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THE UNCOUPLED EXTRUSION OF Na^+ THROUGH THE Na^+ PUMP

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SUMMARY

An ouabain-sensitive or cyanide-sensitive Na^+ efflux into media nominally free of Na^+ and K^+ has been reported in a variety of animal cells. One possible explanation is that, under these peculiar circumstances, the Na^+ pump exchanges intracellular Na^+ for K^+ leaking out of the cells, the affinity for K^+ being highest in the absence of external Na^+.

In this paper we explored a number of alternative possibilities concerning the nature of this pump flux in human red cells.

The results showed that:

1. The ouabain-sensitive Na^+ efflux into Na^+ and K^+-free media, is unaffected by the absence of internal K^+ ($[K^+]_i < 1$ mM) in hypotonically resealed ghosts.

2. Intracellular ADP concentrations, low enough to inhibit the ouabain-sensitive Na^+:Na^+ exchange, have no effect on the Na^+ efflux in the absence of Na^+ and K^+ provided there is enough ATP to sustain a normal Na^+:K^+ exchange.

3. Starvation, which reduces the Na^+:K^+ exchange proportionally more than the Na^+:Na^+ exchange, in intact cells, also inhibits the ouabain-sensitive loss into media lacking both, Na^+ and K^+.

4. In the absence of external Na^+, K^+ activates the ouabain-sensitive Na^+ efflux (probably saturated with internal Na^+) from low K^+, hypotonically resealed ghosts, along a saturation-like curve with a K_m of about 100 μM.

5. Low concentrations of Na^+ in the medium inhibit the Na^+ loss through the pump in the absence and also in the presence of saturating concentrations of K^+ (10 mM). The effect of Na^+ is rather inconsistent and the reason for this variable behaviour remains unknown.

It is suggested that this Na^+ efflux through the pump is uncoupled from the inward translocation of a cation and that it may be associated with a Na^+-inhibitable spontaneous dephosphorylation of the Na^+ pump.

INTRODUCTION

Under physiological conditions, the Na^+ pump found in most animal cell membranes, uses energy from the hydrolysis of ATP to translocate intracellular Na^+ in

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exchange for external K^+. In abnormal circumstances, *i.e.* in the absence of K^+ from the medium, external Na^+ activates an exchange of Na^+ through the pump which is associated with a transphosphorylation reaction between ATP and ADP. In this paper we are concerned with a possible third mode of Na^+ extrusion through the Na^+ pump.

When choline, Tris, Mg^{2+} or dextrose isoosmotically replace the usual Na^+ and K^+ salts in the external medium, a measurable Na^+ efflux which is sensitive to CN^- or ouabain is observed in Sepia axons[1], crab nerve[2], squid giant axons[3], red cells[4] and barnacle muscle (Lew, V. L., Atwater, I. and Rojas, E., unpublished results). However, it is not yet clear whether this represents a genuine pump flux different from the known Na^+:Na^+ and Na^+:K^+ exchange. The reason for doubting, its peculiar identity is that at very low concentrations of Na^+ in the medium, the affinity of the pump for external K^+ increases sharply and, in nominally K^+-free media, traces of K^+ coming out of the cells, or a K^+ space bathing the outside surface of the membrane[3] seem to be enough to allow some measure of normal Na^+:K^+ exchange. This view is further strengthened by the fact that similarly low external Na^+ concentrations inhibit the Na^+ efflux into the nominally K^+-free solutions[4].

The only system in which the measurement of ouabain-sensitive reuptake of K^+ was attempted, and with negative results, is the human red cell[4]. However, the experimental error involved in those measurements would have made it impossible to detect a K^+ reuptake through the pump if the stoichiometry of the Na^+:K^+ exchange under these peculiar circumstances had been nearer to 3:1 rather than 1:1 or 3:2. Stronger, although more indirect evidence in support of an uncoupled Na^+ efflux through the pump comes from experiments by Baker[2,5] on crab nerve where a large ouabain-sensitive loss of Na^+ into isotonic dextrose is accompanied by the loss of glutamic and aspartic acids which are the principal anions in crab nerves. Since no such anion loss was observed during the normal Na^+:K^+ exchange it seems safe to conclude that the anions had to follow the unbalanced loss of Na^+ through the pump.

Na^+ could conceivably be exchanging for other cations but evidence obtained in sepia axons, red cells and crab nerve suggest that there is no measurable exchange of Na^+ for either Mg^{2+}, Ca^{2+} or H^+, in different Na^+ and K^+-free media[1,2,4].

It therefore seems that, at least in some cells, there is a distinctly different mode of operation of the Na^+ pump whereby Na^+ extrusion is uncoupled from the inward translocation of an external cation.

In this paper we explore different possibilities concerning the nature of this ouabain-sensitive Na^+ efflux from human red blood cells.

A preliminary account of this work has been published before[6].

METHODS

Na^+ and K^+ fluxes in intact red cells

Preparation of the cells and measurement of the ionic fluxes was as described previously[7]. Particular details of each experiment will be found in the legends to figures and the table.

