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# THE EFFLUX OF SODIUM FROM HUMAN RED BLOOD CELLS

Y. EILAM and W. D. STEIN

*Institute of Life Sciences, The Hebrew University, Jerusalem (Israel)*

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## SUMMARY

1. We have studied the efflux of $Na^+$ from human red blood cells loaded with different concentrations of $Na^+$, osmolarity being maintained by either $K^+$, choline ions or $Mg^{2+}$. The external medium was buffered $MgCl_2$, with or without the addition of KCl, but with no $Na^+$.

2. With $K^+$ or choline internally, and with $K^+$ externally, the efflux of $Na^+$ attained saturation at relatively low levels of internal $Na^+$, the half saturation concentration being 15–25 mM. In contrast, when only negligible concentrations of $K^+$ were present externally there was no clear evidence for saturation of the $Na^+$ efflux in the concentration range studied (up to 140 mM). About half of this flux was ouabain sensitive and this component similarly did not saturate.

3. The active transport of $K^+$ was inhibited in cells loaded with concentrations of $Mg^{2+}$ above 30 mM. In such cells the efflux of $Na^+$ did not saturate in the range of $Na^+$ concentrations studied, both when $K^+$ was added externally or in its absence. $K^+$ did stimulate a one for one exchange of $Na^+$ with $K^+$ and this stimulated efflux sensitive to ouabain, and also did not saturate in the concentration of $Na^+$ studied.

4. It would appear, therefore, that under conditions in which pumping can occur, $Na^+$ efflux is mediated through a high-affinity site while if pumping is prevented, mediated $Na^+$ effluxes can occur on a low-affinity site. This low-affinity site on the inner face of the membrane is nevertheless part of the $Na^+$ transport system (as indicated by its sensitivity to ouabain) but is only revealed when pumping is prevented.

5. Our results do not contradict the recently proposed internal transfer model for the $(Na^+–K^+)$-ATPase which predicts that zero trans $Na^+$ efflux experiments should reveal the presence of a low-affinity site for $Na^+$ at the inner face of the cell membrane, in contrast to the high-affinity site seen under pumping conditions.

## INTRODUCTION

When $K^+$ is present in the external medium, human red blood cells expel $Na^+$ and take up $K^+$, both ions moving against their respective electrochemical gradients[1,2]. The source of energy for this ion pumping is the hydrolysis of ATP. $K_m$, the half-saturation concentration for $Na^+$ transported by this system at the inner face of

Abbreviation: PCMBS, *p*-chloromercuribenzene sulfonic acid.

the membrane, is some 20 mM (refs 3 and 4). When $Na^+$ but not $K^+$ is present in the external solution, the same system[5] catalyses the exchange of $Na^+$ across the cell membrane[4,6]. The kinetics of $Na^+$ efflux under exchange conditions as a function of internal $Na^+$ concentration are complex. The efflux appears to be composed of a component which becomes saturated at a relatively low concentration of $Na^+$ and of a component that is not saturated in the concentration range studied.

When neither $Na^+$ nor $K^+$ is present in the external solution (the "zero trans" condition) there is a net movement of $Na^+$ out of the cell. Little is known about this process. Sachs[4] measured the zero trans efflux from cells loaded with different proportions of $Na^+$ and $K^+$ (in the range from 3–30 mmoles $Na^+$ per l red cells). The kinetics of $Na^+$ efflux as a function of the internal $Na^+$ concentration suggested that $Na^+$ here was being transported by a system having a low maximum velocity and a low $K_m$, the latter similar in magnitude to the site found under pumping conditions. This result, if valid, would be in direct contradiction to the prediction of the recently-proposed internal transfer model[7] for $Na^+$ pumping, where a low affinity site should operate under zero trans conditions (see ref. 15). However, in a "zero trans" experiment, the external medium being very low in $Na^+$, the pumping system is activated by low levels of external $K^+$, $K^+$ influx on the pump being half maximal in these conditions at 0.14–0.18 mM (ref. 8) (with as little as 5 mM $Na^+$ outside, this $K_m$ increases 10-fold). Thus the possibility arises that traces of $K^+$ in the external medium in allegedly "zero trans" conditions ($K^+$ having possibly leaked out from cells loaded to high levels of $K^+$) may activate the pump and hence bring about the appearance of a site having the low $K_m$ characteristic of the pumping system.

It seemed desirable, therefore, to establish the existence of zero trans $Na^+$ efflux under conditions when the activity of the pump is suppressed, and to determine the nature of this efflux.

## METHODS

Fresh human venous blood was collected in citrate. The blood was centrifuged and the plasma and buffy coat removed. The cells were then washed 3 times with isosmotic $MgCl_2$ solution (107 mM).

