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THE INTERACTION OF SODIUM AND POTASSIUM WITH THE SODIUM PUMP IN RED CELLS

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SUMMARY

1. At high internal K concentrations the efflux of Na from red cells increases with internal Na concentration following an S-shaped curve. As internal K is reduced the S-shaped region and the value of internal Na for which the Na efflux is half-maximal are both shifted progressively towards zero.

2. The effects of internal Na on the shape of the Na efflux curves can be quantitatively accounted for if it is assumed that the rate of Na efflux is linearly related to the number of pump units having three identical and non-interacting sites occupied by Na.

3. The effects of internal K on the shape of the Na efflux curves are fully explained if it is assumed that the inner sites for Na of the Na pump also behave as identical and non-interacting sites for internal K, being the K-carrier complexes unable to promote Na translocation. The apparent affinity of the Na pump for internal K is about 50 times less than for internal Na.

4. Internal K not only alters the apparent affinity of the Na pump for Na, but also affects its turnover rate. The turnover rate of Na:K exchange increases with internal K following a curve which saturates at about 30 mM internal K. The turnover rate for Na:Na exchange increases linearly with internal K.

5. The linear dependence of the rate of Na:Na exchange on internal K explains why, when internal Na is increased at the expense of internal K, the rate of Na:Na exchange progressively decreases after passing through a maximum.

6. The effects of external Na on the rate of Na:Na exchange can be satisfactorily explained assuming that they are due to the occupation by external Na of three identical and non-interacting sites on each pump unit. The apparent affinity of the Na pump for external Na is about 160 times less than the apparent affinity for internal Na.

7. Under all the experimental conditions tested, it was found that the relation between flux and cation concentration at one of the surfaces of the cell membrane is altered only by a constant factor by changes in the cation composition at the opposite surface of the cell membrane. This fact strongly suggests that there are no interactions between the inner and outer sites of the Na pump.

8. The effects of inner and outer cations on both the Na:K and the Na:Na exchanges catalysed by the Na pump suggest that cation fluxes are proportional to the number of pump units having its inner and outer sites *simultaneously* occupied by the relevant cations. It seems therefore that sequential models for ion transport do not provide an adequate description of the molecular mechanism of active transport in red cells.

INTRODUCTION

In the presence of non-limiting concentrations of external K the Na pump in red cells drives an exchange of internal Na for external K. When external K is removed the Na pump, instead of exchanging Na for K, catalyses a ouabain-sensitive one to one exchange of Na across the cell membrane (for references see Garrahan, 1970).

One of the possible approaches to understand better the processes involved in the Na:K and Na:Na exchanges catalysed by the Na pump, is to look at the dependence of the rate of these phenomena with the concentration of cations in the intra- and extracellular media. The experiments described in this paper represent an approach of this kind.

The results show that a relatively simple kinetic model provides an accurate quantitative description of the interactions of Na and K with the Na pump in red cells.

METHODS

Freshly drawn human blood from haematologically normal adults was always used. Coagulation was prevented with acid–citrate–dextrose solution. The blood was centrifuged at 1750 *g* for 10 min and the plasma and buffy coat were removed by aspiration. The remaining cells were washed twice with about 10 volumes of a 150 mM-NaCl solution and once with about 10 volumes of a 150 mM-choline chloride solution. After the last wash, the cells were spun down for 15 min at 1750 *g*. All the procedure was carried out at 4° C.

The internal cation composition of the red cells was varied using a procedure essentially similar to that described by Garrahan & Rega (1967). Unless otherwise stated in results, a known volume of washed and packed cells was suspended in an adequate volume of salt media to give a haematocrit of 5%. Depending on the desired internal cation composition, the salt media were prepared mixing suitable proportions of 'Na medium', 'K medium' and 'choline medium'. The 'Na medium' contained (mM): NaCl, 145; $MgCl_2$, 1; Na phosphate (pH 7·4 at 4° C), 2·5; the 'K medium' contained (mM): KCl, 145; $MgCl_2$, 1; K phosphate (pH 7·4 at 4° C), 2·5;

the 'choline medium' contained (mM): choline chloride, 180; $MgCl_2$, 1; *o*-phosphoric acid titrated with Tris base to give a pH of 7·4 at 4° C, 2, 5-*p*-chlormercuribenzene-sulphonate (PCMBS) concentration was 0·1 mM when only Na and K were present and 0·2 mM when choline was present. The use of hypertonic concentrations of choline chloride and of 0·2 mM-PCMBS was found to be necessary to avoid excessive shrinkage of the cells incubated in the presence of choline. The cells were treated with PCMBS during 20 hr at 4° C. The suspending media were renewed once after 7 hr of incubation. At the end of the treatment with PCMBS the cells were spun down at 4° C for 15 min at 12000 *g* and the supernatant was discarded. The cells were resuspended in PCMBS-free media of identical salt composition to that used in the previous step, to give a haematocrit of 10%.

10-mM glucose and 4 mM cysteine were added to the media. The cell suspensions were incubated at 37° C for 30 min. After this step the cells were spun down at 4° C for 5 min at 1750 *g* and suspended in the same incubation media to give a haematocrit of 30%. When Na efflux was measured enough $^{22}NaCl$ was added to give an activity of 10 μC/ml. The cell suspension was incubated at 37° C for 4 hr. At the end of the incubation the cells were washed at least 3 times with about 20 volumes of ice-cold, 150 mM choline chloride solution. After the last wash the cells were suspended in the same wash solution at a haematocrit of around 50%. A portion of this suspension was set aside to measure intracellular K, Na, haemoglobin and haematocrit. The rest of it was spun down and suspended in the final incubation media ready for use.

Measurement of cation movements. Na efflux was calculated from the loss of ^{22}Na after 15, 30 and 45 min incubation, Na and Rb influxes were estimated from the radioactivity taken up by the cells during a 20 min incubation. All incubations were performed at 37°, haematocrit was 2–4%. Estimations were performed at least in duplicate following a procedure essentially similar to that described by Garrahan & Glynn (1967*a*, *b*). All fluxes were related to the volume of original cells assuming that the 541-nm absorbance of packed cells was 284. This mode of expression allowed us to measure the fluxes across a constant membrane area independently of the changes in cell volume due to the different treatments.

Unless otherwise stated in Results incubations were performed in media containing (mM): NaCl, 140; KCl, 10; $MgCl_2$, 1; *o*-phosphoric acid titrated with Tris base to give a pH of 7·4 at 37° C, 2·5; glucose 10 mM. When K was omitted choline chloride or NaCl replaced an equimolar amount of KCl. The suspending media of different concentrations of Na were prepared mixing suitable proportions of the above-mentioned media with media in which all the NaCl was replaced by an equimolar amount of choline chloride. In the Rb influx experiment RbCl was increased at the expense of choline chloride, the media were otherwise identical to the K-containing suspending medium. In the Na influx experiments enough $^{22}NaCl$ was added to give a specific Na activity of 50 μC/m-mole, in the Rb influx experiments enough $^{86}RbCl$ was added to give a specific Rb activity of 5 mC/m-mole. Ouabain-sensitive cation fluxes were calculated as the difference between the movements in the above mentioned media and in media containing 10^{-4} M ouabain.

Intracellular Na and K. Were estimated by flame photometry. The samples were diluted with distilled water to give concentrations within 20% of the standards. The readings of Na were corrected for interference due to K. To avoid errors due to changes in cell water content under different treatments, intracellular Na and K concentrations were always expressed per litre of cell water.

Estimation of intracellular water. The water content of fresh cells was assumed to be 0·7 (v/v) (Savitz, Sidel & Solomon, 1964). The water content (v/v) of cells treated with PCMBS was calculated according to the following equation:

$$\text{Cell } H_2O = 1 - 0{\cdot}3(Hb_x/284), \qquad (1)$$

where Hb_x is the absorbance at 541 nm of packed cells and 284 is the absorbance at the same wave-length of fresh packed cells.

Haematocrit. This was measured in quadruplicate using the capillary micro-haematocrit technique.

Haemoglobin. This was estimated as oxyhaemoglobin by measuring the absorption at 541 nm.

Radioactivity. This was measured with a Packard 'Tri Carb' liquid scintillation counter, using Bray's (1960) solution. In the influx experiments the samples were first deproteinized with trichloroacetic acid (final concentration 5 % w/v). Counting was always continued for a time long enough to include at least 10000 counts.

Sources of materials. ^{86}NaCl and ^{86}RbCl were obtained as sterile isotonic solutions from the Comisión Nacional de Energía Atómica, Argentina.

PCMBS was *p*-chloromercuriphenylsulphonic acid, monosodium salt (Sigma Chemical Co., U.S.A.). PCMBS solutions were prepared immediately before use.

Ouabain (Strophanthin-octahydrate Sigma Chemical Co., U.S.A.) was dissolved on the day of use in the appropriate salt solution. Cysteine was obtained as cysteine hydrochloride from British Drug Houses Ltd and dissolved and neutralized with tris base on the day of use.

All other salts and reagents were A.R. grade. The solutions were prepared in doubly glass-distilled water.

