependence for the extra fluxes. If this difference between prediction and experiment does not result from some experimental error, then the class of models for the ion fluxes suggested by Hodgkin & Huxley may be inapplicable.

INTRODUCTION

The nerve impulse is produced by current carried through the membrane by ions moving down their electrochemical gradients. Establishing the relationship between these currents and the action potential and the identity of the ions involved has been the major achievement in the understanding of the nerve impulse (Hodgkin & Huxley, 1952; Keynes, 1951). However, the exact mechanisms by which the ions cross the membrane is not well understood. In the model tentatively advanced by Hodgkin & Huxley (1952), the conformation of some membrane con-

stituent was altered by the change in potential, and this altered the permeability of the membranes to sodium and potassium.

Recently Landowne (1972) suggested a new mechanism to explain the increases in sodium and potassium permeability that underlie the action potential. It soon became apparent that the new hypothesis can be tested by measuring the temperature dependence of the extra ion fluxes that occur during the action potential. These experiments were begun using *Loligo forbesi* axons obtained in Plymouth and continued with *L. pealii* axons in Woods Hole. We found that the extra fluxes had only small temperature dependences, in agreement with expectations of the new mechanism, and in disagreement with the predictions made by Hodgkin & Huxley (1952). Preliminary reports have been published (Landowne & Cohen, 1972; Landowne, 1973).

METHODS

Refrigerated *L. forbesi* mantles were obtained at the Laboratory of the Marine Biological Association, Plymouth, and *L. pealii* were available live at the Marine Biological Laboratory, Woods Hole. 5–10 cm of the hindmost giant axon was removed together with the adjoining small fibres. In some cases the giant axons were cleaned by removing the small fibres.

Influx into intact axons. Axons from both sides of the same squid were used in pairs. Both axons were placed in the same chamber which contained sea water made radioactive with ^{22}Na. After 5–15 min equilibration time one axon was stimulated for 20–40 min, *L. forbesi* axons were stimulated at 50/sec, *L. pealii* at 30/sec. The externally recorded action potential was continuously monitored on an oscilloscope during this period as it was in all the experiments reported here. The radioactive sea water was removed and replaced with non-radioactive sea water and the control axon was tested for excitability. The nerves were then removed to a dissecting dish, the diameter was measured at several positions along the axon, the end of the axon was cleaned and the contents extruded on to a tared piece of parafilm (Steinbach, 1941). The axoplasm was weighed, assayed for radioactivity and the influx was computed as p-mole/cm^2 from the specific activity of the bathing sea water. The difference between the two axons was taken as the stimulated influx. In *L. pealii* axons, considerable tapering of the axon was observed and taken into account in computing the surface-to-volume ratio. Generally extrusion removed about 80% of the axoplasm as computed from the diameters.

The temperature was reduced in Plymouth by moving the entire apparatus into a constant temperature room (6–7° C). In Woods Hole a small Peltier cooling device was used to cool the chamber, and the temperature in the bath was monitored by a thermistor device.

In some *L. pealii* experiments the proximal half of the axon was cleaned of small fibres and, at the end of the experiment, the two half axons extruded separately. In these cases the proximal portion was extruded, and then the sheath was cut so that the distal portion was not extruded through the proximal sheath.

Influx into perfused axons. In these experiments, *L. pealii* axons were extruded and perfused using the method of Baker, Hodgkin & Shaw (1962). The perfusion fluid contained 570 mM KF, 34 mM-K phosphate buffer, pH 7·4, and 1 mM ethylenediamine tetra-acetic acid, di-sodium salt. The perfusion rate was 50–100 μl./min. The chamber consisted of a narrow trough with five indwelling platinum electrodes

for stimulating and recording, separated by Vaseline seals. The perfused axon was placed in the chamber, ^{22}Na or ^{24}Na added to the central pool and samples of the perfusate collected every 2–3 min (Shaw, 1966). The nerve was stimulated for 4–5 min at 10–30/sec ending at least 1 min before the end of a collection period. A 1 μl. sample of the sea water was taken after each period of stimulation. The results during periods of no stimulation were expressed as p-mole/cm^2.sec, plotted as a function of time, and a least-square fit made to the data at each temperature. The extra influx associated with nerve impulses was calculated by subtracting this least-square estimate of the resting influx at that time.