Na^+ efflux from hypotonically resealed ghosts

The experimental procedure followed, in general, that reported by Simons[8]. Red cells from fresh heparinized blood were washed four times with a solution containing: choline chloride, 150 mM; Tris–HCl (pH 7.7 at 37 °C), 10 mM. After the last wash, the cells were packed ($10000 \times g$, 5 min) and lysed in about 100 vol. of lysing medium containing: $MgCl_2$, 3 mM; Na^+–ATP, 2 mM; Tris–EGTA, 0.05 mM and 1 mM of Tris–HCl (pH 7.7 at 37 °C). In the experiment of Fig. 5 (circles) the concentration of $MgCl_2$ was 5 mM and that of Na^+–ATP, 3 mM. When present in the lysing medium, the concentration of phosphate (as a sodium phosphate buffer, pH 7.4) was 1 mM, that of creatine phosphate (Sigma Chemical Co., St. Louis, U.S.A.) 5 mM, and that of creatine phosphokinase (Sigma Chemical Co., St. Louis, U.S.A.) 25 mg/100 ml. The ATP concentration in the lysing medium which contained creatine phosphate and creatine phosphokinase was 0.8 mM. After 5 min up to 2.5 ml of isotonic $^{24}NaCl$ (Amersham, approximately 1 mCi/ml at the time of use) was added to the lysed cells with magnetic stirring. The reason for not including the isotope in the lysing medium was to avoid labelling of spontaneously resealed ghosts. After 10 min, adequate volumes of 1 M solutions of choline chloride or KCl to give a final total osmolarity of 140 ideal mosmoles, were added, with magnetic stirring. All this procedure was carried out at 0 °C and with intermittent magnetic stirring. When necessary, appropriate amounts of 1 M NaCl were included in the 10 min addition to give a final concentration of Na^+ in the lysing medium of 10 ± 0.5 mM. Resealing was obtained by incubating the cells for 40 min at 37 °C. The resealed ghosts were subsequently washed 6 to 8 times with an ice-cold solution containing: choline chloride, 70 mM and Tris–HCl, (pH 7.7 at 37 °C) 5 mM (basic medium). After washing, the cells were resuspended at an haematocrit of about 0.5–1% in a similar medium which had, in addition, 2 mM $MgCl_2$ and 0.1 mM Tris–EGTA. When present, NaCl and KCl replaced equivalent amounts of choline chloride. In the experiments reported in Fig. 2 and in Fig. 5 (circles), the cells were warmed for 5 min at 37 °C between the second and third, and between the fourth and fifth wash after resealing in order to decrease the initial temperature-dependent lysis during the first 5 min of the final incubation. This lysis was responsible for the release of relatively large amounts of K^+ into the incubation medium.

Aliquots of the cell suspensions were distributed in duplicates or triplicates into 10-ml polycarbonate tubes containing basic medium with or without ouabain. The final ouabain concentration was $7 \cdot 10^{-5}$ M. The tubes were incubated for 0 and 60 min at 37 °C with intermittent stirring by hand. After the incubation the tubes were allowed to cool for 5 min in an ice-bath and centrifuged ($17000 \times g$, 5 min) at 2 °C. Aliquots of the supernatant were assayed for radioactivity. The total activity present initially inside the cells was estimated from a separate sample. The efflux of Na^+ is expressed as the difference between the percent of $^{24}Na^+$ which has come out of the cells in 1 h, in the absence and in the presence of ouabain. The values are reported as the average $\pm$ the standard error of the mean. Duplicate determinations are reported as the mean. The individual values were always within 5% of each other.

Throughout this paper, the assumption is made that the ouabain-sensitive Na^+ and K^+ fluxes correspond to fluxes of these ions only through the Na^+ pump.

Correction of the fraction of Na^+ lost into nominally Na^+ and K^+-free solutions, for the traces of K^+ present at the end of the incubation

The maximum $Na^+:K^+$ exchange was estimated from the fractional Na^+ loss measured in the experiments of Fig. 5 assuming an average K^+ concentration in the final incubation medium equal to the one measured at the end. Allowance was made for differences in V among different experiments. This correction is independent of any assumptions on the stoichiometry of the $Na^+:K^+$ exchange.

Calculation of the saturated Na^+ efflux through the pump with and without change in the intracellular specific activity of tracer Na^+

In all the experiments with hypotonically resealed ghosts the final Na^+ concentration in the lysing medium, and presumably also inside the resealed ghosts, was 10 mM. Since the internal K^+ concentration was low, it is just possible that the pump was saturated with internal Na^+. The presence of Na^+ in the external medium, by diluting the specific activity of intracellular Na^+, will affect the ouabain-sensitive tracer efflux producing an apparent decrease in the saturated pumping rate of Na^+. It is particularly relevant to allow for this effect in the experiment of Fig. 5 (circles) in order to assess the genuine magnitude of the inhibition of the ouabain-sensitive Na^+ efflux produced by external Na^+. The ouabain-sensitive Na^+ efflux in the experiments of Fig. 5 was therefore calculated in the following way.

Let C be the saturated Na^+ efflux through the pump (in mmole/l cell$\cdot h^{-1}$); k, the rate constant of the ouabain resistant Na^+ flux (in h^{-1}); Na_i^+ and Na_0^+, the internal and external Na^+ concentrations (in mmole/l cell); Na^x, the intracellular concentration of tracer $^{24}Na^+$ (in cpm/l cell); superscripts 0 and t are time (0 means $t=0$). It will be assumed that the volume of cell water is equal to the volume of the cells and that it remained constant during the period the flux was measured. The change in Na_i^+ with time will be:

$$\frac{dNa_i^+}{dt} = k(Na_0^+ - Na_i^+) - C$$

and integrating we get:

$$Na_i^t = \left(Na_0^+ - \frac{C}{k}\right)(1 - e^{-kt}) + Na_i^0 \cdot e^{-kt} \tag{1}$$

For the tracer flux we have:

$$\frac{dNa^x}{dt} = -kNa^x - C \cdot S \tag{2}$$

where $S = Na^x/Na_i^+$, represents the internal specific activity of Na^+ (in cpm/mmole). At very low haematocrit, as in the experiments of Fig. 5, Na_0^+ will remain constant. The backflux of tracer can be neglected.

