Ouabain-Insensitive Sodium Movements in the Human Red Blood Cell

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ABSTRACT Red blood cells exposed to ouabain are capable of net Na outflux against an electrochemical gradient; the net outflux is inhibited by the diuretic, furosemide. In ouabain-treated cells, both the unidirectional Na outflux and the unidirectional Na influx are inhibited by furosemide. Furosemide also inhibits the ouabain-sensitive Na-Na exchange accomplished by the Na-K pump in K-free solutions. From the interaction of extracellular K, furosemide, and ouabain with the transport system, it seems possible that the ouabain-insensitive Na outflux is accomplished by the same mechanism that is responsible for the ouabain-sensitive Na-K exchange. The ouabain-insensitive Na outflux is increased by extracellular Na, and the influx increases as the intracellular Na increases. In fresh cells, high extracellular K concentrations decrease the ouabain-insensitive Na outflux and increase the ouabain-insensitive Na influx. When the rate constant for sodium outflux and the rate constant for sodium influx in ouabain-treated cells are plotted against the extracellular K concentration, the curves obtained are mirror images of each other. In starved cells, extracellular K increases the ouabain-insensitive Na outflux as does extracellular Na, and it has little effect on the Na influx.

INTRODUCTION

The Na and K movements in the human red blood cell which are inhibited by the cardiotonic steroids have been well-characterized. It has been found (Glynn, 1956; Post and Jolly, 1957) that the Na outflux and K influx which occur against electrochemical gradients are coupled in that the ouabain-sensitive Na outflux is stimulated by extracellular potassium (K_o). The source of energy for these movements has been found to be ATP (Gardos, 1954; Hoffman, 1962), and the characteristics of the ouabain-sensitive transport system have been correlated with the characteristics of a membrane component which hydrolyzes ATP when both Na and K are present, and which is inhibited by ouabain (Dunham and Glynn, 1961; Post et al., 1960). The

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behavior of the ouabain-sensitive transport system when K_o is absent has been studied; under these circumstances, the system is able to exchange intracellular (Na_c) for extracellular (Na_o) sodium (Garrahan and Glynn, 1967).

Recently, attention has been devoted to Na and K fluxes in human red blood cells which are not inhibited by ouabain. Hoffman and Kregenow (1966) have reported that there is a component of the Na outflux which is insensitive to ouabain, which does not require K_o, but does appear to depend on the presence of Na_o. This component of the Na outflux appeared to accomplish net movements of Na_c against a concentration gradient and was inhibited by the diuretic ethacrynic acid. Hoffman and Kregenow (1966) stated that their data were consistent with the existence of two pumps, one sensitive to ouabain and one insensitive. Lubowitz and Whittam (1969), however, found that, with ouabain present, the Na influx increases with increasing $[Na_c]$. On the basis of this finding, they suggest that the ouabain-insensitive Na outflux which is inhibited by ethacrynic acid does not represent active transport against an electrochemical gradient, but is simply exchange diffusion (Ussing, 1950).

This paper presents some further studies of the ouabain-insensitive Na fluxes, using as an inhibitor the diuretic, furosemide, instead of ethacrynic acid. Furosemide will be seen to have effects similar to those of ethacrynic acid, but has been chosen since it causes less hemolysis than does ethacrynic acid.

METHODS

All salt solutions were made up to an osmolality of 295 ± 5 milliosmols/kg water using reagent grade chemicals, except RbCl and CsCl solutions, which were made with spectrographically pure salts (Johnson, Mathey Company, London, England). Unless otherwise stated, 10% of the volume of all solutions consisted of a glycylglycine-$MgCO_3$ buffer solution (glycylglycine 273 mM, $MgCO_3$ 54 mM, 295 ± 5 milliosmols/ kg water, pH 7.4 ± 0.05 at 37°C). When Na or K was varied in concentration osmolality was maintained by replacing NaCl or KCl solution with an equal volume of a solution composed of 75% isomotic $MgCl_2$ (107 mM) solution and 25% isosomotic sucrose (267 mM) solution. The chloride concentration of this solution was approximately equal to that of an isosmotic NaCl or KCl solution. Crystalline bovine serum albumin, 20 mg/100 cc, was present in all solutions. Furosemide, which was the gift of the Hoechst Pharmaceutical Company, Cincinnati, Ohio, was added to solutions by dissolving the appropriate amount of crystalline furosemide.

Venous blood was obtained from healthy males using heparin (10 μ/ml) as anticoagulant, the cells separated by centrifugation and the plasma and buffy coat removed by aspiration. The cells were then washed three times in $MgCl_2$ solution (107 mM) (by suspension, centrifugation, and aspiration).

Na outflux and Na influx were measured as previously described (Sachs and Welt, 1967; Sachs and Conrad, 1968). The method for determining $[Na_c]$ has also been described (Sachs and Welt, 1967). Cell water was measured by determining the wet

and dry weight of a measured volume of a red cell suspension of known hematocrit (about 50%), and a measured volume of the solution in which the cells were suspended. The calculation is:

$$C.W. = \frac{C - S(1 - Hct)}{Hct}$$

in which C.W. is the cell water (liters/liter RBC), C is the difference between the wet and dry weight of 1 ml of the red cell suspension, S is the difference between the wet and the dry weight of 1 ml of the solution, and Hct is the hematocrit of the suspension.

Intracellular Na and K concentrations were altered by exposure of the cells to p-chloromercuribenzene sulfonate (PCMBS) according to a modification of a method first proposed by Garrahan and Rega (1967). Details of the procedure have been described (Sachs, 1970).

Depletion of intracellular energy stores was accomplished by incubating cells at 37°C in the absence of substrate. Cells were washed four times in $MgCl_2$ solution, and incubated for 17 hr at about 20% Hct in a solution composed of (mM): Na^+ 32; K^+ 134; Cl^- 144; PO_4 13.4; pH 7.4; or for 21 hr in a solution composed of (mM): Na^+ 166; Cl^- 144; PO_4 13.4; pH 7.4. In either case, penicillin (10,000 μ/100 ml) and streptomycin sulfate (0.1 g/100 ml) were used to suppress bacterial growth, and the solution was changed once about 6 hr after incubation began. At the end of the incubation, the cells were separated from the solution, washed three times in $MgCl_2$ solution, and then resuspended at about 50% Hct in a solution containing (mM): Na^+ 166; Cl^- 144; PO_4 13.4; pH 7.4. To half this suspension, inosine 4.2 mM, adenine 1.7 mM, and glucose 10 mM were added; the other half was incubated in the absence of substrate. The cells which had been incubated for 17 hr were incubated for a further 1 hr in these solutions, and the cells which had been incubated for 21 hr were incubated for a further 3 hr.

The symbols used are defined as follows:

$$^{o}M \left(\frac{\text{mmoles Na}}{\text{liter RBC} \times \text{hr}} \right) = {}^{o}k \left(\frac{1}{\text{hr}} \right) \times [\text{Na}_c] \left(\frac{\text{mmoles}}{\text{liter RBC}} \right)$$

where ^{o}M is the Na outflux, ^{o}k the rate constant for the Na outflux, and Na_c the intracellular Na;

$$^{i}M \left(\frac{\text{mmoles Na}}{\text{liter RBC} \times \text{hr}} \right) = {}^{i}k \left(\frac{\text{mmoles Na}}{\text{liter RBC} \times \text{hr}} \times \frac{1}{\text{mM}} \right) \times [\text{Na}_o](\text{mM})$$

where ^{i}M is the Na influx, ^{i}k the rate constant for the Na influx, and Na_o the extracellular Na. It should be pointed out that the units for ^{i}k(mmoles/liter RBC, hr, mM Na_o) are not directly comparable to the units for ^{o}k(hr^{-1}). In order to make them comparable, one would have to calculate the influx into the relevant compartment; i.e., the cell water. Therefore, by dividing the reported values for ^{i}k by a value for cell water (0.7 liter/liter RBC), one obtains a value for ^{i}k in units comparable to the units in which ^{o}k is expressed, hr^{-1}. If the flux ratio equation (Ussing, 1950) holds f_r any

particular flux, it can be shown that $^ok = {}^ik \dfrac{[Cl_i]}{[Cl_o]}$ (Sachs and Welt, 1967) or $^ok = 0.7\,{}^ik$. Therefore, since the two corrections of ^ik cancel out, the values of ^ik reported in the units used here should equal ^ok if the flux ratio equation holds for the fluxes under investigation.

Where differences and standard errors of the mean are given in the text, the differences are in all cases the mean of differences in paired observations in repeat experiments.