Efflux. Cleaned axons were loaded with ^{22}Na or ^{24}Na by stimulating 10–15 min at 100–200/sec at room temperature. Only the central portions of the axons were exposed to radioactive sea water. Non-radioactive sea water was fed at 1–2 ml./min past the axon, which was held in a tube with indwelling platinum electrodes. This solution was collected at 2–10 min intervals and assayed for radioactivity. At the end of the experiment the axon was removed and counted. The results are expressed as the fraction of the radioactivity which leaves the nerve per minute (k_r). Least-square fitting and subtraction gave the additional fraction leaving the nerve per impulse (k_s) (Keynes, 1951). The axon was stimulated at 10–30/sec usually for a 5 min period. Temperature was controlled by a water-jacket and monitored with a thermistor. To bring the sea water to the desired temperature, it ran through a tube through the water-jacket before flowing over the axon.

Chemicals used in the perfusion fluid were all reagent grade. ^{24}Na was obtained as NaCl, ^{22}Na was carrier free. Ouabain was obtained from Sigma. Natural sea water was used.

Radioactivity was assayed either by a well-type gamma scintillation counter or by counting the light pulses emitted by the Cerenkoff effect.

In order to compare the effect of temperature on various processes, Q_{10}s have been calculated by computing the ratio of the measurements at the two temperatures and scaling to a 10° temperature change logarithmically. Thus the Q_{10} of a process X, measured at two temperatures T and $T+\Delta T$, is given by

$$Q_{10} = \left[\frac{x(T+\Delta T)}{x(T)}\right]^{10/\Delta T}.$$

If the flux is higher at higher temperature then the Q_{10} is greater than one. If the flux is lower at higher temperature the Q_{10} has been expressed as a fraction.

RESULTS

Sodium influx in intact fibres

The resting as well as the extra influx of sodium is presented in Table 1 for pairs of axons, one of which was stimulated. The most striking finding was that there was no large change in the extra influx of sodium with a change in temperature. Axons from *L. pealii* show a small increase in extra influx when the nerve was cooled; the Q_{10} was 1/1·4. *L. forbesi*, on the other hand, showed a slight decrease as the temperature was lowered, the calculated Q_{10} was 1·5, but the fluxes at the two temperatures were not different at a 10% confidence level. In both species the extra influx at room temperature was similar to the extra influx found in squid axons by

other investigators (see Fig. 3). However, there was considerable variability in the resting influx measured in the cold and, as this value affects the calculation of the extra influx, it is necessary to consider what effect this could have on the calculation. In the case of *L. forbesi*, if the two experiments which gave high resting influxes are recalculated, using 80 p-mole/cm^2.sec as the resting influx, the mean value for influx in the cold is raised only slightly to $4\cdot2 \pm 1\cdot2$ p-mole/cm^2.impulse. And, even if it is

TABLE 1. The influx of sodium in the intact squid giant axon

L. forbesi				*L. pealii*			
Expt.	Temp. (°C)	Resting influx (p-mole/cm^2.sec)	Extra influx with stimulation (p-mole/cm^2. impulse)	Expt.	Temp. (°C)	Resting influx (p-mole/cm^2.sec)	Extra influx with stimulation (p-mole/cm^2. impulse)
2202	6·5	87	4·4	08M	2·0	15	10·0
2501	6·5	250	3·7	12M	4·0	40	11·3
2502	7·0	76	1·6	18M	6·0	32	9·5
2601	6·5	74	5·6	23MC	6·5	140	5·4
2701	7·5	228	1·5	25MC	7·5	129	7·6
				25MU	7·5	268	2·5
				01JC	6·0	103	9·7
				01JU	6·0	88	11·8
Mean	6·8	143	3·4		5·7	102	8·4
± S.E.		± 39	± 0·8			± 29	± 1·2
2801	20·5	22	8·5	12M	19	75	5·3
2802	21·5	39	9·1	16M	21	48	3·7
01N1	21·0	58	6·9	17M	22	20	6·2
01N2	20·5	63	4·3	26MC	22	16	3·9
02N1	20·0	38	2·6	26MU	22	21	5·2
02N2	20·5	29	4·0	02JC	23	60	7·1
				02JU	23	96	4·9
Mean	20·7	41	5·9		21·7	48	5·2
± S.E.		± 6	± 1·1			± 11	± 0·5

assumed that all of the resting measurements were abnormally high and the resting influx was actually 20 p-mole/cm^2.sec, the calculated extra influx in the cold is only $7\cdot0 \pm 1\cdot3$ p-mole/cm^2.impulse which is still not significantly different from the value at room temperature. Thus we concluded that the temperature dependence of the extra sodium influx in intact axons was small. The mean Q_{10} for all intact axons was $1/1\cdot2$.

