Sodium Movements in the Human Red Blood Cell

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ABSTRACT Measurements were made of the sodium outflux rate constant, $k_{Na}$, and sodium influx rate constant, $i_{Na}$, at varying concentrations of extracellular ($Na_e$) and intracellular ($Na_i$) sodium. $k_{Na}$ increases with increasing $[Na_e]$ in the presence of extracellular potassium ($K_o$) and in solutions containing ouabain. In $K$-free solutions which do not contain ouabain, $k_{Na}$ falls as $[Na_e]$ rises from 0 to 6 mM; above 6 mM, $k_{Na}$ increases with increasing $[Na_e]$. Part of the Na outflux which occurs in solutions free of Na and K disappears when the cells are starved or when the measurements are made in solutions containing ouabain. As $[Na_i]$ increases from 0 to 6 mM, $k_{Na}$ decreases, suggesting that sites involved in the sodium influx are becoming saturated. As $[Na_e]$ increases, $k_{Na}$ at first increases and then decreases; this relation between $k_{Na}$ and $[Na_e]$ is found when the measurements are made in high Na, high K solutions; high Na, $K$-free solutions; and in $(Na + K)$-free solutions. The relation may be the consequence of the requirement that more than one Na ion must react with the transport mechanism at the inner surface of the membrane before transport occurs. Further evidence has been obtained that the ouabain-inhibited Na outflux and Na influx in $K$-free solutions represent an exchange of $Na_e$ for $Na_i$ via the Na-K pump mechanism.

INTRODUCTION

It has recently become clear that the Na movements in the human red blood cell which are inhibited by the cardiotonic steroids, such as ouabain, are considerably more complicated than had been thought. In addition to the Na outflux which occurs in the presence of extracellular potassium ($K_o$), evidence has recently been presented that there occurs, in $K$-free solutions, an exchange of intracellular ($Na_i$) for extracellular ($Na_e$) sodium (Garrahan and Glynn, 1967 a). The exchange has been shown to be inhibited by $K_o$ and by ouabain, and the outflux component at least has been shown to require intracellular ATP (Garrahan and Glynn, 1967 a, c). Another ouabain-sensitive Na outflux has been shown to occur in $Na$-free solutions, and this component disappears at $[Na_e]$ of about 4 mM (Garrahan and Glynn, 1967 a, c). In addition, evidence has been presented that, in the presence of saturating $[K_o]$, the Na outflux is
greater in solutions containing Na than it is in Na-free solutions (Hoffman and Kregenow, 1966; Lubowitz and Whittam, 1969) when Na is replaced by Mg, although no such effect was observed by Garrahan and Glynn (1967 a) when choline replaced Na.

It seemed that further information might be gained about these processes by examining the relation between the magnitude of these fluxes and [Na] and [Na\textsubscript{0}]. It also seemed desirable to obtain further evidence that the ouabain-sensitive Na outflux and Na influx in K-free solutions are indeed coupled, and are mediated by the same mechanism that is responsible for the ouabain-sensitive Na-K exchange. For this purpose, the influence on these processes of two manipulations which alter the magnitude of the Na-K exchange, variations in [Na\textsubscript{0}] and depletion of intracellular energy stores, was determined. In addition, a comparison was made of the relation between [K\textsubscript{0}] and the magnitude of the Na outflux and of the Na influx. Finally, the effect of varying quantities of ouabain on the Na outflux in the presence and in the absence of K\textsubscript{0} was determined.

METHOD

The solutions used were made up of reagent grade chemicals to an osmolality of 295 ± 5 milliosmols/kg water. In all cases, glycylglycine-MgCO\textsubscript{3} buffer solution (glycylglycine 273 mM, MgCO\textsubscript{3} 54 mM, 295 ± 5 milliosmols/kg water, pH 7.4 ± 0.05 at 37°C) accounted for 10% of the volume of the solutions, and crystalline bovine serum albumin was present at a concentration of 20 mg/100 ml solution. When Na or K was varied in concentration, NaCl or KCI solution was replaced by an equal volume of a solution composed of 75% MgCl\textsubscript{2} solution and 25% isosmotic sucrose solution. The Cl concentration of this solution was about the same as that of an isosmotic NaCl or KCl solution.

Venous blood was obtained from healthy males with heparin as anticoagulant. The cells were separated by centrifugation and plasma and buffy coat removed by aspiration; the cells were then washed (by suspension, centrifugation, and aspiration) three times in isosmotic (107 mM) MgCl\textsubscript{2} solution, and were used as appropriate.

Unidirectional Na outflux and Na and K influx were measured by methods previously described (Sachs and Welt, 1967; Sachs and Conrad, 1968). Outflux measurements were made by exposing cells for 2–3 hr to a solution containing \textsuperscript{24}Na. The cells were then removed from this solution, washed four times to remove extracellular \textsuperscript{24}Na, and then placed in the solution in which the measurements were made. Samples of the suspension were taken four or five times over a 1\frac{1}{2} hr period, the cells removed, and the supernatants saved for counting; a sample of the whole suspension was also counted. The outflux rate constant was calculated from these values as previously described (Sachs and Welt, 1967). For influx measurements, cells were added to solutions containing either \textsuperscript{24}Na or \textsuperscript{42}K, and samples of the cells were taken \frac{1}{2} and 1\frac{1}{2} hr after addition of the cells to the solutions. The cells were washed free of extracellular isotope, hemolyzed, and counted. The influx was calculated from the uptake of \textsuperscript{24}Na or \textsuperscript{42}K over the 1 hr period. When Na influx was measured, the results were corrected
for *Na loss by the cells over the course of the experiment. For this purpose, values for Na outflux simultaneously determined were used (Sachs and Conrad, 1968).

