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THE PARTITION OF SODIUM FLUXES IN ISOLATED TOAD OOCYTES

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SUMMARY

1. The rate constant for Na efflux from the oocyte calculated from (d/dt) $(\ln [Na^*]_i])$ is only approximately 52% of that calculated from $(d/dt)[(\ln(d[Na^*]_i)dt)]$. The difference may be interpreted by supposing that 48% of the internal Na of the oocyte is either bound to proteins or sequestered in cell organelles.

2. The mean rate constant for Na efflux was $6{\cdot}4 \times 10^{-3}$ min^{-1} corresponding to an apparent Na efflux rate of 13·3 p-mole/cm^2.sec. When this is corrected for the increase in surface area produced by microvilli the true efflux rate is 1·1–1·3 p-mole/cm^2.sec.

3. The action of ouabain (1–5 μM) appears to involve two different effects: (*a*) there is 48–65% inhibition of the membrane Na pump, and (*b*) there is a release of some of the sequestered Na in the cell.

4. Removal of external K causes a 40% reduction in Na efflux although this value may be an underestimation owing to the presence of K which has leaked from the cell and may be retained near the cell surface.

5. Raising the external K concentration to 15 mM reduces the inhibitory effect of ouabain by approximately a half.

6. It was concluded that the Na pump in the toad oocyte may have a slightly lower level of activity than that in frog muscle, but that its general properties are similar to those in frog muscle and some other animal cells.

INTRODUCTION

The amphibian oocyte has many unusual features. It is large, up to 2 mm in diameter, and has an exceptionally large nucleus or germinal vesicle; this has recently been used in studies of the permeability of and electrical potential difference across the nuclear membrane, and in experiments on the effect of nuclear transfer between cells. The surface of the oocyte shows a remarkable specialization in the development of microvilli which may increase the surface area by about 10–12 times; this may be a

compensatory mechanism for the otherwise unfavourable surface/volume ratio of such a large cell.

Single oocytes of *Bufo bufo* have been employed in detailed kinetic studies of Na fluxes because of the convenience of their large size and regular geometry (Dick & Lea, 1964). The results were best interpreted by assuming that Na was carried on a single site carrier and that the apparent interaction between Na influx and efflux was not real but probably due to the presence of a specifically 'bound' fraction of the internal Na which interchanged more readily with other Na ions than with Li ions. Since these conclusions conflict with some current interpretations of Na fluxes in amphibian muscle (Keynes & Swan, 1959; Keynes, 1965; Mullins & Frumento, 1963; Harris, 1965), some other aspects of Na fluxes in oocytes were explored so that comparison with muscle could be drawn in the hope of identifying specific differences or similarities between oocytes and muscle fibres.

In the present study the effect on Na efflux of the cardiac glycoside ouabain was studied and also the effect of variation in the external K concentration. Different interpretations have recently been given to the interaction between the effects of ouabain and external K. Charnock & Post (1963) found that ouabain blocked the K-dependent phosphorolysis of an Na-ATPase complex. On the other hand Horowicz & Gerber (1965) attributed the stimulation of Na transport by external K to a depolarization of the cell membrane by K so that the inhibition of the Na pump by ouabain could not be attributed to a competition with K ions. The influence of external K concentration on the change in Na flux produced by ouabain was therefore studied in the oocyte. The effect of ouabain on the oocyte resting membrane potential was also measured.

These studies revealed further evidence for the presence of a sequestered fraction of the internal Na. The existence of this fraction was found to complicate greatly the study of ouabain and K effects since these could not be assumed to act purely on the cell membrane. The possibility of a change in the amount of sequestered Na had to be considered and it appeared that this actually occurred in some circumstances.

METHODS

Preparation of oocytes. Single oocytes of the toad *Bufo bufo* were isolated and prepared as previously described (Dick & Lea, 1964).

Preparation of solutions. The solutions used are shown in Table 1. Radioactive solutions containing ^{24}Na were prepared in the same way as previously (Dick & Lea, 1964). Radioactive solutions containing ^{22}Na were prepared by drying down 0·2 mc $^{22}NaCl$ in a solid watch-glass and then adding 1 ml. of a mixture containing the additional NaCl and other constituents needed to make up Ringer solution. The pH of all non-radioactive solutions (other than trypsin solution) was adjusted to pH $7·4 \pm 0·1$ with 100 mM-NaOH or 100 mM-

HCl. Ouabain (Sigma Chemical Co. or B.D.H.) was sometimes first dissolved in alcohol before adding to the appropriate saline solution so that a final alcohol concentration of 0·08 % (v/v) was present in the experimental solutions. A similar amount of alcohol was added to solutions for control measurements. Other ouabain solutions were made by dissolving directly in Ringer solution.

Measurement of Na efflux. The manipulation of the oocyte and the technique of radioactive assay were as previously described. The initial total Na content of the oocyte was estimated approximately from the initial content of radio-sodium as previously described, with a probable error of ± 25 % (Dick & Lea, 1964, p. 71).

Two different techniques were used to measure Na efflux.

(1) The total Na* content of the oocyte was measured by mounting the whole living cell at intervals in a micro-well scintillation phosphor sandwich (Dick & Lea, 1964, p. 58).

(2) The Na* escaping from the oocyte in a given time interval was collected and assayed. The assay of ^{22}Na and sometimes also ^{24}Na was performed by keeping the cell for a measured time in 0·5 ml. of Ringer solution in a solid watch-glass and then transferring the cell, pipetting the 0·5 ml. of fluid on to a planchet, rinsing with 0·5 ml. of solution and adding this to the planchet, drying this sample and counting under a Geiger counter. Loss of radioactive solution during transfer to the planchet was less than 0·1 %. In other experiments the oocyte was kept in 100 μl. of Ringer solution in a scintillation phosphor chamber and the activity which escaped into this in a given time interval was directly assayed in a scintillation counter after mixing the solution and removing the oocyte in 1–2 μl. of solution with a braking pipette (Holter, 1943). Sometimes both of these techniques, (1) and (2), were employed simultaneously.

TABLE 1. All concentrations in mM except for glucose

	NaCl	KCl	$CaCl_2$	$MgCl_2$	Na_2HPO_4	NaH_2PO_4	Glucose (g/l.)
Ringer solution	107	2·5	2	1	2·4	0·4	1
K-free Ringer solution	109	—	2	1	2·4	0·4	1
High K Ringer solution	94·5	15	2	1	2·4	0·4	1

Before the effects of inhibitors or of change of K concentration were measured, each oocyte used was exposed to non-radioactive Ringer solution (control solution) for at least 2 hr to allow the attainment of a steady state in which the exponential law was obeyed and a steady average rate constant could be estimated. On transfer into the K-free solution or out of an inhibitor solution two rinses were employed to ensure complete removal of the K ion or the inhibitor from the oocyte surface. Since the oocyte was transferred in approximately 10 μl. and each receiving solution was approximately 3 ml., the theoretical dilution factor was 1:90,000.