RESULTS

Ouabain-Insensitive Na Influx and Outflux. Table I presents the results of experiments designed to compare the effects of ouabain, of furosemide, and

TABLE I
EFFECT OF FUROSEMIDE ON THE SODIUM
OUTFLUX AND SODIUM INFLUX

Solution	^ok		^ik	
	$K_o\ 0$	$K_o\ 16\ \text{mM}$	$K_o\ 0$	$K_o\ 16\ \text{mM}$
	$hr^{-1} \pm SEM$		$mmoles/liter\ RBC \times hr \times mM\ Na_o \pm SEM$	
Na_o 0.35 mM	0.081±0.011	0.217±0.019	0.0369±0.0012	0.0261±0.0005
Na_o 0.35 mM, ouabain 10^{-4} M	0.055±0.008	0.041±0.004	0.0217±0.0008	0.0276±0.0014
Na_o 0.35 mM, furosemide 10^{-3} M	0.035±0.006	0.228±0.015	0.0245±0.0022	0.0173±0.0008
Na_o 0.35 mM, ouabain 10^{-4} M, furosemide 10^{-3} M	0.025±0.004	0.025±0.004	0.0175±0.0016	0.0173±0.0017
Na_o 128 mM	0.197±0.022	0.260±0.029	0.0230±0.0015	0.0197±0.0006
Na_o 128 mM, ouabain 10^{-4} M	0.101±0.004	0.100±0.004	0.0169±0.0006	0.0206±0.0006
Na_o 128 mM, furosemide 10^{-3} M	0.103±0.013	0.240±0.033	0.0154±0.0007	0.0135±0.0004
Na_o 128 mM, ouabain 10^{-4} M, furosemide 10^{-3} M	0.063±0.004	0.065±0.004	0.0139±0.0005	0.0137±0.0004

Solutions in which the measurements were made contained (mM): Glycylglycine 27; Mg^{++} 5; K^+ 16 or Mg^{++} 8 and sucrose 7; Na^+ 128 or Mg^{++} 64 and sucrose 54; Cl^- 144; glucose 10. The measurements were made using fresh cells. n (number of experiments) = 5. Na_c was about 11 mmoles/liter RBC.

of ouabain and furosemide together on ^ok and ^ik. The determinations were made at Na_o 0.35 and 128 with and without K_o 16 mM. When the measurements were made in high Na, K-free solutions, furosemide reduced both ^ok and ^ik whether or not ouabain was present. The furosemide reduction of ^ok in solutions without ouabain (0.197 − 0.103 = 0.094, SEM 0.013) was, however, considerably greater than the reduction of ^ok produced by furosemide when the measurements were made in solutions containing ouabain (0.101 − 0.063 = 0.038, SEM 0.002). The same pattern was apparent when the effect of furosemide on ^ik was measured in the same solutions. In ouabain-free solu-

tions, furosemide reduced ^ik more $(0.0230 - 0.0154 = 0.0076,$ sem $0.0011)$ than it did in solutions containing ouabain $(0.0169 - 0.0139 = 0.0030,$ sem $0.0003)$. Garrahan and Glynn (1967) have presented evidence that the ouabain-sensitive Na outflux and influx in high Na, K-free solutions is an exchange of Na_c for Na_o carried out by the Na-K pump. Since the effect of furosemide is greater in the ouabain-free solutions than in the solutions containing ouabain, furosemide must be inhibiting a portion of the ouabain-sensitive Na-Na exchange carried out by the Na-K pump. In K-free solutions, therefore, furosemide affects both the ouabain-insensitive Na fluxes and fluxes accomplished by the Na-K pump.

The situation was, however, quite different when the measurements were made at K_o 16 mM. At this concentration of K_o, the Na-K pump is maximally stimulated, and Garrahan and Glynn (1967) have shown that the ouabain-sensitive Na-Na exchange no longer operates. In K_o 16 mM, Na-free solutions, furosemide reduced ^ok only when ouabain was present. Furosemide did not reduce ^ok in K_o 16 mM, ouabain-free solutions $(0.217 \, [-\text{furosemide}] - 0.228 \, [+\text{furosemide}] = -0.011,$ sem $0.006)$, although it did reduce ^ok in ouabain-free solutions without K $(0.081 - 0.035 = 0.046,$ sem $0.012)$. In K_o 16 mM, high Na solutions, furosemide reduced ^ok to some extent $(0.020,$ sem $0.007)$, but much less than the reduction obtained in the K-free solutions $(0.094,$ sem $0.013)$. Furosemide, therefore, lowered ^ok under all circumstances when ouabain was present, but, in solutions without ouabain, it reduced ^ok in K-free solutions (when the pump is functioning as an Na-Na exchanger) but not in high K solutions (when the pump is functioning as an Na-K exchanger). In high K solutions, furosemide reduced ^ik whether or not ouabain was present.

Lubowitz and Whittam (1969) have suggested that, superimposed upon the ouabain-sensitive movements of Na mediated by the Na-K pump, there is also an ouabain-insensitive Na-Na exchange. Since, in K_o 16 mM solutions, furosemide lowered ^ik by about the same amount whether or not ouabain was present, but significantly lowered ^ok only in solutions containing ouabain, it is unlikely that the furosemide reduction of ^ik reflects the inhibition by furosemide only of an ouabain-insensitive Na-Na exchange. Further evidence that this is not the case can be obtained from the furosemide effect in K-free solutions containing ouabain. Values for ^oM and ^iM calculated from the experiments reported in Table I are presented in Table II. In high Na solutions, the furosemide inhibition of ^oM $(0.53,$ sem $0.06)$ was little different from the furosemide inhibition of ^iM $(0.38,$ sem $0.04)$. In low Na solutions, however, furosemide inhibited ^oM $(0.28,$ sem $0.06)$ despite the lack of a comparable decrease in ^iM.

In low Na solutions containing ouabain, ^ok is greater than ^ik. It seemed, therefore, worthwhile to determine whether the ouabain-insensitive mecha-

nism is capable of accomplishing net transport against an electrochemical gradient. For this purpose, cells with normal $[Na_c]$ were incubated in solutions in which $[Na_o]$ was low, but higher than $[Na_c]$. Since Cl is apparently at thermodynamic equilibrium across the red cell membrane (Van Slyke et al., 1923), it can be concluded that the inside of the red cell membrane is negative relative to the outside, and therefore a solution with a $[Na_o]$ higher than $[Na_c]$ will result in a higher electrochemical potential for Na outside the cell. The bulk of the solution was made up of isosmotic $MgCl_2$-sucrose solution, isosmotic choline Cl solution, or isosmotic KCl solution, and all solutions contained ouabain 10^{-4} M. One-half hour after incubation began, red cell samples were taken for determination of $[Na_c]$ and cell water, and the determinations

TABLE II

EFFECT OF FUROSEMIDE ON ^oM AND ^iM

	^oM	^iM
	mmoles/liter RBC × hr ± SEM	mmoles/liter RBC × hr ± SEM
Na_o 0.35, ouabain 10^{-4} M		
No furosemide	0.53±0.11	0.0076±0.0008
Furosemide 10^{-3} M	0.26±0.05	0.0061±0.0006
Δ	0.28±0.06	0.0015±0.0005
Na_o 128, ouabain 10^{-4} M		
No furosemide	1.32±0.10	2.16±0.08
Furosemide 10^{-3} M	0.80±0.08	1.78±0.06
Δ	0.52±0.06	0.38±0.04

The fluxes were calculated from the data of Table I. The solutions were K-free.

were repeated 8 hr later; there was no change in cell water over the course of the experiment. The results of one of five such experiments are shown in the upper part of Table III. The cells lost Na against an electrochemical gradient in solutions in which the major cation was Mg or choline, but not in solutions in which K was the major cation. The effect of furosemide on the ouabain-insensitive net Na outflux is shown in the bottom part of Table III (one of four such experiments). Furosemide inhibited the net Na loss in the $MgCl_2$-sucrose solution, but had no effect on the cells incubated in the KCl solution. Furosemide, therefore, inhibited a net Na outflux against an electrochemical gradient in ouabain-treated cells.