Alteration of intracellular cation concentrations was achieved by a modification of a method described by Garrahahan and Rega (1967). Cells were suspended at about 5% hematocrit in a solution containing (mM): PO₄ 3.4, Mg⁺⁺ 1.0, p-chloromercuribenzenesulfonic acid (PCMBS) 0.1, Cl⁻ 147, glucose 5, and varying concentrations of Na and K to make up a total of 150 mM. The suspension was stored at 4°C for 20 hr, at which point the cells were removed from the suspension, and resuspended in a solution identical to the first except for the absence of PCMBS and the presence of dithiothreitol 2 mM, adenine 3 mM, and inosine 10 mM. The suspension was incubated for 1 hr at 37°C after which the cells were removed from the suspension, washed four times in isosmotic MgCl₂ solution, and used as appropriate. Preliminary studies indicated that cells treated in this way had values of Na outflux, Na influx, and ouabain-insensitive Na outflux and influx indistinguishable from those obtained in the same batch of cells which had been stored at 4°C in a solution which did not contain PCMBS or dithiothreitol.

In experiments in which energy-depleted cells were used, depletion was accomplished by incubating cells at 37°C for 18 hr in the absence of substrate. Cells were washed five times in isosmotic NaCl solution, then placed at a hematocrit of about 20% in a solution containing (mM): K⁺ 134, Na⁺ 32, Cl⁻ 154, PO₄ 14, pH 7.4. In addition, penicillin 1 million U/liter and streptomycin sulfate 1 g/liter were present to suppress bacterial growth. The cells were incubated for 18 hr at 37°C, and then were separated from the solution, washed three times in isosmotic NaCl solution, and resuspended in a solution containing (mM): Na⁺ 166, Cl⁻ 154, PO₄ 14, pH 7.4. In half the cells, adenine 1.7 mM, inosine 4.2 mM, and glucose 20 mM were added in order to replete intracellular energy stores; the other half was not exposed to substrate. After a further 1 hr incubation, the cells were separated from the solution, washed four times in isosmotic MgCl₂ solution, and used as appropriate.

Intracellular Na concentration was estimated as previously described (Sachs and Welt, 1967).

The symbols used are defined as follows:

\[
\frac{\text{mmoles}}{\text{liter RBC} \times \text{hr}} = \frac{1}{\text{hr}} \times \text{[Na] (mM)}}
\]

where \( 'M_{Na} \) is the Na outflux, \( 'k_{Na} \) the rate constant for the Na outflux, and Naᵢ the intracellular Na;

\[
\frac{\text{mmoles}}{\text{liter RBC} \times \text{hr}} = \frac{\text{mmoles}}{\text{liter RBC} \times \text{hr}} \times \frac{1}{\text{mm}} \times \text{[Na] (mM)}}
\]

where \( 'M_{Na} \) is the Na influx, \( 'k_{Na} \) the rate constant for the Na influx, and Naₑ the extracellular Na. \( 'M_{K} \) (mmoles/liter RBC × hr) is the potassium influx, and the definition of \( 'k_{K} \) (mmoles/liter RBC × hr × mm), the rate constant for K influx, is analogous to that of \( 'k_{Na} \).
RESULTS

The Effect of Na\textsubscript{o} on the Na Outflux and the Na Influx

Fig. 1 represents the relation between $k_{\text{Na}}$ and [Na\textsubscript{o}]. In K-free solutions (middle curve), $k_{\text{Na}}$ at first decreases with increasing [Na\textsubscript{o}], and then increases; the behavior of the Na outflux under these circumstances has been reported by Garrahan and Glynn (1967a). In solutions containing K 16 mM (upper curve) $k_{\text{Na}}$ increases with increasing [Na\textsubscript{o}]; evidence for the increase in the Na outflux under these circumstances has previously been presented (Hoffman and Kregenow, 1966; Lubowitz and Whittam, 1969). The lower curve demonstrates the effect of [Na\textsubscript{o}] on the magnitude of $k_{\text{Na}}$ in the presence of ouabain. The ouabain-insensitive Na outflux increases in the presence of Na\textsubscript{o}, an effect which was also reported by Hoffman and Kregenow (1966) and by Lubowitz and Whittam (1969). The difference between the Na outflux in K-free solutions with and without ouabain (i.e., the difference between the middle and lower curves of Fig. 1) was ascribed by Garrahan and Glynn (1967a) to a ouabain-sensitive exchange of Na\textsubscript{o} for Na\textsubscript{e} accomplished by the Na-K pump.
Table I presents the results of experiments in which the effect of Na⁺ on kNa in solutions with and without ouabain was measured. The stimulation by Na⁺ is significantly greater in the ouabain-free solutions than it is when ouabain is present. An explanation for this behavior is proposed in the discussion.