In the absence of external Na^+, S remains constant:

$$S = \frac{Na^{x0}}{Na_i^0}$$

and integrating Eqn 2 we get:

$$C = \frac{k \cdot Na_i^0}{1 - e^{-kt}} \left(e^{-kt} - \frac{Na^{xt}}{Na^{x0}} \right) \tag{3}$$

The value of k is obtained from the tracer flux in the presence of ouabain. When $C = 0$,

$$\frac{dNa^x}{dt} = -kNa^x$$

and integrating we get:

$$k = -\frac{1}{t} \ln \left(\frac{Na^{xt}}{Na^{x0}} \right)_{(+ouabain)} \tag{4}$$

In the presence of external Na, S is no longer constant:

$$S = \frac{Na^{xt}}{Na_i^t}$$

and Eqn 2 now integrates to:

$$C = \frac{k \cdot Na_0}{\left(kt + \ln \frac{Na_i^t}{Na_i^0} \right) \Big/ \left(1 - kt + \ln \frac{Na^{xt}}{Na^{x0}} \right)} \tag{5}$$

where k is obtained from the ratio Na^{xt}/Na^{x0} measured in the presence of ouabain, as before (Eqn 4).

In order to calculate C in the presence of Na^+ in the medium, Eqns 1 and 5 have to be solved simultaneously for Na_i^t and C. This was done by trial and error. The values of C are reported in Fig. 5. $Na_i^{t=1\,h}$ was 7.38 mM at $[K^+]_0 = 10$ mM and 8.75 mM at $[K^+]_0 = 0$.

With this method, the value of the saturated Na^+ efflux through the pump, C, is corrected for the dilution of the specific activity of intracellular Na^+.

The values of the experiment of Fig. 5 (squares) were "normalized" to coincide with those of Fig. 5 (circles). The original value of C at $[K^+]_0 = 10$ mM was 2.46 mmole/l cell $\cdot h^{-1}$.

RESULTS

Is internal K^+ required for the efflux of Na^+ into Na^+ and K^+-free media?

Under physiological conditions in human red cells, two different cation exchanges take place through the Na^+ pump, namely an exchange of internal Na^+ for external K^+ (Na^+:K^+) and an exchange of internal K^+ for external K^+ (K^+:K^+)[7,9]. In non-sequential models of the Na^+ pump it is conceivable that the outward translocation of Na^+ and K^+ could take place simultaneously on separate units at each pump site. In the absence of external Na^+ and K^+, the release of Na^+

from the pump into the medium could possibly be associated with the inward translocation of unreleased K^+, internal K^+ shuttling forwards and backwards through the "K^+ unit" of the pump.

The hypothesis that the ouabain-sensitive Na^+ efflux may depend on the functioning of the mechanism responsible for the normal $K^+ : K^+$ exchange was tested in two different ways: (a) Inosine, which is known to inhibit (7) the ouabain-sensitive K^+ efflux through the pump by lowering the internal P_i levels, might be expected to decrease the ouabain-sensitive Na^+ efflux in media lacking Na^+ and K^+, if the previous view is correct; (b) Hypotonically resealed ghosts containing less than 1 mM K could be considered a good approximation to K^+-free cells. Simons[8] recently showed that at low internal Na^+, the internal K_m for K^+ is about 10 mM in a similar

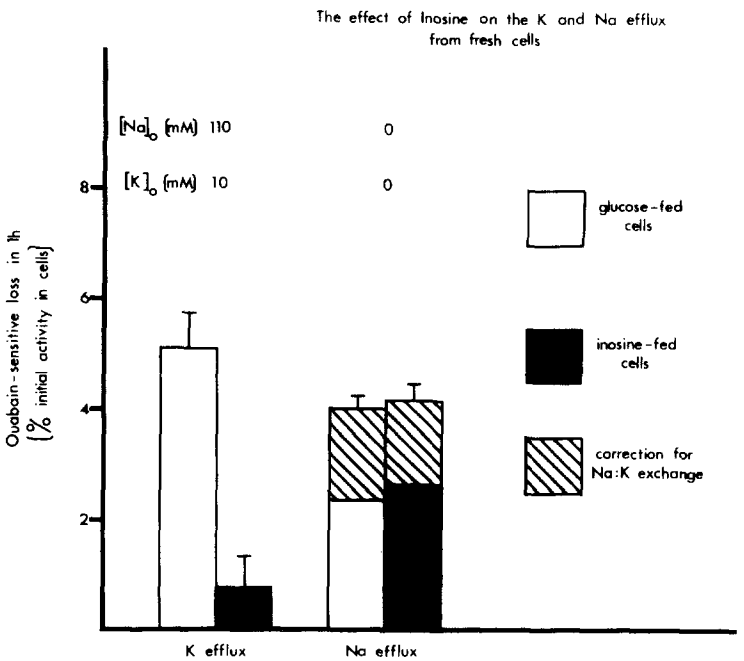

Fig. 1. The effect of inosine on the ouabain-sensitive K^+ and Na^+ efflux from intact red cells. Fresh cells were washed 3 times with about 4 vol. of a solution containing NaCl, 150 mM and Tris–HCl (pH 7.6 at 37 °C), 10 mM. One third of the cells were then resuspended in the same solution and ^{42}KCl and glucose were added. $^{24}NaCl$, KCl and glucose was added to the remaining cells. The final concentrations were: KCl, 5 mM; glucose, 6 mM. The haematocrit was about 20%. The cells were incubated 6 h at 37 °C, washed 6 times with an ice-cold solution containing 150 mM choline chloride and 10 mM Tris–HCl (pH 7.6 at 37 °C) and distributed into tubes containing this solution or isotonic NaCl and KCl to give the final concentrations of Na^+ and K^+, indicated in the figure. Inosine and glucose were present in the final incubation medium at a concentration of 6 mM. When present the concentration of ouabain was $7 \cdot 10^{-5}$ M. The tubes were incubated for 1 h at 37 °C. K^+ efflux was measured in 4-duplicates and Na^+ efflux in triplicate samples. The final haematocrit was about 0.8%. The fractional loss of ^{24}Na in 1 h in a medium containing 110 mM Na^+ and 10 mM K^+ was 26.1% in the glucose fed cells and 27.7% in the presence of inosine.

type of ghosts. If the ouabain-sensitive Na^+ efflux into Na^+ and K^+-free media requires internal K^+ or represents any sort of $Na^+:K^+$ exchange it should be absent in a quasi K^+-free system like this.