Effect of $[Na_c]$ and $[Na_o]$ on the Ouabain-Insensitive ^ok and ^ik Further characterization of the ouabain-insensitive ^ok and ^ik was attempted by determining their response to alterations in $[Na_c]$ and $[Na_o]$. In Fig. 1, the ouabain-insensitive ^ok is plotted as a function of $[Na_c]$ in cells in which $[Na_c]$ was altered by exposure of the cells to PCMBS. The upper curve represents the results of

measurements made in a high Na solution; $^o k$ falls with rising [Na_c], which suggests that the process is saturable, and the affinity of the mechanism for Na appears to be strong. Hoffman and Kregenow (1966) have reported similar findings. When furosemide is added to the same solutions (middle curve), $^o k$ is inhibited, but there is still a decrease in $^o k$ as [Na_c] increases; furosemide does not completely abolish the ouabain-insensitive $^o k$. The lower curve represents the relation between $^o k$ and [Na_c] in Na-free solutions; a saturable ouabain-insensitive component of $^o k$ is present in Na-free solutions.

TABLE III

OUABAIN-INSENSITIVE NET Na OUTFLUX AGAINST
AN ELECTROCHEMICAL GRADIENT

Na_o, mM	Na_c in mmoles/liter RBC water					
	13.4		18.1		22.4	
	½ hr	8½ hr	½ hr	8½ hr	½ hr	8½ hr
MgCl₂-sucrose	10.3 (0.0210)	7.8 (0.0150)	9.9 (0.0207)	7.9 (0.0155)	11.0 (0.0216)	8.8 (0.0170)
Choline Cl	10.6 (0.0211)	6.8 (0.0126)	10.7 (0.0210)	7.5 (0.0139)	10.8 (0.0214)	8.2 (0.0152)
KCl	10.5 (0.0219)	12.0 (0.0249)	10.9 (0.0220)	10.9 (0.0283)	11.1 (0.0224)	13.3 (0.0284)

Na_o, mM	Na_c in mmoles/liter RBC water				
	22.6			22.6	
	Furosemide 0			Furosemide 10^{-4} M	
	½ hr	8½ hr		½ hr	8½ hr
MgCl₂-sucrose	10.5 (0.0253)	8.3 (0.0201)		9.1 (0.0236)	10.9 (0.0259)
KCl	10.6 (0.0241)	11.8 (0.0298)		8.9 (0.0229)	10.8 (0.0314)

Solutions in which the measurements were made contained (mM): Glycylglycine 27; Mg^{++} 5; and Mg^{++} 66-61 and sucrose 56-50, or choline⁺ 132-122, or K⁺ 132-122; Cl⁻ 144; glucose 10. Ouabain 10^{-4} M was present in all solutions. Numbers in parentheses are Na_c given in mmoles/g hemoglobin.

In Fig. 2, $^i k$ is plotted as a function of [Na_o]. Ouabain was not present in the solutions in which these measurements were made, but K 16 mM was included in the solutions. The data of Table I indicate that the Na influx is equal in solutions containing K whether or not ouabain is present. $^i k$ falls with increasing [Na_o], so that the process seems to be saturable, but the affinity for Na is low.

The results of experiments to determine the effect of alterations in [Na_c] on the magnitude of the ouabain-insensitive $^o k$ and $^i k$ are recorded in Table IV. The [Na_c] was altered by exposure of the cells to PCMBS, and the determination of the Na fluxes was made in high K solutions since the ouabain-insensitive $^i k$ is greatest with K as the major extracellular cation (see below). There is little change in the magnitude of $^i k$ as [Na_c] increases from 4.1 to 19.4

mmoles/liter RBC, but $^i k$ increases somewhat (0.0094, SEM 0.0014) as $[Na_c]$ rises from 18.7 to 89.4 mmoles/liter RBC. When furosemide is present, $^i k$ does not increase as the $[Na_c]$ increases. The increase in the ouabain-insensitive $^i k$ as $[Na_c]$ rises is similar to the findings of Lubowitz and Whittam (1969). However, the increase in $^i k$ is small considering that Na_o is very low.

The Effect of K_o and of Ouabain on the Sensitivity of $^o k$ to Furosemide   Since K stimulates the Na-K pump and ouabain inhibits it, the inhibition by K in ouabain-free solutions of the furosemide effect on $^o k$ (Table I), and the reversal
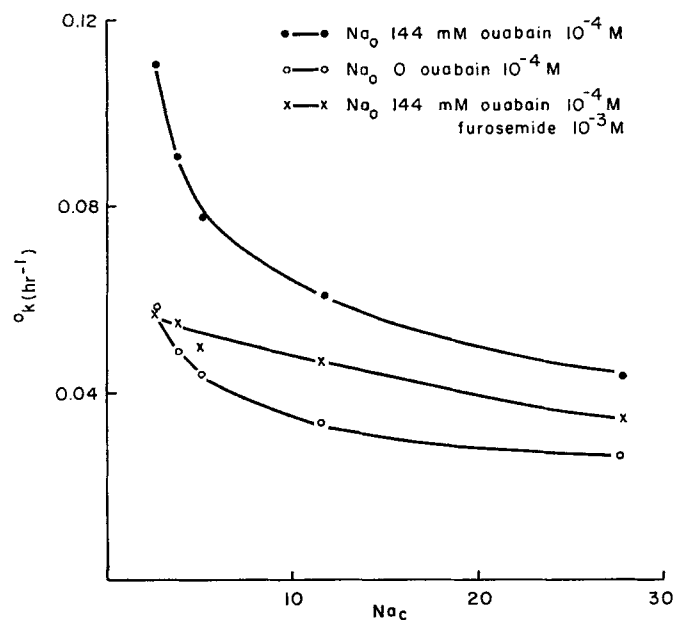

FIGURE 1.   Sodium outflux rate constant ($^o k$) vs. intracellular sodium concentration (Na_c, mmoles/liter RBC). Solutions in which the measurements were made contained (mM): Glycylglycine 27; Mg^{++} 5; Na^+ 144 or Mg^{++} 77 and sucrose 60; Cl^- 144; glucose 10. Intracellular sodium concentration was altered by exposure of the cells to PCMBS.

of the inhibition by ouabain, suggest that the ouabain-insensitive $^o k$ might in some way be related to the ouabain-sensitive Na-K pump. Experiments were performed to determine whether the concentrations of K and of ouabain at which the interaction with furosemide was apparent were the same as the concentrations which produce effects on the Na-K pump.

In Fig. 3, $^o k$ is plotted against $[K_o]$ from measurements made in Na-free solutions free of ouabain with and without furosemide. Fig. 4 is a plot of a similar experiment, but in this case Na_o was 128 mM. The curves in Fig. 4 are shifted to the right compared to those in Fig. 3, but in both cases the difference between the curve in the presence of furosemide and that in its absence is

greatest when K_o is absent, decreases as $[K_o]$ increases, and vanishes when K_o is sufficient to maximally stimulate the Na outflux. The affinity, therefore, for K_o of the sites at which K_o exerts its effect of suppressing the furosemide inhibition of ^ok is the same as the affinity for K_o of the sites at which K_o exerts its effect of stimulating ^ok.

Fig. 5 represents the results of an experiment in which the concentration of ouabain which suppresses the K_o inhibition of the furosemide effect was de-
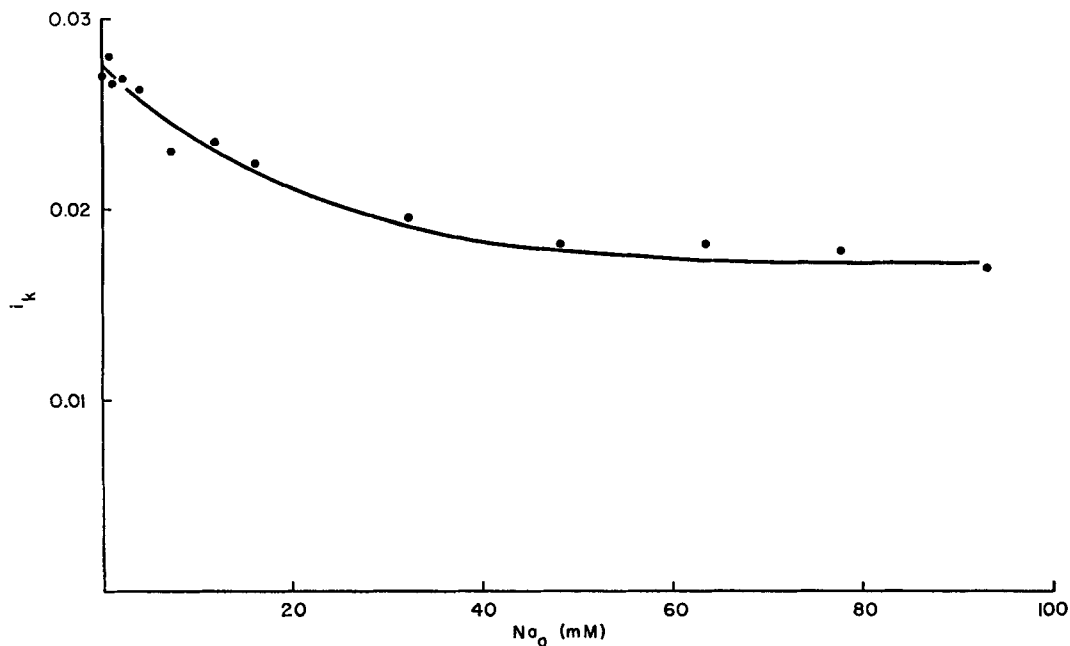