RESULTS

The effect of internal Na on Na efflux

Na:Na exchange. The effects of internal Na concentration on the loss of ^{22}Na from red cells incubated in K-free solutions with and without ouabain are shown in Fig. 1. As internal Na is increased, Na efflux rises along a curve which tends to level off after passing through an inflexion at low internal Na concentrations. At internal Na concentrations higher than 25 mM, the Na efflux in the absence of ouabain seems to increase with internal Na less rapidly than the efflux in the presence of ouabain. At high internal Na concentrations, the difference between the rate constants for Na efflux in the presence and absence of ouabain falls within the experimental error. Therefore the effects of high internal Na concentrations were studied measuring the ouabain-sensitive Na influx since, in the absence of external K, the ouabain-sensitive Na efflux and influx have identical values (Garrahan & Glynn, 1967*a*). The effect of internal Na on the influx of Na in the presence and absence of ouabain is shown in Fig. 2. It is clear that whereas in the presence of ouabain Na influx is independent of internal Na, when the glycoside is absent Na influx first rises and then seems to decrease progressively as internal Na is raised.

Na:K exchange. Fig. 3 shows the efflux of Na as a function of internal Na concentration in cells incubated in media containing 10 mM-K. The experiment was performed on the same batch of cells and at the same time as that of Fig. 1. The efflux curves are similar in shape to those in Fig. 1, except that there is no sign of inhibition of the ouabain-sensitive component at high Na concentrations.

The rate equation for Na efflux as a function of internal Na. One of the possible explanations for the inflexion observed at the base of the Na efflux curves is that more than one site in a pump unit has to be loaded with Na in order to get ion translocation.

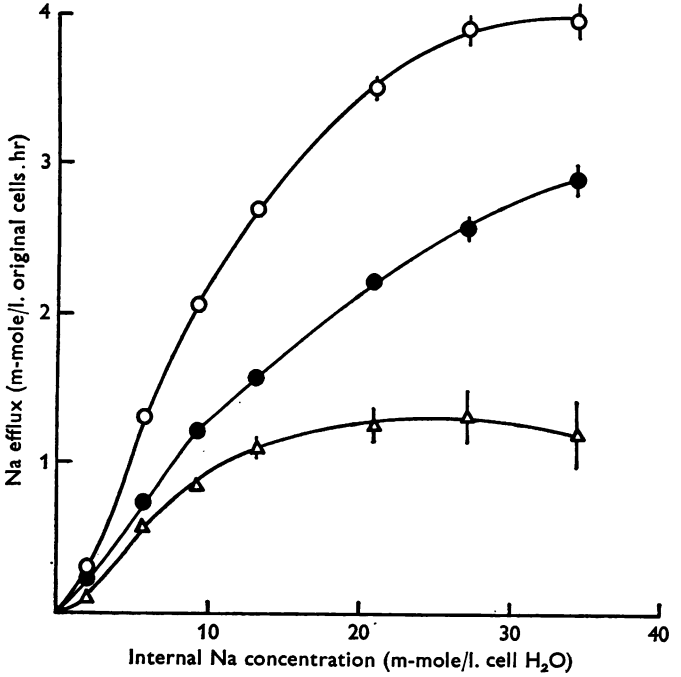

Fig. 1. The effects of internal Na concentration on the efflux of Na from red cells into a K-free medium. ○, total efflux; ●, ouabain-resistant efflux; △, ouabain-sensitive efflux. The vertical lines are the maximum ranges of each measurement, calculated from the maximum and minimum slopes of the efflux curves. When not indicated the range falls within the size of the symbols. Internal Na was increased at the expense of internal K.

If internal Na were always in equilibrium with the Na-site complexes the probability (p) of having a pump unit occupied at one site with Na would be

$$p = 1/(1+K'_{Na}/[Na]_i), \tag{2}$$

where K'_{Na} is the apparent dissociation constant of the Na-site complex and $[Na]_i$ is the internal Na concentration.

The expression for the probability of having more than one site occupied by Na in a pump unit will depend on the number of sites, on the value of K'_{Na} for each site and on whether the occupation of one site modifies the affinity of the rest or not.

In the simplest case, viz. when all the sites have the same affinity and there is no interaction between them, the probability of having n sites occupied by Na on a pump unit will be

$$p = 1/(1+K'_{Na}/[Na]_i)^n. \tag{3}$$

Available experimental evidence (see Whittam & Ager, 1965) seems to indicate that the number of Na or K ions transported per pump cycle is fixed and independent of the cation concentration at either surface of the cell membrane. It seems therefore reasonable to assume that ion translocation will only take place in those pump units which have attained a certain degree (n) of occupation by Na ions. If, as it seems likely at the molecular level, there are no interactions between pump units, Na flux will be linearly related to the fraction of pump units having the degree of saturation required to elicit ion translocation, i.e.

$$M = M'_{max}/(1+K'_{Na}/[Na]_i)^n, \quad (4)$$

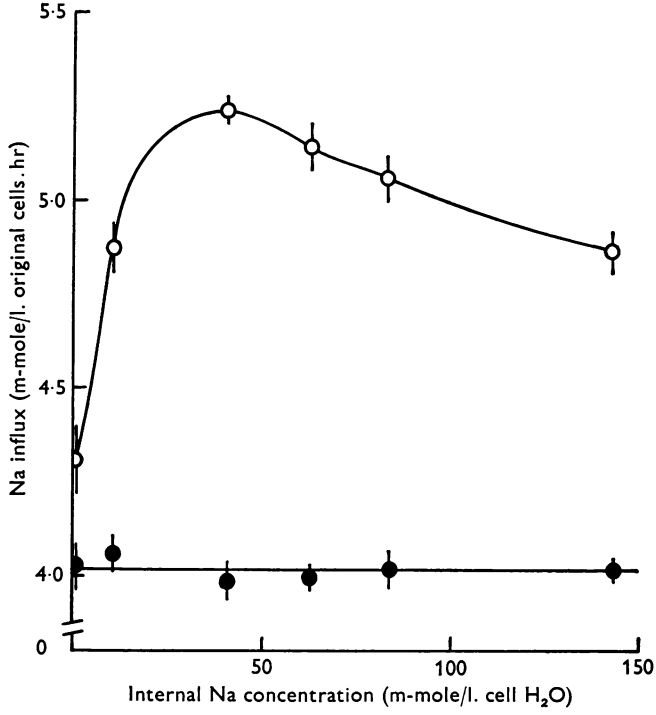

Fig. 2. The effects of internal Na concentration on the influx of Na into red cells suspended in K-free media. ○, total influx; ●, ouabain-resistant influx. Each point is the average of four determinations. The vertical lines show ± 1 S.E. Internal Na was increased at the expense of the internal K.

where M is the flux at a given Na concentration and M'_{max} is the flux at non-limiting internal Na concentration.

Multiplying the nth root of the reciprocal of eqn. (4) by $[Na]_i$ the following expression is obtained:

$$[Na]_i/M^{1/n} = K'_{Na}/M'_{max}{}^{1/n} + [Na]_i/M_{max}{}^{1/n}. \quad (5)$$

If eqn. (4) describes the experimental results, the plot of $[Na]_i/M^{1/n}$ against $[Na]_i$ will yield a straight line with slope $(M'_{max})^{-1/n}$ and intercept at the horizontal axis $-K'_{Na}$ when n = number of sites.

Fig. 4 shows the results of the experiment of Fig. 1 plotted according to eqn. (5). It is clear that (i) for internal Na concentrations between 2 and 15 mM the experimental values of the ouabain-sensitive Na efflux fit a straight line for $n = 3$, whereas for $n = 2$ the points at low Na concentrations curve upwards deviating from a straight line; (ii) over the whole range of Na concentrations tested the plot of the total Na efflux can be

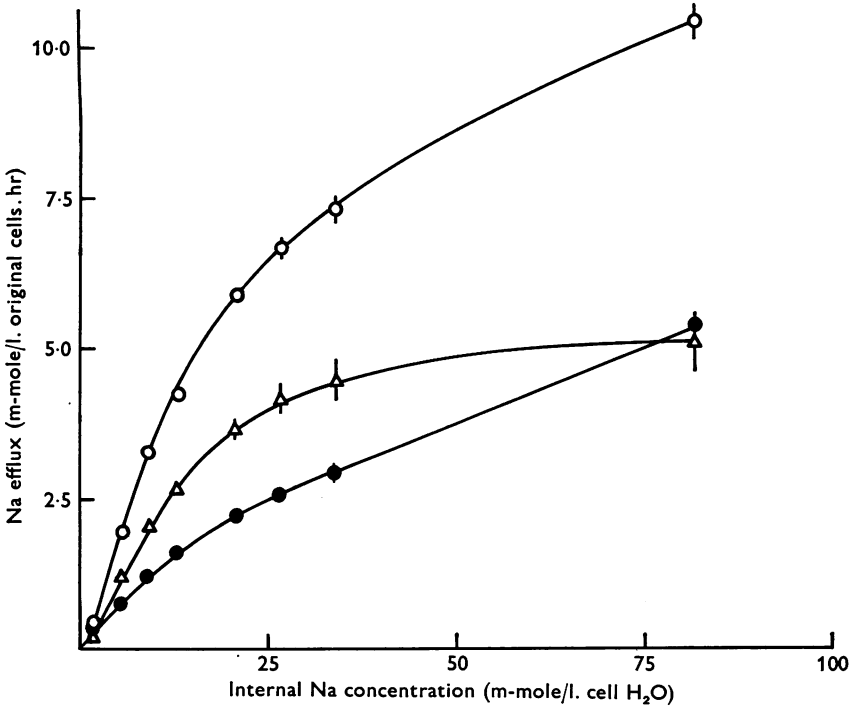

Fig. 3. The effect of internal Na concentration on the efflux of Na into a 10 mM-K medium. ○, total efflux; ●, ouabain-resistant efflux; △, ouabain-sensitive efflux. The vertical lines are the maximum ranges calculated as in the experiment of Fig. 1. When not indicated the ranges fall within the size of the symbols. Internal Na was increased at the expense of the internal K.

fitted by a straight line which cuts the horizontal axis at the same point as that of the plot of the ouabain-sensitive Na efflux.