The effect of inosine on the ouabain-sensitive Na^+ and K^+ efflux from fresh cells, can be seen in Fig. 1. While inosine greatly reduced the ouabain-sensitive K^+ loss into a K^+-containing medium, the ouabain-sensitive Na^+ loss into the Na^+ and K^+-free medium was the same as in the glucose-fed cells.

Fig. 2 shows the ouabain-sensitive fraction of Na^+ lost from hypotonically resealed ghosts containing about 10 mM Na^+ and about 10 or less than 1 mM K^+. In the absence of external Na^+ and K^+, a fractional Na^+ loss equivalent to about 10% of the maximal $Na^+:K^+$ exchange was observed in the low K^+ ghosts. A similar value is obtained with the 10 mM K^+ ghosts if allowance is made for the $Na^+:K^+$ component as evaluated from the K^+-activation curve of Fig. 5. This correction is independent of any assumptions on the stoichiometry of the $Na^+:K^+$ exchange. The small, but significant increase in the $Na^+:K^+$ exchange induced by internal K^+ was observed in two out of three otherwise identical experiments.

The K^+ concentration in the nominally Na^+ and K^+-free medium at the end of the final incubation period was 0.86 μM in the low K^+ ghosts of the experiment reported in Fig. 2 and 0.76 and 0.93 μM in the experiments reported in Fig. 5. In these three experiments, the maximum estimated $Na^+:K^+$ exchange would account for less than 5% of the measured Na^+ loss. In four more experiments in which the ouabain-sensitive Na^+ loss from hypotonically resealed ghosts into Na^+ and K^+-free

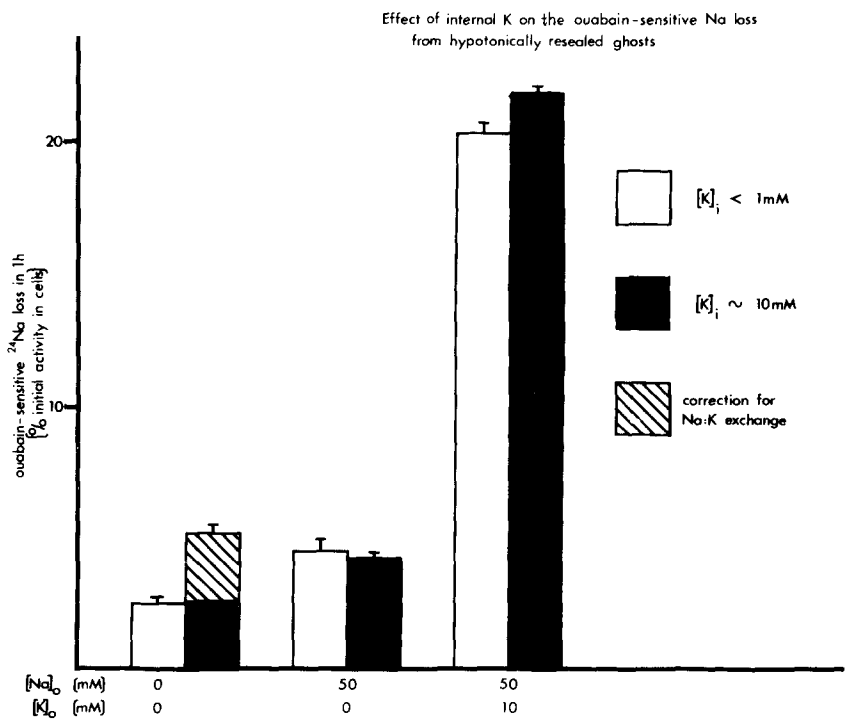

Fig. 2. Effect of internal K^+ on the ouabain-sensitive Na^+ loss from hypotonically resealed ghosts.

TABLE I

COLLECTED MEASUREMENTS OF THE OUABAIN-SENSITIVE ^{24}Na LOSS FROM INTACT CELLS AND HYPOTONICALLY RESEALED GHOSTS INTO Na^+- AND K^+-FREE MEDIA: EFFECT OF LOW CONCENTRATIONS OF Na^+ AND K^+

The correction for $Na^+:K^+$ exchange due to traces of K^+ present at the end of the final incubation was performed as described in Methods.

Type of cell	*Nominal medium concentration (mM)*		*Final concentration in incubation medium (μM)*		*Ouabain-sensitive ^{24}Na loss (% initial activity in cells)*		*Observations*	
	Na^+	*K^+*	*Na^+*	*K^+*	*Measured*	*Corrected for $Na^+:K^+$ exchange*		
Hypotonically resealed ghosts	0	0	17	0.86	2.49	—	$[K^+]_i < 1$ mM	Expt of Fig. 2
	0	0	—	12.2	5.20	2.62	$[K^+]_i \approx 10$ mM	
	0	0	18	0.75	2.29	—		
	5	0	—	1.1	0.44	—	Expt of Fig. 5 (circles)	
	0	10	—	—	19.6	—		
	5	10	—	—	13.8	—		
	0	0	18	0.93	2.56	—	Expt of Fig. 5 (squares)	
	0	10	—	—	21.1	—		
	0	0	—	4.0	3.12	2.60		
	5	0	—	6.6	1.83	—		
	0	0	17	6.2	2.67	1.62		
	5	0	—	6.6	0.38			
	0	10	—	—	18.7			
	5	10	—	—	14.0			
	0	0	—	6.5	2.90	1.80		
	5	0	—	5.4	0.29	—	$1 \text{ mM} < [K^+]_i < 2$ mM	

	0	0	19	3.3	2.13	1.63		
	5	0	—	—	0.66			
	0	0	18	2.0	2.47	—	Control	Expt of Fig. 3
	5	0	—	3.7	0.85	—		
	0	0	23	2.6	2.38	—	+creatine phosphate	
	5	0	—	4.1	1.01	—	+creatine phosphokinase	
Intact cells	0	0	15	10	4.03	2.36	Glucose-fed cells	Expt of Fig. 1
	0	0	18	9	4.18	2.67	Inosine-fed cells	
	0	0	—	13	3.90	2.00	Inosine-fed cells	
	5	0	—	11	1.91	—		
	0	0	23	12	5.01	3.11	Inosine-fed cells	
	5	0	—	11	2.86	—		
	0	10	—	—	24.6	—		
	5	10	—	—	23.9	—		
	0	0	—	—	1.89	—	Choline medium	Expt of Fig. 6
	5	0	—	—	0.01	—		
	0	0	—	—	1.95	—	Mg^{2+} medium	
	5	0	—	—	1.26	—		

media was measured, the final K^+ concentration in the medium varied between 3.3 and 6.5 μM (Table 1). The reason for this is a variable, unexplained, temperature-dependent lysis of part of the cell population.