### (*a*) *Loading procedure*

Loading the cells with different concentrations of cations was essentially by the method of Garrahan and Rega[9] as modified by Sachs[4]. Cells at 5–7% hematocrit were incubated for 18 h at 2–4 °C in 3.4 mM phosphate buffer ($NaH_2PO_4$–$Na_2HPO_4$) of pH 7.4, 1 mM $MgCl_2$, 20 mM glucose, 0.167 mM *p*-chloromercuribenzene sulfonic acid (PCMBS), together with various concentrations of the required cations, to a total osmolarity of 295 mosM. The following combinations of ions were used: NaCl–KCl (Fig. 1), NaCl–choline chloride (Figs 2, 3), NaCl–$MgCl_2$ (Fig. 5, Tables III and IV), NaCl–KCl–$MgCl_2$ and NaCl–KCl–choline chloride (Table II).

### (*b*) *Sealing procedure*

Loaded cells were centrifuged at 0 °C and resuspended (hematocrit, 10–14%) in: 3.4 mM phosphate buffer (pH 7.4); 20 mM glucose, 1 mM $MgCl_2$, 2 mM dithiothreitol, 3 mM adenine, and 10 mM inosine, together with the same concentration

of ions as in the loading solutions. The suspensions were incubated at 37 °C with gentle shaking for 1.5 h. Cells were then centrifuged down and washed 3 times with cold 107 mM $MgCl_2$ solution and once with the appropriate "external" medium, which was either 30 mM Tris–HCl buffer (pH 7.4) and 92 mM $MgCl_2$, or 30 mM Tris, 81 mM $MgCl_2$ and 16 mM KCl (with or without 0.1 mM ouabain), and finally resuspended at 30–50% hematocrit in the same cold external medium. These loaded cells served to measure the rate of efflux and to determine the concentration of cations within the cells.

*(c) Measurement of rate of efflux*

2 or 3 ml loaded cells were pipetted into 50 ml of prewarmed (37 °C) "external" solution, mixed and subdivided into four centrifuge tubes, leaving a fifth sample to determine hemoglobin. Two tubes were immediately centrifuged to determine the zero time concentration of $Na^+$ in the supernatant, and two incubated with gentle shaking at 37 °C for the required experimental time before centrifugation. All centrifugations were in a precooled Sorvall centrifuge at 10000 rev./min, for 10 s after the centrifuge reached the final speed. Part of the supernatant was removed and used later to determine the $Na^+$ and $K^+$ concentrations (using an Eppendorf flame photometer) and to determine the concentration of hemoglobin in order to correct for hemolysis. The amount of $Na^+$ liberated by hemolysis during the experiment was estimated by measuring the absorbance of the supernatant of the samples (zero time and the final time) at 420 nm and comparing the difference with the absorbance and concentration of $Na^+$ in a similar sample of loaded cells subjected to complete hemolysis. The correction never exceeded 5%. The difference between the amount of $Na^+$ in the supernatant at the final time and zero time, after correction for hemolysis, was used to calculate the rate of $Na^+$ efflux per l red cells at isotonicity. The concentration of cells in a sample was measured by determining hemoglobin in the fifth sample (see above) and the relation between the amount of hemoglobin and the volume of cells at isotonicity was determined in a separate experiment. We used plastic tubes, washed several times with distilled water to remove traces of $Na^+$, in all stages in the $Na^+$ determination.

In several early experiments efflux was measured at 30-min intervals up to 90 min. Efflux was linear with time during this period. The duration of the experiment was normally 60 or 45 min so the rates reported can be regarded as initial rates.

*(d) Measurement of the concentration of cations within the cells*

Samples of 0.1 ml from the loaded cells were hemolysed in 50 ml of distilled water, the membranes removed by centrifugation and the concentration of $Na^+$ and $K^+$ determined in the supernatant. The cell volume ratio was determined by drying and weighing samples of cells and measuring the hematocrit of a parallel sample. In some experiments, especially in cells loaded with high $Mg^{2+}$, the cells shrank somewhat after loading. Therefore, it was important to measure cell water to cell volume ratio in each sample. The concentration of cations in the cells was calculated per unit cell water.

*(e) $K^+$ influx*

In several experiments $K^+$ influx was determined simultaneously with $Na^+$

efflux. The experiment proceeded as in (c) but after removing the supernatant in order to determine the $Na^+$ concentrations, the cells in the pellets were now washed 3 times with isotonic $MgCl_2$ solution and samples from the pellets were hemolysed in distilled water. The membranes were precipitated, the concentration of $K^+$ determined by a flame photometer and the concentration of hemoglobin by absorbance. $K^+$ influx was calculated as the difference in the concentration of $K^+$ per l red cells at zero time and at the final time.

To check the validity of the loading procedure, we compared the rate of efflux from loaded cells with fresh cells at the same $Na^+$ concentration. We did not detect any difference.