The *L. forbesi* experiments were performed on axons with the small fibres still adhering. If the movement of sodium through this space were temperature dependent there could be an effect on the influx across the

axon membrane. To test this possibility several experiments were performed with *L. pealii* axons in which either the entire length of the central chamber or one half of it had been cleaned, and the axoplasm from the cleaned and uncleaned portions extruded separately. The experiments with cleaned portions are marked C in the Table and did not show any significant difference in the extra influx associated with impulses. It should be noted that no measurements were made with internal electrodes so we have no measure of fatigue.

Strictly the data presented in Table 1 is the average influx over the entire period in the bath. The true unidirectional influx can be obtained by correcting for the efflux of radioactivity which occurs during this period (Keynes, 1951). This correction factor was calculated from averaged data from the efflux experiments and is 8 and 10 % for *L. forbesi*, in the cold and at room temperature respectively. The corresponding values for *L. pealii* are 11 and 23 % due to the large resting efflux from two axons at room temperature. Computation of the Q_{10}s involves the ratio of two readings so, from this source, the error in the Q_{10}s is less than the error in the individual values, being less than 2 % for *L. forbesi* and less than 7 % for *L. pealii.*

Species differences. The differences in Q_{10} between *L. forbesi* and *L. pealii* axons are not easily explained. Keynes & Lewis (1951) found less sodium influx/impulse when the *Sepia* axon was stimulated at rates above 150/sec. While most of our experiments with *L. forbesi* were done at 50 impulses/sec and those with *L. pealii* were at 30/sec, still it seems unlikely that this difference in rate can account for the difference between the observed stimulated fluxes in the cold. In Plymouth, axons were dissected from refrigerated mantles whereas in Woods Hole, squids were available alive. Also in *L. forbesi* the axons were considerably larger than in *L. pealii.* Both of these variables were present in Keynes & Lewis's experiments, the axons with the lower fluxes were larger and were left for longer periods after decapitation, so that size and internal sodium concentration may provide part of the explanation.

Influx in perfused fibres

The measurements made on intact fibres indicated a small temperature dependence of the extra influx, but there was some scatter between fibres, so it seemed useful to seek a method of measuring the extra influx at various temperatures on a single axon. This was done on *L. pealii* axons using the method of Shaw (1966) except that a fluoride perfusion fluid was used.

Fig. 1 shows the result of such an experiment. The nerve was stimulated for 3 min at 30 shocks/sec. In most cases the extra influx was seen during two collecting periods, occasionally some of the radioactivity was seen in the third collection period, as in the second stimulation in Fig. 1. Although the extra influx was somewhat larger at low temperatures, it seems clear from Fig. 1 that the temperature dependence will not be large. The resting influx data of each temperature were fitted by the least squares method and the extra influx/impulse was calculated by subtracting the least square estimate of the resting influx during the period indicated by

the filled symbols from the observed influx and dividing by the total number of impulses. As shown in Table 2, the mean extra influx/impulse measured in perfused axons was similar to those in intact axons. The Q_{10} calculated from the means at high and low temperatures was 1/1·4. The temperature dependence was also calculated for each experiment where bracketed measurements were made, first at low temperature, then at room temperature and again at low temperature. For example, two values of the temperature coefficient were obtained from the axon in Fig. 1 (axon 16J). The first is

$$\{6\cdot3/[(6\cdot2+11\cdot9)/2]\}^{10/13} = 1/1\cdot32.$$

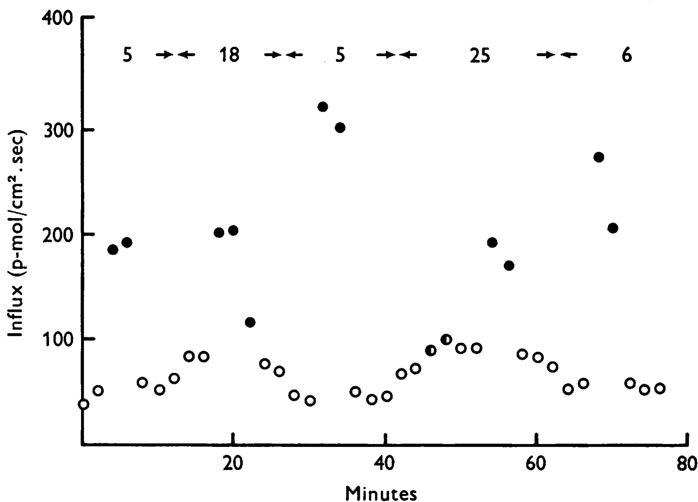

Fig. 1. The influx of sodium into a perfused squid giant axon. During the periods indicated by the filled symbols the axon was stimulated for a total of 3 min beginning at the start of the first filled symbol. Each symbol represents a 2-min collection period, and the difference between the filled and the open (resting) symbols was taken as the extra influx associated with nerve impulses. During the periods indicated with half-filled symbols (◐) the axon received subthreshold stimuli which did not produce propagated impulses. The numbers at the top indicate the temperature in °C. *L. pealii*, axon diameter 500 μm.

