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# NON-PUMPED SODIUM FLUXES IN HUMAN RED BLOOD CELLS EVIDENCE FOR FACILITATED DIFFUSION

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## SUMMARY

Unidirectional and net $Na^+$ fluxes modified by changes in internal $Na^+$ concentration ($[Na^+]_i$) were studied in human red blood cells incubated in $K^+$-free solutions containing $10^{-4}$ M ouabain. An increase in $[Na^+]_i$ brought about (a) a reduction in net $Na^+$ gain, (b) no change in $Na^+$ influx, (c) a reduction in the rate constant for $Na^+$ efflux and (d) an increase in $Na^+$ efflux. Similar reductions in net $Na^+$ gain were observed when the changes in $[Na^+]_i$ were carried out at constant $[K^+]_i$. In addition, the rate constant for $^{42}K^+$ efflux was not affected by changes in $[Na^+]_i$. The electrical membrane potential (as determined from the chloride distribution ratio) was also constant. Furosemide ($10^{-3}$ M) increased the net $Na^+$ gain in high $[Na^+]_i$ cells and had no effect on those with low $[Na^+]_i$. External $K^+$ at 50 mM concentration reduced $Na^+$ efflux and increased $Na^+$ influx: the magnitude of these effects was dependent on the intracellular $Na^+$. The reduction in net $Na^+$ gain as $[Na^+]_i$ increased was unaffected by depletion of cellular ATP to values below 10 $\mu$mol/l cells, and this effect was independent of the depletion method used.

## INTRODUCTION

The movement of $Na^+$ across the erythrocyte membrane falls into two main categories [1, 2], that which is inhibited by cardiac glycosides (generally referred to as ouabain-sensitive) and that which is not. For the ouabain-sensitive fluxes it has been established that with an external $K^+$ concentration ($[K^+]_o$) larger than 5 mM there is an exchange of internal $Na^+$ for external $K^+$, resulting in net fluxes of both ions against an electrochemical gradient (i.e. the $Na^+/K^+$ pump). In $K^+$-free solutions this switches to an $Na^+/Na^+$ exchange [3, 4]. There is agreement that these fluxes are all produced through the enzymatic mechanism of the $Na^+$ pump, either as a complete cycle ($Na^+/K^+$ exchange) or as a partial cycle ($Na^+/Na^+$ exchange) [3, 5].

Abbreviation: PCMBS, *p*-chloromercuribenzene sulfonate.

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The picture is not so clear with regard to the ouabain-insensitive fluxes. The portion of these fluxes which is inhibited by ethacrynic acid and furosemide has been considered as either active transport with the ability to produce net $Na^+$ transport against a gradient [6, 7], or as an exchange diffusion with an $Na^+/Na^+$ exchange on a 1:1 basis and no net movement of substrate in either direction [8, 9]. Finally, others [10] have considered the totally ouabain-insensitive $Na^+$ movements as a simple leak, producing net fluxes only in favor of a gradient.

According to the concepts discussed above, when red cells are incubated in $K^+$-free solutions containing ouabain, the following possibilities exist:

(a) The $Na^+$ pump is the only energy requiring mechanism for $Na^+/K^+$ translocation and ouabain inhibits it completely. In this case, one is left with only passive $Na^+$ and $K^+$ fluxes.

(b) The $Na^+$ pump is the only energy-requiring system, but ouabain does not inhibit it completely. One is then left with a combination of passive and residual active $Na^+$ and $K^+$ fluxes.

(c) Ouabain completely inhibits the $Na^+/K^+$ pump but there is another energy-requiring system for $Na^+$ translocation. Again, the remaining fluxes will be a combination of active and passive ones.

To analyse the passive mechanisms for $Na^+$ transport, all active components must be eliminated. For possibility (a) above, this is accomplished by ouabain treatment. For (b) and (c), the remaining pumping mechanism (either a second pump or a classical pump not completely inhibited by ouabain) could be eliminated by depleting the cells of their energy supply.

If all pumps are absent, the net $Na^+$ gain should behave like a simple leak: if it does not do so, there must be a component in the remaining passive fluxes which does not follow a 1:1 stoichiometry, implying either that a general type of facilitated diffusion system exists, or that the $Na^+$ movements through the membrane have some kind of $Na^+$-membrane interaction of a complex nature. In rat red blood cells there is experimental evidence suggesting the existence of a facilitated diffusion system for passive $Na^+$ translocation [11]. It was the aim of the present work to repeat and extend these earlier experiments using human red blood cells. A brief report of this work has already been made [12].

## METHODS

*Solutions.* All solutions were made with de-ionized water and reagent grade chemicals. Choline chloride was recrystallized from a hot ethanol solution. Glucose (11 mM), ouabain ($10^{-4}$ M) and furosemide ($10^{-3}$ M) were added as solid just before use. The general composition of the solutions was (mM): $MgCl_2$, 1; orthosphosphoric acid (titrated with Tris to give a pH of 7.4 at 37 °C), 2.5; NaCl+KCl, 150. When choline chloride was used as a main cation replacement it was added in an amount to match 150 mM. In the $(Na^+ + K^+)$-free $Mg^{2+}$ buffer solution the principal cations were replaced with 108 mM $MgCl_2$. All solutions were $Ca^{2+}$ free.

*Cation loading.* Changes in the internal cation composition were accomplished by using the method of Garrahan and Rega [13] as modified by Sachs [14]. In some cases (see below), the Nystatin method proposed by Cass and Dalmark [15] was selected. The cells were washed three times with $K^+$-free $Mg^{2+}$ buffer and incubated

at 50 % hematocrit in $K^+$-free $Na^+$ buffer (high-$Na^+$ cells) or in 150 mM $K^+$ buffer (low-$Na^+$ cells), either with 5 $\mu$Ci/ml suspension of $^{22}Na^+$ ($Na^+$ efflux cells) or without it ($Na^+$ influx and net $Na^+$ flux cells). After five additional washes in cold $K^+$-free $Mg^{2+}$ buffer, samples for initial radioactivity and cation content were taken. When $K^+$ efflux was to be measured, after resealing the cells were incubated for 3 h in $K^+$-free $Mg^{2+}$ buffer at about 50 % hematocrit and 37 °C with 10 $\mu$Ci $^{42}K^+$/ml suspension.