*Materials*

Choline chloride was recrystallized before use as described by Garrahan and Glynn[6]. All other reagents were "Analar".

## RESULTS

*(A) Experiments on cells loaded with NaCl and KCl*

This first group of experiments was designed to be as close as possible to the conditions of Sachs[4]. Thus we measured the efflux of $Na^+$ from cells loaded with mixtures of NaCl and KCl. In the upper curve of Fig. 1, KCl was present externally to a concentration of 16 mM. In the lower curve no $K^+$ was added externally. In both cases buffered $MgCl_2$ served to maintain the osmolarity of the external medium. The efflux of $Na^+$ in the presence of added external $K^+$ is saturable and apparently sigmoidal. To take account of the apparent co-operativity here, with respect to internal $Na^+$, the data were analysed (Table I) by plotting $S^2/v$ against $S^2$, rather than using the first power of $S$. ($S$ is the concentration of substrate within the cell, $v$ the rate of efflux of $Na^+$.) The data fell on a straight line and a linear regression of this plot gave a half-saturation concentration of 27 mM and a maximum velocity of 7.3 mmoles/l red cells per h. The data from an experiment of Sachs (Fig. 5 in ref. 4), similar to our experiment but with 128 mM $Na^+$ externally in place of $Mg^{2+}$ (and 16 mM KCl) gave by a linear regression of the plot of $S^2/v$ against $S^2$ (our analysis) a half-saturation concentration of 21 mM and a maximum velocity of 11 mmoles/l red cells per h (Table I).

The lower curve of Fig. 1 shows the efflux of $Na^+$ in the absence of added external $K^+$ (a "zero trans" experiment). This experiment is similar to that performed by Sachs (Fig. 7 in ref. 4) but covers a wider concentration range. The plot of $S^2/v$ against $S^2$ gives by linear regression (Table I) a half-saturation concentration of 38 mM and a maximum velocity of 3.8 mmoles/l red cells per h. Our similar analysis of the data of Sachs (Fig. 7 in ref. 4) gives a half saturation concentration of 12 mM and a maximum velocity of 1.6 mmoles/l red cells per h. We determined the concentration of $K^+$ in the external medium at the end of our "zero trans" experiment and found values ranging from 0.028–0.089 mM. In contrast Sachs reported external $K^+$ concentrations of 0.01 mM although it is not clear whether these were the initial or final concentrations of $K^+$. The varying external $K^+$ in our experiments must have arisen as a result of leakage of $K^+$ during the course of the experiment from these cells, loaded as they were with high and different concentrations of $K^+$. In the next section we describe experiments designed to overcome this difficulty.

TABLE I

KINETIC PARAMETERS OF $Na^+$ EFFLUX

The parameters were obtained by linear regression of $S/v$ against $S$ or $S^2/v$ against $S^2$ as indicated. When the plot of $S/v$ against $S$ showed clearly an upward curvature at low substrate concentration (suggesting co-operativity) the kinetic parameters were calculated from a plot of $S^2/v$ against $S^2$. When the plot of $S/v$ against $S$ was apparently linear, the parameters were calculated from the simpler form of $S/v$ against $S$. (In some cases an analysis according to $S^2/v$ against $S^2$ was added for comparison.)