Five such calculations had a mean value of $1/1\cdot6 \pm 0\cdot1$ (S.E.). These Q_{10}s were again small, and similar to those found on intact axons. Thus we conclude that the extra sodium influx associated with nerve impulses has a low temperature dependence, both in intact and perfused axons.

The temperatures were measured with a thermistor in the bath approximately 1 mm away from the axon. Because the internal perfusion fluid was initially at room temperature and flowed through only 1 cm of axon we are not certain that the

actual temperature of the membrane was the same as that measured in the bath. An error introduced by this uncertainty would tend to make the fluxes appear less temperature sensitive than they actually were.

As can be seen in Fig. 1 and Table 2 there was generally an increase in both the resting and the stimulated influx during the course of the experiment which might be due to slow deterioration of the axon. A second possible cause could be an increase in the area exposed to the radioisotope. Samples were taken from all five pools at the end of experiment and often one of the pools adjoining the centre one would have substantial radioactivity. This could account for up to 40% error in the estimation of the area and thus the absolute value of the fluxes. It has considerably less effect on the estimate of the temperature dependence, as any slow change in the effective area would be averaged out by the bracketing procedure.

The first measurement of extra influx in each experiment was lower than later measurements on the same axon. While it is possible that this is due to incomplete mixing, this seems unlikely unless the mixing requires or is enhanced by stimulation.

TABLE 2. The influx of sodium in perfused squid axons

Expt.	Temp. (°C)	Time (min)	Resting influx (p-mole/ cm^2.sec)	Stimulated influx (p-mole/ cm^2. impulse)	Q_{10} of stimulated influx
14J	4	10	14	7·1	
15J	5	8	17	5·9	
	5	36	56	11·8	
	5	44	62	9·9	
16J	5	8	48	6·2	
	5	36	46	11·9	
	6	72	58	8·2	
17J	4	8	7	2·0	
	5	36	28	8·5	
	4	64	35	6·5	
Mean	5		37	7·8	
± S.E.			± 6	± 0·9	
14J	22	30	52	7·7	
15J	22	22	75	3·2	1/1·80
	22	60	119	4·4	
	22	67	125	5·2	
16J	18	22	81	6·3	1/1·32
	25	58	88	4·2	1/1·56
17J	21	22	32	2·6	1/1·53
	23	50	46	2·8	1/1·70
Mean	22		77	4·6	1/1·56
± S.E.			± 12	± 0·6	

The Q_{10} is given next to the high-temperature measurements which were bracketed by two low-temperature measurements.

Sodium efflux

L. forbesi. Axons were loaded with radioactive sodium and then washed in flowing sea water which was collected and assayed for radioactivity. The measurements are expressed as the fraction of the label which left the axon per minute when the axon was at rest (k_r) and the extra loss (k_s), the fraction lost per impulse. Fig. 2 shows an experiment and clearly there was no large variation in the extra efflux as the temperature was changed. In this particular experiment the axon was exposed to 10^{-5} M ouabain. The Q_{10} of the stimulated efflux measured in four bracketed experiments in the presence of ouabain was $1/1{\cdot}3 \pm 0{\cdot}1$ and in three experiments in the absence of ouabain it was $1/1{\cdot}0 \pm 0{\cdot}1$.

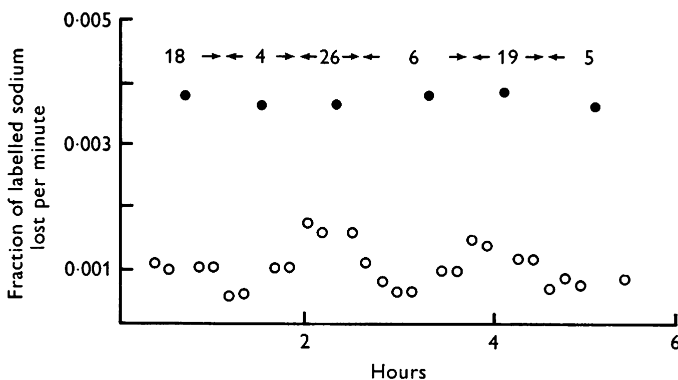

Fig. 2. The rate of efflux of sodium from the squid giant axon at rest (○) and when stimulated at 25/sec for the first 5 min of the 10-min collection period (●). The axon was loaded with ^{24}Na by stimulation 3 hr before the start of this record. 10^{-5} M ouabain was present from $t = -10$ min. The numbers at the top indicate the temperature in °C. *L. forbesi,* axon diameter 600 μm.