The conclusion from these experiments is that the ouabain-sensitive Na^+ efflux into Na^+ and nominally K^+-free media requires the presence of neither internal nor external K^+.

Comparison between the behaviour of the ouabain-sensitive Na^+ loss into solutions free of Na^+ and K^+ and that of the Na^+:Na^+ and Na^+:K^+ exchanges

Since the extrusion of Na^+ through the Na^+ pump in the absence of external Na^+ and K^+ is a continuous process and not a brief transient one, the pump must return to the original conditions after each translocating cycle. There are two known alternative return routes for the pump mechanism after releasing its Na^+ to the external medium: it could either come back as if it were loaded with Na^+ (or indeed loaded with unreleased Na^+, but less than during the outward trip), or it could move back as if it were loaded with K^+, as during the normal Na^+:K^+ exchange. Since the Na^+:Na^+ exchange is associated with a transphosphorylation reaction between ATP and ADP[10,11] and depends on the intracellular ADP concentration, the pump coming back through the Na^+ route might be expected to show the same requirement for ADP as the Na^+:Na^+ exchange does. If, on the other hand, the pump moves

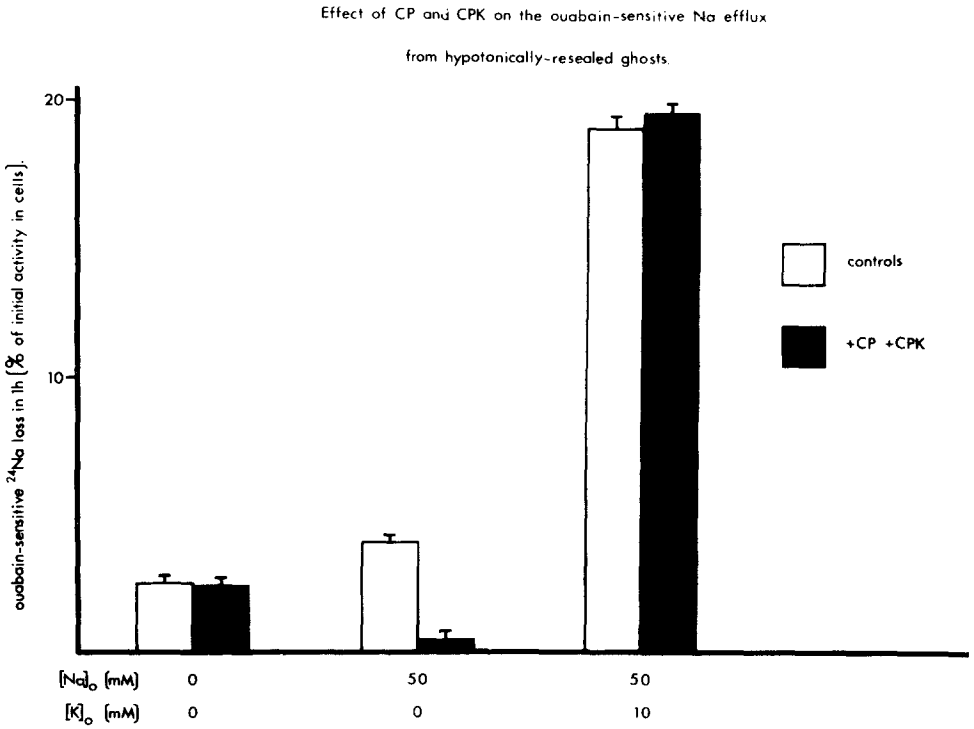

Fig. 3. Effect of lowering the intracellular ADP concentration on the ouabain-sensitive Na^+ efflux from hypotonically resealed ghosts. Creatine phosphate (CP) and creatine phosphokinase (CPK) were used to buffer the intracellular ADP concentration (see ref. 10 and Methods).

back through the K^+ route it will be expected to show a requirement for ATP in the way of an $Na^+:K^+$ exchange.

Fig. 3 shows the result of an experiment performed with hypotonically resealed ghosts containing less than 1 mM K^+. The ghosts that were resealed in the medium containing the nucleotide buffering system and that had, presumably, an ADP concentration[10] low enough to inhibit a substantial proportion of the $Na^+:Na^+$ exchange, showed no difference in relation to the Na^+ loss into the Na^+ and K^+-free medium, compared with the unbuffered controls. Further evidence for a $Na^+:K^+$-like behaviour is presented in Fig. 4. Starvation inhibits the $Na^+:K^+$ exchange more than the $Na^+:Na^+$ exchange in intact human red cells[7]. Although the intracellular concentration of both, ATP and ADP, falls during starvation, the ATP level falls proportionally faster and so does the $Na^+:K^+$ exchange. The ouabain-sensitive Na^+ efflux into the Na^+ and K^+-free medium behaved in a similar way to the $Na^+:K^+$ exchange. Similar results in intact cells were obtained by Sachs[12] and by Garrahan and Glynn (personal communication).

