Ouabain-Uninhibited Sodium Transport in Human Erythrocytes

EVIDENCE AGAINST A SECOND PUMP

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ABSTRACT Others have concluded that a second Na "pump" (active Na outflux) exists in human erythrocytes. This second pump was said to be ouabain-insensitive, unlike the classic ouabain-sensitive Na-K pump. An alternative explanation is that "pump II" is Na exchange diffusion. These hypotheses were examined in the present experiments, utilizing ^{22}Na influx and outflux measurements, net Na fluxes, and ATPase determinations. Ouabain-uninhibited Na outflux was reduced 0.58 ± 0.05 mmol/liter cells per h when extracellular Na (Na_o) was replaced by Mg. Ethacrynic acid or furosemide produced similar decrements of outflux (0.50 mmol) in the presence of ouabain and Na_o. However, these diuretics had minimal inhibitory effects on outflux in the absence of Na_o suggesting that they inhibited principally the Na_o-dependent outflux. Whereas this ouabain-uninhibited portion of outflux was dependent on Na_o, it was independent of K_o. Contrary to expectations, Na influx did not change when intracellular Na was altered. No uphill, net Na transport (ouabain-uninhibited) could be demonstrated under a variety of circumstances. Furosemide at high concentrations inhibited ATPase, reducing both ouabain-sensitive and ouabain-insensitive enzyme at 1.0 mM concentration while showing no effect on ATPase at 0.05–0.1 mM concentration. The effects of furosemide on ATPase and on Na flux were dissociable on a dose-response curve. Energy depletion for 22 h practically eliminated the Na_o-dependent, diuretic-inhibited Na outflux. Activation energies and temperature coefficients for the diuretic-inhibited outflux were one-half the values for the classic ouabain-inhibited pump.

These data are interpreted as evidence against a second Na pump. Exchange diffusion accounts adequately for most of these observations; however, the ouabain-insensitive fluxes may be complex and composed of several processes.

INTRODUCTION

Sodium transport has been studied intensively in human erythrocytes (RBC).[1] Agreement exists generally that most Na outflux from human erythrocytes is accomplished through active, uphill transport. This process is stimulated by intracellular Na (Na_i) and extracellular K (K_o), is dependent upon the hydrolysis of ATP by ATPase, and is inhibited by digitalis glycosides (e.g., ouabain) (1, 2). Recently Hoffman and Kregenow (3, 4) have concluded that a second mode of active Na outflux exists in human erythrocytes. This transport mechanism is stimulated by extracellular Na (Na_o), is not inhibited by ouabain, but is inhibited by ethacrynic acid and may not utilize ATP as a source of energy. Sachs (5) has elaborated upon these findings and concluded also that a ouabain-uninhibited pump, capable of net Na outflux against a Na gradient, was present in human erythrocytes. On the other hand, Lubowitz and Whittam (6) and Dunn (7) have reached the conclusion that the ouabain-uninhibited Na outflux did not accomplish net Na transport against an electrochemical gradient and therefore did not merit the name pump II as used by Hoffman. Lubowitz and Whittam (6) and Dunn (7) have attributed this ouabain-uninhibited Na outflux to exchange diffusion (8) (a bidirectional flux requiring Na on each side of the membrane and achieving no net Na transport). This issue is of some importance since

[1] *Abbreviations used in this paper:* RBC, erythrocytes; TNC, trinitrocresol.

a ouabain-uninhibited Na pump would be unusual; however, Whittembury and Proverbio (9) and Proverbio, Robinson, and Whittembury (10) have recently proposed a second (i.e., insensitive to ouabain) Na pump in renal tubular cells and Robinson (11) has suggested that a similar mechanism exists in intestinal mucosa.

The present paper examines in greater detail the meaning of ouabain-uninhibited Na transport in human erythrocytes. The data are interpreted to support the concept that most of the ouabain-uninhibited, diuretic-inhibited Na outflux is the result of an exchange diffusion process rather than a second pump.

