J. Physiol. (1969), **200**, *pp.* 431–458
With 14 *text-figures*
Printed in Great Britain

THE INFLUENCE OF CALCIUM ON SODIUM EFFLUX IN SQUID AXONS

BY P. F. BAKER, M. P. BLAUSTEIN,*
A. L. HODGKIN AND R. A. STEINHARDT†

From the Laboratory of the Marine Biological Association, Plymouth, the Physiological Laboratory, University of Cambridge, and the Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge

(*Received* 5 *August* 1968)

SUMMARY

1. Previous work has shown that the sodium efflux from the axons of *Loligo forbesi* increases when external sodium is replaced by lithium.

2. The increase in efflux in lithium was unaffected by ouabain but was abolished by removal of external calcium; in these respects it differed from the potassium-dependent sodium efflux which was abolished by ouabain but not reduced by removal of external calcium.

3. Strontium but not magnesium could replace calcium in activating the ouabain-insensitive sodium efflux; lanthanum had an inhibitory effect.

4. Replacing all the external NaCl by choline chloride or dextrose gave a rise in Na efflux which was abolished by ouabain but not by removal of external calcium.

5. The rise in Na efflux resulting from partial replacement of NaCl by dextrose or choline chloride consisted of two components one of which was ouabain-insensitive and calcium-dependent and the other was inhibited by ouabain but calcium-insensitive.

6. The ouabain-insensitive component of the Na efflux was activated by low concentrations of Na, Li or K but inhibited by high concentrations of Na and to a lesser extent Li. The inhibiting effect of high Na was of the kind expected if these ions displace calcium from an external site.

7. The ouabain-insensitive component of the Na efflux was abolished by cyanide, had a Q_{10} of 2·7; and was roughly proportional to $[Na]_i^2$. It was much more variable in magnitude than the ouabain-sensitive, potassium-dependent component of the sodium efflux.

8. The calcium influx increased five to fortyfold when external NaCl

* USPHS Special Fellow; present address: Department of Physiology, Washington University School of Medicine, St Louis, Missouri, U.S.A.

† NSF post-doctoral Fellow; present address: Department of Zoology, University of California at Berkeley, Berkeley, California, U.S.A.

was replaced by LiCl or dextrose, the increase for Li being larger than the increase for dextrose.

9. The calcium influx from Na, Li or dextrose sea water was increased three to tenfold by increasing the internal Na about fourfold.

10. The experiments provide evidence for a coupling between an inward movement of calcium and an outward movement of sodium.

INTRODUCTION

The starting point for this investigation was the observation that the sodium efflux from axons of *Loligo forbesi* increases when external sodium is replaced by lithium, choline or dextrose (Caldwell, Hodgkin, Keynes & Shaw, 1960*b*). A number of surprising points came to light when this effect was studied in detail. In the first place, although ouabain removed 60–80 % of the sodium efflux into sea water and greatly reduced its potassium sensitivity (Caldwell & Keynes, 1959), it had little effect on the increment in efflux resulting from the change from sodium to lithium sea water (Baker, Blaustein, Manil & Steinhardt, 1967). However, this component of the efflux did depend on the presence of calcium in the external medium and seemed to be linked to the influx of calcium. It is therefore possible to make a clear distinction between a ouabain-insensitive sodium efflux which depends on external calcium, and the ordinary Na–K coupled pump which is inhibited by ouabain and is little affected by external calcium. The calcium-dependent component of the sodium efflux was small in sea water containing the normal amount of sodium, but it increased markedly when the sodium concentration was reduced, and, unless allowed for, would lead to erroneous conclusions about the nature of the Na–K system. A further complication is that the results obtained when lithium replaced sodium differed in several respects from those obtained when choline replaced sodium, or dextrose replaced sodium chloride.

The finding that external calcium influenced a component of the sodium efflux led naturally to an investigation of the effect of external and internal sodium concentration on calcium movements. The first paper of this series deals with the properties of the calcium-dependent sodium efflux, and with the influence of sodium concentration on calcium influx. The second (Baker, Blaustein, Keynes, Manil, Shaw & Steinhardt, 1969) is concerned with the ouabain-sensitive fluxes of sodium and potassium, and the third (Blaustein & Hodgkin, 1969) with the effect of cyanide and other agents on calcium efflux.

METHODS

Material. Giant axons with diameters varying between 600 and 1000 μ were isolated and cleaned by the usual methods.

Procedure for injecting ^{22}Na and measuring Na efflux. The method of injection was similar to that described by Caldwell, Hodgkin, Keynes & Shaw (1960*a*), except that a Hamilton microsyringe which delivered 0·18 μl./cm replaced the injector described by Hodgkin & Keynes (1956). Sodium effluxes were sometimes measured by the method of Caldwell *et al.* (1960*a*), but in most cases the axon was removed from the injection cell, attached to a vertical plastic rod and transferred through a series of test-tubes containing 6 ml. of the different test solutions. The axon and rod were moved up and down by a motor at 30/min in order to stir the solution. As a rule the axon was in each tube for 3 min and three samples were taken in each test solution. Equilibration was usually complete by the end of 3 min, as can be seen from Fig. 3. In some experiments a flow method of determining sodium efflux

TABLE 1. Composition of stock solutions (mM)

Row	Solution	K^+	Na^+	Li^+	Ca^{2+}	Mg^{2+}	Cl^-	HCO_3^-	Dextrose
A	10-K, Na	10	462·5	—	11	55	602	2·5	—
B	0-K, Na	0	472·5	—	11	55	602	2·5	—
C	0-K, Li	0	2·5	470	11	55	602	2·5	—
D	0-K, Dextrose	0	2·5	—	11	55	132	2·5	740
E	0-K, Na, 0-Ca	0	472·5	—	—	66	602	2·5	—
F	0-K, Na, 0-Ca (100 Mg)	0	402·5	—	—	100	600	2·5	—

A is Na sea water, B is K-free Na sea water, C is K-free Li sea water, etc. Choline sea water was similar to Li sea water but with choline instead of Li. pH 7·8–8·0. Intermediate concentrations were made by mixing stock solutions.

was employed. Here, solution flowed past the axon at 1–2 ml./sec; samples were collected at intervals of 10 sec and the ^{22}Na content was measured after reducing the volume to 8 ml. by evaporation (see Baker *et al.* 1969).

Calcium influx measurements. The uptake of ^{45}Ca was measured by the method described by Hodgkin & Keynes (1957). Cleaned axons were sometimes used, but in most cases the lightly-cleaned nerve trunk was immersed in about 1·5 ml. of solution containing ^{45}Ca for 20 min. At the end of this time it was washed with sea water and after one end had been rapidly cleaned, axoplasm was extruded on to tared polythene film, weighed and dispersed in sea water on a planchette containing 0·7 ml. artificial sea water. Calcium was then precipitated with oxalate, water removed by evaporation under a lamp, and ^{45}Ca counted with an anti-coincidence counter of conventional design. The ^{45}Ca content of the uptake solutions were determined, in essentially the same way, before and after every measurement of efflux. Uptake solutions were made up at frequent intervals and were rarely used more than 2 or 3 times.

Solutions and chemicals. Table 1 gives the composition of some of the solutions and the nomenclature used in describing them.

Lithium chloride was obtained from the Fisher Scientific Company, U.S.A. Ouabain, (strophanthin-G) was normally applied at 10^{-5} M. (cf. Baker *et al.* 1969).

Specpure NaCl or KCl, supplied by Johnson Matthey & Co., was used for injection.

Miscellaneous points. Unless otherwise stated, measurements were made at 20 °C and the axons were excitable at the end of the experiment.

Na and K in extruded axoplasm were determined in the usual way with a flame photometer.

RESULTS

Na efflux

Distinction between ouabain-sensitive and insensitive Na efflux. The sodium efflux from squid axons can be increased by raising external potassium or by replacing external sodium with lithium. Figure 1 shows that the two effects were roughly additive; in this experiment, replacing external sodium by lithium increased the rate constant for sodium efflux by about 2×10^{-3} min^{-1} in both K-free and 10 mM-K media.

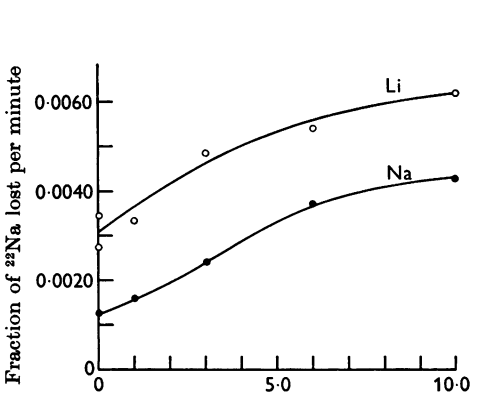

Fig. 1

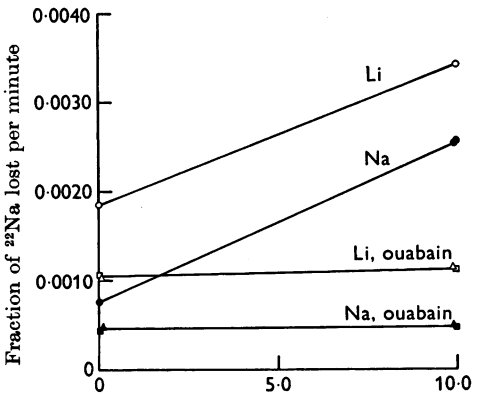

Fig. 2

Fig. 1. Effect of varying external potassium concentration on sodium efflux with Na (●) or Li (○) in the external medium. Unpoisoned axon. Abscissa: concentration of potassium in artificial sea water. Ordinate: fraction of ^{22}Na lost per minute. Axon diameter, 610 μ.

Fig. 2. Effects of Li, K and ouabain on sodium efflux. Abscissa: external potassium concentration. Ordinate: fraction of ^{22}Na lost per minute. ●, Na sea water; ○, Li sea water; ■, Na sea water, 10^{-5} M ouabain; □, Li sea water, 10^{-5} M ouabain; ▲, Na sea water, 10^{-3} M ouabain; △, Li sea water, 10^{-3} M ouabain. Axon diameter, 860 μ.

The increase in sodium efflux associated with replacing external sodium by lithium had the interesting property that it was unaffected by ouabain at concentrations much higher than those needed to remove most of the potassium-sensitive component of the sodium efflux. In Fig. 2 the effect of external potassium concentration on sodium efflux is greatly reduced by 10^{-5} M ouabain, but the difference between the sodium and lithium points is little affected by either 10^{-5} or 10^{-3} M ouabain. This conclusion is supported by the experiments in Table 2, from which it can be seen that the increment in rate constant for the change from external sodium to lithium was little altered by 10^{-5} M ouabain; whereas the increment for the

change from 0 mM-K to 10 mM-K was reduced by a factor of about 25. Since the lithium effect usually decreased during the course of the experiment it is thought that the difference between the normal and ouabain values in Table 2 was caused by a progressive decline rather than by any real inhibition.