Both $^{24}NaCl$ and $^{22}NaCl$ were used as tracers (Codes SGS–1P and SKS–1, respectively, Radiochemical Centre, Amersham). Only measurements of efflux were done with $^{22}NaCl$ and the final ^{22}Na content of the oocyte was assayed by drying the cell on a planchet and counting under a Geiger counter.

Measurement of total Na concentration in oocyte. Oocytes, whose diameter had been measured with an eyepiece micrometer, were briefly washed in distilled water, transferred to weighed sample tubes containing distilled water and allowed to undergo osmotic lysis for 24 hr. The Na concentration in the sample was measured with an EEL Flame Photometer. Details of this technique and evidence that no significant loss of Na occurred during washing have been given by Dick & Lea (1964).

Potential measurements. For each measurement two glass micro-electrodes were used each filled by capillarity with 3 M-KCl. Tip diameters were 2 μ and only those electrodes were used which possessed tip potentials of < 2 mV and resistance < 50 MΩ. The oocytes were held in the cell holder by suction on the end of a glass capillary of tip diameter approxi-

mately 50 μ. One electrode was inserted into the oocyte and the other used as a reference electrode. Each electrode was connected to a calomel half-cell by a polythene tube filled with 3 M-KCl in agar. The potential difference between the half-cells was measured and recorded with a Keithley electrometer amplifier in conjunction with a Bausch & Lomb pen recorder. The flow of Ringer solution through the cell holder was begun after impalement of the oocyte.

Methods of calculation and theoretical aspects of tracer Na efflux

Two methods were used for the calculation of the results from the efflux data:

(1) ln $[Na^*]_i$ was plotted against time,

(2) ln $d/dt\,[Na^*]_i$ was plotted against time,

where $[Na^*]_i$ at any given time during efflux was obtained by summing the final Na* count and all the Na* effluxes following the stipulated time and dividing the result by the cell volume.

If only a single compartment were involved, the process would be an exponential one and for a given cell both plots would have the same gradient. The respective gradients, (d/dt) ln $[Na^*]_i$ and (d/dt) ln $(d/dt)/[Na^*]_i$ were found to differ, the first giving a value much lower than the second. In general this implies that Na* is not being lost from a single compartment. This is consistent with the findings of Dick & Lea (1964), who concluded that approximately 25 % of the internal Na lies in a state secluded from the remainder with which it exchanges at a definite rate. The previous theory has been modified as it appears that the exchange of sequestered Na with internal free Na is slower than it was previously thought to be.

The relation between the two plots is important because it can be used to estimate the fraction of 'free' intracellular Na and because it is necessary to decide which method of calculation is appropriate for the evaluation of the activity of the membrane pump.

The derivation of a simple approximate relation between the two gradients requires two assumptions:

(1) that the sites of sequestered Na are distributed uniformly throughout the cytoplasm. (The validity of this assumption is considered in the discussion.)

(2) that the exchange of labelled Na between the cytoplasmic free and the sequestered fractions is sufficiently slow to be neglected for the purpose of this calculation.

The efflux of Na* then depends only on the free Na* in the cytoplasm and may be written:

$$\frac{d\,[Na^*]_i}{dt} = -k[Na^*]_2 \qquad (1)$$

$$= -k\,([Na^*]_i - [Na^*]_3), \qquad (2)$$

where k is the rate coefficient for exchange between cytoplasm and external solution, $[Na^*]_i$ represents the total internal sodium, $[Na^*]_2$ represents the free Na and $[Na^*]_3$ represents the sequestered Na. So

$$\frac{d}{dt}\ln\frac{d[Na^*]_i}{dt} = \frac{1}{[Na^*]_i - [Na^*]_3}\frac{d}{dt}([Na^*]_i - [Na^*]_3)$$

$$= \frac{1}{[Na^*]_i - [Na^*]_3}\frac{d}{dt}[Na^*]_i$$

$$= -k \text{ (from equation (2))} \qquad (3)$$

(since $[Na^*]_3$ is assumed to be a constant for the purpose of this estimation).

Consider now the identity

$$\frac{d}{dt}\ln[Na^*]_i = \frac{1}{[Na^*]_i}\frac{d\,[Na^*]_i}{dt}$$

$$= -k\,\frac{[Na^*]_i - [Na^*]_3}{[Na^*]_i} \text{ (from equation (2)).} \qquad (4)$$

Equation (3) shows that the gradient of the plot of ln (d/dt) $[Na^*]_i$ against time gives the 'membrane' rate coefficient k whereas equation (4) shows that the gradient of the plot of

ln $[Na^*]_i$ against time gives a quite different value, not strictly a constant at all but legitimately assumed to be so for the present estimation. Moreover the ratio of the gradients given by equations (3) and (4) gives the following relation:

$$\frac{d/dt \ln [Na^*]_i}{d/dt \ln \frac{d[Na^*]_i}{dt}} = \frac{[Na^*]_i - [Na^*]_3}{[Na^*]_i} \quad (5)$$

$$= \frac{[Na^*]_2}{[Na^*]_i}. \quad (6)$$

This ratio is used to give an estimate of the amount of free Na* in the oocyte. The precision of the estimate will, however, be low, owing to the approximate nature of the physical assumptions and mathematical manipulations underlying the formula.