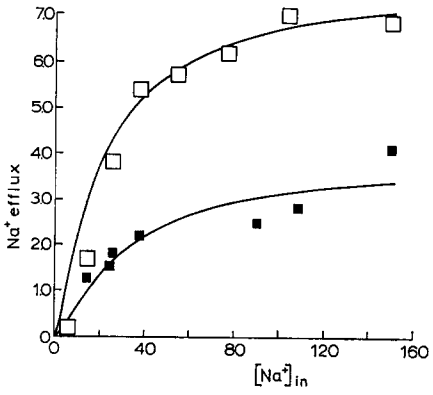
| *Data taken from* | *Cations within the cells* | *Cations in external medium* | *Data plotted as* | *Slope** | *Intercept** | *Derived $K_m$**** | *Derived $V$*** |
|---|---|---|---|---|---|---|---|
| Sachs, Fig. 5 in ref. 4 | $Na^+$, $K^+$ | 128 mM NaCl 16 mM KCl | $S^2/v$ against $S^2$ | 0.088 ± 0.0003 (7) | 38.2 ± 2.8 (7) | 20.8 ± 1.5 | 11.3 ± 0.8 |
| Fig. 1, upper curve | $Na^+$, $K^+$ | 16 mM KCl, buffered $MgCl_2$ | $S^2/v$ against $S^2$ | 0.138 ± 0.001 (8) | 97.2 ± 6.8 (8) | 26.6 ± 1.9 | 7.3 ± 0.5 |
| Fig. 2, upper curve | $Na^+$, choline | 16 mM KCl, buffered $MgCl_2$ | $S^2/v$ against $S^2$ | 0.149 ± 0.001 (6) | 32.8 ± 3.6 (6) | 14.8 ± 0.5 | 6.7 ± 0.7 |
| As above, Fig. 2 | As above | As above | $S/v$ against $S$ | 0.13 ± 0.003 (6) | 1.94 ± 0.14 (6) | 15.0 ± 1.1 | 7.7 ± 0.5 |
| Fig. 3, upper curve | $Na^+$, choline | 16 mM KCl, buffered $MgCl_2$ | $S/v$ against $S$ | 0.148 ± 0.003 (5) | 1.58 ± 0.18 (5) | 10.7 ± 1.3 | 6.8 ± 0.8 |
| Sachs†, Fig. 7 in ref. 4 | $Na^+$, $K^+$ | Buffered $MgCl_2$ | $S^2/v$ against $S^2$ | 0.62 ± 0.01 (5) | 86.9 ± 9.3 (5) | 12.0 ± 1.3 | 1.6 ± 0.2 |
| Fig. 1 lower curve | $Na^+$ $K^+$ | Buffered $MgCl_2$ up to 0.09 mM KCl | $S^2/v$ against $S^2$ | 0.263 ± 0.009 (7) | 389 ± 96 (7) | 38.4 ± 9.5 | 3.8 ± 0.9 |
| As above | As above | As above | $S/v$ against $S$ | 0.23 ± 0.01 (7) | 9.62 ± 0.96 (7) | 42.2 ± 4.8 | 4.4 ± 0.4 |
| Fig. 2 lower curve | $Na^+$, choline | Buffered $MgCl_2$ 0.01 mM KCl | $S/v$ against $S$ | 0.15 ± 0.01 (16) | 21.8 ± 0.8 (16) | 143 ± 11 | 6.5 ± 0.2 |

* Standard error (number of duplicate observations).

** The fractional error in $v$ is taken as the fractional error in the intercept.

*** The fractional error in $K_m$ is obtained as the square root of the sum of the squares of the fractional error of the slope and the intercept.

† In contrast to the other experiments listed in the table the internal concentration of $Na^+$ here did not exceed 43 mmoles $Na^+$ per l cell water.



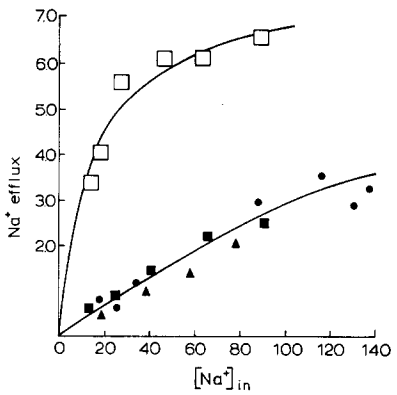




Fig. 1. Rate of $Na^+$ efflux, $v$, in mmoles/l red cells per h, *versus* internal $Na^+$ concentration $S$, in mmoles/l cell water. Cells are loaded with $Na^+$ as indicated in the figure with $K^+$ added to the loading solution to maintain osmolarity. External medium was: zero trans medium (■) 30 mM Tris–HCl buffer (pH 7.4) and 92 mM $MgCl_2$; $K^+$ containing medium (□), 30 mM Tris–HCl buffer (pH 7.4), 81 mM $MgCl_2$ and 16 mM KCl. Each point is the mean of duplicates. The mean deviation between duplicate samples was ±2.6%, the maximal deviation being ±5.0%.

Fig. 2. As Fig. 1, but cells are loaded with $Na^+$ and choline. Solid symbols, various experiments in zero trans medium; open symbols, an experiment in $K^+$ containing medium. The mean deviation between duplicate samples was ±3.0%, the maximal deviation being ±7.3%.

*(B) Experiments on cells loaded with NaCl and choline chloride*

In order to minimize the leakage of $K^+$ into the external medium, we substituted choline for the internal $K^+$. Thus cells were loaded (as described in Methods) with different proportions of NaCl and choline chloride, the choline being added to maintain a constant osmolarity. Cells obtained after loading contained only about 10 mM $K^+$. The concentration of $K^+$ in the external solution at the end of a zero trans experiment was now only 0.01 mM.

The first set of experiments was designed to test whether the presence of choline chloride inside the cell significantly alters the activity of the transport system. Fig. 2, upper curve, shows the efflux of $Na^+$ from cells loaded with different proportions of NaCl and choline chloride, when the external solution was buffered with $MgCl_2$ containing 16 mM KCl. In these experiments it was not possible to determine whether or not there is co-operativity between $Na^+$ since the very lowest $Na^+$ concentrations were not studied. Nevertheless, in order to compare these results with those of the previous figure, we have again analysed the data (Table I) on a plot of $S^2/v$ against $S^2$ as well as on the simple $S/v$ against $S$ form. The linear regression of $S^2/v$ against $S^2$ gives a half-saturation concentration of 15 mM and a maximum velocity of 6.7 mmoles/l red cells per h. It would appear, on comparing Figs 1 and 2, when $K^+$ is added externally, that choline does not alter greatly the activity of the pump (the effect of internal $K^+$ may well be to compete with $Na^+$ for efflux). Choline can then be used to reduce the problems caused by the presence of $K^+$ in the loading solutions.