*Sodium and potassium fluxes.* $Na^+$ and $K^+$ fluxes and intracellular cations were determined as described elsewhere [11, 16]. For $Na^+$ influx and efflux, one single measurement was taken after 1 h incubation: for $Na^+$ net fluxes, the incubation lasted 1 h for low-$Na^+$ cells and 4 h for those high in $Na^+$. $K^+$ efflux was determined by 1 h incubation taking three points at 20 min intervals. To measure the cation content, the erythrocytes were washed with a ($K^+ + Na^+$)-free solution. After immersing the tubes for 5 min in ice cold water, the cells were washed three times with at least 10 vols of ice cold $K^+$-free $Mg^{2+}$ buffer containing $10^{-4}$ M ouabain. Although this method has been previously shown to produce no detectable $Na^+$ loss in rat red cells [11], it was checked in high-$Na^+$ human red cells and, within the resolution of the method, there was no appreciable $Na^+$ loss during washing. All experiments were carried out in duplicate and, in some net flux experiments, in triplicate and quadruplicate.

*Red cell volume.* The volume of cells was estimated by lysing them in 10 ml de-ionized water and then comparing the absorbance at 541 nm of the unknown hemolysate with that of known hematocrit. The hemoglobin cyanide method was used [17]. However, this method is the equivalent of expressing the value per any arbitrary amount of hemoglobin and does not indicate whether individual cells have changed their volume during the experimental procedure. If a large volume change occurred, it would affect the cation measurements: this possibility was examined using Van Allen tubes. Changes in cell volume were investigated in both low- and high-$Na^+$ cells. Samples were taken in quadruplicate. No appreciable volume change had occurred in either case and, as usual, no detectable hemolysis could be demonstrated.

*Red cell chloride.* After 15 min in the incubation solution at 37 °C and about 50 % hematocrit, duplicate samples were taken for $Cl^-$ analysis and microhematocrit and water content determinations. This procedure is described elsewhere [11, 16].

*ATP depletion in low- and high-$Na^+$ cells.* The ATP depletion method described by Glynn et al. [18] and the cation loading one proposed by Cass and Dalmark [15] were adopted with modifications. After five washes in 150 mM $K^+$ buffer, the cells were incubated at 37 °C and 10 % hematocrit. Incubation solutions for low-$Na^+$ cells consisted of 140 mM KCl and 10 mM Tris/phosphate (pH 7.4 at 37 °C), either with glucose (for ATP-containing cells) or with 5 mM inosine and 5 mM iodoacetamide (for ATP-depleted cells). Solutions for high-$Na^+$ cells contained 50 mM NaCl, 90 mM KCl and 10 mM Tris/phosphate, again with either glucose or inosine plus iodoacetamide. After 2 h incubation the tubes were transferred to room temperature with the addition of 1.35 M sucrose (0.2 ml/10 ml suspension) and Nystatin (0.1 ml/10 ml suspension). The Nystatin was previously dissolved in methanol (5 mg/ml methanol). The cells were then centrifuged, washed twice with the appropriate solution without Nystatin and four times with $K^+$-free $Mg^{2+}$ buffer.

Samples were then taken for both initial cation content and ATP content estimations.

The ATP depletion by starvation was accomplished by incubating the cells for 24 h in a glucose-free solution of 50 mM $K^+$, 90 mM $Na^+$, 10 mM Tris/phosphate containing 10 000 units/100 ml penicillin and 0.1 g/100 ml streptomycin: incubation was performed at 37 °C and about 10 % hematocrit with the solution being changed every 6 h. Half of the cells remained in the same solution for 3 h (ATP-depleted) and the other half spent an additional 3 h in a $K^+$-free 150 mM $Na^+$ solution containing 10 mM glucose, 4.2 mM inosine and 1.8 mM adenine to restore the ATP levels (repleted cells). After washing four times in 150 mM KCl, the cation loading was performed by the Nystatin method, as previously described. The cells were then washed and the initial ATP and cation content determined. ATP was assayed by the enzymatic method of Sigma Co., permitting determinations of concentrations as low as 10 μmol/l cells with about 20 % error.

*Chemicals.* Ouabain was obtained from Sigma Co.: furosemide was generously supplied by Hoechst Pharmaceuticals Inc.

## RESULTS

### *Na influx, efflux and net gain as a function of* $[Na^+]_i$

These results are summarized in Table I. As $[Na^+]_i$ was increased from 2.26 to about 42 mmol/l cells, there was a noticeable reduction in the net $Na^+$ gain and in the rate constant for $Na^+$ efflux, but no appreciable modification in $Na^+$ influx. Despite the reduction in the rate constant, the $Na^+$ efflux increased with a tendency

TABLE I

EFFECT OF INCREASING $[Na^+]_i$ ON $Na^+$ INFLUX, NET $Na^+$ GAIN AND RATE CONSTANT FOR $^{22}Na^+$ EFFLUX IN HUMAN RED BLOOD CELLS INCUBATED IN $K^+$-FREE $Na^+$ BUFFER CONTAINING $10^{-4}$ M OUABAIN

The cells were treated with PCMBS and loaded with $^{22}Na^+$ as described in Methods. To obtain the observed net $Na^+$ gain, cell $Na^+$ was determined by flame at $t = 0$ and after 1 h incubation for cells with the lowest $[Na^+]_i$, and for the others after 4 h incubation. All results are normalized to 1 h. The calculated net gain was obtained from the unidirectional fluxes which in all cases were taken during a 1 h period. All fluxes were determined simultaneously. Each value is the mean ±the S.E.M. Number of experiments is shown in parenthesis.