It seems therefore that, at low internal Na concentrations, both the total and the ouabain-sensitive Na efflux in the absence of external K, depend on internal Na in the way predicted by eqn. (4) when n is assumed to be equal to 3, giving the same values of K'_{Na} for both kind of fluxes. As a result of the inhibition that begins to set in at high internal Na, the experimental points of the ouabain-sensitive Na efflux at Na concentra-

tions higher than 15 mM curve upwards diverging from a straight line. The lack of deviation from a straight line of the plot of the total efflux probably results from the fact that the inhibition at high Na concentrations of the ouabain-sensitive component, cancels the opposite effect that would have been exerted by the non-saturable component (Sachs, 1970) of the total efflux.

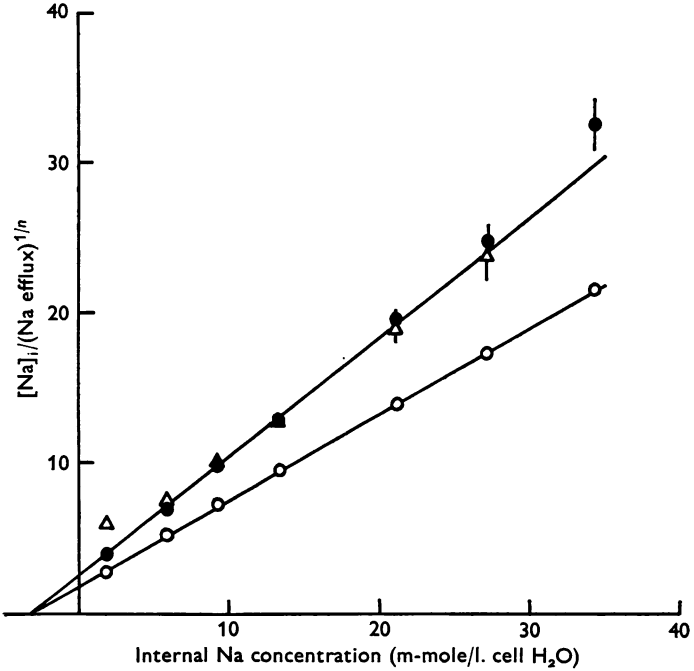

Fig. 4. The effect of internal Na concentration on the total (○) and ouabain-sensitive (●, △) Na efflux from red cells suspended in K-free media, plotted according to eqn. (5). The ouabain-sensitive efflux was plotted assuming $n = 2$ (△) and $n = 3$ (●). K'_{Na} was 3·2 mM. Essentially similar results were obtained in five further experiments, all of which yielded K'_{Na} values not significantly different from 3·2 mM. The vertical lines are the maximum ranges calculated as in the experiment in Fig. 1. When not indicated the range falls within the size of the symbols.

Fig. 5 shows the effect of internal Na on Na efflux in the presence of external K plotted according to eqn. (5). Results show that in the whole range of internal Na concentrations tested (2–80 mM) the ouabain-sensitive efflux can be fitted by a single straight line when $n = 3$, as it is to be expected if the flux varies with internal Na in the way predicted by eqn. (4). When $n = 2$ the points at low Na concentration curve upwards diverging from a straight line. Since the ouabain-sensitive efflux in the presence of external K shows no sign of inhibition at high Na concentrations

(see Fig. 3) results in Fig. 5 support the view that the deviation of the rate of Na:Na exchange from the behaviour predicted by eqn. (4), at internal Na concentrations larger than 15 mM is caused by the inhibition of this phenomenon at high Na concentrations.

When plotted according to eqn. (5) for $n = 3$, the total Na efflux in the presence of external K and at relatively low internal Na concentrations, can also be fitted by a straight line with the same intercept on the hori-

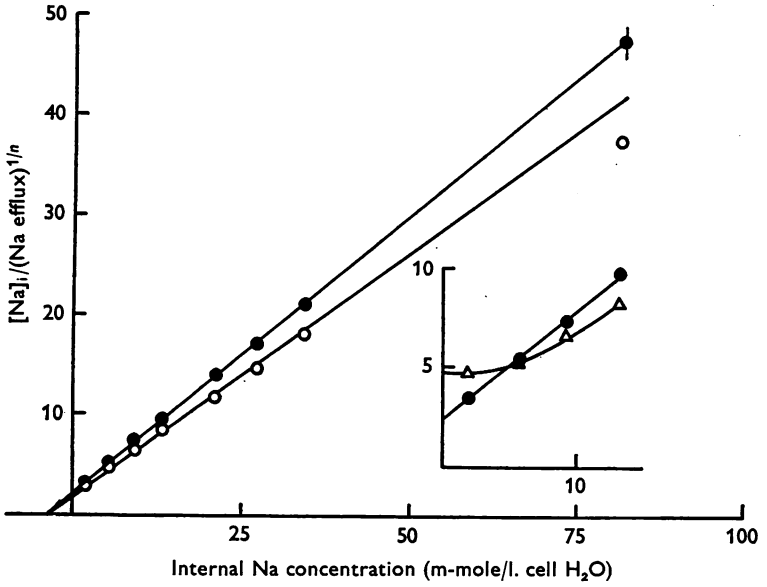

Fig. 5. The effect of internal Na concentration on the total (○) and ouabain-sensitive (●) Na efflux from red cells suspended in 10 mM-K media, plotted according to eqn. (5) assuming $n = 3$. The inset in the Figure shows the initial part of the ouabain-sensitive efflux plotted assuming $n = 2$ (△) and $n = 3$ (●). K'_{Na} was 3·2 mM. Essentially similar results were obtained in three further experiments, all of which yielded K'_{Na} values not significantly different from 3·2 mM. The vertical lines are the maximum ranges calculated as in the experiment of Fig. 1. When not indicated the range falls within the size of the symbols.

zontal axis than the ouabain-sensitive efflux (Fig. 5). In this case, however, presumably due to the increasing weight of the non-saturable component of the total efflux, the values at high internal Na concentrations curve downwards diverging from a straight line.

Comparison of Fig. 5 with Fig. 4 shows that the apparent dissociation constant for internal Na for both the ouabain-sensitive and the saturable component of the total Na efflux is the same, regardless of the presence or absence of K in the suspending media.

The effect of external Na on Na:Na exchange

The experiments shown up to now suggest that the effects of internal Na on the shape of the Na efflux curves can be accounted for if it is assumed that Na efflux is linearly related to the number of pump units having three identical and non-interacting sites occupied by internal Na. If this statement were true it seems likely that a similar kinetic scheme should govern

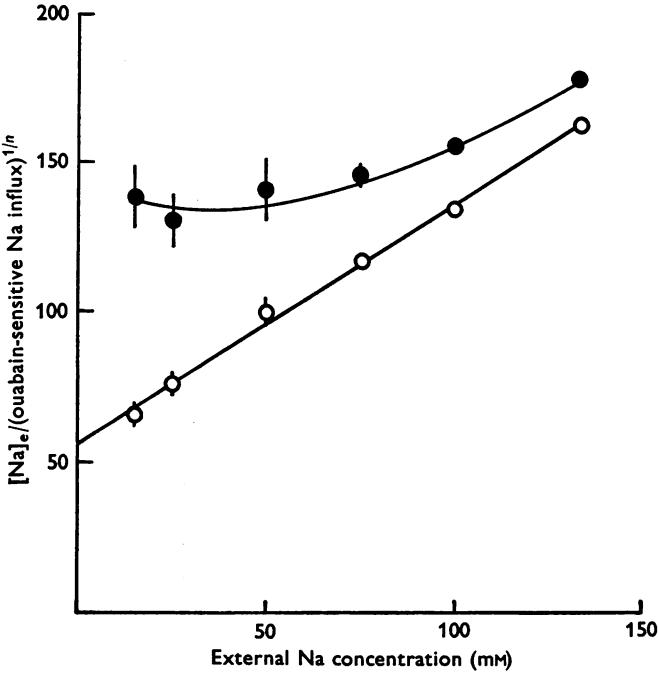

Fig. 6. The effect of external Na concentration on the ouabain-sensitive influx of Na into red cells suspended in K-free media. The results are plotted according to an equation similar to eqn. (5) but with external Na as the variable. n was assumed to be 2 (●) or 3 (○). The vertical lines are maximum ranges. When not indicated the ranges fall within the size of the symbols.

Na:Na exchange when studied as a function of *external* Na concentration. Moreover, as the apparent affinity for external Na of the mechanism responsible for Na:Na exchange seems to be considerably lower than that for internal Na (Garrahan & Glynn, 1967*a*), the study of the dependence of this phenomenon on external Na allows a much more stringent comparison between a two- and a three-site kinetic scheme.

In the experiment shown in Fig. 6 the ouabain-sensitive Na influx, measured in cells suspended in K-free media containing different amounts

of Na, is plotted according to eqn. (5). It is evident that the experimental points fit a straight line for $n = 3$ and diverge from a straight line for $n = 2$.