FIGURE 2.   Sodium influx rate constant (^ik, mmoles/liter RBC $\times$ hr $\times$ mM Na_o) vs. extracellular sodium concentration. Solutions in which the measurments were made contained (mM): Glycylglycine 27; Mg^{++} 21; sucrose 13; K^+ 16; Na^+ 96-0.2 or Mg^{++} 0-48 and sucrose 0-40; Cl^- 144; glucose 10. $Na_c = 10.4$ mmoles/liter RBC.

termined. Cells were incubated for 1 hr at 37°C in K-free solutions containing varying concentrations of ouabain. After the incubation, the cells were removed and washed four times to remove extracellular ouabain. The cells were then added to solutions in which ^ok was determined, either with K_o alone, or with both K_o and furosemide. ^ok is plotted against the concentration of ouabain to which the cells had been exposed during the preincubation. At low ouabain concentrations, ^ok in solutions with furosemide is somewhat higher than in solutions without furosemide; this was an occasional finding, and the reason for it is not apparent. As the ouabain concentration increases, ^ok in solutions without furosemide decreases, and ^ok in solutions with furosemide decreases at

a more rapid rate. At the point at which ouabain has almost completely eliminated the K_o-activated ^ok, it has also eliminated the inhibition by K_o of the furosemide reduction of ^ok. It is, therefore, apparent that the sites at which ouabain and K exert their effects on the interaction of furosemide with the system are either the same as the sites at which K_o and ouabain interact with the Na-K pump, or are different sites but with the same affinities for K_o and ouabain.

TABLE IV

EFFECT OF [Na_c] ON
OUABAIN-INSENSITIVE ^ok AND ^ik

Na_c, mmoles/liter RBC	4.1		19.4	
	^ok	^ik	^ok	^ik
	$hr^{-1} \pm SEM$	mmoles/liter RBC $\times$ hr $\times$ mM Na_0 $\pm SEM$	$hr^{-1} \pm SEM$	mmoles/liter RBC $\times$ hr $\times$ mM Na_0 $\pm SEM$
	0.032 ± 0.003	0.045 ± 0.004	0.032 ± 0.005	0.048 ± 0.005
Furosemide 10^{-3} M	0.021 ± 0.002	0.026 ± 0.003	0.022 ± 0.003	0.028 ± 0.006
Na_c, mmoles/liter RBC	18.7		89.4	
	^ok	^ik	^ok	^ik
	$hr^{-1} \pm SEM$	mmoles/liter RBC $\times$ hr $\times$ mM Na_0 $\pm SEM$	$hr^{-1} \pm SEM$	mmoles/liter RBC $\times$ hr $\times$ mM Na_0 $\pm SEM$
	0.028 ± 0.002	0.043 ± 0.006	0.024 ± 0.003	0.052 ± 0.008
Furosemide 10^{-3} M	0.022 ± 0.002	0.024 ± 0.003	0.024 ± 0.001	0.024 ± 0.003

Solutions in which the measurements were made contained (mM): Glycylglycine 27; Mg^{++} 5; Na^+ 0.2; K^+ 144; Cl^- 144; glucose 10. Ouabain 10^{-4} M was present in all solutions. $n = 4$.

Since the Na-K pump ceases to operate in cells with very low [Na_c] or in cells which have been depleted of energy stores, the effect of these manipulations on the K-furosemide interaction was determined. In Table V, the results of experiments in which ^ok was determined in low [Na_c] and normal [Na_c] cells are presented. In the normal [Na_c] cells (15.6 mmoles/liter RBC) K_o stimulates ^ok, furosemide in K_o-free solutions decreases (0.060 − 0.041 = 0.019, SEM 0.003) it, but, in solutions containing K_o, furosemide has no effect (0.298 − 0.298 = 0, SEM 0.005). These results are the same as those found in fresh cells. In the low [Na_c] cells (3.3 mmoles/liter RBC), K_o does not stimulate ^ok, furosemide in K_o-free solutions decreases it, and, in solutions containing K_o, the furosemide effect is inhibited, but not completely. Although the [Na_c] was low enough to almost completely eliminate the K_o stimulation of ^ok, K_o was able to partially inhibit the furosemide effect. Apparently it is not necessary for the Na-K pump to be fully operating in order for K_o to inhibit the furosemide effect.

Table VI records the results of the same experiments using cells which had been depleted of intracellular energy stores by incubation for 18 hr in the absence of substrate. The depleted cells show very little K_o-stimulated ^ok, furosemide reduces ^ok slightly, and K_o apparently does not reverse the furosemide effect. In the repleted cells, K_o stimulates ^ok, furosemide reduces it in
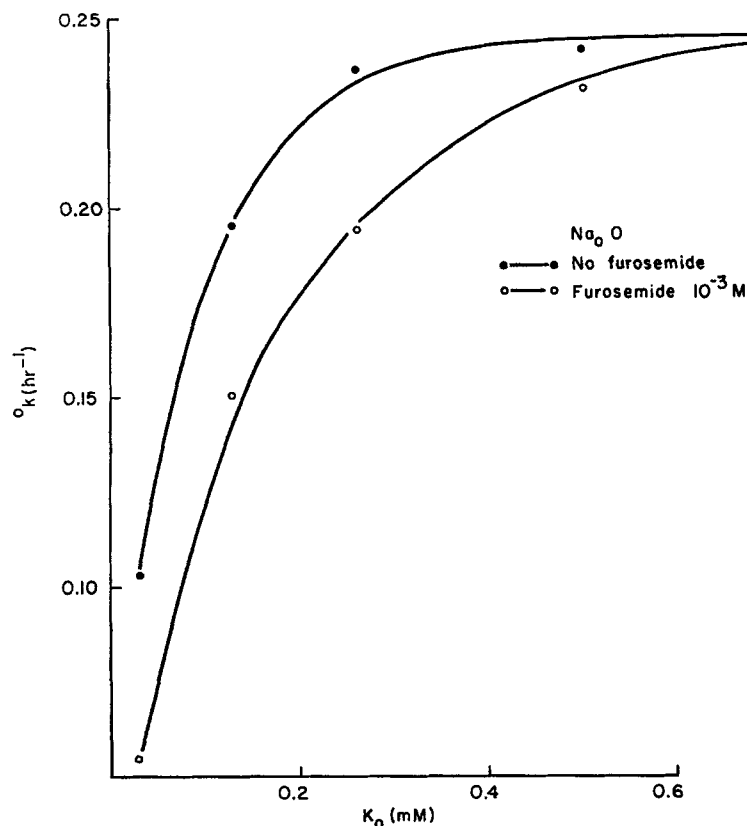

FIGURE 3.   Sodium outflux rate constant (^ok) vs. extracellular potassium concentration (K_o) in the presence and in the absence of furosemide. Solutions in which measurements were made contained (mM): Glycylglycine 27; Mg^{++} 77; sucrose 60; K^+ 0-0.72; Cl^- 144; glucose 10. Na_c = 9.3 mmoles/liter RBC.

K_o-free solutions, but K_o only partially reverses the furosemide effect. Although the repleted cells have resynthesized the energy source necessary to support the K_o-stimulated ^ok, they have not resynthesized to the same extent whatever it is that is necessary for K_o to inhibit the furosemide effect.