| $[Na^+]_i$ (mmol/l cells) | Unidirectional $Na^+$ influx (mmol/l cells/h) | Net $Na^+$ gain | | $k_{Na}^e$ ($h^{-1}$) |
|---|---|---|---|---|
| | | Observed (mmol/l cells/h) | Calculated* (mmol/l cells/h) | |
| 2.26±0.13 (15) | 2.74±0.18 (9) | 2.30±0.10 (14) | 2.44±0.22 (5) | 0.135±0.011 (8) |
| 10.46±0.66 (3) | 2.51±0.20 (4) | 1.17±0.12 (3) | 1.59±0.06 (3) | 0.074±0.008 (3) |
| 24.66±0.71 (11) | 2.64±0.22 (5) | 0.88±0.11 (11) | 1.19±0.08 (5) | 0.053±0.003 (3) |
| 41.81±1.49 (7) | 2.66±0.09 (7) | 0.51±0.09 (7) | 0.57±0.09 (6) | 0.050±0.003 (7) |

* Calculated on the basis of unidirectional fluxes.

toward saturation. The differences between values of net gain were all statistically significant, and the rate constant for efflux seemed to reach a plateau. When compared, the net fluxes predicted from the influx and efflux values and those obtained from the actual flame data agreed well at both the lowest and highest $[Na^+]_i$: however, the agreement was not as good for the intermediate values. At the lowest $[Na^+]_i$ all fluxes were measured after 1 h incubation and the agreement found was as expected. For all other $[Na^+]_i$ the incubation was 4 h for net fluxes and 1 h for unidirectional fluxes. The net changes were normalized to 1 h and thus the values for net fluxes are an average over the whole period rather than the true initial ones. Since $Na^+$ efflux changes together with $[Na^+]_i$, the difference between calculated and actual net values will first increase as $[Na^+]_i$ increases, and then decrease at high $[Na^+]_i$.

Thus, both the calculated and the actual net $Na^+$ fluxes are markedly reduced as $[Na^+]_i$ is increased in human red blood cells incubated in nominally $K^+$-free $Na^+$ buffer solution containing $10^{-4}$ M ouabain. This reduction can be predicted from Figs 1 and 2 of Garay and Garrahan [19], and was found in rat red blood cells [11]. Some reduction (though not statistically significant) is shown in the work of Dunn [9]. The present results do not support Post's finding [10] that net $Na^+$ gain is independent of $[Na^+]_i$.

It has been reported [8] that a small fraction of $Na^+$ influx (about 18 %) is stimulated by increasing the intracellular $Na^+$. The present work, however (in agreement with that of Beaugé and Ortiz [11] and Garay and Garrahan [19]), failed to show any dependence of $Na^+$ influx on $[Na^+]_i$. The modifications in the net $Na^+$ gain were then solely a consequence of an increased loss.

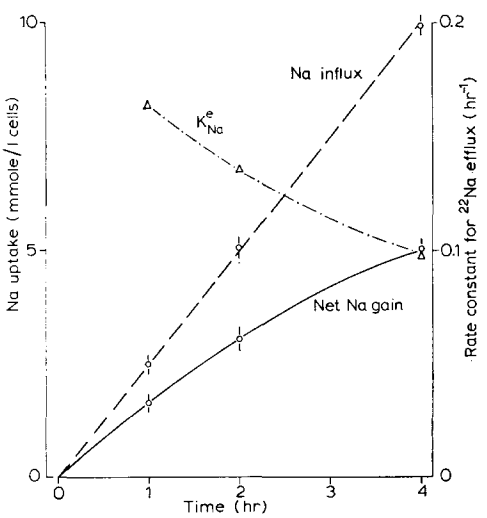


Fig. 1. $Na^+$ influx, net $Na^+$ gain and rate constant for $Na^+$ efflux as a function of time in human red blood cells not treated with PCMBS or nystatin. Prior to the flux measurements the cells were incubated for 12 h at 37 °C and about 15 % hematocrit in 150 mM KCl containing 11 mM glucose, 10 000 units/100 ml penicillin and 0.1 g/100 ml streptomycin with 2 $\mu$Ci/ml of $^{22}Na^+$ (efflux cells) and without it (influx and net flux cells). After 5 washes with cold $K^+$-free $Mg^{2+}$ buffer containing ouabain, all fluxes were determined simultaneously, as described, at 1, 2 or 4 h. Each point is the mean $\pm$S.E.M. of a single experiment done in quadruplicate. The initial $[Na^+]_i$ was 2.45 $\pm$ 0.05 mmol/l cells.

The results reported in this section are from cells treated with *P*-chloromercuribenzene sulfonate (PCMBS). A later section deals with results from cells whose cation loading was accomplished with the use of Nystatin. Since it could be argued that a particular behavior of the $Na^+$ fluxes may be induced by the pretreatment, it would be desirable to reproduce these findings in untreated cells. Thus, freshly drawn cells were first incubated for 12 h in an $Na^+$-free, 150 mM $K^+$ solution (see legend of Fig. 1), reducing the $[Na^+]_i$ to $2.45 \pm 0.05$ (S.E.M.) mmol/l cells ($n = 4$). In this condition, labelled $Na^+$ influx and efflux as well as net $Na^+$ gain were determined during a period of 4 h (see Methods). The results of one experiment done in quadruplicate are shown in Fig. 1. They exactly reproduce those found in PCMBS-treated cells. Whereas the increase in $[Na^+]_i$ did not affect $Na^+$ influx, it reduced the net $Na^+$ gain and the rate constant for $^{22}Na^+$ efflux, although the efflux of $Na^+$ was increased. No hemolysis could be detected at 1 or 2 h and it was below 1 % at 4 h. These results fully agree with those obtained in rat red cells [11] and do not support the notion that human red cells are pump-leak systems as previously suggested [10].