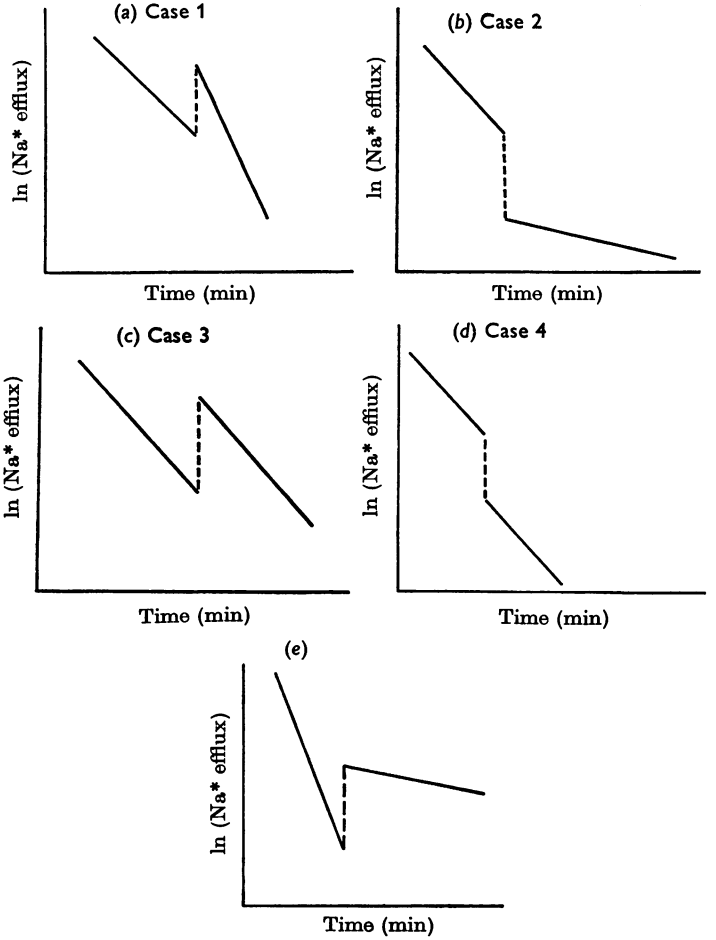

Fig. 1*a*, *b*. The effect on Na* efflux of increasing (*a*) or diminishing (*b*) membrane permeability when $k_{23} \gg$ or $\ll k_{21}$.

Fig. 1*c*, *d*. The effect on Na* efflux of increasing (*c*) or decreasing (*d*) the rate of exchange between the compartments of internal Na.

Fig. 1*e*. The effect on Na* efflux of an increase in free internal Na together with partial saturation of the membrane pump.

In estimating the activity of the Na pump, the gradient (d/dt) ln $(d[Na^*]_i/dt)$ was used, as the more accurate measure of it.

Since the probable existence of a secluded fraction of Na in compartment 3 introduces serious complications into the interpretation of the effects of ouabain and external K on Na efflux, it is important to consider the kinetic results of various possible simple effects of external agents on Na transfer. Four possible cases will be considered:

Cases (1) *and* (2). The effective permeability of the cell membrane to Na is suddenly increased or diminished by an effect on either active or passive Na transfer. It is supposed that no change occurs in the sequestered Na*; i.e. in the notation of Dick & Lea (1964), $k_{23} \gg$ or $\ll k_{21}$ and remains constant. (k_{23} represents the rate coefficient of exchange between external and free internal Na.) The resulting kinetic changes are illustrated in Fig. 1*a* and *b*. In case (1) the rate of Na efflux is suddenly increased and subsequently the rate constant for Na loss (given by the slope of the graph) is increased: in Case (2) the opposite effects occur.

Cases (3) *and* (4). A sudden increase or decrease occurs in the rate of exchange between the compartments of internal Na, i.e. changes from $k_{23} \ll k_{21}$ to $k_{23} \gg k_{21}$ or vice versa. It is assumed that no change in membrane permeability occurs and that the active membrane Na pump remains unsaturated throughout. The kinetic results are illustrated in Fig. 1*c* and *d*. A sudden increase or decrease in the rate of Na efflux occurs but the efflux constant (given by the slope of the graph) remains unchanged.

These are, of course, idealized simple effects and combinations of these may readily occur. For example, if in Case (3) the liberated Na_3, by increasing the total free internal Na, caused partial saturation of the Na pump, a not improbable effect, the result would be a reduction in the over-all rate constant for Na loss and reduction of the slope as if a reduction of membrane permeability had occurred. The result is illustrated in Fig. 1*e*.

RESULTS

Comparison of different methods of calculating rate constants of Na efflux. Rate constants calculated from $(d/dt)/(\ln[Na^*]_i)$ and from $(d/dt)/(\ln(d[Na^*]_i/dt))$ are shown in columns 3 and 4 of Table 2. It is readily seen that the former are much smaller than the latter. The average ratio between the two rate constants is 0·52, implying that approximately 52 % of the internal Na is free and that the remaining 48 % lies sequestered in compartment 3. However, as noted above (see equation (4)), (d/dt) $(\ln [Na^*]_i)$ is not strictly a constant; the above estimate of $[Na^*]_3$ is thus dependent on the rather crude approximation that $[Na^*]_i - [Na^*]_3/[Na^*]_i$ does not alter greatly during the part of the experiment in which (d/dt) $(\ln [Na^*]_i)$ was estimated. This estimate of the percentage of $[Na]_3$ must therefore be regarded as no more than a rough approximation.

Effect of ouabain on Na efflux. The efflux of labelled Na into inactive Ringer solution was first measured at approximately $\frac{1}{2}$ hr intervals and the rate constants estimated before and after treatment with ouabain in concentrations of 1 μM, 3 μM and 5 μM. The results of typical experiments are illustrated in Fig. 2*a* and *b*. An important feature of these results is the absence, in most cases, of a sudden reduction of Na efflux in the form of a step change on treatment with ouabain although there was a reduction in the slope of the efflux curve. (This reduction was significant since con-

trol experiments showed no change of slope between the first 200 min and the remainder of the experiment (see Table 3).) In many cases there was even a slight increase of Na efflux immediately after treatment. The pattern

TABLE 2. Comparison of different methods of calculating the rate constant of Na* efflux

Cell no.	Initial Na concn. (mM)	Rate constant calculated from $\frac{d}{dt}(\ln[Na]_i)$ (min^{-1}) ($\times 10^{-3}$)	Rate constant calculated from $\frac{d}{dt}\left\{\ln \frac{d[Na]_i}{dt}\right\}$ (min^{-1}) ($\times 10^{-3}$)	Ratio
1·1	16	3·2	4·5	0·71
1·2	23	2·1	6·7	0·34
1·3	13	3·3	6·8	0·49
2·1	19	4·8	8·0	0·60
2·2	9	2·5	4·6	0·45
2·3	11	2·8	5·1	0·55
2·4	12	3·1	6·9	0·45
2·5	17	3·5	4·0	0·88
3·1	13	2·6	7·3	0·36
3·2	9	2·2	6·3	0·35
3·3	10	1·9	5·6	0·34
3·4	18	4·7	6·3	0·75
3·5	10	3·1	5·8	0·53
4·1	24	3·4	4·7	0·72
4·2	9	4·0	6·9	0·58
4·5	10	1·3	7·3	0·18
			Mean	0·52