Another relevant question that may be asked when studying the effects of external Na on Na:Na exchange is whether the inhibition observed at high internal Na concentrations also appears at high *external* Na concentrations. This question cannot be answered by experiments performed in the

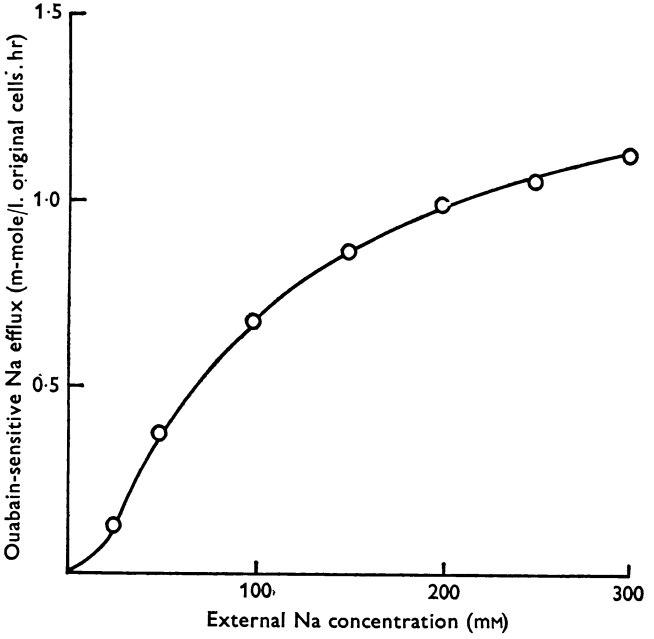

Fig. 7. The effect of external Na concentration on the ouabain-sensitive efflux of Na from red cells suspended in K-free media. Cells were treated with PCMBS in a medium containing (mM): NaCl, 40; KCl, 260; $MgCl_2$, 1; *o*-phosphoric acid titrated with Tris base to pH 7·4 (at 4° C), 2·5; PCMBS, 0·2. The final incubation media contained (mM): NaCl, 300; $MgCl_2$, 1; *o*-phosphoric acid titrated with Tris base to pH 7·4 (at 37° C), 2; glucose, 10. The different levels of external Na were attained replacing NaCl with equimolecular amounts of choline chloride. The cells contained 74% (v/v) of water and 21 m-mole Na and 279 m-mole K/l. cell water. For other details see Methods. The continuous line represents an equation similar to eqn. (4) taking $n = 3$; $K'_{Na} = 31$ mM and $M'_{max} = 1{\cdot}52$ m-mole/l. original cells.hr.

isotonic range of Na concentrations, since within this range Na:Na exchange continually increases with external Na showing no sign of saturation (Garrahan & Glynn, 1967*a*). To extend the useful range of external Na concentrations use was made of the fact that, when suspended for 24 hr in hypertonic saline media containing PCMBS, cells with normal volume but in osmotic equilibrium with the hypertonic media are obtained

(Garrahan & Rega, 1967). In the experiment shown in Fig. 7 red cells were equilibrated in a 300 mM saline media containing PCMBS and the ouabain-sensitive Na efflux was measured in media containing from 25 to 300 mM-Na. It is clear that for the whole range of external Na concentrations tested the ouabain-sensitive Na efflux obeys an equation similar to eqn. (4) with $n = 3$ and an apparent dissociation constant for external Na (K'_{Na}) of 31 mM, not showing any sign of inhibition at high external Na concentrations.

The effect of internal K on Na:Na and Na:K exchanges

Ouabain-sensitive Na efflux was measured as a function of internal Na concentration in cells containing three different intracellular levels of K. In the cells containing low or intermediate K concentrations intracellular K was replaced with choline. In the high K cells, Na was increased at the expense of internal K. The experimental results were plotted according to eqn. (5) assuming $n = 3$. Straight lines were obtained and from their slopes and intercepts the values of M'_{max} and K'_{Na} were calculated for each experimental condition.

Fig. 8*a* and *b* compares the experimental results for Na efflux *vs.* internal Na concentration with the theoretical curves obtained substituting the calculated values of K'_{Na} and M'_{max} into eqn. (4). It is clear that (i) both in the presence (Fig. 8*a*) as well as in the absence (Fig. 8*b*) of external K, eqn. (4) adequately describes the experimental results for the three intracellular K concentrations tested; (ii) as intracellular K is reduced the efflux curves are progressively shifted to the left becoming less sigmoid; (iii) the efflux curves tend to saturation values which decrease as intracellular K is reduced.

The effect of intracellular K on K'_{Na}. The values of K'_{Na} which fit the efflux curves of Fig. 8*a* and *b* were plotted against the intracellular K concentration. Results in Fig. 9 show that the K'_{Na} values for both Na:Na and Na:K exchanges fall on a single straight line with positive intercept and slope. This behaviour is that expected if the three inner sites for Na of the Na pump behaved as identical and non-interacting sites for K, being the K-carrier complexes unable to promote Na translocation. If this were the case, the straight line in Fig. 9 would represent the equation

$$K'_{Na} = K_{Na}(1 + [K]_i/K_K), \tag{6}$$

where K_{Na} is the apparent equilibrium constant for the dissociation of Na from a site and K_K is the apparent equilibrium constant for the dissociation of K from a site. The values of both constants can be obtained from the plot of K'_{Na} *vs.* K. In the case shown in Fig. 9, K_{Na} was 0·19 and K_K 9 mM, which seems to indicate that the inner sites of the Na pump are 47 times

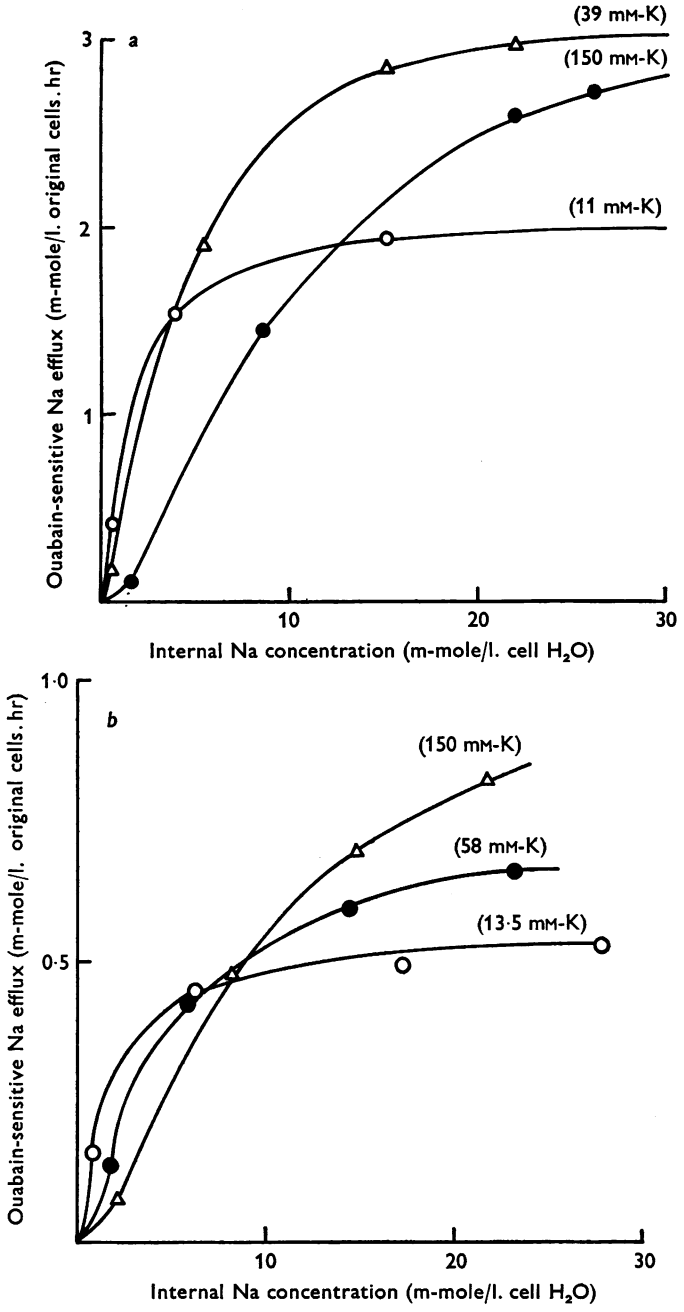

Fig. 8*a* and *b*. The effects of internal Na concentration on the ouabain-sensitive efflux of Na from cells containing three different levels of internal K. The cells were suspended in either 10 mM-K (Fig. 8*a*) or in 0 mM-K (Fig. 8*b*) media. The continuous lines are theoretical curves obtained as described in the text. The water content of the cells was: 65% (v/v) in the low K cells; 68% in the intermediate K cells and 73% in the high K cells. The figures in brackets are the concentrations of K in the cell water.

more selective to Na than to K. Both K_{Na} and K_K are independent of the presence or absence of external K, strongly suggesting that the state of occupation of the outer sites of the Na pump has no effect on the properties of its inner sites.