*Membrane potential at different* $[Na^+]_i$

In order to compare the actual findings with those predicted by electrodiffusion, the electrical membrane potential must be known (see Discussion). Table II shows the results of three experiments in which electrical membrane potential was estimated from the $Cl^-$ distribution ratio. As can be seen, changing $[Na^+]_i$ had no effect on membrane potential: taking into account both low- and high-$Na^+$ cells, the average was $-9.7$ mV, which compares well with values reported in the literature [20].

TABLE II

ELECTRICAL MEMBRANE POTENTIALS IN LOW- AND HIGH-$Na^+$ HUMAN RED BLOOD CELLS INCUBATED IN $K^+$-FREE $Na^+$ BUFFER CONTAINING $10^{-4}$ M OUABAIN ESTIMATED ON THE BASIS OF THE $Cl^-$ DISTRIBUTION RATIO

Cells were treated with PCMBS solutions as described in Methods. The increase in $[Na^+]_i$ was accomplished at the expense of a reduction of $[K^+]_i$. After 15 min in the final incubation solution ($K^+$-free 150 mM $Na^+$ buffer containing ouabain at 37 C) duplicate samples were taken for chloride, microhematocrit and cell water determinations. Each value is the mean ± S.E.M. of three experiments.

| $[Na^+]_i$ (mmol/l cells) | $[Cl^-]_i$ (mM) | $[Cl^-]_o$ (mM) | $E_m$ (mV) |
|---|---|---|---|
| $2.08 \pm 0.035$ | $107 \pm 4.6$ | $151 \pm 1$ | $-9.1 \pm 1.12$ |
| (3) | (3) | (3) | (3) |
| $47.00 \pm 1.22$ | $103 \pm 5.2$ | $151 \pm 1$ | $-10.3 \pm 1.36$ |
| (3) | (3) | (3) | (3) |

*Net* $Na^+$ *gain and* $K^+$ *efflux as a function of* $[Na^+]_i$ *at constant* $[K^+]_i$

From the results in the first section, the ouabain-insensitive net $Na^+$ fluxes do not seem to be a simple leak, and it would seem that some kind of carrier-mediated transport or complex ion-membrane interaction may exist. When incubated in $K^+$-free $Na^+$ buffer with ouabain, the cells gain $Na^+$ and lose $K^+$, possibly indicating the involvement of some $Na_o^+/K_i^+$ exchange. As electroneutrality must be preserved, the net $Na^+$ gain must be accompanied by either a net anion gain or a net cation loss, and

TABLE III

EFFECT OF CHANGES IN $[Na^+]_i$ AT CONSTANT $[K^+]_i$ ON THE NET $Na^+$ GAIN AND $K^+$ EFFLUX IN HUMAN RED BLOOD CELLS INCUBATED IN $K^+$-FREE $Na^+$ BUFFER CONTAINING $10^{-4}$ M OUABAIN

The cation loading was performed as described in Methods, using PCMBS. The loading solutions had KCl + choline chloride for the low $[Na^+]_i$ cells and KCl + NaCl for those with high $[Na^+]_i$, so the final $[K^+]_i$ was similar in both groups. After being resealed they were loaded with $^{42}K^+$ and $K^+$ efflux and net $Na^+$ gain determined. Each value is the mean ± S.E.M. of 4 experiments carried out in duplicate.

| $[Na^+]_i$ (mmol/l cells) | $[K^+]_i$ (mmol/l cells) | Net $Na^+$ gain (mmol/l cells/h) | $k_K^e$ ($h^{-1}$) |
|---|---|---|---|
| 1.69 ± 0.18 | 72.4 ± 2.8 | 2.24 ± 0.18 | 0.0174 ± 0.0019 |
| (4) | (4) | (4) | (4) |
| 39.23 ± 1.49 | 72.7 ± 1.7 | 0.73 ± 0.12 | 0.0183 ± 0.0025 |
| (4) | (4) | (4) | (4) |

$K^+$ is the likely candidate in the latter case. Also, with the loading techniques used so far, $Na^+$ replaced $K_i^+$; the observed effects on $Na^+$ fluxes could have been consequences of either the increase in $[Na^+]_i$ or the reduction in $[K^+]_i$. To clarify these points, experiments were performed on cells with both low- and high-$[Na^+]_i$ at constant $[K^+]_i$. All loading solutions had the same $[K^+]$ and the difference in $Na^+$ was matched with choline chloride. The general procedure was as described in Methods. Net $Na^+$ gain and $^{42}K^+$ efflux were determined in $K^+$-free 150 mM $Na^+$ buffer containing $10^{-4}$ M ouabain. Table III summarizes these results. The net $Na^+$ gain was reduced 3-fold when $[Na^+]_i$ was increased from 1.7 mmol/l cells to 39 mmol/l cells; in addition, the absolute values of the gain (2.24 mmol/l cells/h and 0.74 mmol/l cells/h) were not statistically different from the values in Table I for comparable $[Na^+]_i$. This indicates that the reduction in the net $Na^+$ gain was not a consequence of a reduction in the cell $K^+$. The last column of Table III shows that the rate constant for $^{42}K^+$ efflux was not affected when $[Na^+]_i$ was increased about 20-fold. For an $Na_o^+/K_i^+$ exchange, if the translocation mechanism has specific affinities for $Na^+$ and $K^+$, $K^+$ efflux should be reduced when $[Na^+]_i$ is increased. However, it is also possible that monovalent cations other than $K^+$ could be translocated, perhaps on a molar fraction basis (i.e. no specific affinity besides $Na^+$). Only this mode of operation would satisfy the present findings, and it would mean that an $Na_o^+/K_i^+$ exchange indeed exists under conditions where just the physiological cations are present.