TABLE 3. Rate constants in control experiments

Rate constant before 200 min. (min^{-1}) ($\times 10^{-3}$)	Rate constant after 200 min. (min^{-1}) ($\times 10^{-3}$)	Difference
8·0	7·2	−0·8
6·4	5·2	−1·2
9·4	11·1	+1·7
6·4	9·5	+3·1
7·3	5·4	−1·9
		Mean +0·18 ± 0·95

of response to ouabain thus does not fit any of the simple cases already considered, so that it may be concluded that more than one effect must operate at the same time. It is posssible that the combined processes of release of sequestered Na from compartment 3 and saturation of the membrane pump as discussed in Case (3) (Fig. 1 *c*) might account for the data, but in view of the considerable evidence of inhibition of the membrane Na pump and of membrane Na- and K-dependent ATPase by ouabain it seems more likely that the effect of ouabain is a combination of membrane pump inhibition (Case 2) and release of sequestered Na (Case 3). Since in Case (3) there is no change in slope of the graph (i.e. in membrane rate constant) provided that it is assumed that no saturation

occurs, all changes in membrane slope may be attributed to inhibition of the membrane pump by ouabain. Some of the data of this type from one series of experiments are shown in Table 7. Figure 3 shows the change produced by ouabain in the rate constant for Na efflux (calculated from $(d/dt)/(\ln d[Na^*]_1/dt)$ plotted against the initial rate constant). The regression lines for the 1 μM and 3 μM and 5 μM ouabain concentrations show intercepts ($(1{\cdot}8 \pm 1{\cdot}1) \times 10^{-3}$ min^{-1}, $(0{\cdot}59 \pm 0{\cdot}79) \times 10^{-3}$ min^{-1} and

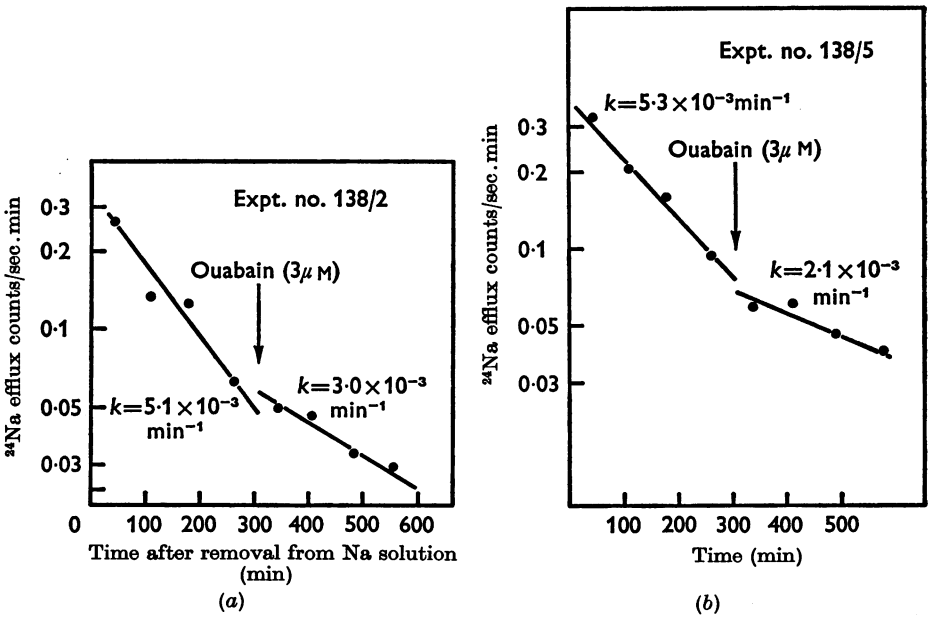

Fig. 2*a*, *b*. The abscissa shows the time in minutes and the ordinate the ^{24}Na efflux rate in counts/sec.min on a logarithmic scale. While there is a considerable reduction of the gradient of the graph on application of ouabain, there is little or no corresponding 'step' reduction in the absolute rate of efflux.

$(-0{\cdot}56 \pm 1{\cdot}7) \times 10^{-3}$ min^{-1}, respectively) on the x-axis, suggesting that no significant fraction of the Na efflux remains unaffected by ouabain. The mean fractional inhibition of the part of the efflux affected by ouabain is given by the slopes of the regression lines, i.e. $0{\cdot}65 \pm 0{\cdot}17$, $0{\cdot}52 \pm 0{\cdot}09$ and $0{\cdot}48 \pm 0{\cdot}17$, for 1 μM, 3 μM, and 5 μM ouabain. Thus there is no clear relation between the degree of inhibition and the concentration of ouabain in the range of concentrations used.

However, the importance of the assumption that no saturation of the membrane pump occurs in these experiments must be borne in mind, since any effect of saturation will be interpreted as membrane inhibition by the above analysis.

Effect of ouabain on total Na content of oocytes. A batch of thirty measured oocytes was divided into six samples, each containing five cells. Two samples were taken initially, washed in distilled water, and prepared for flame photometry. Two samples were placed in Ringer solution containing ouabain (either 5 or 10 μM) and two were maintained in Ringer solution as controls. After 4 hr all four samples were measured,

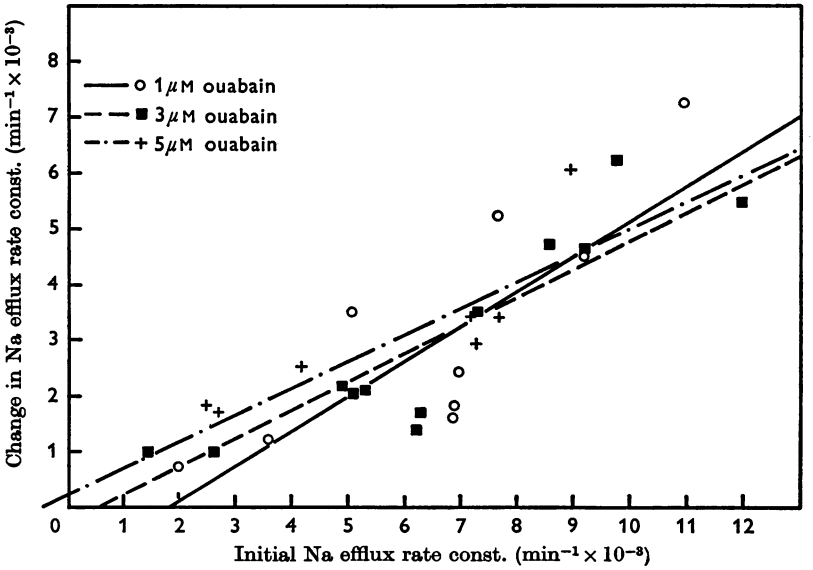

Fig. 3. The change in Na* efflux rate constant (as measured by (d/dt) (ln d [Na*]/dt) produced by 1, 3 or 5 μM ouabain is shown plotted against the initial Na* efflux rate constant. The slope of the graph gives the average fractional inhibition at each concentration.