In Fig. 2, kNa and kNa, determined simultaneously in cells from the same

| Na⁺ 9.3 mmoles/liter RBC |
|--------------------------|
| K₀ 16 mM, ouabain 0      |
| K₀ 0, ouabain 10⁻⁶ M     |

| Na⁺ | K⁺ 16 mM, ouabain 0 | K₀ 0, ouabain 10⁻⁶ M |
|-----|---------------------|---------------------|
| Na₀ 128 mM | 0.379±0.025          | 0.102±0.006         |
| Na₀ 0   | 0.282±0.021          | 0.067±0.010         |
| Δ      | 0.097±0.009          | 0.035±0.007         |

Δ 0.062 ± 0.0061, p < 0.001

Solutions in which the measurements were made contained (mM): Glycylglycine 27; Mg++ 5; K⁺ 16 or Mg++ 8 and sucrose 7; Cl⁻ 144; glucose 10; and either Na⁺ 128 or Mg++ 64 and sucrose 54. n (No. of paired observations) = 6.

Figure 2. Na outflux rate constant (filled circles), kNa (hr⁻¹), Na influx rate constant (open circles), kNa (mmoles/liter RBC × hr × mm Na₀), and K-inhibited kNa (crosses), vs. extracellular Na concentration, Na₀. Solutions in which the measurements were made contained (mM): Glycylglycine 27; Mg++ 69-66; sucrose 53-51; K⁺ 16, or Mg++ 8 and sucrose 7; Na⁺ 0-6; Cl⁻ 144; glucose 10. Values for the K-inhibited kNa were obtained by subtracting values for kNa obtained in solutions containing K 16 mM from those obtained in K-free solutions. The measurements were made using fresh cells; Na₀ 7.1 mmoles/liter RBC.
batch using K-free solutions, are plotted as a function of [Na]; in addition, the difference between $k_{Na}$ in K-free solutions and $k_{Na}$ in solutions containing K 16 mM is also plotted. The difference corresponds to the portion of the Na influx which was attributed by Garrahan and Glynn (1967 c) to a Na-Na exchange by the Na-K pump. The decline in $k_{Na}$ (also seen in the middle curve of Fig. 1) is accompanied by a fall in $k_{Na}$ as [Na] increases from 0 to 5 mM. The early decline in $k_{Na}$ suggests that some component of the transport mechanism saturates with Na at the same time that $k_{Na}$ into low Na solutions decreases; the decline in $k_{Na}$ is complete at about 5 mM Na so that the affinity for Na of the saturable component of the Na influx must be quite high. A mechanism which might account for the simultaneous fall in $k_{Na}$ and $k_{Na}$ will be discussed below. In Fig. 3, $k_{Na}$ is plotted against [Na] over a wide range of Na concentrations, and the difference between $k_{Na}$ in K-free solutions and $k_{Na}$ in solutions containing K 16 mM is also plotted. The K-sensitive $k_{Na}$ first decreases as [Na] rises, reaches a low at about Na 5 mM, then increases as [Na] continues to rise.

The Effect of Na on the Magnitude of the Na Outflux

By determining the form of the relation between $k_{Na}$ and [Na], it seemed that it might be possible to determine whether one or more than one intracellular
Na ion must interact with the transport system before transport occurs; the rationale is discussed below. Fig. 4 represents the results of an experiment in which $k_{Na}$ was measured at varying $[Na]$. The upper curve represents the results of such measurements made at $K_o = 16$ mM and high $Na_i$; the middle curve represents the same measurements made in K-free solutions and at high $Na_i$; the lower curve is the result of experiments in which the measurement was made in solutions containing ouabain and high $Na_i$. A curve similar to the upper curve of Fig. 4 was reported by McConaghey and Maizels (1962); they measured net outflux of Na, using cells in which $[Na]$ was altered by exposure of the cells to lactose solutions. In Fig. 5, the Na outflux is plotted as a function of $[Na]$; the data are from the experiment plotted in Fig. 4; in Fig. 6, the upper curve represents the difference between $k_{Na}$ in solutions containing K $16$ mM and $k_{Na}$ in solutions containing ouabain, and is the response of the outflux rate constant for K-activated Na outflux to alterations in $[Na]$. The lower curve is the difference between $k_{Na}$ in K-free solutions and $k_{Na}$ in solutions containing ouabain, and represents the response of the outflux rate constant for the ouabain-sensitive, $Na_i$-dependent Na outflux to altera-
Figure 5. Na outflux, $^\circ M_{Na}$ (mmoles/liter RBC $\times$ hr) vs. intracellular Na concentration, $Na_c$ (mmoles/liter RBC), calculated from the data of Fig. 4.

Figure 6. Ouabain-sensitive Na outflux rate constant, $^\circ k_{Na}$ ($hr^{-1}$), vs. intracellular Na concentration, $Na_c$ (mmoles/liter RBC). The upper curve is the difference between $^\circ k_{Na}$ measured in solutions containing K 16 mM and $^\circ k_{Na}$ measured in solutions containing ouabain $10^{-4}$ M, and the lower curve is the difference between $^\circ k_{Na}$ measured in K-free solutions and $^\circ k_{Na}$ measured in solutions containing ouabain $10^{-4}$ M. In each case, the results of three different experiments are plotted. The solutions in which the measurements were made are the same as those used in the experiments recorded in Fig. 5.
tions in [Na\textsubscript{i}]. In both cases, \( k_{Na} \) is not a monotonically decreasing function of [Na\textsubscript{i}], but appears to increase at first and then decrease as [Na\textsubscript{i}] increases.

The results of experiments in which the Na outflux was measured as a function of [Na\textsubscript{i}] in Na-free solutions are plotted in Fig. 7; [K\textsubscript{i}] was less than 0.01 mM. The left side of the figure depicts the response of \( k_{Na} \) to increasing [Na\textsubscript{i}]; in the absence of ouabain \( k_{Na} \) at first increases and then decreases as [Na\textsubscript{i}] increases, and the rate constant for the ouabain-sensitive Na outflux in Na-free solutions exhibits the same relation to [Na\textsubscript{i}]. The right side of the figure is a plot of \( M_{Na} \) vs. [Na\textsubscript{i}] from the same experiment.