*Effect of furosemide and $[K^+]_o$ on $Na^+$ fluxes in low- and high-$Na^+$ red cells*

Ouabain-insensitive $Na^+$ fluxes in red cells are affected by furosemide and $[K^+]_o$, but the experimental evidence is conflicting. Dunn [9] reported the same net $Na^+$ gain in cells with normal $Na^+$ incubated in 5 mM $K^+$ $Na^+$, with ouabain alone and with ouabain + furosemide: this agreed with the reduction observed in the magnitude of influx and efflux measured separately. On the other hand, from a separate estimation of influx and efflux, the data of Sachs [7] (his Table II) predicts a larger $Na^+$ gain in the presence of furosemide + ouabain as compared with ouabain

TABLE IV

EFFECT OF $10^{-3}$ M FUROSEMIDE ON NET $Na^+$ GAIN IN LOW- AND HIGH-$Na^+$ HUMAN RED BLOOD CELLS INCUBATED IN $K^+$-FREE $Na^+$ BUFFER CONTAINING $10^{-4}$ M OUABAIN

The loading procedure and net flux determinations were as described in Methods. PCMBS was used to alter the cell membrane's permeability to cations. Both ouabain and furosemide were added to the solution just before use and dissolved by stirring.

| $[Na]_i$ (mmol/l cells) | Net $Na^+$ gain | | |
|---|---|---|---|
| | Ouabain (mmol/l cells/h) | Ouabain + furosemide (mmol/l cells/h) | Difference (mmol/l cells/h) |
| 2.08 | 2.35 | 2.20 | −0.15 |
| 2.61 | 1.92 | 1.86 | −0.06 |
| 1.79 | 2.18 | 2.19 | 0.01 |
| Mean 2.16 | 2.15* | 2.08* | −0.07 |
| ±S.E.M. 0.24 | 0.13 | 0.11 | 0.05 |
| 37.5 | 0.84 | 1.42 | 0.58 |
| 38.9 | 0.65 | 1.22 | 0.57 |
| 37.4 | 0.61 | 1.23 | 0.62 |
| Mean 37.9 | 0.70** | 1.29** | 0.59 |
| ±S.E.M. 0.5 | 0.07 | 0.07 | 0.02 |

* $P > 0.2$.
** $P < 0.002$.

alone in cells of normal $[Na^+]_i$ incubated in $K^+$-free conditions. The effects of furosemide on the ouabain-insensitive net $Na^+$ gain in low-and high-$Na^+$ red cells are summarized in Table IV. The net $Na^+$ gain was not affected by furosemide in cells with low $[Na^+]_i$, but it was increased about 2-fold when $[Na^+]_i$ was elevated. As one of the mechanisms for an increased net gain is a reduction in efflux, the furosemide effect could be taken as an indication that there is a furosemide-sensitive $Na^+$ efflux which is activated by $[Na^+]_i$. However, the fact that even in normal and low-$Na^+$ cells a furosemide inhibition of both $Na^+$ influx and $Na^+$ efflux has been demonstrated [7, 9] suggests that this is not the case. On the other hand, external $K^+$ has been shown to reduce the furosemide effect [7]. However, in $Na^+$-containing media the $[K^+]$ must be at or around 0.5 mM in order to detect any antagonism. In the four cases where the $K^+$ accumulation in the incubation media was checked, it amounted only to $0.077 \pm 0.005$ mM (S.E.M.) after 1 h incubation for low-$Na^+$ cells, and $0.107 \pm 0.006$ mM (S.E.M.) after 4 h incubation for high-$Na^+$ cells. This suggests that the reduced furosemide sensitivity in low-$Na^+$ cells is not a consequence of external $K^+$ accumulation.

It has been reported [7] that, in the presence of ouabain, external $K^+$ reduced $Na^+$ efflux and increased $Na^+$ influx: these effects of $K^+$ were influenced by the ATP content of the cells. The results in Table V support and extend these observations, as they show that the modifications of the ouabain-insensitive $Na^+$ fluxes caused by external $K^+$ are also a function of the $[Na^+]_i$. Thus, the rate constant for $Na^+$ efflux was significantly reduced in low-$Na^+$ cells ($P < 0.02$ for paired values) and

TABLE V

EFFECT OF $[K^+]_o$ ON THE RATE CONSTANTS FOR $Na^+$ INFLUX AND EFFLUX AND ON THE NET $Na^+$ GAIN IN LOW- AND HIGH-$Na^+$ HUMAN RED BLOOD CELLS INCUBATED IN 100 mM $Na^+$ BUFFER SOLUTIONS CONTAINING $10^{-4}$ M OUABAIN

The cationic composition of the cells was modified by the PCMBS method incubating them in 150 mM KCl (low-$Na^+$ cells) or in a solution of 50 mM NaCl, 100 mM KCl (high-$Na^+$ cells) for 24 h in the cold as described. In every case a simultaneous determination of $^{22}Na^+$ influx and efflux and net $Na^+$ gain was performed. The results are the mean ±S.E.M. of 4 experiments carried out in duplicate. $[Na^+]_i$ was 2.23 mmol/l cells in low-$Na^+$ cells and 25.6 mmol/l cells in high-$Na^+$ cells.

| Low-$Na^+$ cells | | | High-$Na^+$ cells | | |
|---|---|---|---|---|---|
| $K^+$-free | 50 mM $K^+$ | Difference | $K^+$-free | 50 mM $K^+$ | Difference |
| Net $Na^+$ gain (mmol/l cells/h) | | | | | |
| 1.62 | 1.64 | 0.02* | 0.33 | 0.62 | 0.30** |
| ±0.13 | ±0.09 | ±0.06 | ±0.08 | ±0.04 | ±0.09 |
| $k_{Na}^i$ (mmol · $l^{-1}$ cells · $h^{-1}$ · $mM^{-1}$) | | | | | |
| 0.0239 | 0.0243 | 0.0004* | 0.0225 | 0.0258 | 0.0033** |
| ±0.0013 | ±0.0014 | ±0.0012 | ±0.0010 | ±0.0010 | ±0.0010 |
| $k_{Na}^e$ ($h^{-1}$) | | | | | |
| 0.120 | 0.096 | −0.024** | 0.048 | 0.045 | −0.003* |
| ±0.012 | ±0.008 | ±0.004 | ±0.003 | ±0.003 | ±0.002 |