L. pealii. The temperature dependence of the stimulated efflux in *L. pealii* axons was similar to *L. forbesi.* In three bracketed experiments the Q_{10} was found to be $1/1{\cdot}3 \pm 0{\cdot}1$. In addition, Dr P. de Weer kindly repeated this experiment in his apparatus (de Weer, 1970) using axons which had been microinjected with ^{22}Na, and he obtained Q_{10}s of $1/1{\cdot}0$ and $1/1{\cdot}4$ in two axons both in the presence of 10^{-5} M strophathadin.

The rate constant for the stimulated efflux was about twice the size of that measured in *L. forbesi.* This cannot be accounted for solely in terms of the diameter, for the *L. forbesi* axons were only 35 % larger on the average. The data from both species is not significantly different from the k_s of 12×10^{-6} found by Keynes (1951) in *Sepia* axons, taking into account the smaller diameter (194 μm).

TABLE 3. The efflux of sodium from squid giant axons

L. forbesi

Expt.	Temp. (°C)	Time (min)	$k_r \times 10^3$ (min^{-1})	$k_s \times 10^6$ ($impulse^{-1}$)	Diam. (μm)
30N	6	30	3·5	4·4	570
	5	110	1·4	6·0	570
01D	4	130	2·1	5·0	525
03D	6	40	4·6	3·0	—
	6	130	1·6	4·0	—
06D	3	90	2·5	2·7	690
09D	5	180	1·0	3·8	785*
	5	290	0·8	3·6	785*
	5	410	0·8	2·2	785*
10D	4	150	0·7	5·1	570*
	6	270	0·7	4·1	570*
	5	390	0·7	3·8	570*
Mean	5·0			4·0	642
± S.E.				± 0·3	± 34
30N	30	70	4·5	4·2	570
01D	25	85	7·7	2·7	525
03D	24	90	5·4	4·0	—
06D	24	40	5·6	3·5	690
	24	130	3·7	2·9	690
	24	180	3·2	2·3	690*
07D	24	35	2·9	1·8	690
	24	90	1·8	1·6	690*
09D	30	350	1·2	1·7	785*
10D	26	200	1·5	2·6	570*
Mean	25·5			2·8	656
± S.E.				± 0·3	± 27

L. pealii

Expt.	Temp. (°C)	Time (min)	$k_r \times 10^3$ (min^{-1})	$k_s \times 10^6$ ($impulse^{-1}$)	Diam. (μm)
07A	0	25	2·6	8·8	450
	0	135	1·0	8·9	450
08A	2	25	2·6	5·6	500
	4	125	1·5	6·4	500
09A	2	45	2·5	9·4	450*
	3	135	0·5	8·2	450*
10A	1	50	2·4	5·5	—*
	1	65	2·1	6·9	—*
Mean	1·7			7·5	467
± S.E.				± 0·6	± 11
07A	24	80	6·3	7·7	450
08A	21	75	6·5	6·5	500
09A	22	90	3·5	5·9	450*
Mean	22·3			6·5	467
± S.E.				± 0·4	± 17

* Indicates 10^{-5} M ouabain was present in the bathing medium.

The mean Q_{10} of the stimulated sodium efflux from all experiments was 1/1·2, and thus both the extra influx and the extra efflux associated with action potentials had a very small temperature dependence.

Ouabain. Although the difference in Q_{10}s is not statistically significant, it did appear that ouabain reduced the stimulated efflux and that the magnitude of this effect was temperature dependent. Thus if one computes that means from the data presented in Table 3 the extra sodium efflux was 13, and 55 % higher in the absence of ouabain at 5 and 23° C respectively. Only the high temperature difference is statically significant ($P < 0·1$). The ouabain experiments were performed on slightly larger axons which would, because of the smaller surface-to-volume ratio, be expected to have a lower efflux (Keynes, 1951). If the results are corrected for the variation in diameter the extra efflux was 7 and 52 % higher at the two temperatures. In two axons the stimulated efflux at 24° C was measured before and after treatment with ouabain and was 22 % higher before ouabain. This was consistent with the finding of Hodgkin & Keynes (1955) who report that dinitrophenol, which inhibited the sodium pump, reduced the stimulated influx and efflux of sodium by 10–20 % at 18° C.