The increased Na outflux as [Na\textsubscript{i}] increases (Table I and the upper curve in Fig. 1) was observed in cells with normal Na\textsubscript{o}, about 9 mmoles/liter RBC. In view of the shape of the upper curve of Fig. 4, it seemed possible that the response of the Na outflux to the presence of Na\textsubscript{o} might arise from variations in the magnitude of Na\textsubscript{i}; the way in which this might occur will be discussed below. Table II presents the results of experiments in which \( k_{Na} \) was measured in solutions with and without Na using cells with high Na\textsubscript{i}; there is no increase in \( k_{Na} \) at high Na\textsubscript{i} in ouabain-free solutions, although the effect of Na\textsubscript{o} is still apparent in the ouabain-treated cells. Table III presents the results...
TABLE II
EFFECT OF Na⁺ ON ³kNa

| Na⁺  | ³kNa  | Na⁺ | ³kNa  |
|------|-------|-----|-------|
| 16.7 mmoles/liter RBC | hr⁻¹ ± SEM | 42.0 mmoles/liter RBC | hr⁻¹ ± SEM |
| Na⁺ 128 mM | 0.275 ± 0.021 | Na⁺ 0 | 0.281 ± 0.027 |
| ∆ | -0.006 ± 0.010 | ∆ | -0.013 ± 0.009 |
| Na⁺ 128 mM, ouabain 10⁻⁴ M | 0.038 ± 0.003 | Na⁺ 0, ouabain 10⁻⁴ M | 0.018 ± 0.003 |
| ∆ | 0.020 ± 0.001 | ∆ | 0.006 ± 0.002 |

Solutions in which the measurements were made contained (mM): Glycylglycine 27; Mg²⁺ 5; K⁺ 16; Cl⁻ 144; glucose 10; and either Na⁺ 128, or Mg²⁺ 64 and sucrose 54. In both sets of cells Na⁺ was altered by exposure of the cells to PCMBS. n = 4.

TABLE III
EFFECT OF Na⁺ ON ³kK

| Na⁺ | ³kK  | Na⁺ | ³kK  |
|------|------|-----|------|
| 128 mM | 0.280 ± 0.031 | 80.7 | 0.291 ± 0.047 | 74.7 |
| ∆ | -0.011 ± 0.008 | | | |
| Na⁺ 128 mM, ouabain 10⁻⁴ M | 0.090 ± 0.006 | 87.1 | 0.017 ± 0.003 | 84.5 |
| ∆ | 0.013 ± 0.002 | | | |

Solutions in which the measurements were made contained (mM): Glycylglycine 27; Mg²⁺ 5; K⁺ 16.5; Na⁺ 128, or Mg²⁺ 65 and sucrose 54; K⁺ 16.5; Cl⁻ 144; glucose 10. Na⁺ was altered by exposure of the cells to PCMBS. n = 4.

of experiments in which ³kK was measured in high and low Na solutions. Na⁺ was raised above normal prior to the determination of ³kK by exposure of the cells to PCMBS. Using cells with a Na⁺ of 79 mmoles/liter RBC, ³kK in the presence of high Na⁺ was no greater than that in the absence of Na⁺. Although the average Na⁺ of the cells studied in the Na-free solutions was lower, the response of ³kK to changes in [Na⁺] is quite small at these high levels, so that a difference of 6 mmoles/liter RBC results in little difference in the value of ³kK. The ouabain-insensitive ³kK is higher in the cells exposed to high levels of Na⁺ than it is in those exposed to Na-free solutions.

The Ouabain-Sensitive Na-Na Exchange

Garrahan and Glynn (1967 a) have presented evidence that the Na outflux which occurs into Na-containing, K-free solutions represents an exchange of Na⁺ for Na⁺ mediated by the same mechanism as that which is responsible for
Na-K exchange when K is present. Several predictions can be made from this hypothesis, and it seemed worthwhile to determine whether they would prove correct.

If the Na-Na exchange hypothesis is correct, then, as \([K_o]\) is increased, the Na outflux and K influx should increase and the Na influx decrease at similar rates. Garrahan and Glynn (1967 c) have shown that this is the case when the K influx in Na-containing solutions is compared with the Na influx at varying \([K_o]\). Since Na competes with K and shifts the curve of K influx or Na outflux vs. \([K_o]\) to the right, the rate at which the Na influx decreases with increasing \([K_o]\) should be greater when Na is very low than when it is high. Fig. 8 represents the results of an experiment designed to test this prediction; \(\dot{k}_{\text{Na}}\) and \(\dot{k}_{\text{Na}}\) were measured simultaneously in the same batch of cells in solutions with and without Na and at varying \([K]\). It can be seen that, at high Na, both the curve of \(\dot{k}_{\text{Na}}\) vs. \([K_o]\) and the curve of \(\dot{k}_{\text{Na}}\) vs. \([K_o]\) are shifted to the right, in agreement with the prediction.