Fig. 2 (lower curve) shows the results of the zero trans efflux of $Na^+$ as a function of internal $Na^+$ concentration. The $K^+$ concentration in the external solution ranged between 0.005 mM at zero time and 0.010 at the final time. As can be seen from the figure, the kinetics of efflux here are quite different from those in the presence

of external $K^+$ (upper curve), there being no clear evidence for saturation in the concentration range studied. We analysed the data according to the simplest model ($S/v$ against $S$), since there was no suggestion of co-operativity. A linear regression of this plot (Table I) gave a half-saturation concentration of 143 mM and a maximum velocity of 6.5 mmoles/l red cells per h. A similar analysis of the data of the upper curve gave a half-saturation concentration of 15 mM and a maximum velocity of 7.7 mmoles/l red cells per h. It must be emphasised that since the zero trans data are all below the half-saturation concentration, the numerical value for the parameters of this efflux should not be taken literally. It is clear, however, from the analyses collected in Table I that the kinetics of efflux here are quite different from the efflux in the presence of external $K^+$.

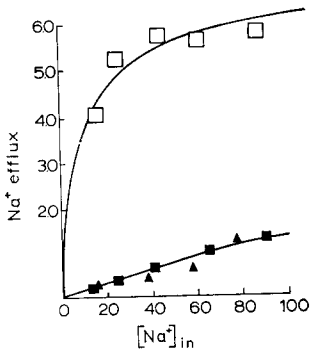



Fig. 3. Ouabain-sensitive component of $Na^+$ efflux. Conditions as in Fig. 2. For each experiment each point is the difference between the $Na^+$ efflux measured in the presence and absence of 0.1 mM ouabain on the same batch of loaded cells. The corresponding data in the absence of ouabain are given in Fig. 2, a consistent system of symbols being used.

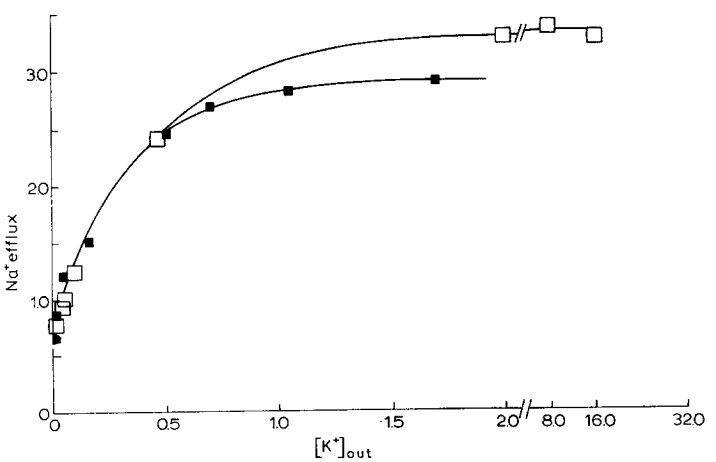



Fig. 4. Rate of $Na^+$ efflux (mmoles/l red blood cells per h) *versus* external $K^+$ (mM). Internal $Na^+$ is 14 mmoles/l cell water (□) and 12 mmoles/l cell water (■), both contained choline-chloride up to osmolarity. External solutions contained 30 mM Tris–HCl buffer (pH 7.4), indicated concentration of KCl, and $MgCl_2$ up to osmolarity. The mean deviation between duplicate samples was ±3.5%, the maximal deviation being ±9.4%.

Since the efflux of $Na^+$ under zero trans conditions for cells loaded with choline does not appear to saturate in the range of concentrations studied, it is difficult to assess how much of this flux is mediated by a transport system and how much is merely a diffusional leak. Therefore, we studied this efflux in the presence and absence of the cardiac glycoside ouabain. Fig. 3 shows the ouabain-sensitive component of the $Na^+$ efflux from cells loaded with $Na^+$ and choline, both in the presence and absence of added external $K^+$. The data in the presence and absence of ouabain were always obtained at the same time using the same batch of loaded cells. (Fig. 2 includes the points obtained in the absence of ouabain in these experiments). It is clear that in the presence of added external $K^+$, $Na^+$ efflux half-saturates at a relatively low level of internal $Na^+$ (some 10 mM), while in the absence of added $K^+$ the flux does not appear to saturate in the concentration range studied.