The effect of calcium on the ouabain-insensitive component of the sodium efflux. Figure 3 illustrates the action of ouabain and calcium-free solutions on the sodium efflux into sodium and lithium and sea water. To begin with, ouabain was absent and the nerve was in potassum-free sodium sea

TABLE 2. Effect of ouabain on K-dependent and Li-stimulated Na effluxes. Rate constants in ($min^{-1}/1000$)

A. K-dependent Na efflux

10-K (Na)	0-K (Na)	$\Delta_{10\text{-}K-0\text{-}K}$	10-K (Na) $+10^{-5}$ M ouabain	0-K (Na) $+10^{-5}$ M ouabain	$\Delta_{10\text{-}K-0\text{-}K}$
3·48	0·95	2·53	0·24	0·19	0·05
3·75	1·00	2·75	1·02	0·87	0·15
2·55	0·75	1·80	0·48	0·43	0·05
2·75	1·46	1·29	1·25	1·17	0·08
2·37	1·54	0·83	1·01	0·97	0·04
2·03	0·92	1·11	0·99	0·95	0·04
2·19	0·83	1·36	0·77	0·74	0·03
1·60	0·48	1·12	0·40	0·45	0·05
Mean ± S.E.					
2·59 ± 0·26	0·99 ± 0·13	1·60 ± 0·25	0·77 ± 0·13	0·72 ± 0·12	0·06 ± 0·01

B. Li-stimulated Na efflux

Li (0-K)	Na (0-K)	$\Delta_{Li\text{-}Na}$	Li (0-K) $+10^{-5}$ M ouabain	Na (0-K) $+10^{-5}$ M ouabain	$\Delta_{Li\text{-}Na}$
1·06	0·95	0·11	0·25	0·19	0·06
1·85	0·75	1·10	1·05	0·43	0·62
2·16	0·84	1·32	2·14	0·90	1·24
0·62	0·39	0·23	0·55	0·40	0·15
1·55	0·53	1·02	1·33	0·65	0·68
2·38	1·17	1·21	1·59	0·84	0·75
1·60	1·19	0·41	1·53	0 97	0·56
1·28	0·63	0·65	0·78	0·36	0·42
0·62	0·31	0·31	0·41	0·24	0·17
Mean ± S.E.					
1·46 ± 0·21	0·75 ± 0·11	0·71 ± 0·15	1·07 ± 0·07	0·55 ± 0·10	0·52 ± 0·12

In Tables 2, 3 and 6, each horizontal row refers to a single axon.

water. On replacing sodium with lithium, the efflux increased, but the additional component was abolished, reversibly, by replacing calcium with magnesium. After making measurements at intermediate concentrations of sodium (summarized in Fig. 4) 10^{-5} M ouabain was applied, and the action of lithium and zero calcium was tested again. As can be seen in the second part of Fig. 3 ouabain had little or no action on the lithium effect or on its calcium sensitivity.

Figure 4*A* summarizes the complete experiment. Curve 1 shows the effect of progressive replacement of external sodium by lithium, in the

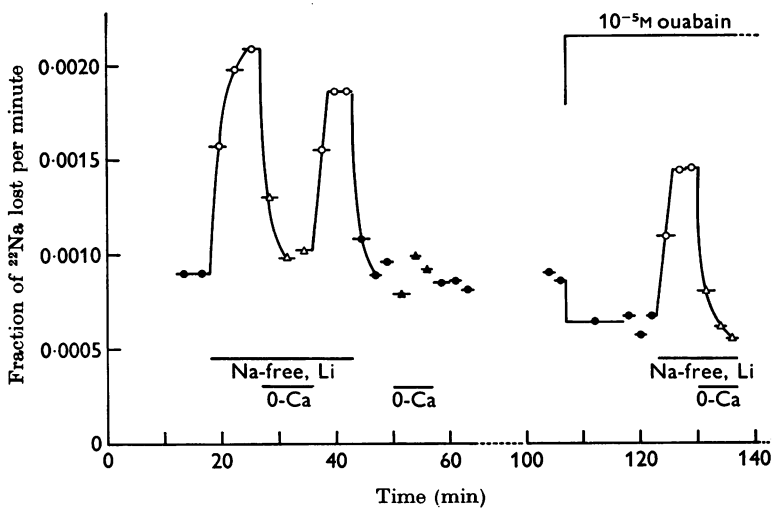

Fig. 3. Part of an experiment showing effects of Ca-free solutions and 10^{-5} M ouabain on sodium efflux into Na and Li sea water. Abscissa: time in min. Ordinate: fraction of ^{22}Na lost per minute. ●, Na, Ca solution containing 473 mM-Na, 0-K, 11 mM-Ca 55 mM-Mg; ○, Li, Ca solution similar with 470 mM-Li replacing 470 mM-Na; ▲, Na, 0-Ca solution containing 403 mM-Na, 0-K, 0-Ca, 100 mM-Mg. △, Li 0-Ca solution similar with 400 mM-Li replacing 400 mM Na. Axon diameter, 770 μ. Further details of this experiment are given in Fig. 4.

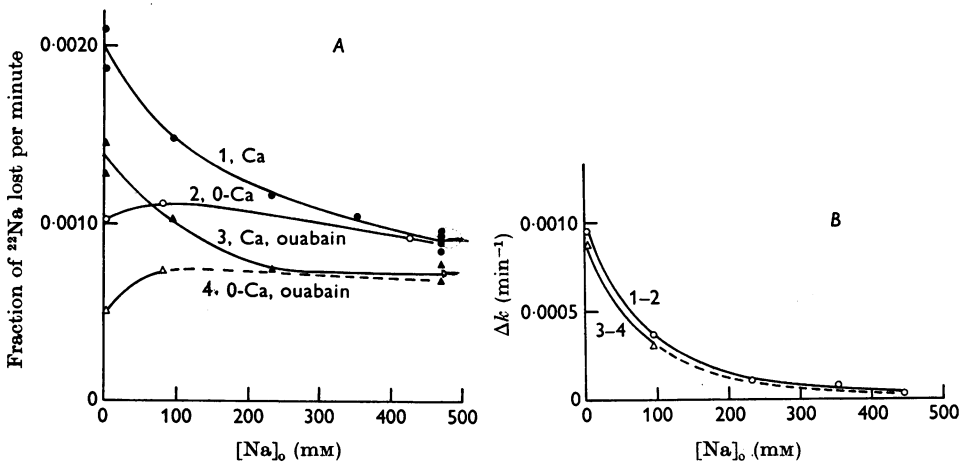

Fig. 4 *A*. Effects of Ca and 10^{-5} M ouabain on Na efflux into mixtures of Li and Na. Abscissa: Na concentration in mM. Ordinate: fraction of ^{22}Na lost per minute. Curve: 1, ● with 11 mM-Ca, 55 mM-Mg; 2, ○ with 0-Ca 100 mM-Mg; 3, ▲ same as 1 but with 10^{-5} M ouabain; 4, △ same as 2 but with 10^{-5} M ouabain. Other details of this experiment are given in Fig. 3.

B. Difference curves showing effect of $[Na]_0$ on calcium-dependent efflux. The ordinate is the rate constant in 11 mM-Ca minus the rate constant in 0-Ca. ○ before ouabain. △ after ouabain.

absence of ouabain and with calcium at its normal concentration of 11 mM. Curve 2 was obtained in the same way but with zero Ca, 100 mM-Mg instead of 11 mM-Ca, 55 mM-Mg. Curve 3 (11 mM-Ca) and curve 4 (0-Ca) correspond to 1 and 2 but were obtained in the presence of 10^{-5} M ouabain. The conclusion from this and other experiments (Table 3) is that the increased efflux associated with the change from external sodium to lithium depends on the presence of calcium but is little affected by ouabain.

TABLE 3. Effect of ouabain on the Ca-sensitivity of Na efflux into Na and Li sea waters. Rate constants in (min^{-1}/1000)

A. Efflux into Na sea water

Na (0-K)	Na (0-K) 0-Ca	Δ_{Ca}	Na (0-K) $+10^{-5}$ M ouabain	Na (0-K) 0-Ca $+10^{-5}$ M ouabain	Δ_{Ca}
0·65	0·54	0·11	0·34	0·28	0·06
0·47	0·46	0·01	0·35	0·29	0·06
0·36	0·42	−0·06	0·33	0·31	0·02
Mean ± S.E.					
0·49 ± 0·09	0·47 ± 0·05	0·02 ± 0·05	0·34 ± 0·01	0·29 ± 0·03	0·05 ± 0·01

B. Efflux into Li sea water

Li (0-K)	Li (0-K) 0-Ca	Δ_{Ca}	Li (0-K) $+10^{-5}$ M ouabain	Li (0-K) 0-Ca $+10^{-5}$ M ouabain	Δ_{Ca}
2·38	1·12	1·26	1·59	0·46	1·13
1·28	0·86	0·52	0·78	0·16	0·62
1·98	1·02	0·96	1·45	0·51	0·94
Mean ± S.E.					
1·88 ± 0·32	1·00 ± 0·08	0·91 ± 0·22	1·27 ± 0·26	0·38 ± 0·10	0·90 ± 0·14

The calcium-dependent component of the sodium efflux is defined as the difference between curves 1 and 2 (before ouabain) or between 3 and 4 (after ouabain); these differences are plotted in Fig. 4*B*. The average curve for the calcium-dependent component is given in Fig. 14, p. 450, where it may be compared with the relation between calcium influx and sodium concentration (Fig. 13).

Attempts to determine the relation between sodium efflux and calcium concentration were complicated by the finding that axons exposed to high calcium tended to lose their lithium sensitivity irreversibly, although in other respects, for example, excitability or potassium-sensitivity of sodium efflux, they were in no way abnormal. The difficulty was avoided by using the flow apparatus, in which sampling times could be shortened to 10 sec. Figure 5 illustrates an experiment of this kind. It shows that, in lithium sea water, calcium activated a component of the sodium efflux along a Michaelis type curve which was half-maximal at 2–3 mM. Replacing half the lithium by sodium reduced the initial slope and increased the apparent Michaelis constant to 20–30 mM. With full sodium only a portion of the curve could be obtained, but if the same limiting value is assumed an apparent Michaelis constant of 50–100 mM is obtained.