Simple competition between Na and K at the carrier sites explains why when Na is increased at the expense of internal K, the efflux curves can be fitted by equations in which the apparent dissociation constant for Na

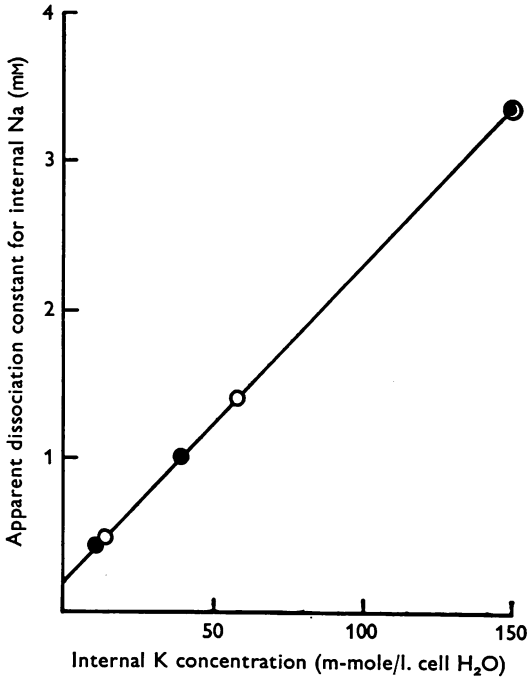

Fig. 9. The dependence with internal K concentration of the K'_{Na} values which fit the efflux curves of Fig. 8*a* (●) and 8*b* (○).

appears to be independent of K concentration. If the expressions for K'_{Na} given in eqn. (6) is substituted into eqn. (4) and $[K]_i$ is expressed as $C-[Na]_i$, where C is the total intracellular concentration of monovalent cations, the following equation is obtained after some rearrangements.

$$M = \frac{M'_{max}/(1-K_{Na}/K_K)^3}{(1+K_{Na}/[Na]_i(1+C/K_K)/(1-K_{Na}/K_K))^3}, \tag{7}$$

which is formally identical to eqn. (4) when $K_K > K_{Na}$. As the ratio K_{Na}/K_K is about 0·02, the terms containing this ratio may be neglected which yields the approximate equation

$$M \cong \frac{M'_{max}}{(1+K_{Na}/[Na]_i(1+C/K_K))^3}. \tag{8}$$

Since C = 150 mM, eqn. (8) validates the use of 150 mM-K as the intracellular K concentration corresponding to the Na efflux curve in the high K cells of the experiments in Fig. 8*a* and *b*.

The effect of intracellular K on M'_{max}. A rather unexpected finding of the experiments of Fig. 8*a* and *b* was that not only the apparent affinity for Na but also the M'_{max} for Na efflux depends on the intracellular concentration of K.

In Fig. 10 the M'_{max} values which fit the efflux curves for Na:K exchange (Fig. 8*a*) are plotted against the intracellular K concentration, together

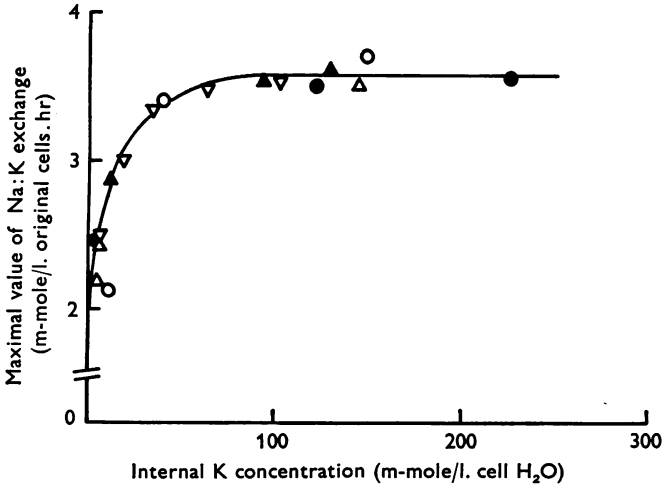

Fig. 10. A comparison of the effects of internal K concentration on the M'_{max} for Na:K exchange of the experiment in Fig. 8*a* (○) with the effects of internal K on the calculated M'_{max} of three experiments in which Na efflux was measured at a fixed internal Na concentration in cells in which different amounts of internal K were replaced by choline (△, ▽, ▲) and one experiment in which internal K was changed at the expense of internal Na (●). In these cases M'_{max} was calculated solving eqn. (4) for M'_{max} using eqn. (6) to calculate K'_{Na} and assuming K_{Na} = 0·19 and K_K = 9 mM (see Fig. 9).

with the M'_{max} values for Na efflux calculated from eqns. (4) and (6), for (i) three experiments in which choline replaced intracellular K and (ii) one experiment in which internal K was increased at the expense of internal Na. It is clear that as internal K is increased M'_{max} first rises and then, at about 30 mM internal K, reaches a steady value. Since this effect is seen both in choline-containing and in choline-free cells it is unlikely to be caused by choline or by the difference in volume between choline and Na-containing cells. The lack of effect on M'_{max} of K concentrations higher than 30 mM explains why most of the efflux curve for Na:K exchange follows an equation with constant M'_{max} (see Fig. 5).

Fig. 11 shows the M'_{max} values which fit the efflux curves for Na:Na exchange (Fig. 8*b*) plotted against the intracellular K concentration, together with the M'_{max} calculated from eqns. (4) and (6) for two experiments in which Na efflux was measured at a fixed internal Na concentration at four different levels of internal K. It is clear that for the whole range of K concentrations tested (5–190 mM) M'_{max} appears to be a linear increasing function of intracellular K concentration. The dependence of the M'_{max} of Na:Na exchange on internal K raises the question: does the inhibition of

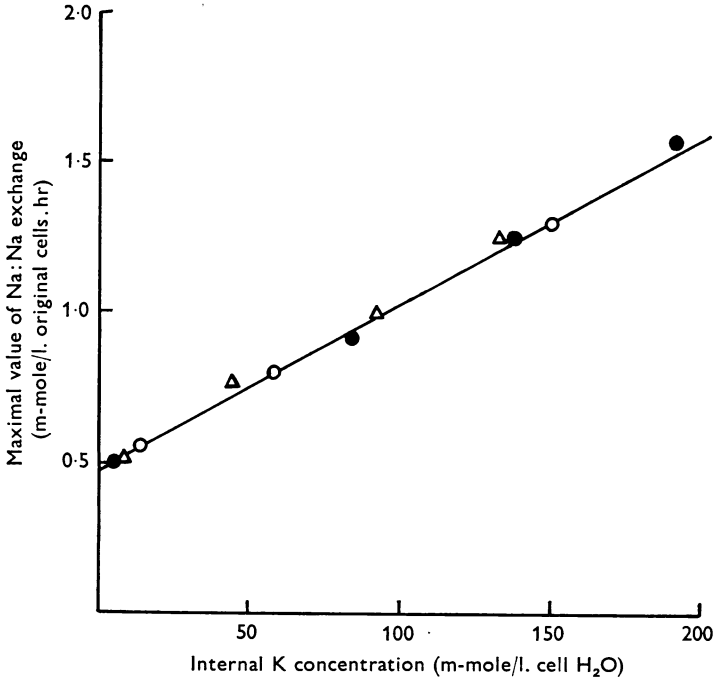

Fig. 11. A comparison of the effects of internal K concentration on the M'_{max} for Na:Na exchange from the experiment in Fig. 8*b* (○) with the effects of internal K on the M'_{max} calculated, as in the experiments of Fig. 10, for two experiments in which Na efflux was measured at fixed internal Na concentration in cells in which different amounts of internal K were replaced by choline (△, ●). The continuous line has a slope of 0·006/hr.

Na:Na exchange which is observed when internal Na is increased at the expense of internal K represent an effect due to the decrease in internal K? To test this the M'_{max} values of the Na influx experiment of Fig. 2 were calculated for each internal K and Na concentration using eqns. (4) and (6) and plotted against internal K. Results in Fig. 12 show that also in this case the M'_{max} for Na:Na exchange appears to be a linear increasing function of internal K concentration with a slope of the same order of

magnitude as that of the experiments in Fig. 11, strongly suggesting that the inhibition of Na:Na exchange at high internal Na concentrations is in fact due to the reduction in internal K. This result, moreover, shows that the effect observed when K is replaced by choline is due to changes in the K concentration and not to the addition of choline or to the difference in volume between the choline and Na-containing cells.

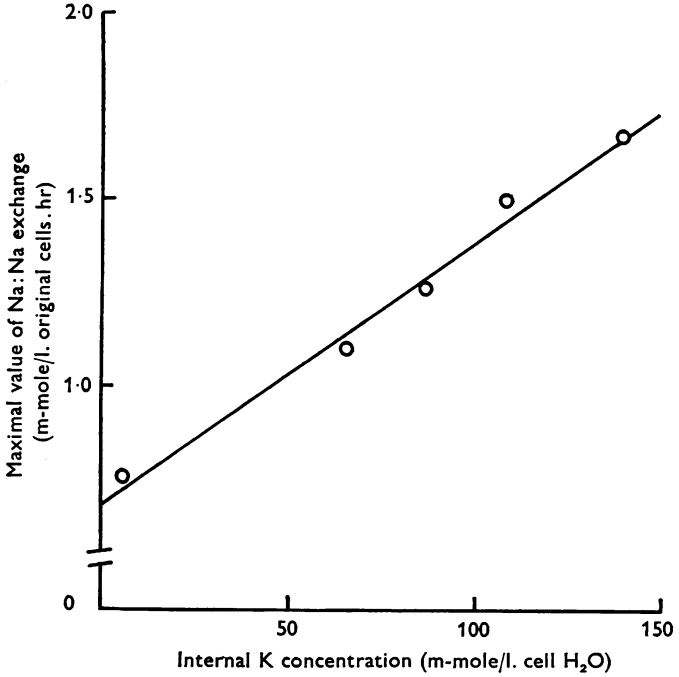

Fig. 12. The effects of internal K on the M'_{max} values of the ouabain-sensitive Na influx of Fig. 2. For each internal cation concentration M'_{max} was calculated as in the experiments of Fig. 10. The continuous line has a slope of 0·007/hr.