Garrahan and Glynn (1967 a) have shown that ouabain inhibits a portion of the Na outflux and of the Na influx; i.e., that portion which is involved in the Na-Na exchange. If this process is mediated by the same mechanism as

![Figure 8](image-url)
that which accomplishes Na-K exchange, it would be expected that a plot of $k_Na$ in solutions containing K vs. ouabain concentration will parallel a plot of $k_{Na}$ in K-free solutions vs. ouabain concentration. Cells from the same batch were preincubated for a 1 hr period in solutions containing varying concentrations of ouabain and no K. The cells were then removed from the solutions, washed four times in MgCl₂ solution, and then placed in solutions in which $k_{Na}$ was measured. In Fig. 9, $k_{Na}$ is plotted against the concentration of ouabain to which the cells were exposed in the preincubation. The decrease

![Graph showing Na outflux rate constant ($k_{Na}$) vs. ouabain concentration](image)

in the rate constant for K-activated Na outflux with increasing ouabain concentration is accompanied by a decrease in the Na outflux in Na-free solutions.

Garrahan and Glynn (1967 c) have shown that the Na-Na exchange requires the presence of intracellular ATP. If the K-inhibited component of the Na influx represents an exchange of this component for a component of the Na outflux, it too should require the presence of intracellular ATP. Table IV presents the results of experiments in which $k_{Na}$ and $k_{Na}$ were measured using cells which were depleted of intracellular energy stores by incubation at 37°C for 18 hr in the absence of substrate, then separated into two parts, one of which was repleted by exposure to adenine and inosine. In the depleted cells, $K_0$ has little effect on either the $k_{Na}$ or $k_{Na}$. 

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**Figure 9.** Na outflux rate constant, $k_{Na}$, vs. ouabain concentration to which the cells were exposed before the measurements were made. Details of the procedure are given in the text. Solutions in which the measurements were made contained (mM): Glycylglycine 27; Mg⁺⁺ 5; Na⁺ 128; Cl⁻ 144; K⁺ 16 or Mg⁺⁺ 8 and sucrose 7; glucose 10.
Finally, it can be predicted from the hypothesis of Garrahan and Glynn (1967a) that, if $N_{a}$ is reduced to a level at which the K-stimulated Na outflux no longer occurs, the K-inhibited Na influx should also disappear; if there is insufficient $N_{a}$ to exchange for $N_{ao}$, the Na influx component of the exchange will be absent. Table V presents the results of experiments in which cells were prepared with low $[N_{a}]$ and with $[N_{ao}]$ of about 18 mmoles/liter RBC by exposure to PCMBS. The low $N_{a}$ cells were only reduced to 3.5 mmoles/liter RBC; Garrahan and Rega (1967) also comment on their inability to lower $N_{a}$ below 2.5 mmoles/liter RBC by this method. The low $N_{a}$ cells display very little K-stimulated $k_{N_{a}}$ and very little K-inhibited $i_{N_{a}}$. The high $N_{a}$ cells exhibit K stimulation of $k_{N_{a}}$ and K inhibition of $i_{N_{a}}$.

**Table IV**

| $N_{a}$ mmoles/liter RBC | $k_{N_{a}}$ mmol/liter RBC/hr | $i_{N_{a}}$ mmol/liter RBC/hr |
|--------------------------|-----------------------------|-----------------------------|
| Repleted cells            | $N_{a}$ 9.1 mmoles/liter RBC | $N_{a}$ 10.5 mmoles/liter RBC |
| $K_{a}$ 0                 | 0.064±0.010                 | 0.039±0.002                 |
| $K_{a}$ 16 mM             | 0.195±0.028                 | 0.021±0.002                 |

Solutions in which the measurements were made contained (mM): Glycylglycine 27; Na$^+$ 0.3; Mg$^{++}$ 69; sucrose 54; K$^+$ or Mg$^{++}$ 8 and sucrose 7; Cl$^-$ 144; glucose 10 (repleted cells) or 0 (depleted cells). $n = 5$.

**Table V**

| $N_{a}$ mmoles/liter RBC | $k_{N_{a}}$ mmol/liter RBC/hr | $i_{N_{a}}$ mmol/liter RBC/hr |
|--------------------------|-----------------------------|-----------------------------|
| $N_{a}$ 3.5               | $N_{a}$ 18.3 mmoles/liter RBC | $N_{a}$ 10.5 mmoles/liter RBC |
| $K_{a}$ 0                 | 0.061±0.006                 | 0.023±0.002                 |
| $K_{a}$ 16 mM             | 0.065±0.002                 | 0.022±0.003                 |

Solutions in which the measurements were made contained (mM): Glycylglycine 27; Na$^+$ 0.3; Mg$^{++}$ 69; sucrose 54; K$^+$ 16 or Mg$^{++}$ 8 and sucrose 7; Cl$^-$ 144; glucose 10. In both sets of cells, $N_{a}$ was altered by exposure of the cells to PCMBS. $n = 5$.