Qualitative experiments showed that strontium could substitute for calcium in maintaining a sodium efflux into lithium sea water, but, as already mentioned, magnesium could not replace calcium. There was some evidence that magnesium might be inhibitory since replacement of magnesium by lithium or dextrose at a low calcium concentration increased the sodium efflux into lithium sea water by about 40 %. The effect was reversible, and was seen in the presence of tetrodotoxin.

Lanthanum, which has certain calcium-like actions in nerve (Takata, Pickard, Lettvin & Moore, 1966; Blaustein & Goldman, 1968), was also tested for its effect on Na efflux. In the absence of ouabain, 0·1 mM-lanthanum irreversibly inhibited the lithium-sensitive Na efflux but not the K-sensitive or dextrose-stimulated Na efflux, nor did it prevent ouabain from producing the usual decrease in Na efflux in sea water. After prolonged exposure to lanthanum, the sodium efflux into K-free Na sea water increased and was reduced by replacing sodium with lithium.

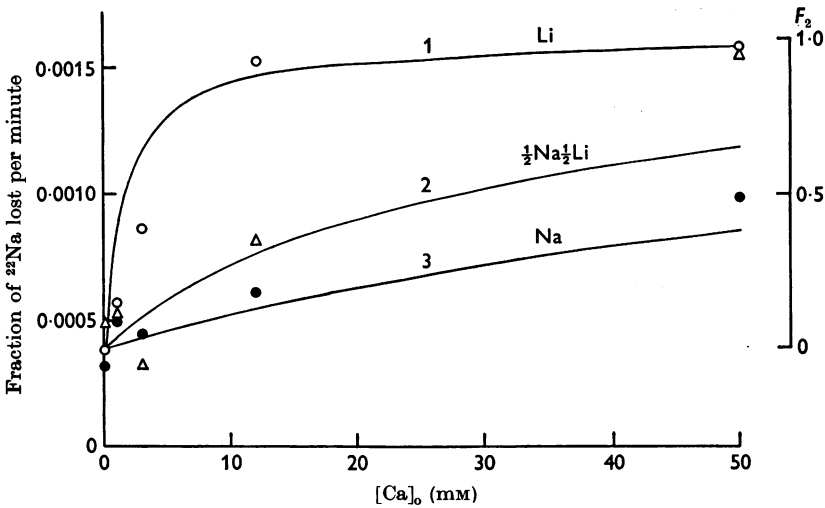

Fig. 5. Effect of external calcium concentration on Na efflux into mixtures of Na and Li. Abscissa: external calcium concentration. Ordinate: fraction of ^{22}Na lost per minute. ○ (curve 1), 400 mM-Li, 2·5 mM-Na; △ (curve 2), 200 mM-Li, 202·5 mM-Na; ● (curve 3), 425 mM-Na. The curves were drawn from eqn. (5), p. 455 with K_m^{Ca} = 1·8, 29 and 90 mM as calculated from eqn. (6), with K_2^{Li} = 100 M and K_2^{Na} = 1·8 M. The same scaling factor was used in all three cases.

The effect of Na_i on the Na efflux into Li sea water. There is general agreement that large changes in the internal sodium concentration of squid axons have little effect on the rate constant for loss of labelled sodium into sea water (Hodgkin & Keynes, 1956; Sjodin & Beaugé, 1967; Brinley & Mullins, 1968). This means that the normal sodium efflux which is mainly due to the ouabain-sensitive Na–K pump is proportional to the internal sodium concentration. In Fig. 6*A* the increment in efflux for the change from K-free to 10 mM-K sea water is plotted against the internal sodium concentration of thirty-one axons. Although there is considerable scatter it appears that the potassium-dependent efflux was roughly pro-

portional to the internal sodium concentration over the range between 15 and 150 mM.

The sodium efflux into lithium sea water, which was calcium-dependent,

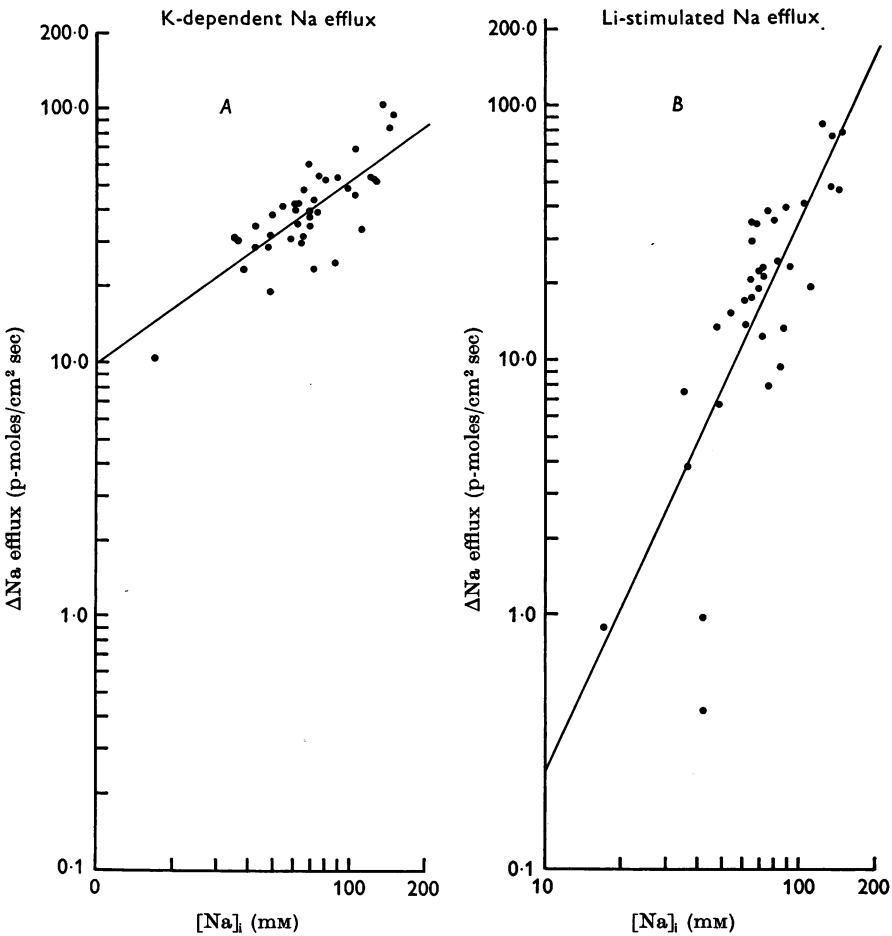

Fig. 6*A*. The effect of internal sodium concentration on the K-dependent sodium efflux. Ordinate: increment in Na efflux when K-free Na sea water was replaced by 10 mM-K Na sea water. Abscissa: internal sodium concentration in mM.

B. The effect of internal sodium concentration on the sodium efflux into lithium sea water. Ordinate: increment in Na efflux when Na sea water is replaced by Li sea water. Abscissa: internal sodium concentration in mM.

The regression lines in *A* and *B* were obtained by the method of least squares using the logarithm of both variables. The slope and S.D. of the lines are: *A*, $0{\cdot}72 \pm 0{\cdot}11$; *B*: $2{\cdot}17 \pm 0{\cdot}30$.

The results were obtained on axons from refrigerated mantles and live squid, axons stimulated in Li (to lower $N[a]_i$) and injected with NaCl (to raise $[Na]_i$). The sodium analyses were performed on distal ends of the axons used for the tracer measurements.

behaved differently. As can be seen from Fig. 6*B*, the increment in efflux for the change from sodium to lithium sea water was roughly proportional to the square of the internal sodium concentration. A similar conclusion can be drawn from Table 4, which shows that on average the sodium efflux into lithium sea water was quadrupled when the internal sodium concentration was increased from 39 to 74 mM by injection. Figure 7 illustrates an experiment in which the curve relating sodium efflux to external sodium concentration was determined before and after injecting sodium chloride into a fresh axon.

TABLE 4. Na efflux into Na or Li sea water before and after raising $[Na]_i$ by injection

Axon	Diam. (μ)		$[Na]_i$ (mM)	Na efflux (p-mole/cm² sec) Na SW	Na efflux (p-mole/cm² sec) Li SW	ΔNa efflux (p-mole/cm² sec)
1	727	Before	42	3·06	3·31	0·25
		After	85	9·80	19·08	9·28
2	818	Before	42	3·01	3·87	0·86
		After	76	9·09	16·36	7·27
3	796	Before	36	3·13	6·85	3·72
		After	72	10·60	31·56	20·96
4	878	Before	35	7·25	14·50	7·25
		After	64	19·89	37·65	17·76
5	898	Not injected	(124)	(25·58)	(110·67)	(85·09)
Mean		Before	39	4·11	7·13	3·02
		After	74	12·35	26·16	13·82

Axons 1 and 2 were from refrigerated mantles and had been stimulated with $3{\cdot}6 \times 10^5$ impulses in Li-sea water to lower $[Na]_i$. Axons 3 and 4 were from freshly killed squid. Axon 5 is a pair to 4 but left 7·5 hr in sea water at 1–2° C before use. Ouabain, 10^{-5} M, was present in all solutions. Axons 4 and 5 were in K-free solutions, the rest in 10 mM-K. Initial $[Na]_i$ values were determined on a length of axon not used in the experiment; values after injection by adding the [Na] injected.

Three experiments carried out in a different way from those in Table 4 gave discordant results. Two axons from refrigerated mantles which had not previously been stimulated in lithium sea water showed large lithium effects before injection. After injecting sodium chloride the efflux into lithium sea water was unaltered in one case and reduced in the other. This may mean that there is an optimum internal sodium concentration or that injection can have a deleterious effect. One axon soaked in lithium sea water for 8 hr gave little lithium effect either before or after injection of sodium chloride. Here, the high internal calcium resulting from a long soak in lithium sea water may have blocked the lithium effect.