It seems therefore that to account for the effects of internal Na and K on the rate of Na:Na exchange the following equation has to be employed.

$$M = \frac{M'_{max_o} + k[K]_i}{(1 + K_{Na}/[Na]_i(1 + [K]_i/K_K))^3}, \tag{9}$$

where M'_{max_o} is M'_{max} when $[K]_i$ is zero. When $[Na]_i + [K]_i = C$ eqn. (9) can be rearranged to give

$$[Na]_i(M'_{max}{}^C - k[Na]_i)/M^{1/3} = [Na]_i + K'_{Na}, \tag{10}$$

where $M'_{max}{}^C$ is the value of M'_{max} when $[Na]_i$ tends to zero. Eqn. (10) was used to replot the Na flux vs. internal Na experiments of Figs. 1 and 2. Results showed that, when k values of the same order of magnitude than those in Fig. 11 were assumed, the experimental points could be fitted by straight lines of unit slope as predicted by eqn. (10).

The effect of internal K on the total Na efflux. As already shown in Figs. 4 and 5 at low internal Na concentrations the total Na efflux obeys the same rate equation as the ouabain-sensitive efflux. Fig. 13*a*, *b* shows that the K'_{Na} and M'_{max} values of the total efflux vary with internal K in the same fashion as the K'_{Na} and M'_{max} values of the ouabain-sensitive Na efflux (cf. Figs. 9–11).

The mechanism of the effect of internal K on M'_{max}. All the experiments in which the effect of internal K on the M'_{max} for Na:Na exchange was observed were performed in cells suspended in media containing 150 mM-Na. Since under these conditions external Na is rate limiting for Na:Na exchange (see Garrahan & Glynn, 1967*a* and Fig. 7 in this paper), the effect of internal K on M'_{max} could have been mediated by changes in either the apparent affinity for external Na or in the turnover rate of the Na pump. To elucidate this point the effect of external Na on the rate of Na:Na exchange was measured in cells containing either 10 or 279 mM intracellular K. Results in Fig. 14 make evident that in both cases the ouabain-sensitive Na efflux *vs.* external Na curves can be fitted by a three-site equation (see eqn. (4)) having the same apparent dissociation constant for external Na in spite of the twentyeight-fold difference in the intracellular K concentration. This result strongly suggests that the apparent affinity for external Na of the Na pump is independent of the internal K concentration and hence that the effect of intracellular K on the M'_{max} for Na:Na exchange is exerted on the turnover rate of the Na pump.

The interaction between the inner and outer sites of the Na pump

Na:K exchange. The effects of external Rb on the ouabain-sensitive Rb influx were studied in cells containing either 9·4 or 65 mM internal Na. Since it has been shown by Sachs & Welt (1967) that the shape of the influx curves of K or the K-like ions (Rb, Li and Cs) can be explained if influx is assumed to be proportional to the number of pump units having two sites occupied by these ions, results were plotted according to eqn. (5) assuming $n = 2$. Fig. 15 shows that the experimental points for both low and high Na cells can be fitted by straight lines which intersect on the base line. It seems therefore that a sevenfold increase in internal Na concentration only affects the M'_{max} for Rb influx leaving unaltered the apparent affinity for Rb of the external sites of the Na pump. This result agrees with the observations of Hoffman & Tosteson (1971) in sheep red cells and of Baker, Blaustein, Keynes, Manil, Shaw & Steinhart (1969) in squid axons who showed that the shapes of the curves relating K influx to external K are independent of the internal composition.

The ratio

$$\frac{M'_{max} \text{ for Rb influx into high Na cells}}{M'_{max} \text{ for Rb influx into low Na cells}}$$

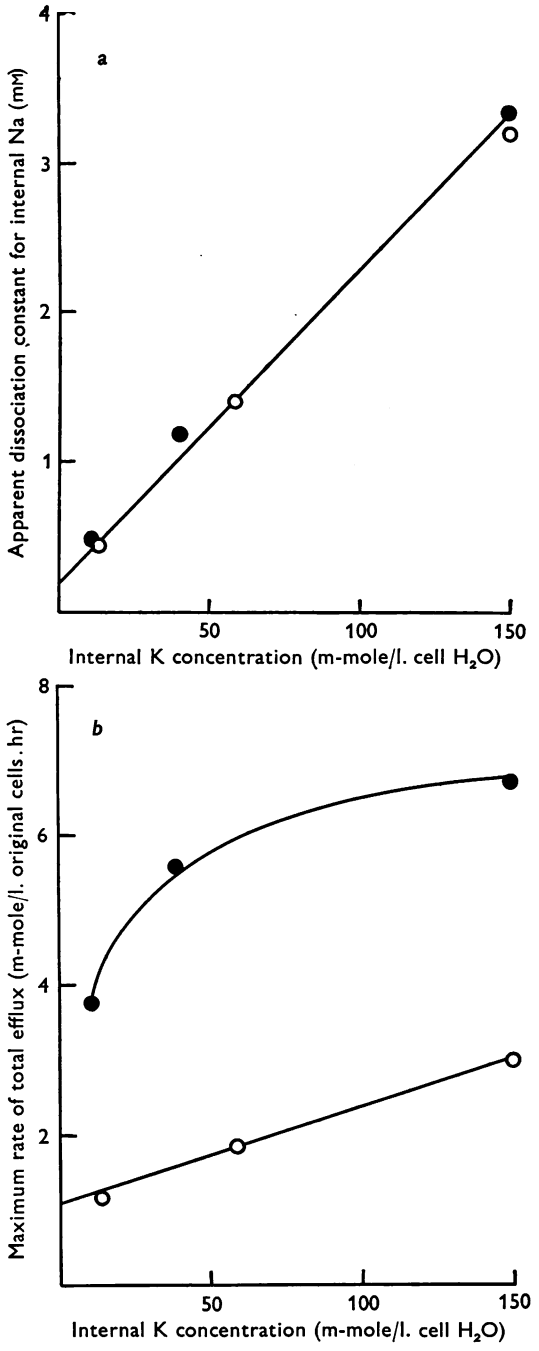

Fig. 13*a*, *b*. The effect of internal K concentration on the K'_{Na} (*a*) and M'_{max} (*b*) for the total Na efflux in the presence (●) and absence (○) of external K.

is 2·21, which is in close agreement with the ratio of the values of the ouabain-sensitive Na efflux at the same two internal Na concentrations (2·18) calculated from eqn. (7) taking $K_{Na} = 0{\cdot}19$ and $K_K = 9$ mM. The agreement between the theoretical and experimental values strongly suggests that the M'_{max} for Rb influx, and thus presumably for K influx,

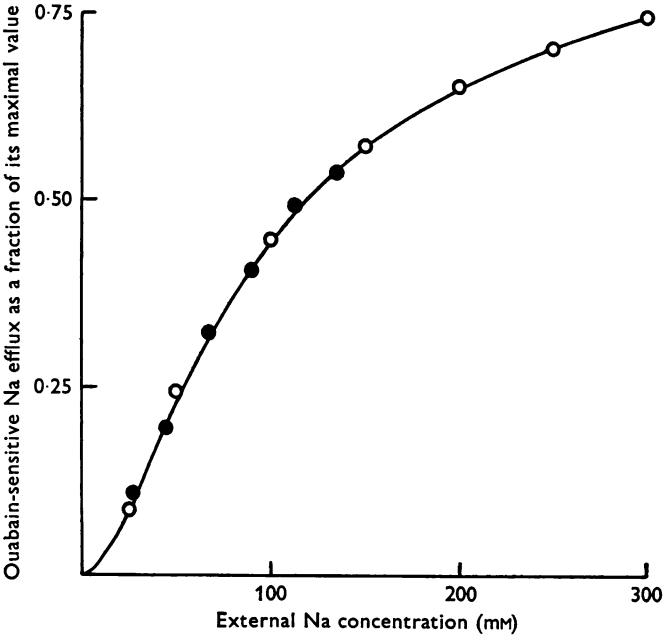

Fig. 14. A comparison between the effects of external Na on the ouabain-sensitive Na efflux into K-free media from red cells containing 10 m-mole K/l. cell water (●) and from cells containing 279 m-mole K/l. cell water (○). Each flux has been expressed as a fraction of its maximum value. The composition of the high K cells was that mentioned in Fig. 7. The low K cells contained 1·9 m-mole Na/l. cell water. Isotonicity inside these cells was maintained with choline. The continuous line represents the theoretical curve obtained using an equation similar to eqn. (4) with $n = 3$ and an apparent dissociation constant for external Na of 31 mM.

varies with internal Na in the same fashion as the ouabain-sensitive Na efflux. It seems therefore that the equations which describe Na efflux in exchange for external K as a function of *internal* cation concentration and the equations which describe K influx in exchange for internal Na as a function of *external* cation concentration can be combined into a product in the rate equation for Na:K exchange as a function of both intra- and extracellular cation concentrations, i.e.

$$M = \frac{M'_{max}}{(1+K'_{Na}/[Na]_i)^3(1+K'_K/[K]_e)^2}, \qquad (11)$$

where K'_{Na} and M'_{max} are functions of the internal K concentration (see Figs. 9 and 10) and K'_K is a function of external Na concentration (see Garrahan & Glynn, 1967*b*; Sachs, 1967).