The Na Outflux in Na-Free Solutions

Garrahan and Glynn (1967b) have shown that the Na outflux which occurs in Na-K-free solutions (Fig. 2) can, in part, be accounted for by the accumu-
lation in the solution of K lost from the cells during the course of the measure-
mments; small amounts of K have much greater effect on the Na outflux in Na-
free solutions than in solutions containing high Na. They point out, however,
that this cannot account for the entire effect. Further evidence that this is the
case can be elicited by measuring $k_{Na}$ in solutions in which the major extra-
acellular cation is tetraethylammonium (TEA), which has been shown to
inhibit competitively the K stimulation of the Na outflux (Sachs and Conrad,
1968), although TEA enters the red cells to only a slight extent (Askari,
1966). When TEA is used as a competitive inhibitor of the active K influx, it
is a better inhibitor than is Na (Sachs, 1967). If $k_{Na}$ in Na-free solutions is
entirely due to the accumulation of K, it might be expected that TEA will
completely eliminate it since the amount of K estimated by Garrahan and
Glynn (1967 b) to be present under similar circumstances was quite small.
The results of experiments to determine the effect of TEA on $k_{Na}$ are shown
in Table VI. The ouabain-sensitive Na outflux into TEA solutions is smaller
than that into Mg solutions (corresponding to the portion of this outflux
attributable to accumulated K), but it has not disappeared. Moreover, the
decrease in the ouabain-sensitive $k_{Na}$ is due to an increase in the ouabain-
insensitive $k_{Na}$ in TEA solutions rather than a decrease in $k_{Na}$ in ouabain-free
TEA solutions.

The inhibition of the Na outflux in Na-free solutions by ouabain (Garrahan
and Glynn, 1967 a) suggests that it is mediated by the same system as that
which is responsible for the Na-K exchange and the Na-Na exchange. A
further indication that this is the case arises from the fact that the form of the
relation between the magnitude of this outflux and $[Na]$ (Fig. 7) is similar to
the form of the relation between the K-stimulated Na outflux and $[Na]$ (Fig. 5). Finally, if the Na outflux into Na-free solutions and the K-stimulated
Na outflux are manifestations of the same system, the Na outflux into Na-free

| Major extracellular cation | $k_{Na}$ 7.0 mmoles/liter RBC |
|---------------------------|-----------------------------|
| Mg$^{++}$                 | $0.101 \pm 0.008$           |
| Mg$^{++}$, ouabain $10^{-4}$ | $0.064 \pm 0.007$           |
| TEA$^{+}$                 | $0.100 \pm 0.010$           |
| TEA$^{+}$, ouabain $10^{-4}$ | $0.083 \pm 0.008$           |

| TEA$^{+}$, ouabain $10^{-4}$ | $0.112 \pm 0.002$           |
| TEA$^{+}$, ouabain $10^{-4}$ | $0.096 \pm 0.001$           |

Solutions in which the measurements were made contained (mM): Glycylglycine 27; Mg$^{++}$ 5;
Cl$^{-}$ 144; Mg$^{++}$ 72 and sucrose 60, or TEA$^{+}$ 144; glucose 10.
EFFECT OF DEPLETION ON \( ^{\alpha}K_{Na} \) IN Na-FREE SOLUTIONS

|                | Repleted cells | Depleted cells |
|----------------|----------------|----------------|
|                | \( hr^{-1} \pm SEM \) | \( hr^{-1} \pm SEM \) |
| \( K_0 0 \)    | 0.078±0.006    | 0.045±0.005    |
| \( K_0 0, \text{ouabain} 10^{-4} M \) | 0.049±0.005 | 0.045±0.004 |
| \( K_0 16 \text{mM} \) | 0.160±0.013 | 0.051±0.007 |

Solutions in which the measurements were made contained (\( \mu M \)): Glycylglycine 27; Mg\( ^{++} \) 69; sucrose 54; \( K^+ \) 16 or Mg\( ^{++} \) 8 and sucrose 7; Cl\( ^{-} \) 144; glucose 10 (repleted cells) or 0 (depleted cells). \( n = 5 \).

solutions should disappear in cells which are depleted of intracellular energy stores. In Table VII, the results of experiments designed to determine whether this is the case are presented. In the repleted cells, K stimulates the Na outflux and, in Na-K-free solutions, ouabain inhibits the Na outflux. In the depleted cells, however, there is very little K-stimulated Na outflux, and the ouabain-inhibited Na outflux in Na-K-free solutions has disappeared.

**Ratio between the Ouabain-Sensitive Na Outflux and the Ouabain-Sensitive Na Influx**

Garrahan and Glynn (1967 a) found that the ratio between the ouabain-sensitive Na outflux and the ouabain-sensitive Na influx in \( K_0 \)-free solutions was not significantly different from 1.0. This differs from the value of 1.5 previously reported (Post and Jolly, 1957) for the ratio between the ouabain-sensitive net Na outflux and the ouabain-sensitive net K influx. The results of simultaneous measurements of the ouabain-sensitive Na outflux and Na influx to determine the ratio of \( ^{\alpha}M_{Na} \) to \( ^{\alpha}M_{Na} \) are presented in Table VIII. The measurements at \( Na_0 \) 12 mmoles/liter RBC were made using fresh cells, and the measurements at \( Na_0 \) 20 and 88 mmoles/liter RBC were made using cells in which the \( Na_0 \) was altered by exposure of the cells to PCMBS. In each case, the ratio of the mean \( ^{\alpha}M_{Na} \) to the mean \( ^{\alpha}M_{Na} \) was greater than 1.0. The mean difference between the ouabain-sensitive \( ^{\alpha}M_{Na} \) and \( ^{\alpha}M_{Na} \) for all the measurements recorded in Table VIII (\( n = 18 \)) was 0.35, SEM 0.13, \( p < 0.02 \). However, as shown in Table VIII, the ratio found varied considerably from individual to individual, and it seems possible that there is no fixed ratio, but that the ratio found depends on circumstances which are not apparent.