METHODS

22Na and net fluxes and intracellular cations. Whole blood was obtained from normal human volunteers and used immediately. The techniques of measuring intracellular Na and K and of determining ^{22}Na outflux and influx have been described previously (7, 12). Unless otherwise specified flux solutions contained: 130 mM NaCl; 5.0 mM KCl; glycylglycine-MgCO$_3$ buffer, pH 7.4, at 37°C, 27 and 4.4 mM respectively; 10 mM glucose; 1.2 mM phosphate (as Na$_2$ HPO$_4$-NaH$_2$PO$_4$, pH 7.4); and 0.1 g/100 ml albumin. Na outflux was always measured immediately before Na influx studies so that the outflux loss of ^{22}Na was corrected for in the influx calculations (7). Whenever extracellular Na (Na$_o$) or K (K$_o$) was altered, 295 mosmol MgCl$_2$ was added to preserve isosmolarity. Ouabain,[2] ethacrynic acid,[3] and furosemide[4] were added as a water concentrate (ouabain) or as the dry powder. The net Na fluxes were done in solutions similar to the ^{22}Na flux solutions except that Na$_o$ and K$_o$ were varied as described in the Results section. Adenine, 3 mM, and inosine, 10 mM, were used to maintain intracellular ATP (13) in the 16 h net Na outflux experiments when Na$_o$ was 22 and 42 mM but not when Na$_o$ was "zero."

Alterations of intracellular Na (Na$_i$). Na$_i$ was increased or decreased through the use of trinitrocresol (TNC)[5] an agent which reversibly increases cation and decreases anion permeability of the erythrocyte membrane (14). It is not known whether transmembrane potential difference is normal after the exposure to TNC. The cells were exposed to 1.0 mM TNC for 14-24 h at 2-3°C. The extracellular solution was entirely NaCl if high Na$_i$ was desired and entirely KCl if normal or low Na$_i$ was desired. In two of four such experiments the extracellular solutions also contained 10 mM glucose, 1 mM sodium phosphate buffer, 3 mM adenine, and 10 mM inosine. These additions did not influence any of the measured parameters of flux as compared with the two incubations without these substances. Intracellular K (K$_i$) varied inversely with Na$_i$. After the incubation with 1.0 mM TNC the cells were washed three times with NaCl or KCl (for high and low Na$_i$ respectively) and at least five times with isosmotic MgCl$_2$. Washing was discontinued when the yellow color of the TNC could not be detected in the wash solutions. The effects of TNC were entirely reversible as judged by the similarity

[2] Sigma Chemical Co., St. Louis, Mo.
[3] Merck, Sharp & Dohme, West Point, Pa.
[4] Hoechst Pharmaceuticals, Somerville, N. J.
[5] Eastman Kodak Co., Rochester, N. Y.

of influx and outflux rates for Na transport (c.f. Table I) with normal values. This agrees with similar findings by Gunn and Tosteson (14). Na$_i$ ranged from 2.1 to 68.3 mM in the TNC-exposed cells. In other studies using TNC, RBC content of ATP was 0.8-1.0 mmol/liter cell. In three additional studies Na$_i$ was altered in stored bank blood (approximately 21-30 days old) using the method of Lubowitz and Whittam (6). This technique had two disadvantages: (a) Na$_i$ was not increased as greatly as with TNC; (b) since the normal Na$_i$ group of cells was incubated with substrates for metabolism and the high Na$_i$ group was not, the ATP content of the normal Na$_i$ cells exceeded that of the high Na$_i$ cells.

ATPase. RBC membrane ghosts were prepared from fresh cells washed three times with 295 mosM MgCl$_2$. The cells were lysed in 10 mM Tris-HCl and 1 mM EDTA solution. The ghosts were washed three to five times with 15 mM Tris-HCl solution (pH 6.7-6.8) and frozen. All centrifugation was carried out in a refrigerated centrifuge. Membrane protein was measured according to Lowry, Rosebrough, Farr, and Randall (15). The reaction mixture contained 80 mM NaCl, 30 mM KCl, 3 mM MgCl$_2$, 25 mM Tris HCl (pH 7.4-7.6), 0.25 mM EDTA, and 2.65 mM ATP. Membrane ATPase was measured in this medium over a 60 min incubation at 37°C in a shaker-water bath and the reaction was stopped with 2.5% cold trichloroacetic acid. The inorganic phosphate released was determined with the method of Gomori (16). The values of ATPase were expressed as nanomoles phosphate released per milligram membrane protein over 1 h.