It seemed possible that the difference between the kinetics of $Na^+$ efflux under "zero trans" conditions in the experiments of Fig. 1 and those of Fig. 2 (Table I) was due to the presence of low levels of external $K^+$, arising by leakage from the $K^+$-loaded cells. To explore this possibility we loaded cells with $Na^+$ and choline, and measured the effect of added external $K^+$ on the rate of $Na^+$ efflux. The cells were loaded with a relatively low $Na^+$ concentration (12 or 14 mM) and the external solutions contained a range of $K^+$ concentrations. Fig. 4 shows the rate of $Na^+$ efflux as a function of external $K^+$ concentration. There is clearly a marked stimulation of $Na^+$ efflux by external $K^+$. To analyse the form of this stimulation we obtained an estimate of the $Na^+$ efflux in the absence of $K^+$ by drawing a straight line between the first three points in each curve and extrapolating this line to zero external $K^+$. The "no $K^+$" values obtained in this way were 0.64 mmole/l red cells per h for cells loaded with 14 mM $Na^+$ and 0.57 mmole/l red cells per h for cells loaded with 12 mM $Na^+$. We then subtracted these values from the total $Na^+$ efflux at each value of external $K^+$ and obtained an estimate of $\Delta v$, that component of sodium efflux due to activation by external potassium. The reciprocal plots of these derived values ($S/\Delta v$ against $S$, where $S$ is now the concentration $K^+$) yielded straight lines, $K_m$ values of 0.19 and 0.22, in the two experiments, being derived by a linear regression. These values for $K_m$ are close to the value of 0.14–0.19 mM reported by Garrahan and Glynn[8] for the effect of external $K^+$ on $K^+$ influx, when $Na^+$ is absent from the external solution. It is thus reasonable to assume that the efflux of $Na^+$ brought about by external $K^+$ occurs by the transport system working in its pumping mode, and that the difference between the results of Figs 1 and 2 can be explained by the difference between the levels of external $K^+$.

*(C) Experiments on cells loaded with NaCl and $MgCl_2$*

To gain further information about the system we have in addition investigated the effect of substituting $Mg^{2+}$ for the internal $K^+$. There are some indications that the rate of ATP hydrolysis by microsomal ATPases is reduced at higher levels of $Mg^{2+}$ (refs 10 and 11). It was shown in our laboratory that the rate of ATP hydrolysis by microsomal ATPase decreased when the concentration of $Mg^{2+}$ in the medium was raised between 5 and 30 mM but that the rate remained stable at this low level when the concentration of $Mg^{2+}$ was increased further in the range 30–100 mM (Tolkovsky, A., personal communication). It seemed possible, therefore, that in cells loaded with a high concentration of $Mg^{2+}$, the activity of the pump might be inhibited, but that the zero trans efflux might proceed normally.

TABLE II

NET $K^+$ UPHILL INFLUX IN CELLS LOADED WITH DIFFERENT CATIONS

$Mg^{2+}$ or choline as indicated were added to maintain internal osmolarity. External osmolarity was balanced by the addition of 30 mM Tris and $MgCl_2$.

| *Cations in cells* | *$K^+$ concn* | | *$Na^+$ concn* | | *Net $K^+$ influx into the cells (mmoles/l red cells per h)* |
|---|---|---|---|---|---|
| | *In (mmoles/l cell water)* | *Out (mM)* | *In (mmoles/l cell water)* | *Out (mM)* | |
| $Na^+$, $K^+$, $Mg^{2+}$ | 40.6 | 28.7 | 35.1 | 26.5 | 1.3 |
| $Na^+$, $K^+$, choline | 59.1 | 45.0 | 24.9 | 25 | 6.4 |
| $Na^+$, $K^+$ | 51.0 | 34.0 | 92.7 | 113.4 | 4.1 |

To test whether high $Mg^{2+}$ levels inhibited pump function, we proceeded as follows: Cells were loaded with the following cations: $Na^+$, $K^+$, $Mg^{2+}$; $Na^+$, $K^+$, choline; $Na^+$, $K^+$. The concentration of $K^+$ in the external medium was in each case below its concentration in the cells and we measured the net influx of $K^+$ against its concentration gradient. Table II shows that when cells are loaded with high $Mg^{2+}$, the active uptake of $K^+$ and hence presumably, the activity of the pump, is largely inhibited.