Garrahan and Glynn (1967 c) have reported that high \( Na_0 \) cells do not exhibit a ouabain-sensitive Na outflux in K-free, high Na solutions unless they are depleted of ATP or contain high concentrations of phosphate. In the experiments reported in Table VIII, the mean ouabain-sensitive Na outflux does not decrease when \( Na_0 \) rises from 20 to 88 mmoles/liter RBC. Neither the ATP nor the phosphate content of the cells used here was measured.
TABLE VIII
RATIO OF OUABAIN-SENSITIVE \(^{22}M_{Na}\) TO OUABAIN-SENSITIVE \(^{42}M_{Na}\) IN K\(_{o}\)-FREE SOLUTIONS

| Na\(_{e}\), mmole/liter RBC | 12    | 20    | 88    |
|---------------------------|-------|-------|-------|
| \(^{22}M_{Na}\)         | \(^{42}M_{Na}\) | \(^{22}M_{Na}/^{42}M_{Na}\) | \(^{22}M_{Na}\) | \(^{42}M_{Na}\) | \(^{22}M_{Na}/^{42}M_{Na}\) | \(^{22}M_{Na}\) | \(^{42}M_{Na}\) | \(^{22}M_{Na}/^{42}M_{Na}\) |
| 1.44 | 1.37 | 1.05 | 2.94 | 2.28 | 1.29 | 2.06 | 1.70 | 1.21 |
| 1.09 | 1.77 | 0.62 | 2.69 | 2.07 | 1.30 | 2.82 | 1.90 | 1.48 |
| 1.25 | 0.81 | 1.54 | 1.70 | 1.54 | 1.10 | 0.76 | 0.75 | 1.01 |
| 1.04 | 0.99 | 1.05 | 2.11 | 2.49 | 0.85 | 2.60 | 2.19 | 1.19 |
| 1.58 | 1.51 | 1.05 | 1.93 | 1.28 | 1.51 | 3.60 | 1.84 | 1.56 |
| 1.68 | 0.68 | 2.40 | 1.39 | 1.34 | 1.04 |       |       |     |
| 0.53 | 0.44 | 1.20 |       |       |       |       |       |     |

Mean ± SEM
1.23±0.15 | 1.08±0.18 | 1.14 | 2.13±0.24 | 1.83±0.21 | 1.16 | 2.37±0.47 | 1.68±0.24 | 1.41±0.34

Solutions in which the measurements were made contained (mm): Glycylglycine 27; Mg\(^{2+}\) 5; Na\(^{+}\) 144; Cl\(^{-}\) 144; glucose 10. The measurements
using cells with Na\(_{e}\) 12 were made in fresh cells, and the measurements using cells with Na\(_{e}\) 20 and 88 were made in cells exposed to PCMBs.
Each recorded measurement is from a different individual.
However, after exposure to PCMBS, the cells were incubated for 1 hr in a solution containing 10 mM inosine, 3 mM adenine, and 10 mM glucose; it would be expected that this treatment would raise intracellular ATP and lower phosphate (Whittam and Wiley, 1968; Glynn and Lüthi, 1968), and these were the circumstances under which Garrahan and Glynn (1967 c) found little ouabain-sensitive Na-Na exchange. A major difference in the experimental procedure is the use of reconstituted ghosts by Garrahan and Glynn (1967 c) and the use of PCMBS-treated intact cells in the present experiments.

**DISCUSSION**

The relation between $k_{N_a}$ and $[N_a]$ is of the same form whether measured in (Na + K)-free solutions; high Na, K-free solutions; or high Na, high K solutions. This correspondence suggests that all three processes are effected by the same system. The curves obtained for the relation cannot be described by a Michaelis-Menten model requiring that the interaction of a single Na ion with the system results in the activation of the transport process. Such a model can be described by the relation:

$$^{o}M_{N_a} = \frac{a}{b + [N_a]} \tag{1}$$

where $^{o}M_{N_a}$ is the active sodium outflux, and $a$ and $b$ are constants. Dividing both sides of the equation by $[N_a]$ results in:

$$^{o}k_{N_a} = \frac{a}{b + [N_a]} \tag{2}$$

It can be seen that this relation predicts that $^{o}k_{N_a}$ continually decreases as $[N_a]$ increases; this is not the case for the curves of Figs. 6 and 7. It has previously been shown (Sachs and Welt, 1967) that the relation between both the active K influx and Na outflux and $[K_a]$ can be described by a model in which it is required that two K ions interact with the system before transport occurs, and the total number of K sites is limited so that the process demonstrates saturation kinetics. Such a model may be used to describe the active sodium outflux as a function of $[N_a]$:

$$^{o}M_{N_a} = \frac{a}{b + [N_a] + [N_a]^2} \tag{3}$$

where $a$, $b$, and $c$ are constants. Dividing both sides of this equation by $[N_a]$:

$$^{o}k_{N_a} = \frac{a}{[N_a] + b + [N_a]} \tag{4}$$
The behavior described by this relation of $k_{Na}$ as $[Na]$ increases will be that demonstrated in Figs. 6 and 7; i.e., at first $k_{Na}$ will increase with increasing $[Na]$, and then it will decrease. The model described here assumes that two Na ions must interact with the system before transport occurs, but the conclusion will be qualitatively similar using a model which requires that three or more ions must be simultaneously present. It does not seem possible to decide between these possibilities on the basis of the data presented here. The form of the relation between $k_{Na}$ and $[Na]$ when the measurement is made in the presence of ouabain, either in high Na (Fig. 4) or Na-free (Fig. 7) solutions, is quite different from the form of the relation when the measurement is made in the absence of ouabain, and can be adequately described by equation 2. This suggests that the saturable Na outflux measured with ouabain present is mediated by a different system from that which mediates the ouabain-sensitive fluxes, or, if the same system is responsible for both phenomena, ouabain markedly alters its properties so that it is able to perform Na transport when a single Na ion has interacted with it.