The Effect of Extracellular Cation on the Magnitude of the Ouabain-Insensitive ^ok and ^ik   Experiments were performed to determine the magnitude of the ouabain-insensitive ^ok and ^ik in solutions in which the major extracellular
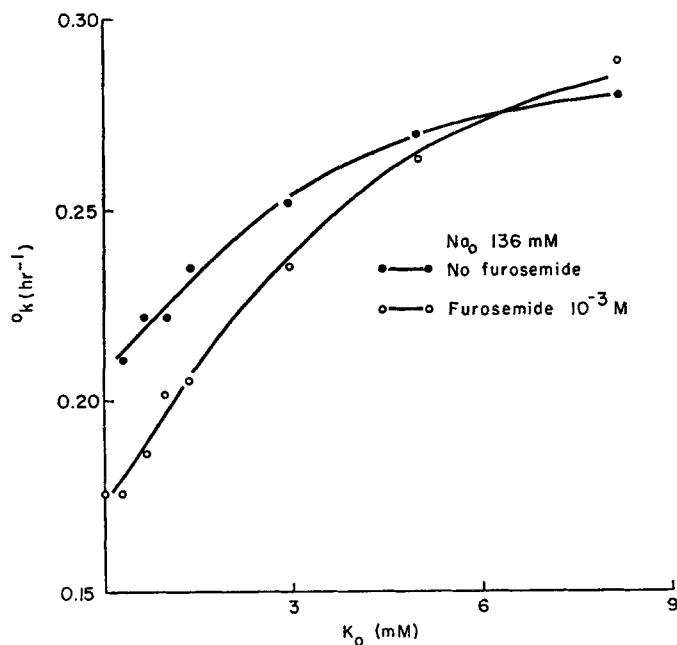
FIGURE 4. Sodium outflux rate constant (^{o}k) vs. extracellular potassium concentration (K_o) in the presence and in the absence of furosemide. Solutions in which the measurements were made contained (mM): Glycylglycine 27; Mg^{++} 9-5; sucrose 3-0; K^+ 0-8; Na^+ 136; Cl^- 144; glucose 10. Na_c = 8.4 mmoles/liter RBC.

cation was varied, and the results are presented in Table VII. Both ^{o}k and ^{i}k are approximately the same in solutions in which the major cation is Mg or Ca. In Rb or K solutions, ^{o}k is lower and ^{i}k higher than in Mg or Ca solutions. In Cs solutions, ^{o}k is higher and ^{i}k lower than in K solutions. When Na is the major extracellular cation, ^{o}k is higher than it is in any other solution, and ^{i}k is lower, although the effect on ^{i}k can be attributed to saturation of the ouabain-insensitive Na transport mechanism at high [Na_o]. In Li solutions, ^{o}k is high but somewhat lower than in Na solutions, and ^{i}k is lower than in any other solution. It is possible that part of the decrease in ^{i}k under this circumstance can be attributed to substitution of Li for Na at the Na transport mechanism, and therefore depression of ^{i}k by saturation at high concentrations of Li. When Rb is the major extracellular cation, furosemide depresses ^{o}k to some extent, and markedly depresses ^{i}k. In Li solutions, furosemide has a marked effect on ^{o}k, and a smaller effect on ^{i}k.

In Fig. 6, ^{o}k is plotted as a function of [K_o] in solutions with and without Na_o. Ouabain was present in all solutions. In both circumstances, K_o reduces the ouabain-insensitive ^{o}k, and the effect is maximal at about 50 mM K_o. Garrahan and Glynn (1967 a) and Glynn et al. (1970) have reported no effect
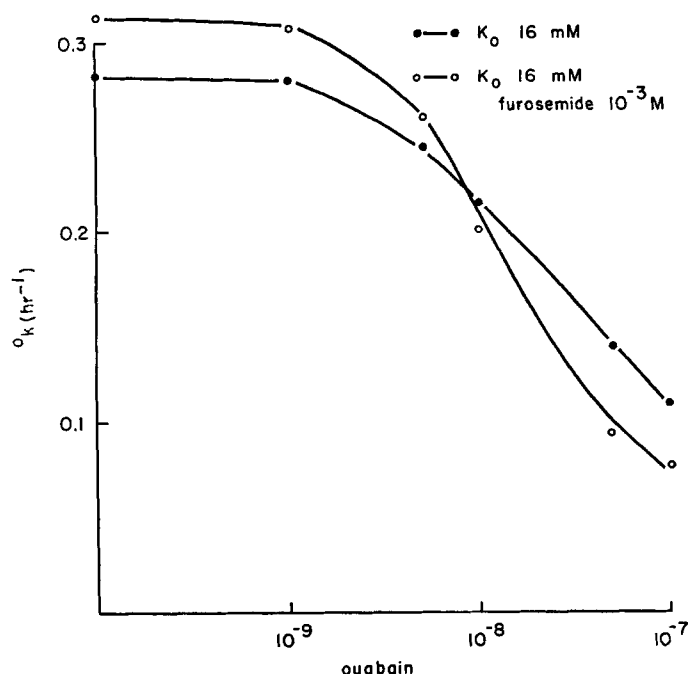
FIGURE 5.   Sodium outflux rate constant $(^o k)$ vs. concentration of ouabain (M) to which the cells were exposed prior to the measurement of $^o k$. Solutions in which the measurements were made contained (mM): Glycylglycine 27; Mg^{++} 5; Na^+ 128; K^+ 16; Cl^- 144; glucose 10. The experimental procedure is described in the text. Na_c = 7.9 mmoles/liter RBC.

of K_o on the ouabain-insensitive sodium outflux either in high or low sodium solution; the concentrations of K which they used were low, and it is possible that this may have contributed to the discrepancy. Furthermore, in their low Na solutions, osmolality was maintained by choline, and in the present experiments it was maintained by Mg; it is possible that the response of $^o k$ to alterations in K_o is different in high choline solutions than in high magnesium solutions. Fig. 7 presents the results of an experiment in which $^o k$ and $^i k$ were determined simultaneously in solutions containing ouabain; both $^o k$ and $^i k$ are plotted against $[K_o]$. As $^o k$ decreases, $^i k$ increases, and the curves are approximately mirror images of each other. Garrahan and Glynn (1967 *b*) have previously reported that the ouabain-insensitive influx is increased by K_o, but in their studies the effect was maximal at a K concentration of 10 mM. It is possible that the higher concentration of K necessary to maximally stimulate $^o k$ in the present experiments is due to the fact that these experiments were performed in a low Na, high Mg solution; the experiments of Garrahan and Glynn were done in a high Na solution.

In order to eliminate the possibility that replacement of NaCl or KCl solu-

TABLE V

K_o-FUROSEMIDE INTERACTION
IN CELLS WITH LOW [Na_c]

Extracellular cation	^ok	
	Na_c 3.3 mmoles/liter RBC	Na_c 15.6 mmoles/liter RBC
	$hr^{-1} \pm SEM$	$hr^{-1} \pm SEM$
K_o 0	0.085±0.014	0.060±0.011
K_o 16 mM	0.093±0.009	0.298±0.045
K_o 0, furosemide 10^{-3} M	0.040±0.003	0.041±0.010
K_o 16 mM, furosemide 10^{-3} M	0.072±0.004	0.298±0.043

Solutions in which the measurements were made contained (mM): Glycylglycine 27; Mg^{++} 69; sucrose 54; Cl^- 144; K^+ 16 or Mg^{++} 8 and sucrose 7; glucose 10. Intracellular sodium concentrations were altered by exposure of the cells to PCMBS. $n = 5$.

TABLE VI

K_o-FUROSEMIDE INTERACTION IN
REPLETED AND DEPLETED CELLS

Extracellular cation	^ok	
	Repleted cells	Depleted cells
	$hr^{-1} \pm SEM$	$hr^{-1} \pm SEM$
K_o 0	0.078±0.006	0.045±0.005
K_o 16 mM	0.160±0.013	0.051±0.007
K_o 0, furosemide 10^{-3} M	0.045±0.003	0.037±0.002
K_o 16 mM, furosemide 10^{-3} M	0.116±0.005	0.039±0.003

Solutions in which the measurements were made contained (mM): Glycylglycine 27; Mg^{++} 69; sucrose 54; Cl^- 144; K^+ 16 or Mg^{++} 8 and sucrose 7; glucose 10 (repleted cells) or 0 (depleted cells). Cells were depleted by incubation in the absence of substrate for 18 hr. The intracellular sodium concentration was 9.6 mmoles/liter RBC. $n = 5$.

tion with $MgCl_2$-sucrose solution might contribute to the observed effects, experiments were performed in which choline Cl solution was used as the replacement solution; the results are recorded in Table VIII. In the upper part of the table, the ouabain-insensitive ^ok and ^ik in solutions in which the major extracellular cation was Mg, choline, or K are recorded. ^ok in the all K solution is lower, and ^ik is higher, than in either the Mg or choline solution, but because of the variability in the data, the effect of K on ^ok was not statistically significant. Two experiments were therefore performed in which the ouabain-insensitive ^ok was determined in quintuplicate in all Na, all K, and all choline solutions. ^ok is higher in the all Na solution than in the all choline solution, and lower in the all K solution than in the all choline solution. The effects of Na_o and K_o are not, therefore, dependent on the replacement of NaCl and KCl solutions with $MgCl_2$-sucrose solution.