Fig. 5 shows the efflux of $Na^+$ from cells (loaded with different proportions of $Na^+$ and $Mg^{2+}$) in the absence and in the presence of 16 mM $K^+$ in the external medium. Under both conditions, the efflux of $Na^+$ does not appear to saturate in the range of internal $Na^+$ concentrations studied. The efflux in the absence of added

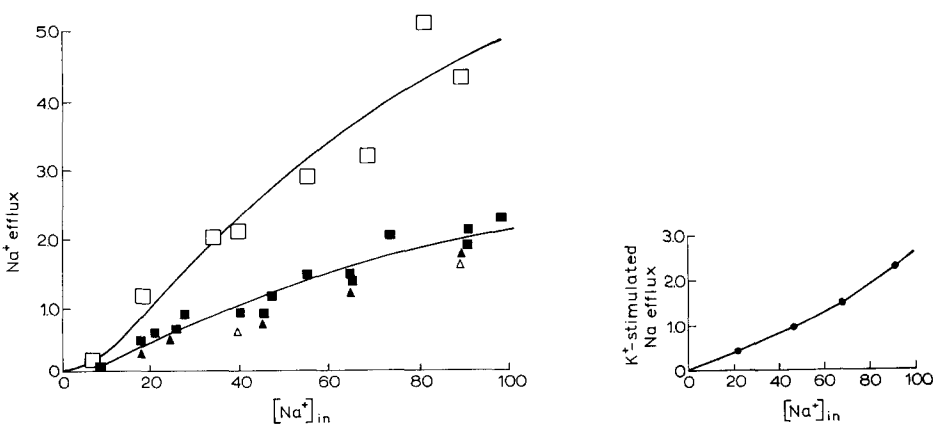



Fig. 5. As Fig. 1 but cells are loaded with NaCl and $MgCl_2$. Solid symbols: experiments in zero trans medium; open symbols: experiments in $K^+$ containing medium; squares: experiments in the absence of ouabain; triangles: experiments in the presence of 0.1 mM ouabain. The mean deviation between duplicate samples was $\pm 2.8\%$, the maximal deviation being $\pm 8.4\%$.

Fig. 6. $K^+$-sensitive component of $Na^+$ efflux from cells loaded with $Na^+$ and $Mg^{2+}$. Each point is the difference between the rate of $Na^+$ efflux in the presence and in the absence of 16 mM KCl in the external medium. Data are from Table II

$K^+$ is largely insensitive to the addition of ouabain. Therefore, it is difficult to assess how much of this flux is mediated and how much is merely due to a diffusional leak. That part of the flux which is stimulated by external $K^+$ is evidently mediated. To study this increment further, we loaded cells with different concentrations of NaCl and $MgCl_2$, split each sample into two parts and measured the efflux of $Na^+$ in the presence and absence of added $K^+$. The results of this experiment are recorded in Table III and the resulting $K^+$-stimulated efflux plotted in Fig. 6. It is clear from this figure that this flux does not saturate in the concentration range studied. This increment is, however, sensitive to ouabain (Fig. 5).

To study further the component of $Na^+$ efflux induced by external $K^+$, we performed the following experiments (Table IV): Cells were loaded with 40 and

TABLE III

RATE OF $Na^+$ EFFLUX FROM CELLS LOADED WITH HIGH $Mg^{2+}$ IN PRESENCE AND ABSENCE OF $K^+$ IN THE EXTERNAL MEDIUM

External solutions as in Fig. 5. The mean deviation between duplicate samples was $\pm 2.4\%$, the maximal deviation being $\pm 4.8\%$.

| *Concn of $Na^+$ in the cells (mmoles/l cell water)* | *Rate of $Na^+$ efflux (mmoles/l red cells per h)* | | *Percentage stimulation by $K^+$* |
|---|---|---|---|
| | *No $K^+$ in external medium* | *16 mM $K^+$ in external medium* | |
| 20.6 | 0.35 | 0.80 | 230 |
| 45.4 | 0.67 | 1.66 | 230 |
| 67.6 | 1.21 | 2.74 | 230 |
| 91.1 | 1.87 | 4.16 | 220 |

TABLE IV

RATES OF $Na^+$ EFFLUX AND $K^+$ INFLUX IN CELLS LOADED WITH HIGH $Mg^{2+}$

External solutions as in Fig. 5. The mean deviation between duplicate samples was $\pm 3.2\%$, the maximal deviation being $\pm 6.4\%$.

| | *Cells loaded with 40.6 mmoles/l cell water, $Na^+$* | *Cells loaded with 90.6 mmoles/l cell water, $Na^+$* |
|---|---|---|
| Rate of $Na^+$ efflux (mmoles/l red cells/h) | | |
| (1) −ouabain, $-K^+$ | 0.90 | 1.82 |
| (2) −ouabain, $+K^+$ | 2.05 | 4.28 |
| (3) +ouabain, $+K^+$ | 0.65 | 1.51 |
| $Na^+$ efflux induced by $K^+$ | | |
| (2) − (1) | 1.15 | 2.46 |
| $K^+$ influx (mmoles/l red cells per h) | | |
| (2) −ouabain | 1.38 | 2.57 |
| (3) +ouabain | 0.04 | 0.03 |

90 mM $Na^+$, osmolarity being maintained by the addition of $Mg^{2+}$. The efflux of $Na^+$ was measured in the presence and in the absence of $K^+$ and the rates of $Na^+$ efflux induced by $K^+$ were determined by difference. In the same cells the rates of $K^+$ influx were measured. As Table V indicates, the amount of $Na^+$ efflux induced by $K^+$ is approximately equal to the concominant $K^+$ influx, both fluxes being inhibited by ouabain. Hence internal $Na^+$ is exchanged one for one with external $K^+$. It would appear from Fig. 6 that this exchange flux is mediated by a site saturating only at high levels of $Na^+$.