TABLE 4. Effect of ouabain on total Na concentration of oocytes

Expt. No.	Ouabain concn. (μM)	Initial Na concn. (mM)	Mean (mM)	Na concn. control of control cells (mM)	Mean (% of initial)	Na concn. of ouabain-treated cells (mM)	Mean (% of initial)	Difference (% of initial)
1	10	10·9 12·0	11·4	9·0 9·5	81	8·5 8·6	75	−6
2	5	12·8 18·4	15·6	9·3 14·1	75	9·7 8·9	60	−16
3	5	13·5 17·5	15·5	28·0 18·4	150	13·9 13·4	88	−62
4	10	36·4 23·6	30·1	25·0 24·3	82	19·1 30·0	82	0
					Mean difference (%)			−20·8 ± 14·1 (0·25 > P > 0·10)

washed and prepared for flame photometry. Four such experiments are summarized in Table 4. There is a decrease in the Na content of cells exposed to ouabain as compared with the controls, the average reduction being approximately 21 %. However, the variation of the results is so great that the probability integral associated with the significance of the decrease is between 0·1 and 0·2 so that the result can only be regarded as suggestive.

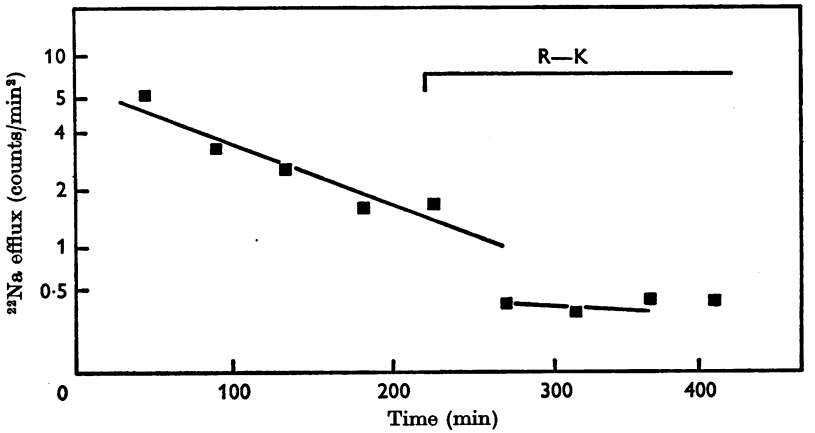

Fig. 4. The ^{22}Na efflux rate in counts/min^2 is shown plotted on a logarithmic scale against time. The application of K-free Ringer solution (R–K) causes a sudden reduction in Na efflux along with a reducton in the slope of the graph.

The effect on Na efflux of the removal of external K. In these experiments the Na efflux was measured directly and total $[Na^*]_i$ estimated at time t by adding to the final $[Na^*]_i$ count all the subsequent efflux counts. The result of a typical experiment is shown in Fig. 4. There are two features which distinguish these experiments from the ouabain ones. First, there is a sharp reduction of Na efflux on removing external K, but not on applying ouabain and, secondly, the gradient of the graph of log. (efflux) against time is almost reduced to zero on removing K, whereas after application of ouabain a significant negative gradient remains. Table 5 shows the change of efflux which took place after treatment. Since a change of efflux did not appear immediately after removal of external K (see Fig. 4), but after an interval of approximately 20 min, the graphs for efflux before and after removal of external K were extrapolated so as to overlap at this time and the step change of efflux measured as if it took place instantaneously 20 min after removal of external K.

In this case the change of efflux produced by K removal was not measured by the change in $(d/dt)/(\ln d[Na^*]_i/dt)$ since the almost zero final

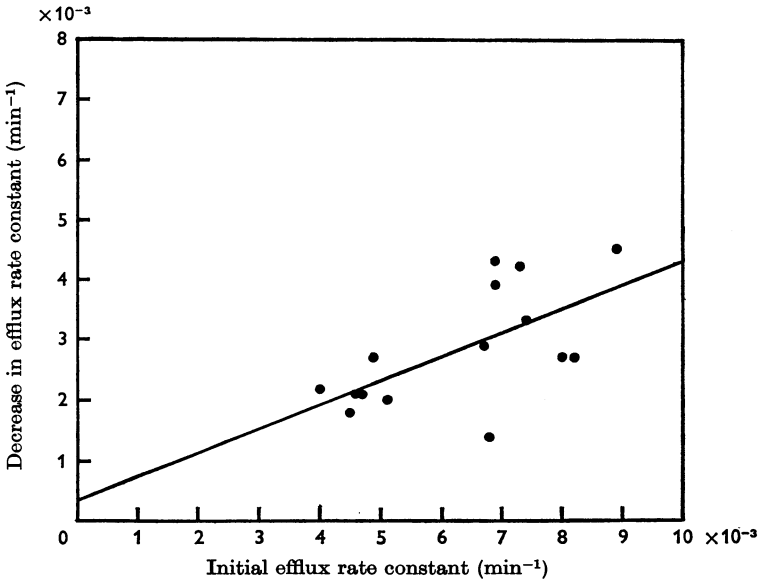

Fig. 5. The decrease in ^{24}Na efflux rate constant caused by withdrawal of ext. K is shown plotted against the initial efflux rate constant. The slope of the graph gives the average fraction of K-linked Na efflux.

TABLE 5. Effect of removal of external K on Na efflux

Cell no.	Initial Na concn. (mM)	Initial Na efflux rate constant (min^{-1}) ($\times 10^{-3}$)	Decrease of Na efflux rate constant* (min^{-1}) ($\times 10^{-3}$)
1·1	16	4·5	1·8
1·2	23	6·7	2·9
1·3	13	6·8	1·4
1·4	11	8·2	2·7
1·5	9	8·9	4·5
2·1	19	8·0	2·7
2·2	9	4·6	2·1
2·3	11	5·1	2·0
2·4	12	6·9	3·9
2·5	17	4·0	2·2
4·1	24	4·7	2·1
4·2	9	6·9	4·3
4·3	14	7·4	3·3
4·4	10	4·9	2·7
4·5	10	7·3	4·2

* Measured from the 'step' decrease of efflux which took place 20 min after removal of ext. K.

value of this gradient indicated the presence of some other factor. The relation between the 'step' change of efflux rate constant (computed as described above) to the initial efflux rate constant is shown in Fig. 5. The slope of the graph is $0{\cdot}40 \pm 0{\cdot}14$ but this may well be less than the

fraction of the active efflux linked to external K (see Discussion, p. 305). The intercept on the x-axis is $(-0{\cdot}9 \pm 1{\cdot}5) \times 10^{-3}$ min^{-1}, not significantly different from zero, so that there appears to be no fraction of Na efflux unaffected by external K.