Na:Na exchange. Cells loaded with four different Na concentrations were incubated in K-free media having four different levels of Na. Fig. 16*a* and *b* shows the ouabain-sensitive Na efflux plotted according to eqn. (5), as a function of either internal (Fig. 16*a*) or external (Fig. 16*b*) Na concentrations.

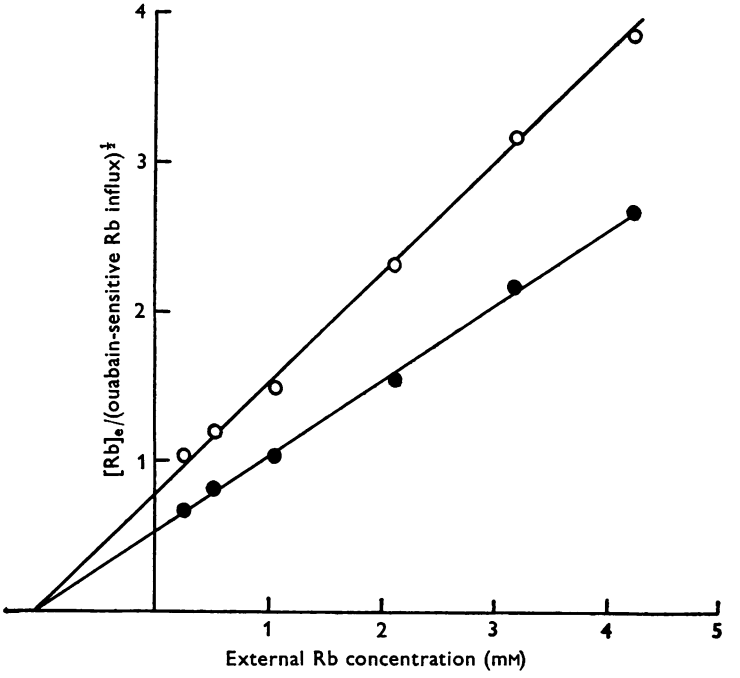

Fig. 15. The effect of the external concentration of Rb on the ouabain-sensitive influx of Rb into red cells containing either 9·4 (○) or 65 (●) m-mole Na/l. cell water. The results are plotted according to an equation similar to eqn. (5) but with external Rb as the variable.

The results show that:

(i) For each level of external Na the experimental points for Na efflux *vs.* internal Na concentration fit straight lines which cut the same point on the horizontal axis. Hence external Na affects the M'_{max} but not the apparent affinity for internal Na of the pump.

(ii) The same result is obtained when Na efflux is plotted against external Na for each level of internal Na. Hence internal Na only affects the M'_{max} but not the apparent affinity for external Na of the pump.

(iii) When the Na efflux at non-limiting external Na concentration,

calculated from the slopes of the graphs in Fig. 16*b*, is plotted against internal Na concentration, a straight line is obtained which cuts the horizontal axis at the same point as the rest of the Na efflux *vs.* internal Na curves (Fig. 16*a*). It seems therefore that the M'_{max} for Na efflux as a function of external Na depends on *internal* Na in the same fashion as the Na efflux does at any fixed external Na concentration.

(iv) Essentially similar results are obtained when the Na efflux at non-limiting internal Na, calculated from the slopes of the graphs in Fig. 16*a*, is plotted against external Na concentration (Fig. 16*b*). Hence the M'_{max} for Na efflux as a function of internal Na depends on *external* Na in the same fashion as the Na efflux does at any fixed internal Na concentration.

These results clearly show that the terms $1/(1+K'_{Na}/[Na])^3$ which adequately describe the kinetics of Na:Na exchange when Na concentration is kept constant at one of the surfaces of the cell membrane, can be combined into a product in the rate equation for Na:Na exchange as a function of both *intra-* and *extra*cellular Na, at constant intracellular K, i.e.

$$M = \frac{M_{max}}{(1+K'_{Na}/[Na]_i)^3(1+K''_{Na}/[Na]e)^3}, \qquad (12)$$

where M_{max} is proportional to the true turnover rate of the Na pump. Eqn. (12) reduces to eqn. (4) when Na is kept constant at one of the surfaces of the cell membrane.

Legend for Fig. 16

Fig. 16. (*a*) The effects of internal Na concentration on the ouabain-sensitive Na efflux into K-free media. The results are plotted according to eqn. (5) assuming $n = 3$. The efflux was measured in media containing 52 (○); 76 (●); 102 (△) and 141 (▲) mM external Na and compared with the calculated efflux values for non-limiting external Na concentration (▽). (*b*) The effects of external Na concentration on the ouabain-sensitive Na efflux into K-free media. The results are plotted according to an equation similar to eqn. (5) but with external Na as the variable, assuming $n = 3$. The efflux was measured in cells containing 1·15 (○), 2·70 (●), 5·39 (△) and 9·18 (▲) mM internal Na and compared with the calculated efflux values for non-limiting internal Na concentration (▽). The internal K concentration was 6·5 m-mole/l. cell water. Low internal K concentration was used in order to maximize the difference between the fluxes in the presence and absence of ouabain.

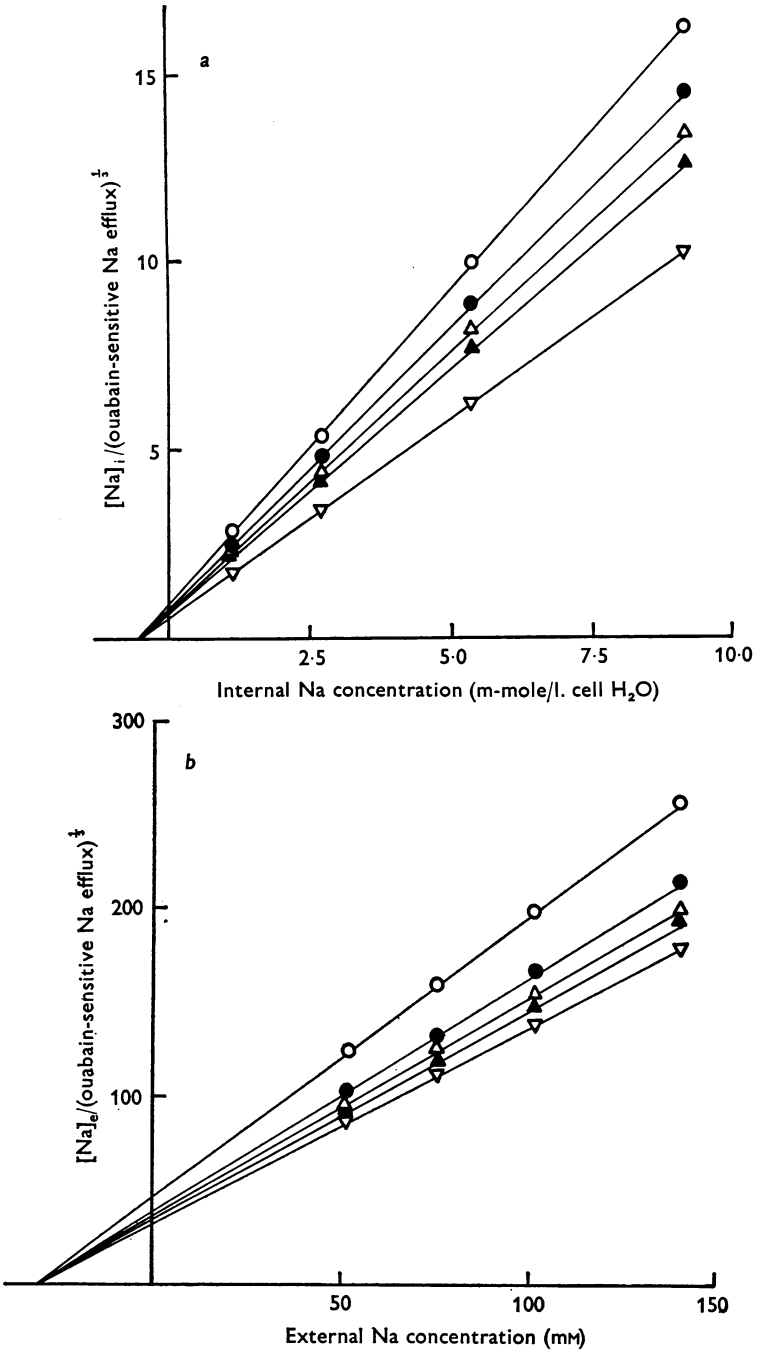

Fig. 16. For legend see opposite page.

DISCUSSION

The shape of the flux curves

The effects of intracellular Na and K. At high internal K concentrations the relation between Na efflux and internal Na concentration, both in the presence and in the absence of external K, is far removed from a rectangular hyperbola, the plot of Na efflux *vs.* internal Na concentration yielding an S-shaped curve (see also Sachs, 1970). As intracellular K is progressively replaced by choline the value of internal Na for which Na efflux is half maximal is shifted towards zero and the efflux curves become progressively less sigmoid. At any constant internal K, the effects of internal Na on the shape of the Na efflux curves can be fully and quantitatively accounted for if it is assumed that:

(i) Facing the inner surface of the cell membrane, each pump unit possesses three identical and non-interacting cation binding sites.