The increase in the ouabain-insensitive ^ik in solutions containing K might be due to a direct stimulation by K of the influx mechanism, or it might result

TABLE VII

EFFECT OF EXTRACELLULAR CATION ON THE MAGNITUDE
OF THE OUABAIN-INSENSITIVE ^{o}k AND ^{i}k

Extracellular cation	^{o}k	^{i}k
	$hr^{-1} \pm SEM$	$mmoles/liter\ RBC \times hr \times mM$ $Na_0 \pm SEM$
Mg^{++} 72 mM, sucrose 60 mM	0.056±0.010	0.024±0.005
Ca^{++} 72 mM, sucrose 60 mM	0.056±0.011	0.021±0.002
Rb^{+} 144 mM	0.034±0.003	0.047±0.005
K^{+} 144 mM	0.037±0.003	0.049±0.004
Cs^{+} 144 mM	0.047±0.006	0.034±0.006
Na^{+} 144 mM	0.102±0.010	0.018±0.001
Li^{+} 144 mM	0.074±0.008	0.014±0.001
Furosemide 10^{-3} M		
Rb^{+} 144 mM	0.025±0.001	0.021±0.003
Li^{+} 144 mM	0.028±0.001	0.011±0.001

Solutions in which the measurements were made contained (mM): Glycylglycine 27; Na^{+} 0.3; Mg^{++} 5; Cl^{-} 144; glucose 10; and the cation concentration indicated above. Ouabain 10^{-4} M was present in all solutions. The measurements were made using fresh cells. Na$_c$ = 9.8 mmoles/liter RBC. $n = 5$.
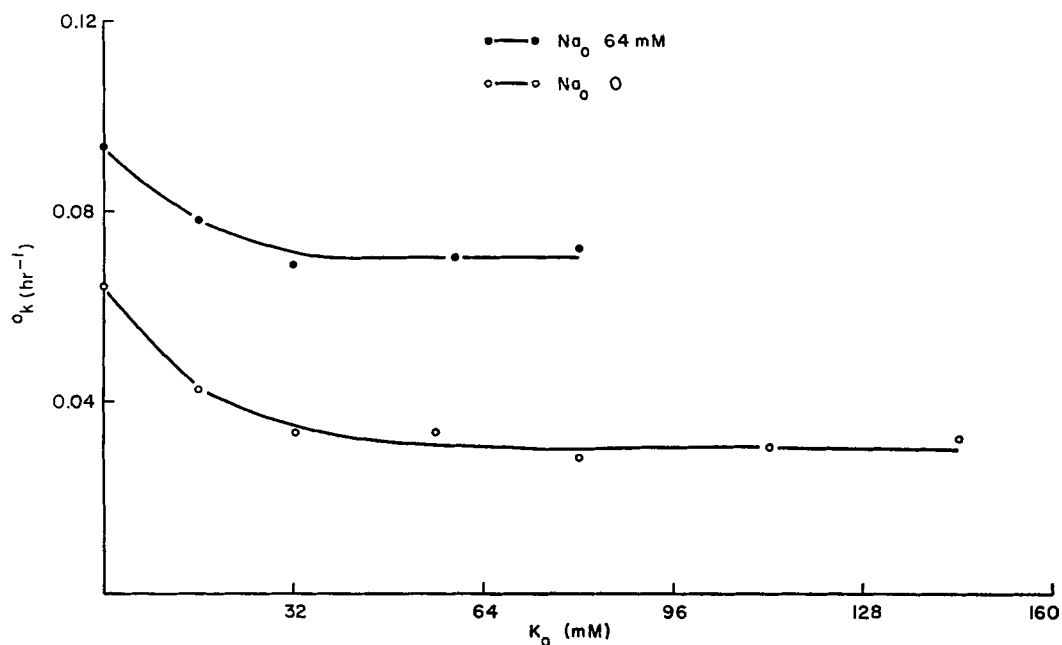

FIGURE 6. Sodium outflux rate constant (^{o}k) vs. extracellular potassium concentration (K$_o$). Solutions in which the measurements were made contained (mM): Glycylglycine 27; Na^{+} 64; K^{+} 0-80; Mg^{++} 45-5 and sucrose 34-0; Cl^{-} 144; glucose 10; or glycylglycine 27; K^{+} 0-144; Mg^{++} 77-5 and sucrose 60-0; Cl^{-} 144; glucose 10. All solutions contained ouabain 10^{-4} M. As the concentration of potassium increases, the concentrations of magnesium and sucrose decrease. Na$_c$ = 9.6 mmoles/liter RBC.
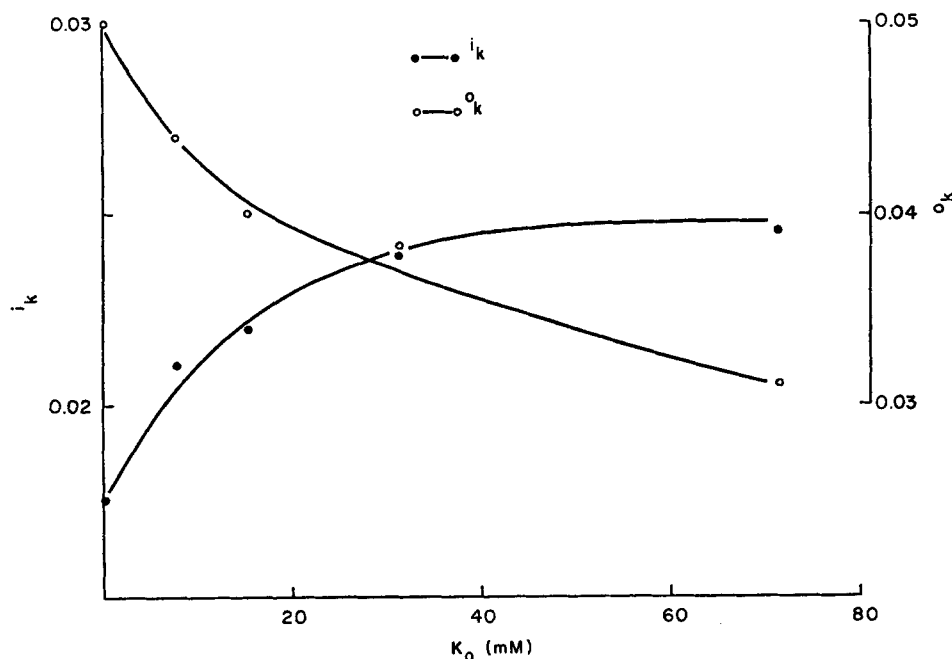
FIGURE 7. Sodium outflux rate constant (^{o}k, hr^{-1}) and sodium influx rate constant (^{i}k, mmoles/liter RBC $\times$ hr $\times$ mM Na_o) vs. extracellular potassium concentration (K_o). Solutions in which the measurements were made contained (mM); Glycylglycine 27; K^+ 0-72; Mg^{++} 77-40 and sucrose 60-30; Cl^- 144; glucose 10. As the concentration of potassium increases, the concentrations of magnesium and of sucrose decrease. Ouabain 10^{-4} M was present in all solutions. Na_c = 7.8 mmoles/liter RBC.

from an increase caused by K in the affinity of the system for Na. In order to distinguish between these possibilities, ^{i}k was measured at Na_o = 96 mM, with and without K_o = 48 mM. It can be seen from Fig. 2 that, at this [Na_o], the influx mechanism is almost saturated so that a change in the affinity of the system for Na_o would result in very little change in ^{i}k. In Table IX, the results of the experiment are presented. In the upper part, it can be seen that, in an Mg solution, K_o has its usual effect of increasing ^{i}k. In the lower part of the table, the effect of K_o in the presence of high Na is seen; ^{i}k increases under these circumstances too (0.0035, SEM 0.0009). The effect of K is to increase the magnitude of the Na influx rather than simply to alter the affinity of the system for Na.