Effect of cyanide, dinitrophenol (DNP) and temperature on Na efflux into Li sea water. The experiments in Table 5 confirm and extend the observations of Caldwell *et al.* (1960*b*) on the action of cyanide on the Na efflux into lithium or sodium sea water. They show that cyanide not only reduced the efflux into lithium sea water to a low level but reversed the direction

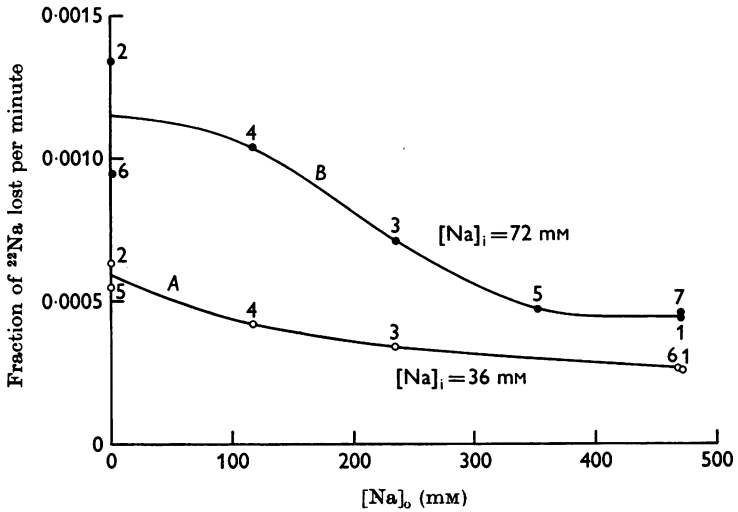

Fig. 7. Effect of raising the internal sodium concentration on the sodium efflux in a fresh axon when external Na is replaced by Li; curve A (○), before the injection of NaCl; curve B (●), after the injection of enough 1 M-NaCl to raise the internal sodium concentration by 36 mM. Same data as axon 3 of Table 4. Axon diameter: 796 μ. Ordinate: fraction of ^{22}Na lost per minute. Abscissa: concentration of sodium (mM) in the bathing medium. The numbers against the points indicate the order in which the values were determined. As mentioned on p. 435 the efflux into Li sea water decreased with time. All the solutions were K-free.

TABLE 5. Effect of 2 mM-CN on Na efflux into Li SW and Na SW

		Rate constant for Na-efflux (min^{-1}/1000)			
Axon	Solution	Normal	In cyanide		After recovery
1	Li (10-K)	3·18	0·24	—	—
	Na (10-K)	2·11	0·32	—	—
	Δ (Li–Na)	1·07	−0·08	—	—
2	Li (0-K)	1·38	0·24	—	—
	Na (0-K)	0·40	0·40	—	—
	Δ (Li–Na)	0·98	−0·16	—	—
3	Li (50-K)	4·33	0·32	—	—
	Na (50-K)	3·78	0·47	—	—
	Δ (Li–Na)	0·55	−0·14	—	—
4	Li (10-K)	3·48	0·37	—	3·11
	Na (10-K)	2·19	0·48	—	1·90
	Δ (Li–Na)	1·29	−0·11	—	1·21
5	Li (10-K)	4·16	0·37	0·27*	0·93*
	Na (10-K)	2·37	0·42	0·33*	0·69*
	Δ (Li–Na)	1·79	−0·05	−0·06*	0·24*
6	Li (0-K)*	1·48*	—	0·22*	0·68*
	Na (0-K)*	0·40*	—	0·32*	0·52*
	Δ (Li–Na)	1·08*	—	−0·10*	0·16*

Parentheses indicate potassium concentration, e.g. 10 mM-K.

* Solutions contain 10^{-5} ouabain.

Measurements were made at 60–90 min after applying cyanide.

of the normal lithium effect. From the last experiment, in which ouabain was present throughout, it appeared that the efflux into lithium sea water was little inhibited for about 30 min in cyanide and was fully inhibited after about 60 min. The tentative conclusion might be that the lithium effect disappears with about the same time course as adenosine triphosphate, ATP (Caldwell, 1960). Further evidence that ATP but not

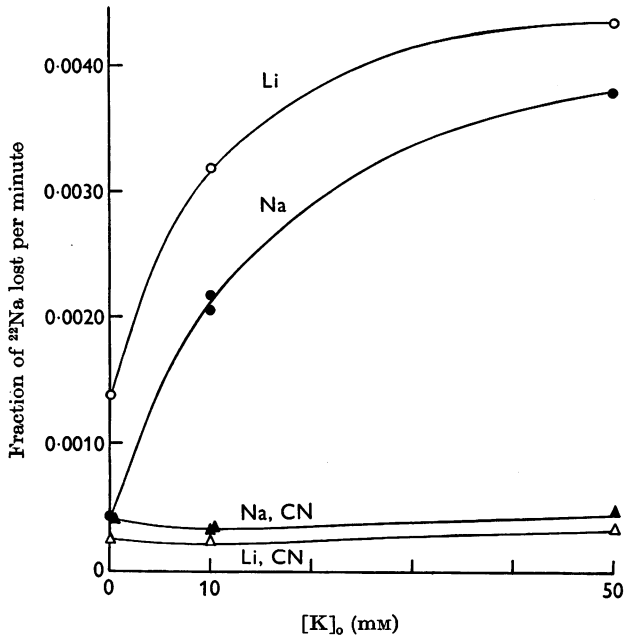

Fig. 8. Effect of 2 mM-CN on Li effect at different potassium concentrations. Abscissa: external potassium concentrations. Ordinate: sodium efflux. ○ in Li sea water. ● in Na sea water. △ in Li sea water after 1–2 hr in 2 mM-CN. ▲ in Na sea water after 1–2 hr in 2 mM-CN. Axon diameter 680 μ.

arginine phosphate is required is provided by the observation that a calcium-dependent sodium efflux into lithium sea water is present in axons partially poisoned with dinitrophenol (Baker *et al.* 1969).

The distinction between ouabain, which abolished potassium sensitivity but not the lithium effect, and cyanide, which abolished both, is brought out by comparing Fig. 8 (cyanide) with Fig. 2 (ouabain).

Cooling the axon from 20° C to 1° C reduced the efflux into Li sea water or Na sea water by a factor of about 7.

The effects on Na-efflux of replacing Na by choline or dextrose. Although the increase in sodium efflux associated with the change from sodium chloride to lithium chloride was sometimes similar in magnitude to that observed with choline chloride or dextrose, most axons gave a smaller

increase in lithium sea water than in the other two solutions. Several axons, particularly some in a group studied in January, showed marked choline or dextrose effects but negligible lithium effects. Further evidence of the distinction was provided by the action of ouabain and calcium-free solutions. Figure 9 illustrates an experiment in which choline replaced sodium.

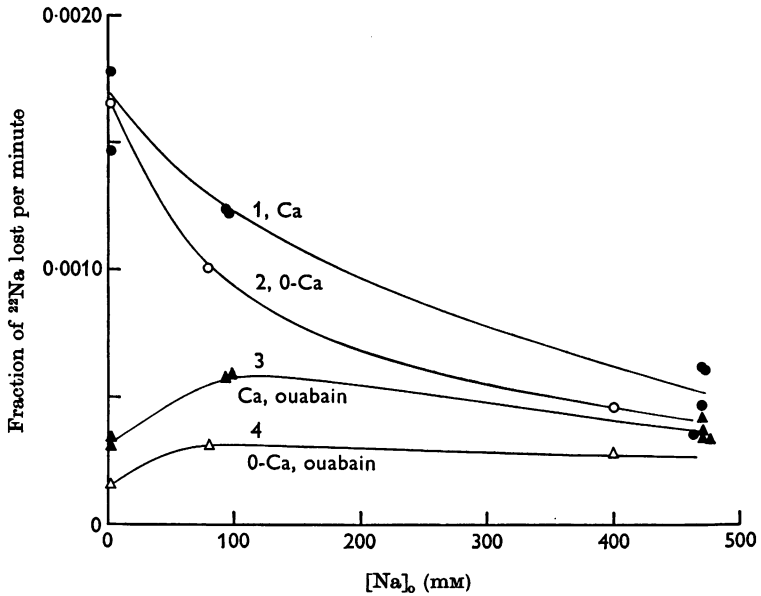

Fig. 9. Effects of Ca-free solutions and 10^{-5} M ouabain on Na efflux into mixtures of choline and Na sea water. Abscissa: Na concentration in mM. Ordinate: fraction of ^{22}Na lost per minute.

Curve 1, ●, with 11 mM-Ca and 55 mM-Mg; curve 2, ○, 0-Ca 100 mM-Mg; curve 3, ▲, same as 1 but with 10^{-5} M ouabain; curve 4, △, same as 2 but with 10^{-5} M ouabain: Axon diameter, 1000 μ.

Before applying ouabain, calcium removal reduced the efflux into 100 mM-Na, but had less effect on the efflux into choline sea water; in this respect the result was clearly different from that with lithium. Again, in contrast to the lithium results, ouabain abolished the extra efflux into choline sea water (Table 6*A*). From curves 3 and 4, which were obtained after ouabain, it is evident that the calcium-sensitive component varied with external sodium in a different way from that described for the change from sodium to lithium. Instead of increasing steadily as the sodium concentration was reduced, the efflux reached a maximum at 100 mM-Na and then declined as the concentration was reduced to zero. Similar results were obtained with dextrose after ouabain, as may be seen from curves 3 and 4 in Fig. 10 and from Table 6*B*. The relation between the calcium-dependent component of the sodium efflux and the sodium or dextrose concentration

is shown by the lower curve in Fig. 14, where it may be compared with the curve relating calcium influx to sodium or dextrose concentration (Fig. 13).

In Fig. 10 where dextrose replaced sodium chloride, the two calcium-free points before ouabain (curve 2) coincided approximately with curve 1. From this one might conclude either that the component represented by the difference between curves 3 and 4 did not appear until ouabain was applied, or that its effect was masked by a relatively small change in the much larger ouabain-sensitive component. For the second explanation to hold quantitatively in the experiment of Fig. 10 calcium removal should increase the ouabain-

TABLE 6. Effect of ouabain on the extra Na efflux into dextrose and choline sea waters. Rate constant in (min^{-1}/1000)

A. Efflux into choline sea water

Choline (0-K)	Na (0-K)	$\Delta_{Choline-Na}$	Choline (0-K) $+10^{-5}$ M ouabain	Na (0-K) $+10^{-5}$ M ouabain	$\Delta_{Choline-Na}$
0·70	0·39	0·31	0·38	0·40	−0·02
2·47	1·34	1·13	0·91	0·93	−0·02
1·61	0·34	1·27	0·29	0·37	−0·04
1·35	0·43	0·92	0·25	0·37	−0·12
1·82	0·70	1·12	0·37	0·54	−0·17
1·83	0·86	0·97	0·66	0·82	−0·16
1·75	0·34	1·41	0·27	0·33	−0·06
Mean ± S.E.					
1·65 ± 0·20	0·63 ± 0·14	1·02 ± 0·13	0·45 ± 0·09	0·54 ± 0·09	−0·08 ± 0·03

B. Efflux into dextrose sea water

Dextrose (0-K)	Na (0-K)	$\Delta_{Dextrose-Na}$	Dextrose (0-K) $+10^{-5}$ M ouabain	Na (0-K) $+10^{-5}$ M ouabain	$\Delta_{Dextrose-Na}$
0·95	0·39	0·56	0·33	0·40	−0·07
2·08	0·64	1·44	0·37	0·59	−0·22
2·16	1·15	1·01	0·56	0·79	−0·23
2·38	0·95	1·43	0·49	0·62	−0·13
2·11	0·34	1·77	0·26	0·28	−0·02
2·35	0·31	2·04	0·26	0·38	−0·12
2·14	0·47	1·67	0·36	0·40	−0·04
1·58	0·78	0·80	0·29	0·58	−0·29
2·79	1·30	1·49	0·75	0·97	−0·22
2·63	0·64	2·01	0·18	0·27	−0·09
1·72	0·59	1·13	0·27	0·58	−0·31
1·88	0·86	1·02	0·47	0·82	−0·35
2·90	0·96	1·94	0·43	0·65	−0·22
2·43	0·75	1·68	0·26	0·40	−0·14
Mean ± S.E.					
2·15 ± 0·14	0·72 ± 0·08	1·43 ± 0·12	0·38 ± 0·04	0·55 ± 0·06	−0·18 ± 0·02

sensitive component by 16 % at 100 mM-Na. Since the effect of removing calcium before ouabain was variable, it was difficult to clear up this question, but the evidence favours the second alternative (Baker *et al.* 1969). When curve 3 was larger relative to curve 1 than in Fig. 10, its effect could be seen before ouabain, since curve 1 was then S-shaped, and, in one instance, had a maximum at 100 mM-Na. In this experiment, which was carried out on a 'poor' axon with little potassium-sensitivity, calcium removal reduced the Na efflux into 100 mM-Na. The tentative conclusion is that the calcium-dependent component, which is clearly seen after ouabain, is present in an unpoisoned axon, but that it cannot be measured satisfactorily in the presence of a large ouabain-sensitive component.