(ii) Each site in a pump unit may bind either Na or K but the apparent affinity for the formation of a Na-site complex is about 50 times larger than for the formation of a K-site complex.

(iii) Only those pump units with their three sites occupied by Na ions are able to promote Na translocation at a measurable rate.

(iv) There is no interaction between pump units. This statement implies that the rate of ion translocation will be linearly dependent on the number of pump units having three sites occupied by Na ions.

(v) The rate of translocation is slow compared with the rate of formation of Na-pump complexes. Therefore the fraction of pump units in conditions to promote ion movements can be calculated on the basis of equilibrium considerations.

The assumption that three Na ions have to combine with a pump unit to induce ion translocation seems to rest on a sound physical basis since all the available experimental evidence indicates that about three Na ions are transported per pump cycle by the Na pump of red cells (Glynn, 1962; Sen & Post, 1964; Whittam & Ager, 1965; Garrahan & Glynn, 1967*d*). The stoicheiometry of the Na pump seems to be independent of the intra- or extracellular ionic composition. This fact validates the use of a three-site hypothesis even under conditions, such as low internal K concentrations, in which the experimental results could have been fitted by a Michaelis-like equation. The remaining assumption employed in the development of the rate equations for Na efflux are the simplest compatible with a three-site kinetics, even so the agreement between experiment and theory was excellent in the whole range of internal and external cation concentrations tested.

Since a kinetic scheme which does not imply interaction between cation

binding sites gives an accurate description of the shape of the Na efflux curves, it appears to be unnecessary, at this stage, to invoke indirect, allosteric, effects between binding sites (see Monod, Wyman & Changeux, 1965) to explain either the deviation of the Na efflux curves from simple Michaelis-like behaviour, or the effects of internal K on the shape of the Na efflux curves.

Though the above-mentioned kinetic model was mainly derived from the analysis of the ouabain-sensitive Na efflux curves, it must be stressed that its validity does not necessarily depend on the complete identification of such fluxes with the Na movements through the Na pump. In fact, all the kinetic properties of the ouabain-sensitive fluxes are reproduced by the total Na efflux when studied at Na concentrations low enough as to make negligible the contribution of the passive membrane permeability to the total efflux. It seems, therefore, that if there were a saturable component in the ouabain-resistant Na efflux (see Sachs (1970) and Figs. 1 and 3 in this paper), it should be sufficiently similar to the saturable component of the total efflux as to yield, when the difference between total and ouabain-resistant Na efflux is plotted against internal Na concentration, a function differing only in a constant factor from the saturable component of the total Na efflux.

The effects of external Na and K. The properties of the external sites for Na of the Na pump seem to be analogous to those of the inner sites, at least to the extent that the effects of external Na on the rate of Na:Na exchange can be satisfactorily explained assuming that they are due to the occupation by external Na of three identical and non-interacting sites on each pump unit. A three non-interacting site model but with sites of different affinity has been proposed by Baker *et al.* (1969) to explain the effects of external cations on the rate of Na:Na exchange in squid axons. The inner and outer sites of the Na pump differ markedly in their affinity for Na. If a three-site model is accepted, the apparent constant for the dissociation of Na from an external site (31 mM) is about 160 times larger than that for the dissociation of Na from an inner site (0·19 mM).

Though they have not been analysed in detail, the effects of external Rb on the rate of Rb uptake, and hence presumably the effects of external K on the rate of K uptake, seem to be adequately described on the assumption that two identical and non-interacting sites have to be occupied in a pump unit to induce Rb uptake (see also Sachs & Welt, 1967; Baker *et al.* 1969).

The effect of internal K on the M'_{max} for Na efflux

A rather unexpected result of the experiments reported in this paper was that replacement of internal K by choline not only affects the shape of the Na efflux curves, but also alters its value at non-limiting internal

Na concentrations. In contrast with the effect of K on the shape of the efflux curve, the effect of K on the M'_{max} for Na efflux depends on whether the Na pump is performing Na:Na or Na:K exchange. The M'_{max} for Na:K exchange increases with internal K following a curve which saturates at about 30 mM internal K, whereas the M'_{max} for Na:Na exchange increases linearly with internal K. There seems to be no clear-cut physical explanation for the mechanism of the effect of K on M'_{max}. Nevertheless several properties of this phenomenon seem worth pointing out, i.e. (i) the observed effect of K cannot be attributed to the intracellular accumulation of choline or to the concomitant changes in cell volume; (ii) the observed effect of K is not dependent on the identification of the ouabain-sensitive Na efflux, with the 'pump' fluxes, since a similar effect is exerted by K on the total Na efflux; (iii) as Na:K exchange was measured in the presence of non-limiting concentrations of external K, and we showed that internal K has no effect on the apparent affinity of the Na pump for external Na, the effect of K on the M'_{max} for Na efflux must be exerted on the turnover rate of the Na pump and not on its apparent affinity for external cations.

It is not yet possible, however, to decide whether the effect of K on the turnover rate of the Na pump is the direct result of its interaction with the Na pump, or if it is the indirect consequence of changes in the metabolic state of red cells brought about by their altered cationic composition.

The inhibition of Na:Na exchange at high internal Na. When internal Na is increased at the expense of internal K the rate of Na:Na exchange after passing through a maximum progresively decreases. This phenomenon was first reported by Garrahan & Glynn (1967*c*) and has been confirmed by the results presented in this paper.

The inhibition of Na:Na exchange at high internal Na concentration has usually been ascribed to the increase in Na concentration and all the hypothetical models that have been proposed to explain its mechanism are based on this assumption (Baker & Connelly, 1966; Garrahan & Glynn, 1967*e*; Stone, 1968; Baker *et al.* 1969; Glynn, Hoffman & Lew, 1971). Observations reported in this paper strongly suggest that the decrease in the rate of Na:Na exchange at high internal Na concentrations is the result of the reduction in the turnover rate of the Na pump due to the decrease in the intracellular K concentration and not caused by a direct effect on the Na pump of high levels of internal Na.

The lack of interaction between the inner and outer sites of the Na pump

One of the interesting observations reported in this paper is the observation that the occupation of pump sites at one of the surfaces of the cell membrane apparently is independent of the nature of the ionic species which are occupying the pump sites at the opposite surface of the cell

membrane and of their degree of saturation. This assertion, which agrees with the observation by Hoffman & Tosteson (1971), in sheep red cells and of Baker *et al.* (1969) in squid axons, who showed that the shape of the K influx curve was independent of the intracellular cationic concentration, is based on the following facts:

(i) At any internal K concentration, the curves relating Na efflux to internal Na concentration measured in K-free media, differ only in a constant factor from those measured in media containing non-limiting concentrations of K.

(ii) Similar results are obtained when Na efflux curves are measured in K-free media additioned with different concentrations of Na.

(iii) Likewise, changes in the intracellular level of Na and/or K only alter by a constant factor the curves relating Na efflux to external Na concentration and the curves relating Rb influx to external Rb concentration.

The independence between the effects of inner and outer cations strongly suggests that there are no interactions between the inner and outer sites of the Na pump. For this assertion to be true it is only necessary to assume that (as seems likely) active cation fluxes are some function of the number of pump units having the adequate degree of saturation as to elicit ion translocation. If this assumption is taken for granted it is clear that to prove the lack of interaction, it will be sufficient to show, as we have done, that when expressed as a fraction of their maximum values, the flux curves become invariant to changes in the cation composition at the opposite surface of the cell membrane. This kind of demonstration is therefore independent of the physical assumptions that were employed to develop our rate equations, since the only demand it places on them is that they should provide an adequate *empirical* description of the shape of the flux curves.

The lack of interaction between the inner and outer sites of the Na pump implies that the over-all rate equations for Na:Na or Na:K exchanges must be of the form

$$M/M_{\max} = X([\mathrm{Na}]_i,[\mathrm{K}]_i)\,Y([\mathrm{Na}]_e,[\mathrm{K}]_e), \qquad (13)$$

where i and e mean intra- and extracellular respectively and $M_{\max}$ is the flux at non-limiting internal and external cation concentration. A similar equation has been proposed by Hoffman & Tosteson (1971) to explain the kinetic properties of the Na pump in sheep red cells. We have shown in this paper that the shape of the flux curves can be quantitatively accounted for if X and Y are taken as *equal* to the fraction of pump units having n identical and non-interacting sites occupied by the relevant cation, where the numerical value of n agrees with what is known of the stoicheiometry

of the Na pump. As the above-mentioned fractions can be equated to probabilities and the product of the probability of two independent events is equal to the probability of the simultaneous occurrence of such events, it follows that in our case the product of X and Y in eqn. (13) must be equal to the fraction of pump units having both its inner and outer sites occupied by the relevant cations. This strongly suggests that, as already proposed by Hoffman & Tosteson (1971) for sheep red cells, active ion translocation in human red cells is the consequence of the *simultaneous* reaction of inner and outer cations with a pump unit (see also Baker & Stone (1966) and Skou, 1971). It would seem, therefore, that sequential models for active transport (see, for instance, Shaw, 1954, Garrahan & Glynn, 1967*a*, Stone, 1968 and Caldwell, 1970), do not provide an adequate explanation for the molecular mechanism of the Na pump in red cells, since in these models inner and outer sites do not exist at the same time on the same pump unit.

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