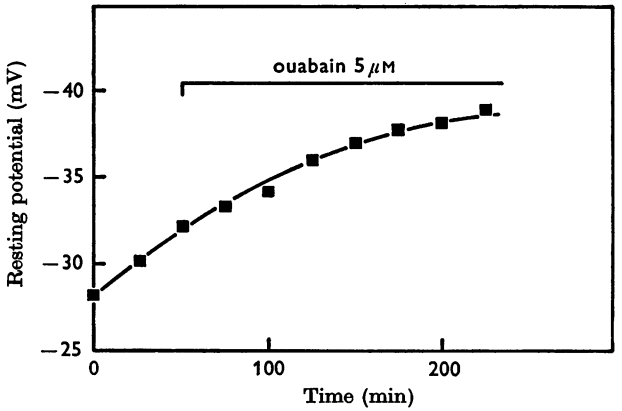

Fig. 6. The resting potential is shown plotted against time. A steady rise occurs, probably due to diffusion of KCl from the electrode tip. Application of ouabain (5 μM) has no marked effect on the resting potential as compared with a control.

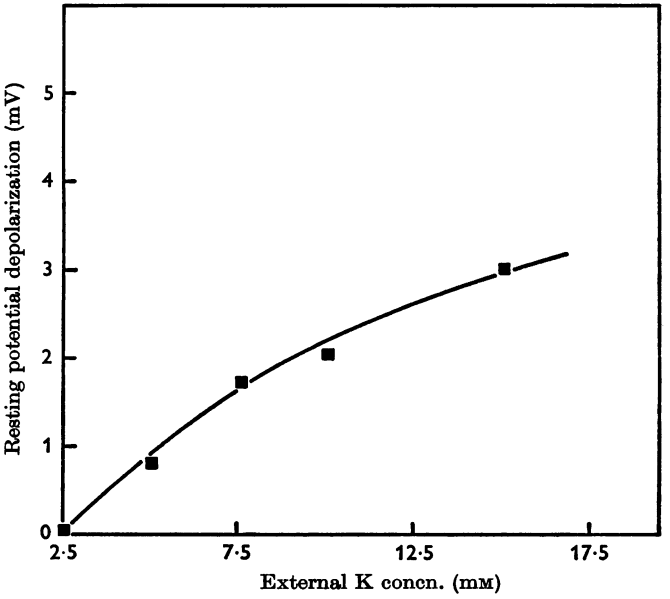

Fig. 7. The depolarizarion (mV) produced by increase of external K is shown plotted against the external K concentration. Depolarization increases with K concentration but much less than predicted by the Nernst equation.

The effect of ouabain and the variation of external K on the resting potential. Approximately 30 min after inserting a micro-electrode into an oocyte, Ringer solution containing 5 μM ouabain was substituted for the normal Ringer solution which was flowing continuously past the oocyte. Figure 6 shows a trace (re-drawn with a smaller time scale) of the resting potential. No change in resting potential which could be attributed to the effect of ouabain was observed during the subsequent 2 hr, although the oocyte slowly hyperpolarized during the experiment, possibly owing to diffusion of KCl into the cell from the micro-electrode tip (see Kerkut & Thomas, 1965).

The effect of the variations of $[K]_0$ on resting potential is shown in Fig. 7. The solutions were applied in descending order of K concentration, alternating with ordinary Ringer solution, followed by re-application of the solution containing 15 mM-K. On each return to ordinary Ringer solution, the membrane potential returned to the base line, which, as already mentioned, rose continuously, probably owing to K leakage from the micro-electrode tip.

TABLE 6. Effect of ouabain and removal of external K on Na efflux

Cell no.	Estimated initial cell Na (mM)	Initial Na rate const. (min^{-1}) ($\times 10^{-3}$)	Final Na efflux rate const. (min^{-1}) ($\times 10^{-3}$)	Change in Na efflux rate const. (min^{-1}) ($\times 10^{-3}$)
104·1	25·3	5·1	0·7	−4·5
104·2	33·0	3·6	0·9	−2·7
104·3	43·0	6·1	1·1	−5·0

The effect on Na efflux of simultaneous application of ouabain and removal of external potassium. The next step was to remove external K from oocytes already inhibited by ouabain and measure the initial Na efflux rate constant, the change in rate constant produced by application of ouabain and the further change in rate constant produced by removal of K. Only three results are available; they are shown in Table 6. The relation between the total change in Na efflux rate constant produced by ouabain and removal of external K together, and the initial Na efflux rate constant was examined graphically. The slope of the regression line was 0·95 and the intercept on the x-axis is $0{\cdot}4 \times 10^{-3}$ min^{-1}. The estimate of the unaffected flux is thus intermediate between previous estimates for the K and ouabain effects separately (and probably not significantly different from zero), but the percentage inhibition of the active flux has risen to approximately 95 %.

The effect on the ouabain inhibited Na efflux of increasing the external K concentration. Table 7 shows the effect of increase in $[K]_0$ from 2·5 to 15 mM. ($[Na]_0$ was reduced by 12·5 mM to keep the solutions isotonic); the inhibi-

tion by ouabain was approximately 50 % reversed. It can be seen that the reversal appears, if anything, to increase with the ouabain concentration so that these experiments do not provide evidence of simple competition between K and ouabain; however, the limited range of ouabain concentrations used and the lack of consistent variation of the degree of the original inhibition with ouabain concentration makes it impossible to draw any firm conclusion concerning K-ouabain competition from the present data.