These results suggest that the ouabain-sensitive Na^+ loss in the absence of external Na^+ and K^+, hydrolyses ATP in the same way as the $Na^+:K^+$ exchange does, and that it is probably associated with a K^+-independent spontaneous dephos-

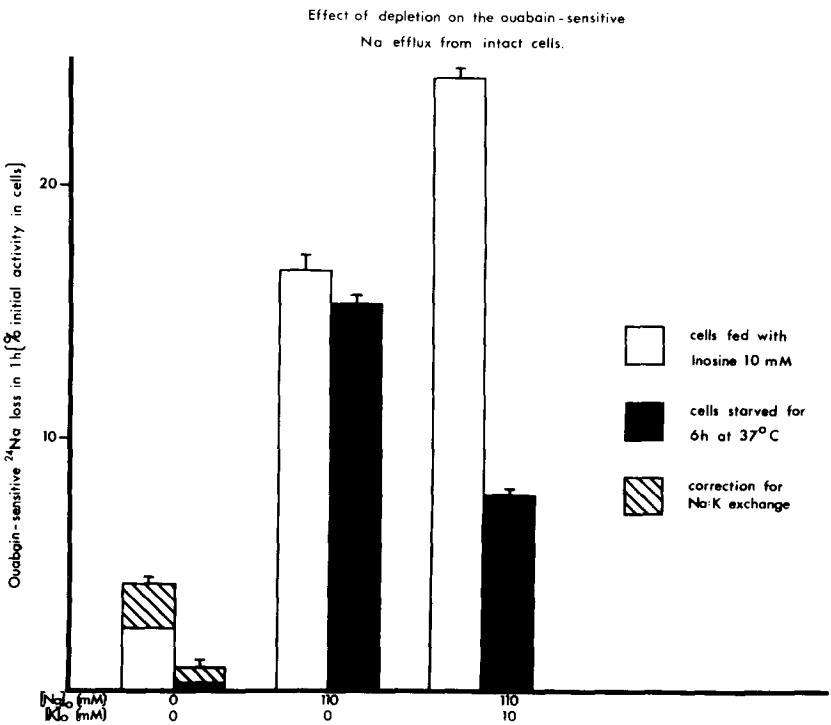

Fig. 4. Effect of starvation on the ouabain-sensitive Na^+ efflux from intact red cells. The procedure was similar to that of the experiment of Fig. 1. All the cells were preincubated together for 6 h at 37 °C, in a medium containing: KCl, 140 mM; $^{24}NaCl$, 10 mM; Tris–HCl (pH 7.6 at 37 °C), 10 mM. Inosine was present only during the final incubation (1 h). The haematocrit was about 1% in the Na^+ and K^+-free medium and about 4% in the presence of Na^+ or Na^+ and K^+.

phorylation of the Na^+ pump. The ouabain-sensitive hydrolysis of ATP that can be expected in red cells is too small to be detected, but Baker[13,5] found a large ouabain-sensitive increase in the internal phosphate level in crab nerves loaded with Na^+ and incubated in Na^+ and K^+-free solutions.

Activation of the Na^+ efflux by external K^+ in the absence of Na^+. Inhibition by external Na^+

External K^+ activates the ouabain-sensitive Na^+ efflux in the absence of Na^+ (Fig. 5) along a Michaelis-type curve with a K_m of about 0.1 mM. The experimental values in Fig. 5 are fitted quite well by an equation of the type

$$F = \frac{F_m[K^+]_0}{K_m + [K^+]_0} + F_0$$

where F is the ouabain-sensitive Na^+ efflux; $[K^+]_0$, the potassium concentration in the medium; $F_m + F_0$, the maximum ouabain-sensitive Na^+ efflux; F_0, the ouabain-sensitive Na^+ efflux in the absence of K^+, and K_m, the concentration of potassium

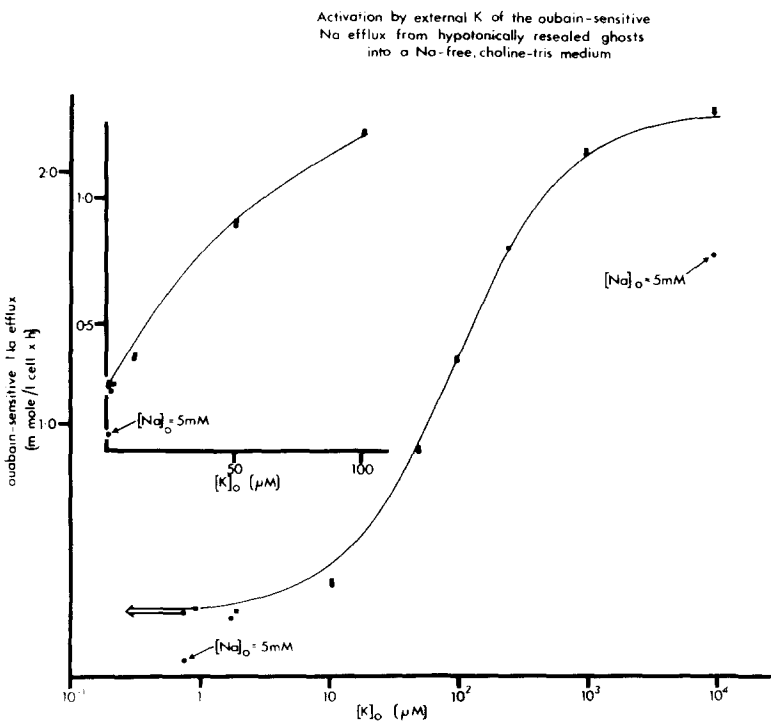

Fig. 5. Activation by external K^+ of the saturated ouabain-sensitive Na^+ efflux from hypotonically resealed ghosts into a Na^+-free, choline–Tris medium. For details see Methods. The insert represents the foot of the activation curve on a linear scale. The continuous line represents the equation:

$$\text{Ouabain-sensitive } Na^+ \text{ efflux} = \frac{1.97 \quad [K^+]_0}{100 + [K^+]_0} + 0.255.$$

Results from two different experiments. The arrow to the left of the points at $[K^+]_0 < 1\ \mu M$, indicates the concentration of K^+ at the beginning of the final incubation.

at which the activated efflux is half-maximal. This equation describes two simple models which are kinetically indistinguishable. The first model admits the existence of an uncoupled component of the Na^+ efflux (F_0) even at saturating K^+ concentrations. The second model assumes that K^+ activates the Na^+ efflux with the same $[K^+]_m$ as it inhibits the uncoupled component.