The effect on sodium efflux of adding cations to dextrose sea water. A useful way of sorting out the effects of different cations is to start with an axon in a dextrose sea water containing ouabain, calcium and magnesium and to determine the effects of replacing dextrose by Li, Na, K or choline. Figure 11*A* shows that choline had virtually no activating effect but Li, Na, and K all increased the sodium efflux at low concentrations. The

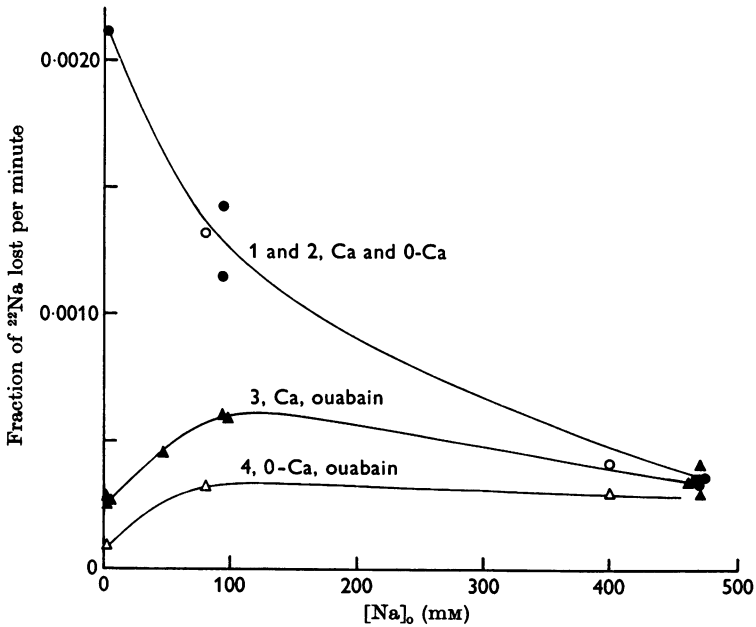

Fig. 10. Effects of Ca-free solutions and 10^{-5} M ouabain on Na efflux into mixtures of dextrose and Na sea water. Abscissa: Na-concentration in mM. Ordinate: fraction of ^{22}Na lost per minute.

Curve 1, ●, with 11 mM-Ca and 55 mM-Mg; curve 2, ○, with 0-Ca 100 mM-Mg; curve 3, ▲, same as 1 but with 10^{-5} M ouabain; curve 4, △, same as 2 but with 10^{-5} M ouabain. Axon diameter, 860 μ.

sodium curve has a maximum at 100 mM but declines to a low value at higher concentrations; high concentrations of lithium also have a slight inhibitory effect. Potassium activates along a simple Michaelis curve with an apparent Michaelis constant of about 50 mM. The corresponding figure for potassium activation of the ouabain-sensitive component is 1 mM or less for solutions containing no sodium or lithium (Baker *et al.* 1969), so the component of sodium efflux which persists after ouabain is evidently much less sensitive to potassium than the ordinary potassium-coupled component of the sodium efflux.

The effect of caesium on the ouabain-insensitive fraction was qualitatively similar to that of potassium or lithium.

The family of curves in Fig. 11*A* can be explained by supposing that the cations potassium, sodium and lithium, but not choline, promote an exchange of external calcium for internal sodium. At high concentrations, sodium ions, and to a small extent lithium ions, had an inhibitory effect, perhaps because two or more sodium ions displace one calcium ion from a carrier. Potassium takes no part in this second reaction.

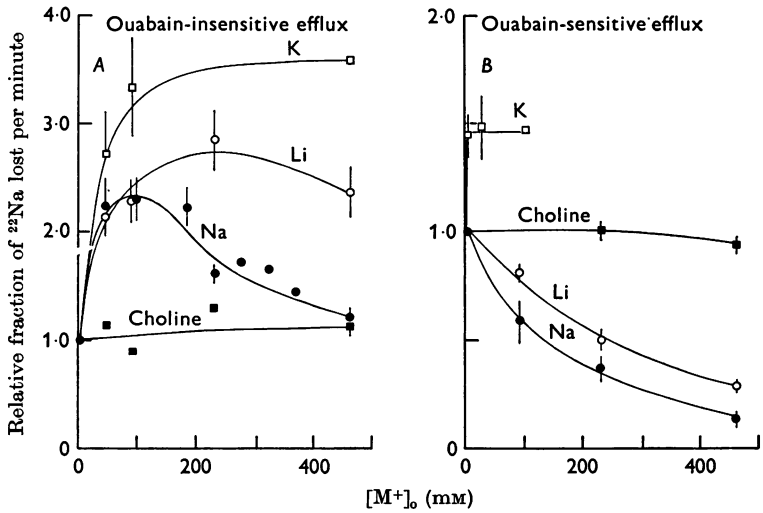

Fig. 11*A*. Effect of cation concentration on sodium efflux in 10^{-5} M ouabain. Abscissa: external monovalent cation concentration in mM. Ordinate: fraction of ^{22}Na lost per minute relative to the fraction lost per minute in dextrose sea water containing 10^{-5} M ouabain. The mean rate constant in dextrose was $0{\cdot}46 \pm 0{\cdot}06$ in $\text{min}^{-1} \times 10^{-3}$. Average values from fifteen experiments; vertical bars are ± 1 S.E. of mean in cases when three or more values were obtained. ●, Na; ○, Li; □, K; ■, choline. Isotonicity maintained with dextrose.

B. Effect of cation concentration on ouabain-sensitive component of sodium efflux, from Baker *et al.* 1969. Abscissa and symbols as in *A*. Ordinate: ouabain-sensitive Na efflux as fraction of that into K-free dextrose sea water. The mean increment in rate constant of the ouabain sensitive component in dextrose was $1{\cdot}77 \pm 0{\cdot}14$ in $\text{min}^{-1} \times 10^{-3}$. On an absolute scale 1 unit in $A \equiv 11{\cdot}5$ p-mole/cm^2 sec and 1 unit in $B \equiv 44{\cdot}3$ p-mole/cm^2 sec.

Figure 11*B*, which is taken from the next paper, gives the corresponding curves for the ouabain-sensitive component of the sodium efflux. A possible explanation of these curves is that sodium and lithium act as competitive inhibitors for potassium and that the high sodium efflux into dextrose or choline sea water results from residual potassium (Baker *et al.* 1969) in the space immediately outside the axolemma.

The curves in Fig. 11*A* are incomplete since they give no information about the calcium sensitivity of the sodium efflux. Figure 12*A* summarizes the results of five experiments in which the effects of adding sodium or

lithium to dextrose sea water were determined in the presence and absence of 11 mM-Ca. The points in Fig. 12*B* give the calcium-dependent component of the sodium efflux as a function of the concentration of sodium or lithium. The curves near the points were calculated from the theory outlined on p. 454.

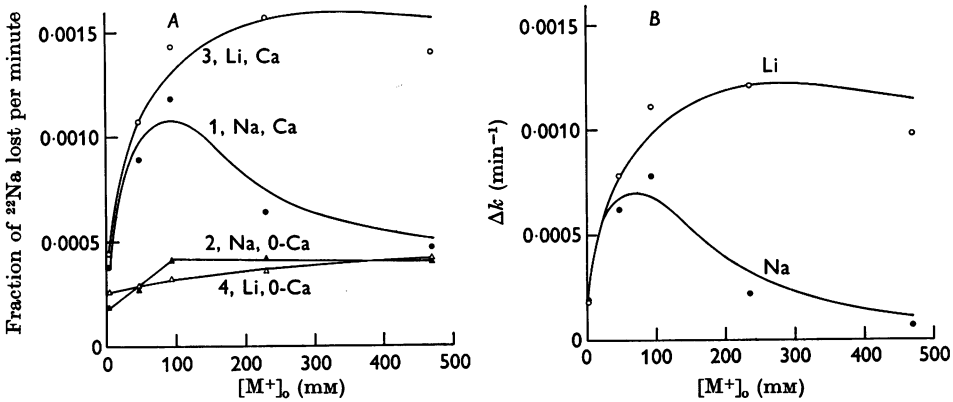

Fig. 12*A*. Effect of cation concentration on Na efflux in 10^{-5} M ouabain in presence and absence of calcium. Mean results from five experiments in which at least two curves were determined. Abscissa: external concentration of Na or Li in mM. Ordinate: fraction of ^{22}Na lost per minute. ● (curve 1), Na + Ca; ▲ (curve 2), Na + 0-Ca; ○ (curve 3), Li + Ca; △ (curve 4), Li + 0-Ca. NaCl or LiCl were replaced with dextrose.

B. Difference curves showing effects of external concentration of Na (●) or Li (○) on the calcium-dependent sodium efflux in 10^{-5} M ouabain. Abscissa, as in *A*. Ordinate: rate constant in 11 mM-Ca minus rate constant in Ca-free sea water; ○, Li; ●, Na. The smooth curves were drawn from eqn. (2), (3), (4) (p. 454) with $K_1^{Li} = K_1^{Na} = 70$ mM, $K_2^{Li} = 100$ M, $K_2^{Na} = 1{\cdot}8$ M, $\alpha = 0{\cdot}136$, $C = 0{\cdot}0014\,\text{min}^{-1}$.