The effect of K_o on ^{o}k and ^{i}k has so far been demonstrated in cells exposed to ouabain. In Table X, the results of experiments in which the effect of K_o on ^{o}k in ouabain-free solutions are presented; the measurements were made in low Na solutions. At K_o = 3.2 mM, ^{o}k is maximally stimulated, and ^{i}k inhibited (Garrahan and Glynn, 1967). At K_o = 144 mM, there is no decrease in ^{o}k, but

<div align="center">

T A B L E   V I I I

OUABAIN-INSENSITIVE ^{o}k AND
^{i}k IN CHOLINE SOLUTIONS

</div>

Extracellular cation	^{o}k	^{i}k
		$mmoles/liter\ RBC \times hr \times mM$
mM	$hr^{-1} \pm SEM$	$Na_0 \pm SEM$
Mg^{++} 72	0.061±0.007	0.023±0.002
Choline^{+} 144	0.084±0.012	0.028±0.002
K^{+} 144	0.040±0.004	0.051±0.005

	Ouabain-insensitive ^{o}k	
	$^{o}k \pm SEM$	
Extracellular cation	Experiment 1	Experiment 2
mM		
Na^{+} 144	0.143±0.0089	0.169±0.0070
Choline^{+} 144	0.117±0.0023	0.132±0.0028
K^{+} 144	0.040±0.0027	0.040±0.0023

Solutions in which the measurements were made contained (mM): Glycylglycine 27; Mg^{++} 5; Na^{+} 0.2; cations recorded in the table; Cl^{-} 144; glucose 10. All solutions contained ouabain 10^{-4} M. The measurements were made using fresh cells, Na_c about 9.2 mmoles/liter RBC. The numbers in the upper part of the table are, as usual, the mean of the values from four different experiments; the numbers in the lower part of the table are from two experiments in which ^{o}k was measured in quintuplicate; the mean and SEM are given.

^{i}k is increased above the value in the K_o 3.2 mM solution, and the value obtained is not different from ^{i}k in high K solutions containing ouabain. Although high K_o in ouabain-free solutions does not decrease ^{o}k, it does markedly increase ^{i}k above its value in low K solutions.

Ouabain-Insensitive ^{o}k and ^{i}k in Depleted Cells   Table XI presents the results of experiments in which the ouabain-insensitive ^{o}k and ^{i}k were measured using cells which had been depleted of energy stores by incubation for 24 hr in the absence of substrate. In the repleted cells, ^{o}k is higher in the Na solution than in the Mg or K solutions. In the K solution, ^{o}k is about the same as it is in the Mg solution, and not lower as is the case in fresh cells. In the depleted cells, however, ^{o}k in the K solution is about the same as it is in the Na solution, and in both solutions it is higher than in the Mg solution. In the repleted cells, the response of ^{i}k to high K is similar to its response in fresh cells; ^{i}k is greater in the K solution than in the Mg solution. With the use of depleted cells, the K_o stimulation of ^{i}k is still present, but it is much reduced. By depleting the cells, therefore, the usual K_o reduction of the ouabain-insensitive ^{o}k is converted to a stimulation, and the usual K_o stimulation of ^{i}k is much reduced.

Experiments were performed to determine whether the ouabain-insensitive ^{o}k in depleted cells continued to be sensitive to furosemide, and the results

TABLE IX

THE EFFECT OF K_o ON THE
OUABAIN-INSENSITIVE ^ok AND ^ik

Extracellular cation	^ok	^ik
mM	$hr^{-1} \pm SEM$	*mmoles/liter RBC* $\times$ *hr* $\times$ *mM* $Na_o \pm SEM$
Choline$^+$ 48, Mg^{++} 48	0.059±0.007	0.022±0.002
K$^+$ 48, Mg^{++} 48	0.034±0.001	0.030±0.002
Choline$^+$ 48, Na$^+$ 96	0.106±0.003	0.018±0.001
K$^+$ 48, Na$^+$ 96	0.090±0.001	0.022±0.001

Solutions in which the measurements were made contained (mM): Glycylglycine 27; Mg^{++} 5; Na$^+$ 0.2; cations recorded in the table; Cl$^-$ 144; glucose 10. All solutions contained ouabain 10^{-4} M. The measurements were made using fresh cells. Na$_c$ about 10.7 mmoles/liter RBC. $n = 4$.

TABLE X

THE EFFECT OF K_o ON ^ok AND ^ik
IN OUABAIN-FREE SOLUTIONS

Extracellular solution	^ok	^ik
	$hr^{-1} \pm SEM$	*mmoles/liter RBC* $\times$ *hr* $\times$ *mM* $Na_o \pm SEM$
K_o 0	0.102±0.005	0.037±0.002
K_o 3.2 mM	0.265±0.012	0.020±0.002
K_o 144 mM	0.276±0.013	0.037±0.004
K_o 144 mM, ouabain 10^{-4} M	0.036±0.002	0.039±0.006

Solutions in which the measurements were made all contained (mM): Glycylglycine 27; Na$^+$ 0.2; Mg^{++} 5; Cl$^-$ 144; glucose 10; the K-free solution contained Mg^{++} 72 and sucrose 60; the K 3.2 mM solution contained Mg^{++} 70 and sucrose 59. The measurements were made using fresh cells. Na$_c$ 7.7 mmoles/liter RBC. $n = 4$.

TABLE XI

OUABAIN-INSENSITIVE ^ok AND ^ik
IN REPLETED AND DEPLETED CELLS

Extracellular cation	Repleted cells		Depleted cells	
	^ok	^ik	^ok	^ik
	$hr^{-1} \pm SEM$	*mmoles/liter* *RBC* $\times$ *hr* $\times$ *mM* $Na_o \pm SEM$	$hr^{-1} \pm SEM$	*mmoles/liter* *RBC* $\times$ *hr* $\times$ *mM* $Na_o \pm SEM$
K$^+$	0.047±0.005	0.033±0.004	0.064±0.008	0.023±0.002
Mg^{++}	0.046±0.004	0.014±0.001	0.046±0.005	0.016±0.002
Na$^+$	0.068±0.007		0.073±0.007	

Solutions in which the measurements were made contained (mM): Glycylglycine 27; Mg^{++} 5; Na$^+$ 0.2; K$^+$ 144; or Na$^+$ 144, or Mg^{++} 72 and sucrose 60; Cl$^-$ 144; glucose 10 (repleted cells) or 0 (depleted cells). All solutions contained ouabain 10^{-4} M. Cells were depleted for 24 hr before use. Na$_c$ 23.4 mmoles/liter RBC. $n = 4$.

are shown in Table XII. In the repleted cells, furosemide inhibited ^ok more when the measurement was made in the high Na solution than when it was made in the high K solution; in the depleted cells, the reduction by furosemide was about the same in the two solutions. In the depleted cells, K_o had the same effect on the ouabain-insensitive ^ok as did Na_o, and ^ok was inhibited by furosemide.

It is clear that, in ouabain-treated cells, ^ok into high Na solutions is greater than it is into Na-free solutions, and the effect is present in both fresh and depleted cells. It was of interest to determine whether the stimulation of ^ok by Na_o occurs only in the presence of ouabain. It has previously been shown (Sachs, 1970) that, with the Na-K pump operating, ^ok into high Na solutions is greater than it is into Na-free solutions provided [Na_c] is normal; the effect disappears if [Na_c] is raised prior to the measurement. Experiments were therefore performed to determine the response of ^ok to alterations in [Na_o] using high Na_c cells in which the Na-K pump was shut off by depletion of intracellular substrate. The results of the experiments appear in Table XIII.

TABLE XII

EFFECT OF FUROSEMIDE ON THE
OUABAIN-INSENSITIVE ^ok IN DEPLETED CELLS

Extracellular cation	^ok	
	Repleted cells	Depleted cells
	$hr^{-1} \pm SEM$	$hr^{-1} \pm SEM$
K^+	0.058 ± 0.006	0.090 ± 0.006
K^+, furosemide 10^{-3} M	0.049 ± 0.003	0.061 ± 0.003
Na^+	0.078 ± 0.003	0.090 ± 0.004
Na^+, furosemide 10^{-3} M	0.060 ± 0.002	0.058 ± 0.003

Solutions in which the measurements were made contained (mM): Glycylglycine 27; Mg^{++} 5; K^+ 144 or Na^+ 144; Cl^- 144; glucose 10 (repleted cells) or 0 (depleted cells). All solutions contained ouabain 10^{-4} M. Cells were depleted for 24 hr prior to use. Na_c 31.7 mmoles/liter RBC. $n = 4$.