The shape of the plot of $k_{Na}$ vs. $[Na]$ may also explain the observed increase in $k_{Na}$ when measured in high Na, high K solutions as compared to the value obtained in Na-free, high K solutions (Table I). These measurements were made using cells with Na at the start of the experiment of about 9 mmoles/liter RBC. Since Na will leak into the cells from the high Na solutions, the cells in which the measurements were made in high Na solutions would be expected to have a higher mean Na over the course of the measurement than the cells in which the measurements were made in Na-free solutions. In 10 experiments in which measurements of Na were made under circumstances similar to those under which the measurements recorded in Table I were made, Na in the high Na, high K solution at 30 min was 10.4 ± 0.8 (SEM) mmoles/liter RBC, and at 90 min 10.6 ± 0.8. In the high K, Na-free solutions, Na at 30 min was 9.1 ± 0.8 and at 90 min 7.6 ± 0.8. The experiments recorded in Table I lasted 90 min. If $k_{Na}$ were related to $[Na]$ by an expression similar to equation 2, and if there were no effect of Na, aside from its effect in raising Na, then the cells in which the measurements were made in high Na solutions (higher Na) would have a lower value of $k_{Na}$ than the cells in which the measurements were made in Na-free solutions. A direct effect of Na, would have to be invoked to explain the observed increase in $k_{Na}$ in high Na solutions. However, at Na, of 9 mmoles/liter RBC, $k_{Na}$ actually increases with increasing Na (Fig. 4), and therefore the observed increase in $k_{Na}$ in high Na solutions using cells which begin with Na of 9 mmoles/liter RBC can be explained by the effect of Na on $[Na]$, and no direct effect of Na need be invoked. This is supported by the observation that, using cells with high Na, in the range in which $k_{Na}$ falls with increasing $[Na]$ (Fig. 4), $k_{Na}$ is not increased in high Na solutions unless ouabain is present.
Garrahan and Glynn (1967 a) have proposed that the relation between [Na\textsubscript{0}] and the magnitude of the Na-Na exchange can be explained by assuming that, in the absence of K\textsubscript{0}, the Na sites can move from the outer to the inner border only when Na is attached and that the affinity for Na at the outer border is low, so that, even at high Na\textsubscript{0}, the [Na\textsubscript{0}] is below the apparent K\textsubscript{m} of the system for Na at the outer surface. Figs. 2 and 3, however, seem to indicate that there are two components of the relation between \( k_{Na} \) and [Na\textsubscript{0}]. At first, \( k_{Na} \), and the portion of \( k_{Na} \) which is inhibited by K\textsubscript{0}, fall rapidly with increasing [Na\textsubscript{0}], and this suggests saturation of the Na sites involved with a high affinity of the sites for Na. After this, \( k_{Na} \) remains fairly constant, and the portion of \( k_{Na} \) which is inhibited by K\textsubscript{0} increases. Addition of ouabain also decreases the value of \( k_{Na} \). It seems clear that both these effects of Na\textsubscript{0} cannot be explained by the interaction of Na at a single set of sites. Once the sites responsible for the early apparent saturation of the Na influx are filled, further increases in Na\textsubscript{0} will not affect them, and the effect of Na\textsubscript{0} in increasing the Na-Na exchange must be accomplished at some other site. It seems inescapable that, in order to account completely for the relation between \( k_{Na} \) and Na\textsubscript{0}, one must assume that Na interacts with two sets of sites at the outer membrane surface.

At low Na\textsubscript{0} concentrations, \( k_{Na} \) and \( k_{Na} \) simultaneously fall with increasing [Na\textsubscript{0}]. Assuming that the two processes are related, one can qualitatively account for this similarity in behavior by supposing that the transport mechanism, when combined with Na, prefers a configuration in which the Na sites are at the outer membrane surface, and, when free of Na, prefers a configuration in which the Na sites are at the inner membrane surface. Under such circumstances, the inward movement of the Na sites would be more rapid when free of Na than when combined with Na, and sites combined with Na would tend to accumulate at the outer membrane surface. When Na\textsubscript{0} is zero, Na sites at the inner membrane surface would pick up Na ions, move to the outer membrane surface, discharge Na, and move to the inner surface as free Na sites. When Na\textsubscript{0} is higher, more of the Na sites at the outer surface would be combined with Na, and therefore less likely to move to the inner surface; since less sites would be available at the inner surface, \( k_{Na} \) would fall. This mechanism will not account for the increased Na outflux and Na influx as Na\textsubscript{0} increases above 6 mM; as discussed above, this seems to indicate an action of Na at some site other than those which are becoming saturated at low Na\textsubscript{0}.

The ouabain-sensitive \( M_{Na} \) in K-free solutions is greater than the ouabain-sensitive \( M_{Na} \) under the same circumstances, and there appears to be a small net loss of Na from the cells by this mechanism. The energy source for this net movement is not clear; Garrahan and Glynn (1967 c, d) have shown that, although ATP is required for the outflux component of the Na-Na exchange
to occur, very little ATP is hydrolyzed under conditions which permit the exchange to proceed. It is possible that the amount of ATP hydrolyzed is enough to support the small net outflux of Na or that some source of energy other than ATP is responsible for net Na outflux under these circumstances.

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