TABLE 7. Effect of raising external K on inhibition by ouabain

Cell no.	Estimated initial Na concn. (mM)	Initial Na efflux rate const. (min^{-1}) ($\times 10^{-3}$)	After ouabain (min^{-1}) ($\times 10^{-3}$)	Change produced by ouabain (min^{-1}) ($\times 10^{-3}$)	After ouabain + 15 mM K (min^{-1}) ($\times 10^{-3}$)	Change produced by 15 mM K (min^{-1}) ($\times 01^{-3}$)
			Inhibition by 1 μM ouabain			
11A1	21	7·0	4·6	−2·4	6·3	+1·7
11A2	19	6·9	5·1	−1·8	5·8	+0·7
11A3	28	6·9	5·3	−1·6	6·3	+1·0
11A4	23	9·2	4·7	−4·5	4·7	0·0
11A5	23	7·7	2·5	−5·2	5·5	+3·0
11A6	27	11·0	3·8	−7·2	4·6	+0·8
				Mean −3·78		Mean +1·20
			Inhibition by 3 μM ouabain			
11B1	28	7·3	3·8	−3·5	4·6	+0·8
11B2	21	6·3	4·6	−1·7	6·9	+2·3
11B3	24	8·6	3·9	−4·7	6·0	+2·1
11B4	23	9·8	3·6	−6·2	4·6	+1·0
11B5	24	9·2	4·6	−4·6	5·8	+1·2
11B6	20	12·0	6·6	−5·4	2·3	−4·3*
				Mean −4·35		Mean +1·48
			Inhibition by 5 μM ouabain			
11C1	27	2·7	1·0	−1·7	5·1	+4·1
11C3	25	7·3	4·4	−2·9	6·6	+2·2
11C5	21	7·7	4·3	−3·4	6·9	+2·6
11C6	21	7·2	3·8	−3·4	6·3	+2·5
				Mean −2·85		Mean +2·85
	Group mean	7·92	Group mean	−3·75	Group mean	+1·73

* Excluded from calculation of mean.

DISCUSSION

The supporting evidence which appeared in the course of the present experiments for Dick & Lea's (1964) hypothesis that there is a sequestered (previously referred to as 'bound') fraction of the internal Na was not anticipated. Nevertheless, some such interpretation proved essential in explaining the complex effects of ouabain which cannot be explained by supposing a simple inhibition of the membrane Na pump. There are two specific difficulties: (1) the absence of a 'step' change of efflux on application of ouabain and (2) the fact that the total cell Na concentration prob-

ably falls after application of ouabain, These are explained by supposing that ouabain causes a release of sequestered Na at the same time as causing inhibition of the Na pump. The fall of total Na concentration may be understood by recollecting that in the tracer Na experiments, Na efflux after ouabain application is regularly higher than that found in control cells or predicted by extrapolation of the antecedent part of the Na efflux graph. The mechanism of the release of sequestered Na produced by ouabain is not known. However, A. W. Rogers, D. A. T. Dick & D. J. Fry (unpublished results) have recently obtained evidence by means of autoradiography of ^{22}Na in frozen sections of oocyte which suggests that the sequestered Na lies in the cytoplasm and not in the nucleus, a finding which narrows the possible sites of sequestration to some degree.

The general hypothesis that there is a sequestered fraction of intracellular Na (i.e. that the activity of Na in the cytoplasm is considerably lower than that expected from flame photometer measurements on the whole cell, assuming an activity coefficient similar to that of Na in free solution, 0·078 at 0·1 M) has been strengthened by some recent findings in nerve and muscle with a Na-specific electrode. Thus Hinke (1961) found in the squid giant axon a Na activity coefficient of 0·457. Similarly, Lev (1964) found a Na activity coefficient of 0·20 in skeletal muscle; Sorokina (1964) obtained a value of 0·56; McLaughlin & Hinke (1966) found a value of 0·16 in single muscle fibres of the giant barnacle. Cope (1965) used the measurement of nuclear magnetic resonance to estimate the Na activity coefficient in muscle and obtained a value of 0·28. Partial sequestration of internal Na does therefore appear to occur in nerve and muscle as well as in the oocyte.

Bearing in mind the assumption underlying the above interpretation of the effect of ouabain, i.e. that no saturation of the Na pump occurs, effects apparently attributable to a 50–60 % inhibition of the membrane Na pump may be compared with ouabain effects in other cells. Inhibition of the Na pump by ouabain has been demonstrated in many different tissues (see Glynn, 1964). Although the degree of inhibition varies with the ouabain and external K concentrations, at ouabain concentrations around 10^{-6} M and at physiological K concentration, the degree of inhibition of the Na efflux found was 50–80 % in frog muscle (Edwards & Harris, 1957; Horowicz & Gerber, 1965), 42 % in mouse ascites tumour cells (Maizels, Remington & Truscoe, 1958) and 80 % in squid giant axons (Caldwell & Keynes, 1959). These figures compare well with the present estimate of 50–60 % inhibition of the active Na pump, although inhibition in squid axon is somewhat greater than in the oocyte.

With regard to the small and statistically non-significant values of the non-affected Na efflux, it must be noted that this is consistent with that

calculated from simple diffusion since, assuming that Na influx is wholly passive and that total Na influx is equal to total Na efflux, then from Ussing's equation (1949) (where z is valency and E, F, R and T have their usual meanings):

$$\frac{\text{passive Na efflux}}{\text{total Na efflux}} = \frac{\text{passive Na efflux}}{\text{passive Na influx}} = \frac{[\text{Na}]_i}{[\text{Na}]_o} \exp\left(\frac{zEF}{RT}\right) \tag{7}$$

$$= \frac{21}{111} \exp\left(-\frac{30\text{ mV}}{25\text{ mV}}\right) = \frac{21}{111} \times 0{\cdot}30 = 0{\cdot}06.$$

Since a passive Na efflux of only 6 % of the total efflux would not be detectable in the present experiments, the agreement may be considered satisfactory. (If free internal Na is only 0·5 $[\text{Na}]_i$, the fraction of passive efflux will be half of this estimate.)

Any change in intracellular Na activity might be expected to be accompanied by a change in resting potential. In the case of the oocyte, however, where no change has so far been observed after treatment with ouabain, it can be shown that the expected change in resting potential would be very small indeed. For example, consider the effect of a change in the intracellular Na activity coefficient from 0·2 to 0·8 on the resting potential given by the constant field equations (Goldman, 1943; Patlak, 1960):

$$E \approx \frac{-RT}{F} \ln\left(\frac{P_K[K]_i + P_{Na}\,[Na]_i\,\gamma_{Na} + P_{Cl}\,[Cl]_o}{P_K[K]_o + P_{Na}\,[Na]_o + P_{Cl}\,[Cl]_i}\right) \tag{8}$$

$$= \frac{-RT}{F} \ln\left(\frac{k_K[K]_i + k_{Na}\,[Na]_i\,\gamma_{Na} + k_{Cl}[Cl]_o}{k_K\,[K]_o + k_{Na}\,[Na]_o + k_{Cl}\,[Cl]_i}\right) \tag{9}$$

where the P's are permeability coefficients, k_{Na} is the passive tracer efflux rate constant, γ_{Na} is the intracellular Na activity coefficient, k_K is the K tracer efflux rate constant and k_{Cl} is the Cl influx rate constant calculated from the efflux rate constant k'_{Cl} using the relation

$$k_{Cl} = k'_{Cl} \frac{[Cl]_i}{[Cl]_o}.$$

By substituting, in equation (10), the values $k_{Na} = 0{\cdot}0003\text{ min}^{-1}$, $k_K = 0{\cdot}004\text{ min}^{-1}$, $k_{Cl} = 0{\cdot}003\text{ min}^{-1}$ (E. J. A. Lea, 1967; in preparation); $[\text{Na}]_i = 21$ mM, $[\text{Cl}]_i = 70$ mM, $[\text{K}]_i = 120$ mM (Dick & Lea, 1964); and $[\text{Na}]_o = 112$ mM, $[\text{K}]_o = 2{\cdot}5$ mM, $[\text{Cl}]_o = 116$ mM (see Table 1); equation (9) becomes:

$$E = -\frac{RT}{F} \ln\left(\frac{0{\cdot}48 + 0{\cdot}01\,\gamma_{Na} + 0{\cdot}35}{0{\cdot}01 + 0{\cdot}03 + 0{\cdot}21}\right) = -29\text{ mV} \tag{10}$$