Ca influx

The effect of external sodium. The results described in the first part of this paper made it important to study the effect of changing external and internal sodium concentration on calcium influx. When possible, comparisons were made on pairs of axons from the same squid. This improved the accuracy of a single comparison since the ratio of the two influxes was within 0·3 of unity if both members of the pair were treated in the same way. However, when describing the effect of several variables it is simpler to give average values, as has been done in Table 7.

The calcium influx from Na sea water averaged 0·15 p-mole/cm^2 sec, and varied between 0·04 and 0·6 p-mole/cm^2 sec in thirty axons. These results were obtained on axons dissected from mantles which had been refrigerated for several hours and the axons themselves were sometimes stored at 0° C for an hour or two before measuring the influx. The influx

was usually high if either the mantle or the axon had been stored for some time. Hodgkin & Keynes (1957) obtained an average calcium influx from artificial sea water of 0·076 p-mole/cm² sec with a range from 0·04 to 0·15 p-mole/cm² sec on five axons from living squid. It is not clear whether the discrepancy between the two results is to be attributed to the use of refrigerated mantles or to some other factor such as the size of the axons or a seasonal variation.

TABLE 7. Effect of cations and ouabain on Ca influx

External solution	Ca influx (p-mole/cm² sec) Mean ± S.E.	Range	*n*
A. Axons from refrigerated mantles			
10-K, Na	0·16 ± 0·03	0·06–0·57	20
10-K, Na + 10^{-5} M ouabain	0·13 ± 0·08	0·06–0·25	3
0-K, Na + 10^{-5} M ouabain	0·14 ± 0·06	0·04–0·46	7
10-K, Li	3·74 ± 0·65	0·90–9·50	14
0-K, Li	5·34 ± 0·78	1·70–9·40	10
0-K, Li + 10^{-5} M ouabain	3·98 ± 0·86	1·40–5·90	5
10-K, dextrose	2·91 ± 0·41	2·50–3·40	2
0-K, dextrose	2·53 ± 0·18	0·80–5·30	6
10-K, dextrose + 10^{-5} M ouabain	2·92 ± 0·18	2·60–3·10	3
0-K, dextrose + 10^{-5} M ouabain	1·78 ± 0·26	1·20–2·50	4
Collected results:			
Na sea water, 0 and 10-K, ± ouabain	0·15 ± 0·02	0·04–0·57	30
Li sea water, 0 and 10-K, ± ouabain	4·33 ± 0·43	0·90–9·50	29
Dextrose sea water, 0 and 10-K ± ouabain	2·46 ± 0·29	0·80–5·30	15
B. Axons from live squid			
10-K, Na	0·23	—	1
10-K, Li	0·66 ± 0·48	0·16–1·60	3

n is the number of axons in each group.

As can be seen from Table 7, neither 10^{-5} ouabain nor K-free sea water had any large effect on the influx from Na sea water. On the other hand, replacing all the sodium by lithium or dextrose increased the influx five to fortyfold. The ratio was lowest when the control influx from sodium sea water was small; from the results in Table 10 it seems likely that a rise in internal sodium increases the calcium influx from both Na sea water and Na-free sea water, but that the effect on the latter is greater than that on the former. Ouabain had no obvious effect on the calcium influx from sodium-free sea water. The effect of reducing external sodium in raising calcium influx in squid axons is consistent with observations on other tissues, for example, frog skeletal muscle (Cosmos & Harris, 1961), frog heart (Niedergerke, 1963), mammalian liver (Judah & Ahmed, 1964), smooth muscle (Goodford, 1967), and crab nerve (Baker & Blaustein, 1968).

The average calcium influx from lithium sea water was 75% greater than that from dextrose sea water and the difference is significant if it is

assumed that the values are from a random population. In order to make certain that there was a real difference, the comparison was made on four pairs of axons. The result (Table 8) was that the influx from lithium sea water was $2{\cdot}72 \pm 0{\cdot}57$ times the influx from dextrose sea water. Potassium-free solutions were used in these experiments and the nerves were equilibrated with the sodium-free solutions for 6 min before exposure to ^{45}Ca. In a preliminary communication (Baker *et al.* 1967) we described results which showed little difference between the calcium influx from lithium or dextrose sea water. In that case there was no direct comparison on paired axons; the solutions contained 10 mM-K and an equilibration time of 3 instead of 6 min was employed.

TABLE 8. Average Ca influx ratios for paired axons

External solutions	Ratio, mean and S.E.	*n*
$\frac{\text{Dextrose sea water}}{\text{0·8 Dextrose sea water}}$	$0{\cdot}74 \pm 0{\cdot}11$	4
$\frac{\text{Li sea water}}{\text{0·8 Li sea water}}$	$1{\cdot}59 \pm 0{\cdot}38$	4
$\frac{\text{Li sea water}}{\text{Dextrose sea water}}$	$2{\cdot}72 \pm 0{\cdot}57$	4

0·8 dextrose sea water contained 20 % Na sea water and 80 % dextrose sea water.
0·8 Li sea water contained 20 % Na sea water and 80 % Li sea water. *n* is the number of pairs.

The relation between sodium concentration and calcium influx is given in Fig. 13. The curve obtained with lithium replacing sodium is different from that found with dextrose replacing sodium chloride. If lithium replaces sodium the influx is maximal with zero sodium. If dextrose replaces sodium chloride the maximum is reached at 0·2 times the sea water concentration and decreases at lower concentrations. There is evidently a striking parallel between the effects of lithium and dextrose replacement of sodium on calcium influx with the corresponding effects on the calcium-sensitive component of the sodium efflux (Fig. 14). In order to check the difference between the lithium and dextrose curves the points at the left-hand end were compared on paired axons. The results which are given in Table 8 were similar to those in Fig. 13.

In a subsequent paper (Blaustein & Hodgkin, 1969) it will be shown that replacing sodium by lithium or dextrose causes a fall in calcium efflux. This means that in Na-free sea water there should be a net entry of calcium. A rise in the calcium content of crab nerve immersed in Na-free solutions has been demonstrated by Baker & Blaustein (1968).

The effect of internal sodium concentration on calcium influx. Two methods of changing the internal sodium concentration were used. In the first method (Table 9) pairs of axons were cleaned and were then stimulated

at 100/sec for 20 min in lithium sea water in order to lower the internal sodium concentration. After the stimulation one member of each pair was injected with enough sodium chloride to raise the internal sodium concentration by 67 mM (pair 1) or 120 mM (pair 2). The control axon was treated in a similar way except that an equivalent amount of potassium chloride was injected. The calcium influx from lithium sea water was then measured; as can be seen from Table 9 the calcium influx in the high sodium axons was two or five times greater than in the low sodium axons.

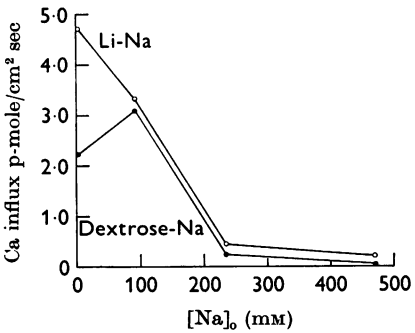

Fig. 13

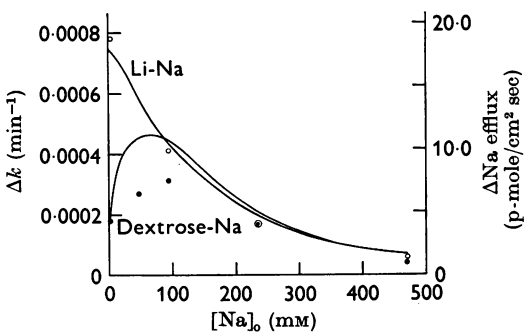

Fig. 14

Fig. 13. Effect of external sodium concentration on calcium influx using lithium, upper curve (○), or dextrose, lower curve (●) as replacement for Na. Average results obtained on fourteen axons from seven squid (Li), or sixteen axons from eight squid (dextrose). Abscissa: sodium concentration in mM. Ordinate: calcium influx. K-free solutions with 10^{-5} M ouabain were used.

Fig. 14. Effect of replacing external Na by Li or dextrose on Ca-dependent Na efflux in the presence of 10^{-5} M ouabain. (○) Average Ca-dependent sodium efflux for Li-Na mixtures (three experiments). (●) Average Ca-dependent sodium efflux for Na-dextrose mixtures (two experiments) in which the efflux in Li sea water was also determined. The mean increment on replacing Na by Li (in the presence of Ca) in these two experiments was the same as in the three experiments from which the Li–Na curve was obtained. Ordinate: left-hand scale, fraction of ^{22}Na lost per minute; right-hand scale, the sodium efflux (p-mole/cm² sec) based on an average diameter for these five axons of 822 μ, and a mean intracellular sodium concentration for all refrigerated mantle axons tested, of 70 mM/kg axoplasm. The smooth curves were drawn from eqns. (2), (3), (5), (6) with the same values of k_1 and k_2 as in Fig. 12; $\alpha = 0{\cdot}24$ and $C = 0{\cdot}0008$ min.$^{-1}$.

In the second type of experiment, the internal sodium concentration was raised by stimulating axons at 100/sec for 40–60 min in sodium sea water. Other axons, usually pairs to these high sodium axons, were stimulated with the same number of shocks in lithium sea water. After the stimulation, calcium influxes were measured in the usual way. In some cases, part of the axon which was not required for measuring calcium influx was analysed for internal sodium. The results in Table 10*A* show that the calcium influx was greater in high than in low sodium axons

irrespective of whether the influx was measured in sodium, lithium or dextrose sea water. However, the effect of increasing internal sodium was relatively greater in lithium or dextrose sea water than in sodium sea water. This would be explained if some of the calcium influx from sodium sea water were insensitive to internal sodium.

TABLE 9. Effect of $[Na]_i$ on Ca influx from Li sea water

1 Axon	2 Initial $[Na]_i$ (mM)	3 Injected [Na] (mM)	4 Final $[Na]_i$ (mM)	5 Ca influx (p-mole/ cm^2 sec)
1	(40)	0	(40)	0·71
1′	(40)	67	(107)	1·58
2	31	0	31	0·84
2′	46	120	166	4·18

Parentheses indicate an assumed value for the initial $[Na]_i$. The figures in column 3 were calculated from the quantity of NaCl injected and the axon diameter.