* $P > 0.2$.
** $P < 0.02$.

slightly, or not at all, affected in high-$Na^+$ cells ($P > 0.2$). Conversely, the rate constant for $Na^+$ influx was significantly increased in cells with high $[Na^+]_i$ ($P < 0.02$ for paired values) and unaffected when $[Na^+]_i$ was low ($P > 0.2$). When considering flux units, it was predicted from these results, and actually shown (see first row of Table V), that external $K^+$ would increase the net $Na^+$ gain in high-$Na^+$ cells much more than in those with low $Na^+$, i.e. low-$Na^+$ cells behave as ATP-repleted ones, whereas high-$Na^+$ cells behave like ATP-depleted cells [7].

The main conclusion that could be drawn from this section is that, since both furosemide and $K^+$ modify the net $Na^+$ gain, they cannot act upon a mechanism which is of the proposed 1:1 type of exchange diffusion [8, 9]. In some cases (as in low-$Na^+$ cells), the lack of an effect on net fluxes would occur simply because, for that particular concentration of $Na^+$ on both sides of the membrane, a similar reduction in influx and efflux is produced. This could suggest that $Na^+$ is translocated by a system of variable stoichiometry.

*Net Na gain in low- and high-$Na^+$ cells as influenced by ATP content*

The procedure for ATP depletion proposed by Glynn et al. [18] was followed with some modifications and Nystatin was used instead of PCMBS for the cation loading of the cells. In addition, one experiment was performed using cells depleted of cellular ATP by starvation (see Methods).

The results of all these experiments, which are summarized in Table VI, clearly indicate that the reduction in the net $Na^+$ gain as $[Na^+]_i$ increases (with the $Na^+/K^+$

TABLE VI

EFFECT OF INTRACELLULAR ATP DEPLETION ON NET $Na^+$ GAIN IN HUMAN RED CELLS WITH LOW- AND HIGH-$Na^+$ INCUBATED IN $K^+$-FREE AND 10 mM $K^+$ $Na^+$ SOLUTIONS CONTAINING $10^{-4}$ M OUABAIN

In the inosine-iodoacetamide-treated cells half were incubated in the presence of 5 mM inosine and 5 mM iodoacetamide while the other half remained as a control. Each group was divided into low-$Na^+$ cells (incubated in 150 mM KCl) and high-$Na^+$ cells (incubated in NaCl + KCl). In the starvation method for ATP depletion, all cells were starved in glucose-free solutions for 24 h. After this period, half the cells were kept in the same solution whereas the other half went into a solution containing 10 mM glucose, 4.2 mM inosine and 1.8 mM adenosine for 3 h at 37 °C (repleted cells). In all cases the cation loading was performed by the nystatin method. See text for details.

| Low-$Na^+$ cells | | | High-$Na^+$ cells | | |
|---|---|---|---|---|---|
| $[ATP]_i$ (mmol/l cells) | $[Na^+]_i$ (mmol/l cells) | Net $Na^+$ gain (mmol/l cells/h) | $[ATP]_i$ (mmol/l cells) | $[Na^+]_i$ (mmol/l cells) | Net $Na^+$ gain (mmol/l cells/h) |
| 2 h inosine-iodoacetamide depletion | | | | | |
| $K^+$-free 150 mM $Na^+$ ($n = 3$) | | | | | |
| 0.740 | 1.27 | 2.64 | 0.770 | 35.26 | 0.61 |
| ± 0.050 | ± 0.16 | ± 0.17 | ± 0.025 | ± 0.82 | ± 0.13 |
| 0.010 | 1.57 | 2.52 | 0.010 | 34.93 | 0.68 |
| — | ± 0.16 | ± 0.10 | | ± 0.72 | ± 0.13 |
| 10 mM $K^+$ 140 mM $Na^+$ ($n = 1$) | | | | | |
| 0.810 | 1.22 | 2.24 | 0.760 | 33.86 | 0.74 |
| 0.010 | 1.89 | 2.27 | 0.010 | 33.67 | 0.72 |
| 27 h starvation and repletion | | | | | |
| $K^+$-free 150 mM $Na^+$ ($n = 1$) | | | | | |
| 0.540 | 2.50 | 2.20 | 0.540 | 32.79 | 0.77 |
| 0.010 | 3.08 | 2.42 | 0.010 | 32.12 | 0.96 |

pump inhibited) was not affected by lowering the ATP content below 10 μmol/l cells. This ATP value is estimated on the basis of the sensitivity of the method used, but according to the results of Glynn et al. [18], values on the order of 1 μmol could be expected. In order to have a more precise evaluation of the extent to which ATP depletion may have affected some existing energy-requiring mechanism, it should be taken into account that the ouabain-sensitive $Rb^+$ influx was non-existent in red cells similarly treated [21] and in those treated in the same way as in the present work (Beaugé and Ortiz, unpublished). It is also interesting to note that the presence of 10 mM $K^+$ in the incubation medium did not influence the results.

In the starved cells, the net $Na^+$ gain in high-$Na^+$ cells was reduced about 2.5-fold as compared with that in low-$Na^+$ cells. However, the absolute value was somewhat larger in ATP-depleted than in ATP-repleted ones. Since in the low-$Na^+$ cells the gain was also larger in the ATP-depleted ones, this could be accounted for by some increase in permeability. Also, as the two methods of reducing $[ATP]_i$ have different biochemical implications, it is quite possible that the non-pumped $Na^+$ fluxes are not dependent on the biochemical composition of the cell.