Energy depletion and activation energy. RBC were incubated in glucose-free solutions for 22 h at 37°C in a shaker-water bath. The medium contained 10 mM NaCl, 135 mM KCl, and glycylglycine-MgCO$_3$ buffer, pH 7.4, at 37°C, 27 and 4.4 mM respectively. In addition, glucose was omitted from the ^{22}Na loading solution before the flux. The effects of temperature on Na outflux were studied at 23, 30, and 37°C. The techniques were identical with those used for the conventional studies at 37°C. The temperature coefficient was defined as the ratio of the flux rates at T and $T + 10$ where T was 23°C for these studies. The activation energies were calculated from a classic Arrhenius plot of log flux against $1/T$ where T was absolute temperature (17).

Definition of terms. Active transport is defined as net transport of an ion against its electrochemical gradient and away from its thermodynamic equilibrium. Passive transport is a downhill process with movement of the ion down its electrochemical gradient toward thermodynamic equilibrium. Pump transport is defined as a carrier-mediated process which is linked stoichiometrically to an intermediate of metabolism. The classic Na-K pump linked to ATP is defined by the extent of inhibition by the cardioactive steroids (digitalis glycosides). Under these terms, pump transport is active only when the ion movements are against the electrochemical gradient and passive when they are down the gradient. Leak refers to a pathway for passive, downhill ion movements which are not linked stoichiometrically to metabolism. Exchange diffusion is considered to be a carrier-mediated transport process, definable through the use of isotopic tracers, which involves the bidirectional exchange of a solute of the same or similar species without the occurrence of net transport in either direction (8). The carrier system for the Na-K pump may function as an exchange diffusion carrier under certain circumstances (18).
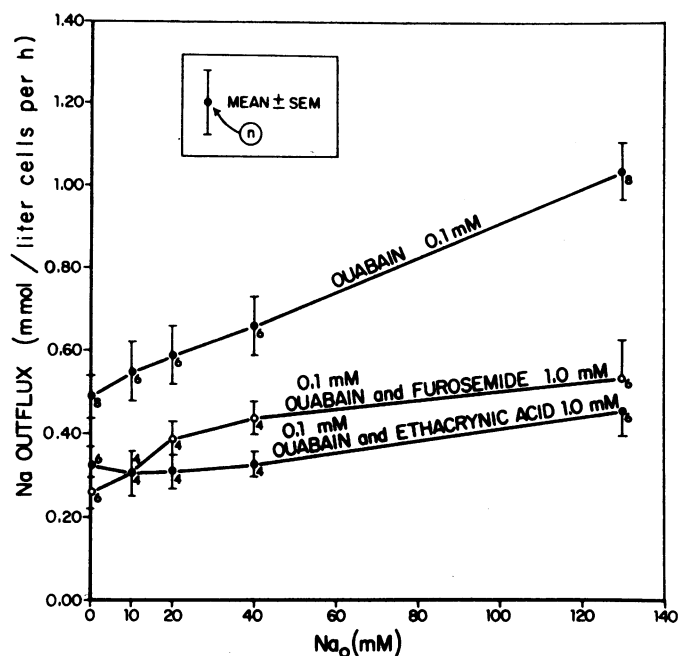
FIGURE 1 Na outflux (^{22}Na) was determined in solutions containing 130 mM NaCl, 5 mM KCl, 27 mM glycylglycine, 4.4 mM MgCO$_3$ (pH 7.4), 1.2 mM sodium phosphate, 10 mM glucose, and 0.1 g albumin/100 ml. The ouabain-uninhibited Na outflux increased progressively as extracellular Na (Na$_o$) was increased from 0.1 to 130 mM. The addition of furosemide or ethacrynic acid to ouabain substantially diminished, but did not eliminate, this Na$_o$-dependent outflux. See text for details.