DISCUSSION

The extra influx of sodium into various squid axons is plotted as a function of temperature in Fig. 3. The general conclusion which can be drawn is that there was only a small change in the extra influx as the temperature was varied. Table 4 is a summary of all the temperature-dependent extra fluxes measured. The values calculated from the predictions of Hodgkin & Huxley (1952, Table 5) are also presented. It can be seen that the experimentally determined temperature dependences are much smaller than predicted.

Although we are not aware of previous measurements of the temperature dependence of sodium influx or efflux, there are two less direct measurements of the net sodium flux. In the rabbit vagus nerve den Hartog & Ritchie (1969) suggested that the amount of sodium gained by the nerve per impulse was largely independent of temperature because the area under the post-tetanic response (which gives a measure of the amount of sodium extruded after a tetanus) did not change markedly with temperature (12–35° C). The duration of the action potential was greatly increased in the cold (Howarth, Keynes & Ritchie, 1968) as was the action potential of the squid axon (Hodgkin & Katz, 1949).

Moore & Adelman (1961) estimated the internal sodium concentration of *L. pealii* axons by measuring the reversal potential for the early current

during a voltage clamp experiment (E_{Na}). They report that stimulation caused a net influx of 1·5 p-mole/cm^2.impulse at 7·5° C, a value much smaller than predicted and consistent with our findings. Thus previous

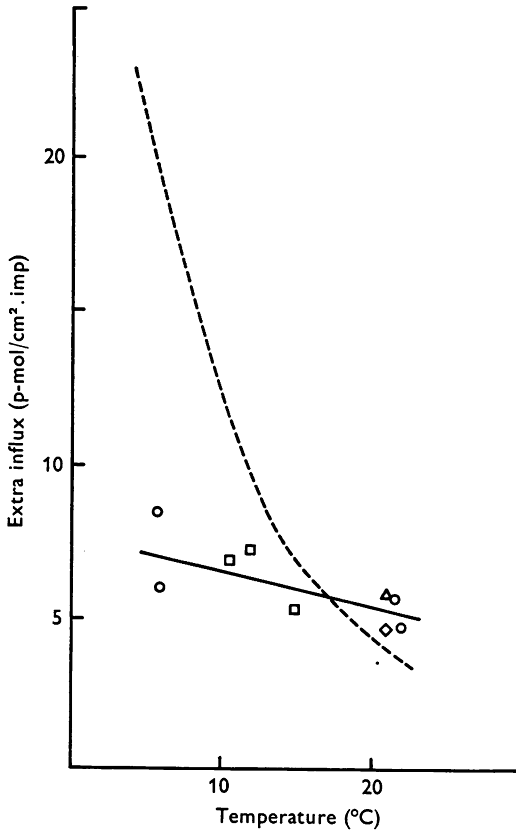

Fig. 3. The extra influx of sodium associated with nerve impulses in squid axons. The straight line was fitted by eye, the curved line represents the prediction of Hodgkin & Huxley (1952) (Q_{10} of 1/3·0). Sources are as follows: (□) Atwater, Bezanilla and Rojas (1970); (◇) Rothenberg (1950); (△) Shaw (1966); (○) this paper.

TABLE 4. The temperature dependence of the extra sodium fluxes associated with nerve impulses in the squid giant axon

	Extra influx (intact)	Extra influx (perfused)	Extra efflux (intact)
Q_{10} (observed)	1/1·2	1/1·6	1/1·2
Q_{10} (Hodgkin & Huxley, 1952)	1/3·0	—	1/3·6

measurements of net sodium flux showed a smaller temperature dependence than predicted from the Hodgkin & Huxley model.

Previous measurements of the stimulated fluxes of ions other than sodium have also indicated small temperature dependences. The Q_{10} of the extra potassium efflux in crab nerves is 1/1·1 (Keynes & Ritchie, 1965) and between 1 and 1/1·6 in rabbit vagus nerves (Keynes & Ritchie, 1965; Howarth, Keynes & Ritchie, 1968), while the temperature dependence of the net potassium flux in crab and squid axons was 1/1·8 (Shanes, 1954). The Q_{10} for calcium influx was 1/1·5 (Hodgkin & Keynes, 1957). In addition to the fluxes of sodium, potassium and calcium, it has been suggested that there might be significant chloride fluxes during the action potential (Cohen, Keynes & Landowne, 1972). Further experiments will be necessary to determine the temperature dependence of the unidirectional potassium and chloride fluxes in squid axons.