In summary, the results of the present section show that, whatever mechanism is responsible for the reduction in the net $Na^+$ gain as $[Na^+]_i$ is increased and the $Na^+/K^+$ pump inhibited, its efficiency is unaffected by the amount of ATP (and possibly other high energy phosphates) present in the cell.

## DISCUSSION

In a preliminary analysis of the possible mechanism involved in the passive $Na^+$ movements, a comparison should be made between the actual findings and those predicted from electrodiffusion. The integration of the electrodiffusion equation for $Na^+$ fluxes gives the following expression [22]:

$$J = P_{Na} f_{Na}([Na^+]_o - [Na^+]_i e^{EF/RT}) \quad (1)$$

where $P_{Na}$ is the membrane permeability coefficient for $Na^+$, $f_{Na}$ is a factor of permeability which depends on the ion charge and the electrical membrane potential, $[Na^+]_o$ and $[Na^+]_i$ are the external and internal sodium concentrations, $E$ is the electrical membrane potential, and $R$, $T$ and $F$ have the usual meanings.

In order to be able to compare the present results with those predicted by Eqn 1, it was desirable to eliminate the unknowns $P_{Na}$ and $f_{Na}$. This was accomplished by using the influx/net flux ratio,

$$\frac{J_i}{J} = \frac{[Na^+]_o}{[Na^+]_o - [Na^+]_i e^{EF/RT}} \quad (2)$$

(A similar approach has been used in the analysis of carrier-mediated transport mechanisms [23].) From the data of Tables I and II, the predicted ratio can be estimated and then compared with the actual findings for the same experimental conditions. Eqn 2 predicts a slight rise in the ratio as $[Na^+]_i$ increases, starting at a value of 1 at $[Na^+]_i = 0$. This rise would be at the expense of a reduction in the net gain with constant $Na^+$ influx. As can be seen in Fig. 2, although the increase in the ratio was a consequence of a reduction in the net gain, it reached a value between 5.4

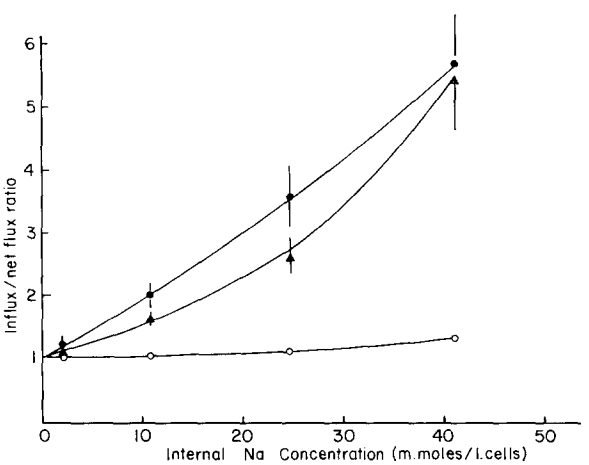


Fig. 2. Influx/net flux ratio in human red blood cells incubated in $K^+$-free $Na^+$ buffer containing ouabain as a function of the intracellular $Na^+$. (○), values expected from electrodiffusion theory on the basis of $[Na^+]_i$ and $[Na^+]_o$ and the transmembrane electrical potential. (●), values obtained from $^{22}Na^+$ influx and the actual net changes from flame data. (▲), values calculated on the basis of unidirectional fluxes. All experimental points are the mean ±S.E.M. of 3–14 experiments. For details see text.

and 5.7 at 42 mmol/l of internal $Na^+$, which is much larger and significantly different from the 1.4 predicted from electrodiffusion. This indicates that human red blood cells cannot be considered just as a pump-leak system for sodium.

For a cell membrane having both an electrodiffusion pathway and another system composed of a limited number of sites for $Na^+$ translocation, the total equation for flux will have one linear plus one saturable term [1]. The ratio influx/net flux thus becomes

$$\frac{J_i}{J} = \frac{P_{Na} f_{Na} [Na^+]_o + SC(influx)}{P_{Na} f_{Na} ([Na^+]_o - [Na^+]_i e^{EF/RT}) + SC(influx) - SC(efflux)} \tag{3}$$

where all symbols have the same meaning as before and SC(influx) and SC(efflux) are the saturable components of $Na^+$ influx and efflux respectively, with no assumptions regarding their structure or kinetic properties. Eqn 3, where the product $P_{Na} f_{Na}$ cannot be dropped, can be used to make some predictions on the behavior of unidirectional and net fluxes as a function of the cell $Na^+$. Any unidirectional flux would have both a linear and a saturable component; this agrees with the reduction in the rate constant for $Na^+$ efflux as $[Na^+]_i$ was increased (Table I, Fig. 1). Eqn 3 also predicts an influx/net flux ratio which, starting from 1 at zero $[Na^+]_i$, will increase with increased $[Na^+]_i$ to values higher than those estimated from electrodiffusion. However, the rise in the ratio could be obtained in different ways depending on the type of saturable mechanism for translocation. Thus, if the saturable component were the type of exchange diffusion proposed by Ussing [24], the terms SC (influx) and SC(efflux) would always be equal, and the ratio would be:

$$\frac{J_i}{J} = \frac{[Na^+]_o + SC(influx)/P_{Na} f_{Na}}{[Na^+]_o - [Na^+]_i e^{EF/RT}} \tag{4}$$

that is, the increase in $J_i/J$ would be mostly a consequence of an increase in the influx term, whereas the net gain would have only a slight reduction. Contrary to the predictions of Eqn 4, the present results show a large decrease in the net $Na^+$ gain and no modification in $Na^+$ influx, indicating that there was no "trans" effect of the solute and that the system was able to produce net movement of the substrate. These findings do not support the idea that red cells can be considered totally [25], or even partially [8, 9], as a pump-leak-exchange diffusion system.

One way to account for the present results would be to postulate that what moves $Na^+$ out of the cell is a different entity from the one which moves it in. As the data indicate no cell energy requirements for these net movements of $Na^+$, the existence of a separate $Na^+$ pump different from the $Na^+/K^+$ ouabain-sensitive pump [6] does seem to be useful as an explanation. That is, if $Na^+$ influx and efflux occur by different mechanisms, both seem to be passive.