## DISCUSSION

The data reported in this work suggest the operation of two classes of sites in the efflux of $Na^+$ from the inner face of the membrane. In what follows we will, for convenience only, refer to a process demonstrating a low $K_m$ (some 15–20 mM) as occurring on a high affinity site, while the mediated fluxes which do not saturate in the concentration range studied will be designated as the low affinity site or sites.

There is now increasing evidence that a good part of the flux of cations under non-pumping conditions is mediated by a system which is insensitive to ouabain[12,13]. We are unable, however, to distinguish between such mediated transport and a diffusional leak, so most of our comments will refer only to the ouabain-sensitive fluxes.

The existence of a high affinity site for $Na^+$, at the inner face of the membrane, under pumping conditions has been shown previously[4], and is confirmed in our work (Figs 1 and 2). The existence of a low affinity site system for $Na^+$ inside the cell as a component of the efflux has not been shown before. Our data show (Fig. 6) that when most of the internal $K^+$ is replaced by $Mg^{2+}$, the ouabain-sensitive efflux in the presence of external $K^+$ occurs largely through a low affinity site. It could be argued that it is the high concentration of $Mg^{2+}$ inside the cells in these experiments that converts a true high affinity site into a site of apparently low affinity. The $Mg^{2+}$ might, for instance, be acting as a competitive inhibitor or allosterically. This may perhaps be the case. However, the experiments recorded in Figs 1, 2 and 3 suggest another possibility. With choline as the added internal cation, the high affinity site is seen normally when external $K^+$ is present (*cf.* upper curves of Figs 1 and 2), so the mere presence of internal choline does not cause the appearance of a low affinity site. (Indeed it could be argued from our data that the absence of $K^+$ removes a competitive effect on $Na^+$ efflux.) Yet a site of much lower affinity becomes operative when negligibly low levels of external $K^+$ are present (Fig. 3), and one might identify this site with the site seen at high levels of internal $Mg^{2+}$.

The analysis of the efflux data obtained under pumping conditions presents little difficulty. The upper curve in Fig. 2 is consistent with high affinity sites being involved in the $K^+$-linked $Na^+$ efflux. In contrast, the data on zero trans $Na^+$ efflux in the presence of internal choline and $Mg^{2+}$ (Figs 2 and 3 lower curves and Figs 5 and 6) are difficult to analyse since the range of concentrations of internal $Na^+$ available to us is limited by the necessity to maintain the physiological osmolarity. One cannot, therefore, obtain meaningful values for the half-saturation concentration and for the maximum velocity of transport through the low affinity site from our experiments.

It is clear, however, that these ouabain-sensitive components of the $Na^+$ effluxes are mediated by the $Na^+$ transport system operating under non-pumping conditions. (An unknown fraction of the ouabain-resistant efflux may also be mediated by this system). It would seem, therefore, that the low affinity site for $Na^+$ at the inner face of the membrane is a part of the transport system and is revealed under conditions in which pumping is prevented. With the switch to pumping conditions, a high affinity site for $Na^+$ is used for $Na^+$ efflux. These findings are strictly consistent with a very simple model for pumping in which a circulating carrier of low affinity for substrate in the absence of coupling to metabolic energy is converted to a high affinity form at the inner membrane surface, on setting up the coupling to metabolic energy. Our recently proposed internal transfer model for $Na^+$ and $K^+$ transport[7] requires similar affinity changes in the presence and absence of energy coupling. The present data are, therefore, consistent with our proposed model but make no distinction between it and the earlier models.

We cannot as yet interpret simply the results obtained in the presence of high $Mg^{2+}$ inside the cell on the basis of the internal transfer model. It is clear, however, that $Na^+/K^+$ exchange under these conditions is different from the $Na^+/Na^+$ exchange studied by previous workers[4], being mediated only through the low affinity site for $Na^+$.

Under non-pumping conditions, the internal transfer model for $Na^+/K^+$ system reduces to the model we have proposed for sugar transport in red cells[14]. It has been shown[15] that this tetramer model predicts a low affinity site for zero trans conditions and both high and low affinity sites under exchange conditions. Had a high affinity site been found to dominate the zero trans efflux this could have been sufficient to reject the internal transfer model.

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