The effect of external Na^+ provides one way of distinguishing between these two possibilities. The presence of 5 mM Na in the medium, which inhibited almost completely the ouabain-sensitive Na^+ efflux in the absence of K^+ also reduced the Na^+ efflux at saturating K^+ levels (Fig. 5). At this concentration of Na^+, K^+ activates the Na^+ efflux with a half maximum of about 0.25 mM[14], and the degree of unsaturation expected from the shift in K_m from 0.1 (Fig. 5) to 0.25 mM is less than 6% of the Na^+ effect in the presence of 10 mM K^+, corrected for the dilution of specific activity (see Methods).

Inhibitory effects of Na^+ at saturating K^+ concentrations were previously observed in crab nerve[5] and in red cells[15]. This action of Na^+ supports the view that even in the presence of saturating amounts of K^+ in the medium part of the Na^+ extruded through the pump involves a mechanism different from the normal $Na^+ : K^+$ exchange.

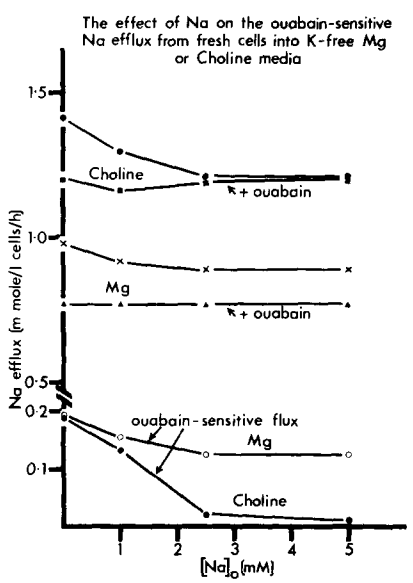

Fig. 6. The effect of Na^+ on the ouabain-sensitive Na^+ efflux from intact cells into K^+-free, Mg^{2+} or choline media. Cells were treated in a similar way to those of Fig. 4. The preincubation was performed in a solution containing: choline chloride, 140 mM; $^{24}NaCl$, 10 mM and Tris–HCl (pH 7.8 at 37 °C), 10 mM. The final incubation medium was either: choline chloride, 150 mM; Tris–HCl (pH 7.8 at 37 °C), 10 mM or $MgCl_2$, 107 mM; Tris–HCl (pH 7.8 at 37 °C), 10 mM. After the preincubation the cells were washed 6 times with about 10–12 vol. of choline or Mg^{2+} medium. In the final incubation medium isotonic Na^+ replaced isotonic $MgCl_2$ or choline chloride to give the final concentrations indicated in the figure. ●—●, controls, choline and ouabain-sensitive difference, choline; ■—■, ouabain-resistant flux, choline; ×—×, controls, Mg^{2+}; ▲—▲, ouabain-resistant flux, Mg^{2+}; ○—○, ouabain-sensitive flux, Mg^{2+}.

However, the evidence is not conclusive since the Na^+ effect is rather inconsistent (Table 1). Some degree of inhibition of the ouabain-sensitive Na^+ efflux into K^+-free media seems to be always present[4,12] but its magnitude varies within a wide range. This is what could be expected if Na^+ had two simultaneous and opposite actions, namely to inhibit the Na^+ loss into K^+-free solutions and to stimulate the Na^+:Na^+ exchange. However, this explanation would fail to account for the poor inhibition obtained in the experiment of Fig. 3 (see Table 1), where the Na^+:Na^+ exchange was nearly absent and where the full inhibition should have been easily seen. Even in the same cells and at the same time (Fig. 6) while the ouabain-sensitive Na^+ efflux into solutions lacking both Na^+ and K^+, was substantially the same, and independent of the composition of the medium, the inhibition by Na^+ was considerably less in the Mg^{2+} containing medium than in the choline one. Incidentally, the variations observed in the ouabain-resistant levels (Fig. 6) could be interpreted in terms of an inhibitory action of Mg^{2+}, in agreement with earlier results[16].

The fit between theory and experiment in Fig. 5 can be improved, specially at the lower K^+ concentrations where there seems to be a slight inflection, using an equation of the type:

$$F = F_m \frac{a[K^+]_0^2}{a[K^+]_0^2 + b[K^+]_0 + 1} + F_0$$

where a and b are constants, the values of which depend on the affinities of two external binding sites for K^+. The best fit is obtained assuming dissociation constants of about 4 and 100 μM. However, no further speculation on this point is justified at present due to the insufficient number of experimental values at the lower part of the curve.

DISCUSSION

The experiments reported in this paper show that in the absence of external Na^+ and K^+, and probably also in the presence of K^+, an uncoupled Na^+ efflux takes place through the Na^+ pump. This flux requires ATP but not ADP and is inhibited to a variable extent by external Na^+.