Thus, the previous measurements of ion fluxes during the action potential had already suggested a small temperature dependence and so our results mainly extend these observations to the unidirectional sodium fluxes.

Possible explanations for the difference between experiment and prediction

As shown in Fig. 3 and Table 4 there is a large difference between the observed influx and the predictions of Hodgkin & Huxley (1952). Because the different types of flux experiments might be expected to produce different types of experimental error, we are inclined to believe that our measurements are not the result of a simple experimental artifact.

Hodgkin & Huxley (1952) ascribed no temperature dependence to $\bar{g}_{Na}$ while experimentally Hodgkin, Huxley & Katz (1952) found the magnitude of the voltage clamp currents had a temperature coefficient of 1·0–1·5. If $\bar{g}_{Na}$ has a temperature coefficient greater than 1·0 then the predicted temperature dependence of the fluxes would be reduced. The temperature dependence of $\bar{g}_{Na}$ and $\bar{g}_{K}$ has been reported in an abstract by J. W. Moore (1958) and a Q_{10} of 1·5 can be calculated from his results. A comparable value (1·6) was found by Chandler & Meves (1970) in axons perfused with sodium fluoride solutions. Fitzhugh & Cole (1964) have recalculated the temperature dependence of the net efflux of potassium. They found a Q_{10} of 1/2·8 if the temperature coefficient of $\bar{g}_{K}$ is 1·0 and 1/2·2 if Moore's (1958) results are used. J. W. Moore and L. E. Moore (personal communication) have since extended the measurements of $\bar{g}_{Na}$ and find variability between preparations, some axons having a temperature coefficient of 1·0. Since we have not simultaneously measured $\bar{g}_{Na}$ and fluxes in our axons and the data from other measurements are

not clear on this point, it seemed best to proceed as if $\bar{g}_{Na}$ had a Q_{10} of 1·0, recognizing that the predicted Q_{10} for action-potential fluxes will need correction if that assumption proves incorrect.

Fitzhugh & Cole (1964) also noted that Shane's (1954) potassium flux data had less temperature dependence than their prediction. They suggest that part of the discrepancy may come from the use of 3·0 as the temperature coefficient for the kinetic parameters in the Hodgkin & Huxley (1952) calculation. For example, if the sodium current inactivated relatively more rapidly in the cold than expected there could be less sodium influx and less potassium efflux. There is no indication of this type of effect in Hodgkin & Huxley's experiments and Hodgkin *et al.* (1952) found a range of values from 2·7 to 3·5. Clearly this possibility should be reinvestigated as well as the temperature dependence of $\bar{g}_{Na}$. If the temperature dependence of the rate constants is consistently smaller and the temperature dependence of $\bar{g}_{Na}$ is consistently greater than found by Hodgkin & Huxley it may be possible to account for the flux experiments reported here. It should be noted that Hodgkin & Huxley computed the action potentials for 6·3° and then scaled them to 18·5°, so that predictions would be expected to be better at low temperature; in fact they greatly overestimate the observed fluxes.

The effects of repetitive stimulation and unstirred layers are difficult to evaluate quantitatively without a more complete knowledge of the barriers to diffusion. The mobility of sodium in water has a Q_{10} of about 1·3, so ions should be able to reach the membrane less easily at low temperature and the effective outside concentration would be a smaller fraction of the bulk concentration at low temperatures than at room temperature. This is however a second-order correction. Frankenhaeuser & Hodgkin (1956) calculate that repetitive stimulation at 50/sec would change the outside sodium concentration by less than 1 %. In any case the unstirred layer is unable to account for the observed fluxes being more than predicted at room temperature and less at low temperature. Cleaned and uncleaned axons showed similar fluxes even though the diffusion paths to the membrane should be qualitatively different. Furthermore, the efflux data showed the same low dependence on temperature, and Frankenhaeuser & Hodgkin (1956) were able to rule out a barrier to diffusion inside the axon.

Even though all our results suggest a temperature dependence much smaller than that predicted from Hodgkin & Huxley theory, still our conclusion that such a discrepancy exists must be considered preliminary, for two reasons. First, even though our experiments very consistently indicated a small temperature dependence, the ion flux and voltage clamp experiments were not carried out on the same axons, and this needs

to be done. Secondly, and perhaps more important, our experiments were done without an internal electrode so that the size and duration of the action potential were not accurately measured.