TABLE XIII

EFFECT OF [Na_o] ON ^ok IN DEPLETED CELLS

Solution	^ok	
	$K_o = 0$	$K_o = 16$ mM
	$hr^{-1} \pm SEM$	$hr^{-1} \pm SEM$
Na_o 0	0.060 ± 0.008	0.062 ± 0.008
Na_o 128 mM	0.095 ± 0.010	0.099 ± 0.010
Na_o 128 mM, ouabain 10^{-4} M	0.088 ± 0.010	0.096 ± 0.010

Solutions in which the measurements were made contained (mM): Glycylglycine 27; Mg^{++} 5; Na^+ 128 or Mg^{++} 64 and sucrose 53; K^+ 16 or Mg^{++} 8 and sucrose 7; Cl^- 144. The cells were depleted of intracellular energy stores by incubation for 24 hr in the absence of substrate. $n = 4$. [Na_c] = 28 mmoles/liter RBC.

There is no stimulation of ^0k by K_o (since there is insufficient substrate for the Na-K pump). ^0k is higher in the high Na solutions than in the low Na solutions, and the effect occurs whether or not ouabain is present. The Na stimulation of ^0k in depleted cells, therefore, does not require the presence of ouabain.

DISCUSSION

The Na movements which occur in the presence of ouabain have been cited by Hoffman and Kregenow (1966) as evidence for a Na pump (which they refer to as pump II) different from the ouabain-sensitive Na-K pump (pump I). An alternative proposal has been advanced by Lubowitz and Whittam (1969) who suggested that these ouabain-insensitive Na movements represent an Na-Na exchange. From the evidence presented here, it is clear that, even in Na-free solutions, an ouabain-insensitive Na outflux occurs which is inhibited by furosemide, and that this outflux increases as $[Na_o]$ is raised. These findings can be interpreted as indicating that there is a system which, in ouabain-treated cells, facilitates a downhill movement of Na_c into Na-free solutions, and a Na-Na exchange in high Na solutions. However, the evidence of net Na_c loss against an electrochemical gradient presented in Table III must mean that the system is capable of active transport even in the presence of ouabain. Furthermore, the Na influx and Na outflux do not always respond in the same way to alterations in the experimental conditions; K_o lowers the ouabain-insensitive Na outflux but increases the Na influx; in solutions containing K, furosemide reduces ^ik to the same extent whether or not ouabain is present, but the reduction of ^0k produced by furosemide depends on the presence of ouabain. Although it is not possible to exclude the possibility that, under some circumstances, an ouabain-insensitive Na-Na exchange occurs, other mechanisms must also be involved.

The mechanism which produces the ouabain-insensitive Na outflux may be an entirely different mechanism from that which accomplishes the ouabain-sensitive Na-K exchange, or both operations may be functions of the same system with different properties under different circumstances. There are two reasons for believing that the latter possibility is the case. First, in addition to inhibiting the ouabain-insensitive Na movements, furosemide apparently inhibits the ouabain-sensitive Na movements which Garrahan and Glynn (1967) have shown occur in K-free solutions, and which are properties of the Na-K pump. Therefore, if the ouabain-sensitive and ouabain-insensitive Na movements are not properties of the same system, but of two independent systems, it must be concluded that furosemide inhibits both of them. Second, the modification of the furosemide effect on ^0k by K_o and by ouabain (Figs. 3-5) indicates that, if the ouabain-sensitive and ouabain-insensitive Na outflux are accomplished by two distinct mechanisms, the affinity of both mechanisms for K and for ouabain must be identical.

The state of the system required in order that K_o can prevent the furosemide effect must be somewhat different from the state required for K_o to stimulate the ouabain-sensitive Na outflux. By using cells in which [Na_c] was so lowered that K_o no longer stimulated the Na outflux (Table V), K_o was nevertheless able to prevent the furosemide effect, although not as completely as in cells with normal [Na_c]. Further, in the experiment in which depleted cells were used (Table VI), the repleted cells were able to achieve Na-K exchange, but the K_o protection against the furosemide effect was incomplete. Apparently it is not necessary that the Na-K pump be operating in order for K to exert its protective effect, and the energy requirements for the protection of the system by K are somewhat different from the energy requirements for the Na-K pump.

The characteristics of the system when it is accomplishing the ouabain-insensitive Na outflux are markedly different from the characteristics of the Na-K pump. K_o is not necessary for the ouabain-insensitive outflux; in fact high [K_o] is inhibitory. Na_o, which apparently does not directly increase ^ok through the Na-K pump (Sachs, 1970), is not necessary for the ouabain-insensitive Na outflux, but the outflux is higher in high Na solutions. Finally, the form of the relation between the ouabain-insensitive ^ok (Fig. 1) and [Na_c] is quite different from the form of the relation between the ouabain-sensitive ^ok and [Na_c] (Sachs, 1970). When ouabain is present, the relation can be described in terms of a classic Michaelis-Menten model without the necessity of postulating that more than one Na_c ion simultaneously interacts with the system; to describe the curve in the absence of ouabain, it is necessary to assume that more than one Na_c ion must simultaneously interact for the system to operate.

Although it seems doubtful that the ouabain-insensitive Na influx and Na outflux represent exchange diffusion of the type described by Ussing (1950), nevertheless they seem to be related. K_o simultaneously lowers the ouabain-insensitive ^ok and increases the ouabain-insensitive ^ik (Table VI), and the curves of ^ok and ^ik vs. [K_o] are mirror images of each other (Fig. 7). The same kind of reciprocal relation is seen in the depletion experiment presented in Table X. In the repleted cells, K_o has little effect on ^ok, but ^ik is increased; in the depleted cells, the effect of K_o on ^ik is small but ^ok is markedly increased. K_o at high concentrations increases ^ik even if the cells are not exposed to ouabain (Table X); it is possible that, in cells not exposed to ouabain, high [K_o] increases the Na influx by converting membrane components which ordinarily move K_o inward into components which move Na inward.

It is necessary to consider the relation between ^ok mediated through the Na-K pump and ^ok mediated through the ouabain-insensitive pathway. It is possible that both processes may operate simultaneously so that, under circumstances which permit operation of the Na-K pump, the total ^ok is the sum of ^ok through the Na-K pump plus ^ok through the ouabain-insensitive path-

way; alternatively, it is possible that ^0k through the ouabain-insensitive pathway occurs only when the Na-K pump is turned off. The experiment of Table XIII would indicate that the latter is the case, at least for the Na_o-stimulated portion of the ouabain-insensitive ^0k. It has been previously shown (Sachs, 1970) that Na_o does not directly increase ^0k when the Na-K pump is operating. When the Na-K pump is shut off, however, either by exposure to ouabain, or as in Table XIII, by depleting the cells, Na_o is capable of stimulating ^0k, and the effect is ouabain-insensitive. The experiment also demonstrates that, in order for this portion of the ouabain-insensitive pathway to operate, it is not necessary that ouabain be present, but only that the Na-K pump be turned off. Further support for the contention that the ouabain-insensitive ^0k occurs only when the Na-K pump is not functioning is provided by the fact that furosemide has no effect if the Na-K pump is operative, but does inhibit ^0k if the pump is inoperative, and from the fact that high $[K_o]$ does not inhibit ^0k with the pump operative, but does inhibit it if the pump is turned off.

Some evidence that a source of energy other than ATP is able to influence the Na transport system has been presented by Askari and Rao (1968). By using reconstituted ghosts, they reported that ghosts which had been resealed and incubated in the presence of inosine, and those which had been resealed in the presence of ATP, both exhibited an ouabain-sensitive Na outflux into solutions containing K. However, in the cells in which ATP was the source of energy, the Na outflux was inhibited by furosemide, but the cells in which inosine was the source of energy were insensitive to furosemide under these conditions. The experiments reported in the present paper on the effect of furosemide on the Na outflux when K_o is present (Table VI), using cells which had been depleted and subsequently repleted, may represent the same phenomenon. The repleted cells may not have accumulated enough of a metabolite, present in fresh cells and in reconstituted ghosts incubated in the presence of inosine, which would permit K_o to protect the transport system from the effect of furosemide. The metabolic state of the cell also appears to affect the ability of the transport system to distinguish between Na and K. In cells depleted of intracellular energy stores by incubation in the absence of substrate for 24 hr, the ability of K_o to decrease ^0k and increase ^ik is lost, and K_o in fact increases ^0k; Na_o increases ^0k in both fresh and depleted cells.

It is attractive to attempt to correlate the ouabain-insensitive Na movements with the phosphorylated forms of the Na + K ATPase which have been reported to be present under some circumstances in red blood cell membranes (Blostein, 1968), and in preparations from other tissues (Charnock et al., 1963; Albers et al., 1963). However, such an attempt seems premature since little is known about the energy source for the net Na movements by the ouabain-insensitive pathway or about the effects produced on the phospho-

rylated intermediates by manipulations which alter the magnitude of the ouabain-insensitive Na movements.

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