The idea was advanced[6] that the uncoupled Na^+ extrusion is associated with some degree of spontaneous dephosphorylation of the pump. Previous studies on the phosphorylated intermediate of the Na pump show a relatively small rate of breakdown of this intermediate in the absence of K^+ from the medium[17]. However, most of these experiments were performed in the presence of Na^+ since it was required for the labelling of the intermediate from [^{32}P]ATP. But, at the same time, Na^+ would presumably have acted on the external surface of the membrane reducing the rate of spontaneous dephosphorylation in the same way as it decreases the uncoupled Na^+ extrusion. Therefore, most of the observed rates of dephosphorylation in the absence of external K^+ correspond to the values already inhibited by Na^+. We could find only two instances in the literature where the rate of breakdown of the phosphorylated intermediate was measured at Na^+ concentrations lower than 2 mM. Post *et al.*[18] report results by Kanazawa *et al.* showing the same rate of breakdown at 0.5 and at 140 mM Na^+. According to Post *et al.*[18], the rate of splitting at 140 mM

Na^+ is about double the rate at 4–16 mM Na^+. The value at 0.5 mM Na^+ would therefore correspond to an increased rate of breakdown. However, Post *et al.* attribute that increase to "extraneous factors". In one experiment, they found no difference in the rate of splitting at 0.5 or 1 mM Na^+ compared with splitting at 16 mM Na^+ after taking precautions to exclude traces of NH_4^+. These meant washing the cold stored enzyme, and it is unlikely that this mild procedure would have suppressed the action of Na^+ on the hydrolysis rate if it were a genuine effect. However, the existence of real differences in the Na^+ effect among enzyme preparations of different origin is a likely possibility as we shall see later. It should be pointed out that no specific precautions were taken in the present experiments with red cells to exclude traces of NH_4^+ from the different media, although freshly-prepared solutions were used each time.

If the uncoupled Na^+ efflux is associated with a Na^+-inhibitable dephosphorylation, one can further predict that that reactive form of the intermediate which is sensitive to dephosphorylation by K^+ and not by ADP, will be inhibited by Na^+.

The possible existence of an uncoupled component of the Na^+ efflux even in the presence of external K^+, suggests that K^+ may not always be rate-limiting for the dephosphorylation of the intermediate in the absence of external Na^+. It is possible that in systems where the magnitude of the uncoupled Na^+ efflux compares with the maximum rates of $Na^+:K^+$ exchange (refs 1, 5 and Lew *et al.* unpublished), the action of external Na^+ is to decrease the spontaneous dephosphorylation rate so that K^+ now becomes rate-limiting. If so, this may represent the mechanism by which the inward translocation of K^+ becomes coupled to the Na^+ extrusion through the pump. In crab nerve, Baker[2] found that, in Na^+-free, choline-artificial sea waters, K^+ inhibited the anion loss with a K_m of about 0.8 mM. If the loss of the ninhydrin-positive material is proportional to the uncoupled Na^+ efflux through the pump, the effect of K^+ represents an inhibition of the uncoupled flux with or without a simultaneous stimulation of the coupled $Na^+:K^+$ exchange. The high levels of P_i (which in crab nerve correlate with the rates of ATP breakdown through the pump) found at 0 and 10 mM K^+ (ref. 5) suggest that in the absence of Na^+, K^+ activated an $Na^+:K^+$ exchange. In this system, K^+ must therefore be rate-limiting for dephosphorylation even in the absence of Na^+.

Na^+ inhibited the anion loss from crab nerve[2] immersed in K^+-free, choline–artificial sea water with a K_m of about 50 mM and a similar value was obtained for the K_i of Na^+ for the inhibition of the pump in the presence of K^+ (ref. 19). It would be of interest to test if high Na^+ is also required to inhibit the spontaneous dephosphorylation rate which presumably parallels the large uncoupled Na^+ efflux in an ATPase preparation of crab nerve. Only if the K_m of Na^+ for phosphorylation is much lower than the K_i of Na^+ for the inhibition of the uncoupled Na^+ efflux would it be possible to observe an inhibition by Na^+ of the $(Mg^{2+}+Na^+)$-dependent ATPase activity. Inhibition of the $(Mg^{2+}+Na^+)$-ATPase activity by relatively high concentrations of Na^+ has been reported earlier by Skou[20] on an enzyme preparation of crab nerve.

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REFERENCES

1 Hodgkin, A. L. and Keynes, R. D. (1955) *J. Physiol. London* 128, 28–60
2 Baker, P. F. (1964) *Biochim. Biophys. Acta* 88, 458–460
3 Baker, P. F., Blaustein, M. P., Keynes, R. D., Manil, J., Shaw, T. I. and Steinhardt, R. A. (1969) *J. Physiol. London* 200, 459–496
4 Garrahan, P. J. and Glynn, I. M. (1967) *J. Physiol. London* 192, 175–188
5 Baker, P. F. (1965) *J. Physiol. London* 180, 383–423
6 Hardy, Jr, M. A., Ellory, J. C. and Lew, V. L. (1973) *J. Physiol. London* 231, 110–111P
7 Glynn, I. M., Lew, V. L. and Lüthi, U. (1970) *J. Physiol. London* 207, 371–391
8 Simons, T. J. B. (1972) *J. Physiol. London* 227, 19P–20P
9 Glynn, I. M. and Lew, V. L. (1970) *J. Physiol. London* 207, 393–402
10 Glynn, I. M., Hoffman, J. F. and Lew, V. L. (1971) *Phil. Trans R. Soc. London Ser. B* 262, 91–102
11 Glynn, I. M. and Hoffman, J. F. (1971) *J. Physiol. London* 218, 239–256
12 Sachs, J. R. (1970) *J. Gen. Physiol.* 56, 322–341
13 Baker, P. F. (1963) *Biochim. Biophys. Acta* 75, 287–289
14 Glynn, I. M. and Lew, V. L. (1969) *J. Gen. Physiol.* 54, 289s–305s
15 Garrahan, P. J. and Glynn, I. M. (1967) *J. Physiol. London* 192, 159–174
16 Rettori, O. and Lenoir, J. P. (1972) *Am. J. Physiol.* 222, 880–884
17 Post, R. L., Kume, S., Tobin, T., Orcutt, B. and Sen, A. K. (1969) *J. Gen. Physiol.* 54, 306s–326s
18 Post, R. L., Hegyvary, C. and Kume, S. (1972) *J. Biol. Chem.* 247, 6530–6540
19 Baker, P. F. and Connelly, C. M. (1966) *J. Physiol. London* 185, 270–297
20 Skou, J. C. (1960) *Biochim. Biophys. Acta* 42, 6–23