The significance of the measurement of extra sodium fluxes. The fluxes we have measured were different from the prediction of Hodgkin & Huxley and therefore do not support the model they have suggested. It must be emphasized that the detailed calculations of Hodgkin & Huxley (1952) were made from electrical measurement and do predict very well this electrical behaviour of the axon, and that it is only the quantitative relationships between electrical measurements and ion flux measurements which is being questioned. Also, we do not wish to suggest that the current is carried by something other than sodium ions but rather question how these ions carry the current. The basic assumption made to convert currents to fluxes is that Faraday's law is applicable as is the case in homogeneous systems. Our experiments suggest that this conversion may not be appropriate so it seems reasonable to question the assumption. Faraday's law cannot be applied when there is a change in the charge distribution between the electrodes. This cannot happen in the aqueous phases but only in or near the membrane. Cole (1949) (cf. Cole & Moore, 1960), Hoyt & Strieb (1971) and Landowne (1972) have proposed models in which the charge distribution (or ion concentration) within the membrane changes. Cole's discussion pertains to potassium movements, but both Hoyt & Strieb (1971) and Landowne (1972) have independently suggested that the sodium ions which move during the nerve impulse might be stored within the membrane prior to the impulse and depleted during the impulse, the membrane (or channel) refilling with sodium during the refractory period. In both of these models the amount stored in the membrane, and hence the amount moved per impulse, is not expected to vary greatly with temperature. In the space-charge limited model (Landowne, 1972, 1973) the change in the duration of the sodium current, and hence the action potential, is produced by a change in the initial distribution of ions. It was proposed that at low temperatures the sodium ion distribution within the membrane rests closer to the outer membrane–aqueous interface than at room temperature and therefore has further to travel within the membrane and produces current in the external circuit for a longer time. Clearly this model must be considered speculative at this time, and the experiments reported here provide only a minimum of evidence that such a model might be correct. The experiments are not adequate to distinguish between the two models (Landowne, 1972; Hoyt & Strieb, 1971) or indeed any other model of the action potential mechanism which relies on stored sodium ions within the membrane. They do argue against any simple 'pore' model where a hole in the membrane opens and

closes with the time course of the Hodgkin–Huxley variables, as this should give the fluxes predicted by Hodgkin & Huxley (1952).

Atwater, Bezanilla & Rojas (1969) and Bezanilla, Rojas & Taylor (1970) have found the ratio of the extra sodium influx to the computed ionic flux to be near unity (range 0·53–1·56). This would seem to indicate that Faraday's law is followed and that one need not consider ion redistribution models suggested above. Further experiments seem necessary, in particular the measurement of the temperature dependence of these fluxes. It will also be necessary to consider the role of sodium inside the axon as Bezanilla *et al.* (1970) have found an increase in the ratio of the sodium influx to the computed flux when sodium was included in the perfusion fluid. Atwater, Bezanilla & Rojas (1970) also measured extra influxes of sodium during action potentials that were less than the predicted amounts at lower temperatures. These values are shown in Fig. 3 as the squares.

The effect of ouabain on the stimulated efflux. In our experiments ouabain reduced the stimulated efflux 5–50 % depending on the temperature. Since the only known action of ouabain on nerve is to inhibit the sodium pump, and since cooling should make this inhibition less apparent, it seemed reasonable to suggest that this is evidence for some interaction between the pump mechanism and the action-potential mechanism. This is not to say that they are the same mechanism for there is considerable evidence that they are not (Hodgkin & Keynes, 1955).

There are several ways in which inhibition of the sodium pump might produce these results. First, if the pump is electrogenic, inhibition of the pump could slightly depolarize the axon, leading to an increased resting sodium inactivation. Then during the nerve impulse there would be a reduced amount of sodium conductance and hence reduced fluxes. De Weer & Gedulig (1973) have reported a small depolarization of *L. pealii* axons on application of strophanthidin which suggests that the effect of ouabain might be explained by such a mechanism.

A second possible source of interaction is from the elevated potassium concentration in the space adjacent to the axon. This would be expected to increase the sodium efflux due to the pump. Using the average values presented in Frankenhaeuser & Hodgkin (1956) one would expect a train of impulses at 25/sec to increase the potassium concentration by about 3 mM. This would increase the ouabain-sensitive sodium efflux by about 20 % (Baker *et al.* 1969). This effect could account for about half of the difference in the extra efflux in the presence and absence of ouabain. This combined with a small depolarization may be sufficient to explain the effect of ouabain of the stimulated effluxes.

A third possible source of interaction could arise if the pump and the

action-potential mechanism fill or deplete the same compartment. This type of interaction is suggested by consideration of the store-charge models because these models propose a third compartment for sodium, the membrane itself. While it seems possible to model this type of interaction in this way, speculation as to the details of such models seems unproductive without further reason to do so.

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