TABLE 10. Effect of $[Na]_i$ on Ca influx

A. Pre-stimulated axons

$[Na]_o$ (mM)	$[Na]_i$ (mM)	Ca influx (p-mole/cm^2 sec)
460	30	0·20 ± 0·03 (8)
460	130	0·64 ± 0·12 (4)
2·5 (Li)	30	0·61 ± 0·09 (4)
2·5 (Li)	130	5·91 ± 0·46 (5)
2·5 (dextrose)	30	0·52 (1)
2·5 (dextrose)	130	4·56 (1)

B. Pre-soaked axons (paired)

Axon	Pre-soaked 70 min in:	Ca influx from Li sea water (p-mole/cm^2 sec)
1	10-K (Na)	2·93
1′	10-K (Li)	1·77
2	10-K (Na)	3·86
2′	10-K (Li)	1·97

C. Fresh and stored axons (paired)

Axon	Time in 10-K (Na) at 1–2° C	Ca influx from Li sea water (p-mole/cm^2 sec)
1	—	0·16
1′	7 hr	0·99
2	—	0·19
2′	7 hr	1·35

In A the pre-stimulation consisted of 1 hr stimulation at 100/sec in Li or Na sea water. Values for $[Na]_i$ are the average values obtained on three pairs of axons.

In A the figures give the number of axons.

Since soaking in lithium sea water almost certainly increased the internal calcium it might be argued that the differences in calcium influx in Table 10*A* were caused by a rise in internal calcium and had nothing to do with changes in internal sodium. Evidence on this

point was obtained by measuring calcium influx into axons which had been soaked, but not stimulated, in solutions containing either sodium or lithium. The results, which are given in Table 10*B*, show that pre-treatment with sodium or lithium did influence the calcium influx, but that the effects were much smaller than when the axons were stimulated in these solutions. It therefore seems likely that the much larger effects in Table 10*A* are caused by the alteration in internal sodium concentration associated with stimulation.

Two axons from living squid which were tested within 85 min from decapitation gave very low calcium influxes from lithium sea water. Pairs to these axons were stored in sodium sea water at 1° C for 7 hr before measuring the influx in lithium sea water. As can be seen from Table 9*C*, storage increased the influx six to sevenfold. Although there is no direct evidence it is possible that the increase in influx was caused by a rise in internal sodium.

The effects of pH and cyanide. The average calcium influx from dextrose sea water was unaffected by raising the pH from 6·1 where the influx in four axons was $1·9 \pm 0·2$ p-mole/cm^2 sec to pH 8·5 where it was $3·0 \pm 0·6$ p-mole/cm^2 sec (four axons). Tris-buffered solutions were used in these experiments.

Short exposures (70 min) to 2 mM-CN reduced the influx in lithium sea water by about 60 % but longer exposures (140–180 min) caused a slight increase in the influx from either sodium or lithium sea water. Details will be given in a subsequent paper (Blaustein & Hodgkin, 1969).

DISCUSSION

Distinction between Ca-dependent and K-dependent Na efflux

The experiments described here show that in the axons of *Loligo forbesi* there is a component of the sodium efflux which depends on the presence of external calcium and is unaffected by ouabain at concentrations much higher than those needed to inhibit the Na–K pump. The calcium-dependent component was small under normal conditions, but it could be increased by raising internal sodium or external calcium, or by lowering external sodium. It was much more variable than the potassium-coupled efflux and was barely detectable in some axons examined late in the season. Both components of the sodium efflux were inhibited reversibly by cyanide, but in most other respects their properties were quite different (Table 11).

The potassium- and calcium-dependent components do not account for the whole of the sodium efflux since addition of ouabain and replacement of calcium with magnesium removed only 70–90 % of the normal efflux. Little is known about the properties of the residual efflux, except that it is too large to be a passive movement of sodium and that it is reduced when external sodium chloride is replaced by lithium chloride or dextrose.

At first sight there seems to be a clear difference between the behaviour of axons from *Loligo forbesi* and *L. pealii*. With *L. forbesi*, replacement of external sodium with lithium usually increases sodium efflux, but with *L. pealii* the effect is either small (0 mM-K_o), absent (10 mM-K_o)—Sjodin & Beaugé (1967) or reversed (low Na_i)—Frumento & Mullins (1964). Since the magnitude of the lithium effect was variable in *L. forbesi* it would not be surprising

to find a species variation. However, before accepting this conclusion it would be desirable to re-examine the lithium effect in *L. pealii* using axons in which the internal sodium had been raised by stimulation, injection or storage.

TABLE 11. Comparison of the ouabain-insensitive and ouabain-sensitive components of the Na efflux (see Baker *et al.* 1969)

Treatment	Ouabain-insensitive Na efflux	Ouabain-sensitive Na efflux
Ouabain	No effect at 10^{-3} M	Inhibited by 10^{-7} M
External monovalent cations	K: activates with low affinity	K: activates with high affinity
	Na: activates with low affinity up to 100 mM and then inhibits	Na: inhibits
	Li: activates with low affinity	Li: inhibits
	Choline: no effect	Choline: no effect
Ca-free media	Markedly reduced	Slightly increased
Internal Na	Approximately proportional to $[Na]_i^2$	Approximately proportional to $[Na]_i$
Stability of the efflux	Tends to decline markedly with time	Little reduction with time
Lanthanum (0·1 mM)	Inhibits	No effect
Cyanide (2 mM)	Inhibits	Inhibits
Dinitrophenol (0·4 mM) pH 8·0	No effect	Increases the extent of Na-Na exchange in 0-K (Na) ASW

Relation between Ca-dependent Na efflux and Ca influx

There were striking parallels between the effects of sodium concentration on the calcium influx and on the calcium-dependent part of the sodium efflux. In both cases, the flux increased markedly when internal sodium was raised, or external sodium was reduced. When lithium replaced external sodium, the calcium influx and the calcium-dependent part of the sodium efflux increased steadily as sodium was reduced. When dextrose replaced NaCl, both curves had a maximum at about 100 mM-Na and declined at lower concentrations. None of these effects were inhibited by ouabain. Since both fluxes were variable and could not be measured on the same axon, it was difficult to determine the coupling ratio between the two movements. From the upper curves in Figs. 13 and 14 (in which Li replaced Na) it appears that entry of one calcium ion is linked to the exit of 3–5 sodium ions. For the lower curves (dextrose replacing NaCl) the ratio Na_{out}/Ca_{in} was between 2 and 4.

A difficulty with this type of hypothesis is the difference in the effect of cyanide on the two variables. The sodium efflux into lithium sea water was reduced to about 10 % after 1 hr in cyanide and remained at a low level, but the calcium influx from lithium sea water was reduced to about 40 % after 70 min and increased slightly after 2–3 hr in cyanide. Possible explanations are that cyanide makes the axon leaky to calcium, promotes exchange diffusion or destroys the normal coupling between sodium efflux and calcium influx. An alternative arises from later experiments, which

indicate that, in a cyanide poisoned axon, ionized calcium may increase ten to twentyfold (Blaustein & Hodgkin, 1969). Since Na^+ and Ca^{2+} compete on the outside of the membrane it is reasonable to suppose that the same thing happens on the inside and that the rise in internal calcium concentration might block outward sodium movement. This could be tested by injecting ethyleneglycol bis (aminoethylether)-N,N'-tetra-acetic acid (EGTA) into a cyanide-poisoned axon.

Possible basis of interaction between Ca and Na

The complicated effects of external sodium on calcium entry and on the calcium-dependent sodium efflux fit at least qualitatively with a model similar to that discussed by Niedergerke (1963) and Lüttgau (1963). Suppose that a carrier molecule R has two sites which will be called X and Y. Calcium and sodium compete at site Y but not at X which combines with monovalent cations only. Magnesium is assumed to take no part in either reaction and Y is occupied either by Ca, Na or some other monovalent cation, i.e. the amount of free Y is negligible. With Na and Ca in the external medium the chemical reactions to be considered are

$$Na + X = NaX, \tag{i}$$

$$2Na + CaY = Na_2Y + Ca. \tag{ii}$$

Since low concentrations of Na have an activating effect in the absence of other cations, it is assumed that NaRCa penetrates more easily than RCa. For such a system one would expect

$$\text{Ca influx} = [A(1\text{-}F_1) + BF_1]\, F_2F_3, \tag{1}$$

where A and B are constants proportional to the permeability coefficients for RCa and NaRCa; F_1 is the fraction of 'external' X combined with Na, F_2 is the fraction of 'external' Y combined with Ca, and F_3 is the fraction of R available to external ions. F_3 will arbitrarily be taken as constant without specifying how this is achieved; there might for example be a fixed number of external sites for R. With this assumption eqn. (1) becomes

$$\text{Ca influx} = G\,(\alpha + F_1)\,F_2, \tag{2}$$

where G is a scaling constant and $\alpha = A/(B-A)$.

Application of the law of mass action to reactions (i) and (ii) gives

$$F_1 = \frac{[Na]_o}{K_1^{Na} + [Na]_o}, \tag{3}$$

$$F_2 = \frac{[Ca]_o\, K_2^{Na}}{[Na]_o^2 + [Ca]_o\, K_2^{Na}} \tag{4}$$

when K_1^{Na} and K_2^{Na} are the equilibrium constants of reactions (i) and (ii); $[Na]_o$ and $[Ca]_o$ are external concentrations of these ions.

Similar equations apply to the other monovalent cations, but the affinity constants vary with the cation. K_1^{Li} and K_1^{Na} were about the same but the inhibitory constant (K_2^{Li}) for lithium, which had only a slight tendency to displace calcium, was much greater than K_2^{Na}. For potassium and caesium, which seemed to have no inhibitory effect, $K_2 = \infty$; for choline, which did not activate or displace Ca, both affinity constants were taken as infinite. The permeability coefficients A (RCa) and B(CRCa) were supposed to be the same for all cations (C) with $B \doteqdot 7A$. The theory could be applied either to the calcium influx or to the calcium-dependent part of the sodium efflux if this is assumed to be proportional to calcium influx.

When considering mixtures of Na, Li and Ca in which $[Na]_o + [Li]_o =$ 460 mM, activation by monovalent cations is constant and nearly complete. On the assumption that Li and Na combine without interaction, which means that the Langmuir principle (Forbes & Roughton, 1931) can be applied, it follows that the effects of Ca, Li and Na should be described by

$$F_2 = \frac{[Ca]_o}{[Ca]_o + K_m^{Ca}}, \tag{5}$$

where

$$K_m^{Ca} = \left\{ \frac{[Li]_o}{\sqrt{K_2^{Li}}} + \frac{[Na]_o}{\sqrt{K_2^{Na}}} \right\}^2. \tag{6}$$

The theory was applied to the calcium-dependent component of the sodium efflux, since there was more information about this quantity than about calcium influx. The affinity constants in the activating reaction were taken as $K_1^{Na} = K_1^{Li} = 70$ mM, and in the inhibitory reaction as $K_2^{Na} = 1{\cdot}8$ M, $K_2^{Li} = 100$ M. The cases considered were (1) the effect of adding Na or Li to dextrose sea water, Figs. 12 and 13, (2) the effect of replacing Na by Li, Fig. 13, (3) the effect of varying $[Ca]_o$ at different $[Na]_o$, Fig. 5. The experimental results were not sufficiently accurate for a rigorous test, and agreement was sometimes qualitative rather than quantitative. However, it is satisfactory that activation by calcium should follow a Michaelis curve, in agreement with eqn. (5), and that K_m^{Ca} should have values that agree roughly with eqn. (6).