It can be seen from equation (10) how little the intracellular Na influences the resting potential and that a change in γ_{Na} from 0·2 to 0·8 would be expected to cause a change in resting potential far too small to be detected by conventional techniques.

The fraction of Na efflux apparently linked to K influx has been found to be $0{\cdot}40 \pm 0{\cdot}14$. This may be compared with 0·31 in the erythrocyte (Glynn, 1956), 0·70 in squid giant axons (Hodgkin & Keynes, 1955) and 0·46 in frog muscle (Edwards & Harris, 1957). While it must be remembered that all of these values may be underestimations of the K-linked Na efflux (see below), the value in the oocyte seems to be similar to those in muscle and the erythrocyte, but lower than that in the squid axon.

Are the ouabain-inhibited and K-linked fractions of the Na efflux additive? In view of the findings that ouabain inhibits 48–65 % of the Na efflux and approximately 40 % is linked to K influx, while the combined effect of ouabain and K removal is 95 % inhibition, the question arises whether these are independent and additive effects. If the theory that ouabain competes with K for a transport activating site on the outside of the cell membrane is rejected (and it will be remembered that no conclusive support for it was found in the present experiments described on p. 301), then there is no difficulty in presuming that their actions are independent. Such a view would be in accordance with the recent findings of Horowicz & Gerber (1965) that high external K concentrations cause a stimulation of ^{24}Na efflux which can be inhibited by ouabain, suggesting that the high K concentration does not interfere with ouabain action. Horowicz & Gerber proposed that the influence of external K on Na efflux was due to its effect on the resting potential and not to any direct action on the Na pump. This proposal is similar to the critical energy barrier theory of Na pump action put forward by Conway, Kernan and their colleagues (Conway, Kernan & Zadunaisky, 1961; Dee & Kernan, 1963). Against this view, however, is the evidence for competition between external K and ouabain or other glycosides which has been presented by several authors (Glynn, 1957; Page, Goerke & Storm, 1964; Post, Merritt, Kinsolving & Albright, 1960). Charnock & Post (1963) and Charnock, Rosenthal & Post (1963) suggested that ouabain interferes with the K-activated dephosphorylation of phosphorylated carrier complex containing Na. In explaining the present data, it might be supposed that the K-free effect was not fully developed since it must be practically impossible to reduce external K to zero owing to leakage from the cell interior. If any space exists for retaining escaping K near the cell surface so that it can be taken up again, the K-free effect will be gravely underestimated. The intervals between the surface microvilli might well serve as such a K-retaining space. Addition of ouabain would then inhibit the still-functioning part of the normal Na–K exchange

pump, and on the competition theory it would be expected that ouabain would be more effective than usual with a reduced external K concentration. Since it seems to be feasible to explain the present data on either view, it must be concluded that it is not yet possible to decide between them.

The suggestion that the fraction of K-linked Na efflux may have been underestimated gains some support from the estimation of Adrian & Slayman (1966) that in a Na-loaded muscle exposed to Rb solutions, over 90 % of the Rb influx (which is almost equal to Na efflux) must be chemically linked to Na efflux. Cross, Keynes & Rybová (1965) have concluded that while a non-linked Na efflux can play a part, in certain muscles K influx must be largely linked to Na efflux.

Comparison of the Na pump of the oocyte with those of muscle and other cells. The mean rate constant for efflux of free Na, averaged over all thirty-nine experiments shown in Tables 2, 5, 6 and 7, was found to be $6{\cdot}4 \times 10^{-3}$ min^{-1} (see Table 7). This value obtained from (d/dt) $[\ln(d[Na^*]_i/dt)]$ compares well with the value of $7{\cdot}3 \times 10^{-3}$ min^{-1} for k_{21} previously found by Dick & Lea (1964) from measurements of $[1/([Na^*]_i - [Na^*]_B)]$ $(d[Na^*]_i/dt)$, where $[Na^*]_B$ is the concentration of 'bound' Na*. When combined with the corresponding values of the free Na, calculated assuming 48 % sequestered Na, the rate constants give an average Na efflux of 63 μmole/min which is equivalent to 13·1 p-mole/cm^2.sec, assuming a mean volume/surface ratio of 125 μ^3/μ^2, for a cell diameter of 750 μ. This estimate is lower than that of 22·8 p-mole/cm^2.sec previously obtained by Dick & Lea (1964) (possibly because the cells used in the present study had on average a lower Na concentration). It must be remembered that this flux rate is calculated as if the surface membrane were smooth. In fact the surface area is greatly increased by microvilli; Bradbury, Dick & Dick (1967) have estimated from electron micrographs that the area is increased approximately 10–12 times in a 750 μ cell. (The previously quoted figure of 35 times given by Kemp (1956) was based on the assumption 'that half of this (surface) area is occupied by the bases of the microvilli'. Kemp gives no grounds for this assumption and careful re-examination of his electron micrographs suggests it is probably an over-estimation). The corrected rate of Na efflux from the oocytes in the present experiments thus works out at approximately 1·1–1·3 p-mole/cm^2.sec, and 1·9–2·3 p-mole/cm^2.sec in the previous experiments of Dick & Lea (1964). These results may be compared with the figure of 3·5 p-mole/cm^2.sec obtained by Hodgkin & Horowicz (1959) for the Na efflux rate of single frog muscle fibres. They suggest that the Na pump in the oocyte membrane may have a somewhat lower level of activity than that in muscle although this probably depends on the degree of Na loading.

Since the percentages of ouabain-inhibited and K-linked Na efflux compare well with previous data, it may be concluded that the properties of the Na pump in the oocyte are generally similar to those found in amphibian muscle and some other animal cells. However, both the ouabain-inhibited and K-linked Na effluxes are rather less in the oocyte than in squid axon.

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