On the other hand, if both influx and efflux of $Na^+$ go by the same pathway, the idea of a carrier-mediated facilitated diffusion is attractive. In this case the translocating structure (which under some conditions can act as an "unbalanced carrier") is able to produce net movements of the substrate in favor of its electrochemical gradient. The unbalanced carrier concept is also quite relevant to flux stoichiometry. Thus, if on an average cycle the carrier can cross in one direction loaded and return unloaded, the stoichiometric ratio would be infinite: in a cycle where the same

number of $Na^+$ is moved in either direction, the stoichiometric ratio would be unity. Unbalanced carrier movements have been proposed to account for the membrane potential sensitivity of $Na^+/Ca^{2+}$ counter transport in squid giant axons where 3 $Na^+$ going in should be required for every $Ca^{2+}$ being transported out of the cell [26]. A similar mechanism has also been recently suggested to explain some of the ouabain-resistant fluxes of $Na^+$ and $K^+$ in nucleated erythrocytes [27, 28].

If the dissipative $Na^+$ movements are the counterpart of the energy-requiring ones, it would be logical to expect that they be based on a type of external $Na^+$/internal $K^+$ exchange. The data presented here are consistent with this hypothesis only if the translocating mechanism has no specific affinity for $K^+$ as compared with other monovalent cations. If external $Na^+$/internal $K^+$ exchange does not occur, a simultaneous transport of NaCl could account for the preservation of the electroneutrality. A net transport of salt with a consequent water transport should produce changes in cell volume: however, a net flux of 2.30 mmol/l cells/h in low-$Na^+$ cells would be equivalent to a volume increase of only about 1.5 %. Since under these conditions a simultaneous net loss of $K^+$ also exists, the percentage should be even smaller and would be within the error of the method.

If one accepts the existence of both a saturable and a leakage pathway, it would be desirable to know what fraction of non-pumped $Na^+$ flux occurs by each of them. This question cannot be answered with precision at present. However, the experimental data have been fitted to Eqn 3 by assuming that the saturable component obeys Michaelian kinetics and some extreme values for each component have been obtained. It is interesting that a relatively good fit was accomplished even for cases where the leak was totally absent ($P_{Na} f_{Na} = 0$) and all fluxes were through the saturable component. For a pure carrier system the $K_m$ would oscillate between 20 and 50 mM. For a combined leak-facilitated diffusion, the range of possible $K_m$ values is from 6 to 20 mM. Thus, the data are compatible with the idea of translocation by a carrier as the sole mechanism responsible for the total dissipative process of $Na^+$ gain. In any case, electrodiffusion would not be higher than 25 % of the total non-pumped fluxes, i.e. if the passive $Na^+$ fluxes in human red blood cells are effected by a combination of a leakage and a facilitated diffusion system with Michaelis-Menten kinetics, the latter would account for at least three quarters of the total $Na^+$ translocation.

## REFERENCES


1 Glynn, I. M. (1957) J. Physiol. Lond. 136, 148–173
2 Hoffman, J. F. (1966) Am. J. Med. 41, 666–680
3 Garrahan, P. J. and Glynn, I. M. (1967) J. Physiol. Lond. 192, 159–174
4 Sachs, J. R. (1970) J. Gen. Physiol. 56, 322–341
5 Glynn, I. M. and Lew, V. L. (1970) J. Physiol. Lond. 207, 393–402
6 Hoffman, J. F. and Kregenow, F. (1966) Ann. N. Y. Acad. Sci. 137, 566–576
7 Sachs, J. R. (1971) J. Gen. Physiol. 57, 259–282
8 Lubowitz, H. and Whittam, R. (1969) J. Physiol. Lond. 202, 111–131
9 Dunn, M. (1970) J. Clin. Invest. 49, 1804–1814
10 Post, R. L., Albright, C. D. and Dayani, K. (1967) J. Gen. Physiol. 50, 1201–1220
11 Beaugé, L. A. and Ortiz, O. (1973) J. Membrane Biol. 13, 165–184
12 Beaugé, L. A. (1974) Fed. Proc. 33, 1592
13 Garrahan, P. J. and Rega, A. (1967) J. Physiol. Lond. 193, 459–466

14 Sachs, J. R. (1974) J. Gen. Physiol. 63, 123–143
15 Cass, A. and Dalmark, M. (1973) Nat. New Biol. 244, 47–49
16 Beaugé, L. A. and Ortiz, O. (1971) J. Physiol. Lond. 218, 533–549
17 Van Kampen, E. J. and Zijlstra, W. G. (1961) Clin. Chim. Acta 6, 538–544
18 Glynn, I. M., Hoffman, J. F. and Lew, V. (1971) Phil. Trans. Roy. Soc. Lond. B 262, 91–102
19 Garay, R. P. and Garrahan, P. J. (1973) J. Physiol. Lond. 231, 297–325
20 Cotterrell, D. and Whittam, R. (1971) J. Physiol. Lond. 214, 509–536
21 Beaugé, L. A. and Adragna, N. (1971) J. Gen. Physiol. 57, 576–592
22 Mullins, L. J. and Noda, K. (1963) J. Gen. Physiol. 47, 117–132
23 Stein, W. D. (1967) in The Movement of Molecules Across Cell Membranes, Academic Pres New York
24 Ussing, H. H. (1949) Physiol. Rev. 29, 127–155
25 Motais, R. (1973) J. Physiol. Lond. 233, 395–422
26 Mullins, L. J. and Brinley, Jr, F. J. (1975) J. Gen. Physiol. 65, 135–152
27 Kregenow, F. M. (1971) J. Gen. Physiol. 58, 372–395
28 Schmidt, W. F. and McManus, T. J. (1974) Fed. Proc. 33, 1457