The theory has defects which in some ways are more interesting than its merits. In the first place, the calcium influx increased more steeply when external sodium was reduced than one would predict for a competition between 1 Ca and 2 Na. Assuming that 3 or 4 Na displace 1 Ca helps here, but seems less plausible physically and gives incorrect predictions for K_m^{Ca}. The second defect is that the model does not explain why the calcium influx should increase tenfold when the internal sodium concentration is

raised from 30 to 130 mM. Formally what is required is that F_3 should increase with $[Na]_i$ but it is difficult to construct a model which gives this result and also explains the action of external sodium. A long molecule which accepts Ca plus a cation on one end and 3 Na on the other is the type of carrier required, but the idea is too speculative to be worth pursuing.

Implications of Na–Ca exchange

The observation that raising the internal sodium concentration increases calcium influx may help to explain the cardiotonic action of cardiac glycosides. Ouabain seems to have no direct effect on calcium movements, but it inhibits the Na–K pump in therapeutic doses and might increase the internal sodium concentration (Wilbrandt, 1966; Glynn, 1964). If heart muscle behaves like squid nerve, a rise in internal sodium should promote calcium influx and increase the internal calcium concentration. Since muscle is probably activated by a release of calcium from the sarcoplasmic vesicles, or by entry of calcium from outside, an increase in the background level of calcium might improve the effectiveness of the action potential in turning on the contractile mechanism (see also Glynn, 1968; and Birks & Cohen, 1968*b*).

A similar argument may apply to the actions of cardiac glycosides on a number of calcium-dependent secretory systems including the release of catecholamines from the adrenal medulla (Banks, 1967), the frequency of miniature end-plate potentials at the neuromuscular junction (Birks & Cohen, 1968*a*, *b*), and the release of insulin from the pancreas (Hales & Milner, 1968*a*, *b*). In these preparations application of ouabain in the presence of external sodium and calcium increases both the basal and stimulus-dependent secretions. The effects of ouabain seem to result from inhibition of the sodium pump because they are not seen in the absence of external sodium and can be mimicked by potassium-free solutions. Since a number of physiological stimuli including a rise in extracellular glucose and amino acids (e.g. Lyon & Crane, 1967; Eddy, 1968) may also lead to an increase in sodium entry and cell sodium, it seems possible that a sodium–calcium exchange may be important physiologically as well as pharmacologically.

The influence of external and internal sodium on calcium influx provides a possible explanation of an observation made by Brinley (1968) on barnacle muscle. He found that replacing external sodium by lithium gave a contracture and an increased sodium efflux when the internal sodium had been raised by injection, but not otherwise. If barnacle muscle behaves like squid nerve, this result is to be expected since the rate at which internal sodium exchanges for external calcium should be greatly increased under the conditions in which Brinley observed a contracture.

Since the calcium influx seems to be linked to part of the sodium efflux, it is natural to suppose that part of the sodium influx might be coupled with calcium efflux. Reuter & Seitz (1968) have shown that calcium efflux from guinea-pig auricle is reduced by removing external sodium, and similar evidence has been obtained in squid axons (Baker, *et al.* 1967; Blaustein & Hodgkin, 1969). This raises the possibility, already considered by Reuter & Seitz (1968), that calcium may be pumped out of cells by a mechanism in which external sodium exchanges for internal calcium. In such a system sodium ions would be moving downhill and could provide part, or perhaps all, of the energy needed to extrude calcium. ATP break-down need not be involved directly, although it must be involved indirectly, since the sodium pump would have to work slightly faster in order to keep pace with the Na–Ca exchange. If this interpretation is correct the calcium influx and sodium efflux studied in this paper might represent movements in a direction opposite to that occurring under normal conditions.

We wish to thank the Director and Staff of the Marine Biological Association for much assistance. We are also indebted to Mr R. H. Cook and Mr S. Cross for designing and building equipment; to Dr R. D. Keynes for assistance in the initial stages of the investigation and to Dr I. M. Glynn for helpful discussion.

REFERENCES

BAKER, P. F. & BLAUSTEIN, M. P. (1968). Sodium-dependent uptake of calcium by crab nerve. *Biochim. biophys. Acta* **150**, 167–170.

BAKER, P. F., BLAUSTEIN, M. P., HODGKIN, A. L. & STEINHARDT, R. A. (1967). The effect of sodium concentration on calcium movements in giant axons of *Loligo forbesi*. *J. Physiol.* **192**, 43–44*P*.

BAKER, P. F., BLAUSTEIN, M. P., MANIL, JACQUELINE & STEINHARDT, R. A. (1967). A ouabain-insensitive, calcium-sensitive sodium efflux from giant axons of *Loligo*. *J. Physiol.* **191**, 100–102*P*.

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

BANKS, P. (1967). The effect of ouabain on the secretion of catecholamines and on the intracellular concentration of potassium. *J. Physiol.* **193**, 631–637.

BIRKS, R. I. & COHEN, M. W. (1968*a*). The action of sodium pump inhibitors on neuromuscular transmission. *Proc. R. Soc.* B **170**, 381–399.

BIRKS, R. I. & COHEN, M. W. (1968*b*). The influence of internal sodium on the behaviour of motor nerve endings. *Proc. R. Soc.* B **170**, 401–421.

BLAUSTEIN, M. P. & GOLDMAN, D. E. (1968). The action of certain polyvalent cations on the voltage-clamped lobster axon. *J. gen. Physiol.* **51**, 279–291.

BLAUSTEIN, M. P. & HODGKIN, A. L. (1969). The effect of cyanide on the efflux of calcium from squid axons. *J. Physiol.* **200**, 497–527.

BRINLEY, F. J. (1968). Sodium and potassium fluxes in isolated barnacle muscle fibres. *J. gen. Physiol.* **51**, 445–477.

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

CALDWELL, P. C. (1960). The phosphorus metabolism of squid axons and its relationship to the active transport of sodium. *J. Physiol.* **152**, 545–560.

CALDWELL, P. C., HODGKIN, A. L., KEYNES, R. D. & SHAW, T. I. (1960*a*). The effects of injecting 'energy-rich' phosphate compounds on the active transport of ions in the giant axons of *Loligo*. *J. Physiol.* **152**, 561–590.

CALDWELL, P. C., HODGKIN, A. L., KEYNES, R. D. & SHAW, T. I. (1960*b*). Partial inhibition of the active transport of cations in the giant axons of *Loligo*. *J. Physiol.* **152**, 591–600.

CALDWELL, P. C. & KEYNES, R. D. (1959). The effect of ouabain on the efflux of sodium from a squid giant axon. *J. Physiol.* **148**, 8–9*P*.

COSMOS, E. & HARRIS, E. J. (1961). *In vitro* studies of the gain and exchange of calcium in frog skeletal muscle. *J. gen. Physiol.* **44**, 1121–1130.

EDDY, A. A. (1968). A net gain of sodium ions and a net loss of potassium ions accompanying the uptake of glycine by mouse ascites-tumour cells in the presence of sodium cyanide. *Biochem. J.* **108**, 195–206.

FORBES, W. H. & ROUGHTON, F. J. W. (1931). The equilibrium between oxygen and haemoglobin. 1. The oxygen dissociation curve of dilute blood solutions. *J. Physiol.* **71**, 229–260.

FRUMENTO, A. S. & MULLINS, L. J. (1964). Potassium-free effect in squid axons. *Nature, Lond.* **204**, 1312–1313.

GLYNN, I. M. (1964). The action of cardiac glycosides on ion movements. *Pharmac. Rev.* **16**, 381–407.

GLYNN, I. M. (1969). The effects of cardiac glycosides on metabolism and ion movements. *Digitalis*. (University of Indiana Symposium, 1966), ed. FISCH, C. & SURAWICZ, B. New York: Grune & Stratton. (In the Press.)

GOODFORD, P. J. (1967). The calcium content of the smooth muscle of the guinea-pig *taenia coli*. *J. Physiol.* **192**, 145–157.

HALES, C. N. & MILNER, R. D. G. (1968*a*). The role of sodium and potassium in insulin secretion from rabbit pancreas. *J. Physiol.* **194**, 725–743.

HALES, C. N. & MILNER, R. D. G. (1968*b*). Cations and the secretion of insulin from rabbit pancreas *in vitro*. *J. Physiol.* **199**, 177–187.

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

HODGKIN, A. L. & KEYNES, R. D. (1957). Movements of labelled calcium in squid giant axons. *J. Physiol.* **138**, 253–281.

JUDAH, J. D. & AHMED, K. (1964). The biochemistry of sodium transport. *Biol. Rev.* **39**, 160–193.

LÜTTGAU, H. C. (1963). The role of calcium ions in excitation-contraction coupling, from *Electrophysiology of the Heart*, ed. TACCARDI, B. & MARCHETTI, G., pp. 87–95. Oxford: Pergamon Press.

LYON, I. & CRANE, R. K. (1967). Studies on *in vitro* transmural potentials in relation to intestinal absorption. III. K^+ inhibition of Na^+-dependent transmural potential of rat small intestine. *Biochim. biophys. Acta* **135**, 61–73.

NIEDERGERKE, R. (1963). Movements of Ca in frog heart ventricles at rest and during contractures. *J. Physiol.* **167**, 515–550.

REUTER, H. & SEITZ, N. (1968). The dependence of calcium efflux from cardiac muscle on temperature and external ion composition. *J. Physiol.* **195**, 451–470.

SJODIN, R. A. & BEAUGÉ, L. A. (1967). The ion selectivity and concentration dependence of cation coupled sodium transport in squid giant axons. *Currents in Modern Biology* **1**, 105–115.

TAKATA, M., PICKARD, W. F., LETTVIN, J. Y. & MOORE, J. W. (1966). Ionic conductance changes in lobster axon membrane when lanthanum is substituted for calcium. *J. gen. Physiol.* **50**, 461–471.

WILBRANDT, W. (1966). The mechanism of action of cardiac glycosides. In *The Myocardial Cell: Structure, Function and Modification by Cardiac Drugs*, ed. BRILLER, S. A. & CONN, H. L., Jr, pp. 297–308. Philadelphia: Univ. of Pennsylvania Press.