RESULTS

Effects of extracellular sodium on sodium outflux. There is general agreement that human RBC, exposed to ouabain, have a substantial decrement of sodium outflux as extracellular sodium (Na$_o$) is removed from the medium (3, 5, 6, 19). Fig. 1 shows data which confirm and extend these observations. When Na$_o$ was reduced from 130 to 0.1–0.2 mM,[6] the ouabain-uninhibited sodium outflux decreased 0.58±0.05 mmol/liter cells per h in three experiments (all values expressed as mean ±SEM). The addition of ethacrynic acid or furosemide to 130 mM Na$_o$, ouabain solutions reduced sodium outflux 0.58 ±0.06 and 0.50±0.07 mmol/liter cells per h respectively. Since the decrements in ouabain-uninhibited Na outflux seen after removal of Na$_o$ or after the addition of ethacrynic acid or furosemide were similar quantitatively, it was possible that they were identical transport mechanisms. In addition, if these maneuvers were defining an exchange diffusion pathway, the inhibitory effects of ethacrynic acid and furosemide should diminish substantially or disappear in zero Na$_o$ solutions. Fig. 1 depicts the results of the experiments which tested this hypothesis. The data generally substantiate the hypothe-

sis. The decrement of outflux attributable to ethacrynic acid and furosemide was reduced to 0.16±0.04 and 0.21 ±0.05 mmol/liter cells per h respectively in zero Na$_o$ solutions. Although the removal of Na$_o$ significantly reduced the effects of these inhibitors, the remaining small action of ethacrynic acid and furosemide was statistically significant with $P < 0.05$ and $P < 0.01$ respectively (paired t test; although mean ±SEM was plotted in Fig. 1 the paired differences were analyzed). Further, the model predicts that if exchange diffusion accounted for most of the ouabain-uninhibited, diuretic-inhibited outflux, then cells incubated with ouabain and ethacrynic acid or ouabain and furosemide should show very little Na$_o$-dependent outflux (i.e., decrement of outflux due to reduction of Na$_o$). Fig. 1 shows that this was the case. The Na$_o$-dependent outflux was diminished from 0.58±0.05 mmol/liter cells per h in ouabain medium to 0.13±0.02 and 0.27±0.04 in ouabain and ethacrynic acid or ouabain and furosemide media respectively. Although the Na$_o$-dependent outflux was reduced substantially the latter values were also significantly different from zero with $P < 0.01$. In summary, approximately two-thirds of the diuretic-induced, ouabain-uninhibited outflux conformed to the exchange diffusion model. However, one-third of the diuretic-inhibited outflux was not dependent on the presence of Na$_o$ and one-third of

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[6] These were "zero" Na solutions but a small, measurable amount of Na was present at the completion of the flux.

the Na_o-dependent outflux was not inhibited by the diuretics.

The effects of intracellular sodium on sodium influx. The exchange diffusion model predicts increased influx when intracellular sodium (Na_i) is increased as well as increased outflux when Na_o is increased. The latter phenomenon has been amply demonstrated as seen in Fig. 1. Lubowitz and Whittam (6) and Lubowitz (20) have reported increased sodium influx when Na_i is increased. Seven experiments were done, four with TNC-treated cells and three with bank blood, in order to test this hypothesis. Bidirectional tracer fluxes and net Na fluxes were measured. In the cells prepared by TNC exposure, the Na_i was increased 3–13-fold (mean increase from 10.1 to 43.1 mM) in four studies (Table I). Despite these significant elevations of Na_i the ouabain-uninhibited Na influx did not increase nor did the furosemide-inhibited influx of Na increase. The increase of Na_i had the expected stimulatory effect on total Na outflux (no inhibitors), ouabain-inhibited outflux, and also, to a smaller extent, on furosemide-inhibited outflux. In a single experiment, Na_i was varied at five different levels in cells from one donor. No change of Na influx was seen over the range of Na_i from 15 to 60 mM. Since these data differed from those of Lubowitz and Whittam (6) and since these authors used outdated bank blood as a source of high Na_i cells, three experiments were designed to replicate those conditions. Because the normal Na_i group must be obtained by incubation of the cells in medium rich in energy precursors, it is clear that the high and normal Na_i groups

	Normal Na_i	High Na_i	
	mmol/liter cells	*mmol/liter cells*	
Na_i	10.1±3.0	43.1±9.2	$P < 0.02$‡
${}^iM_{Na}^{ouab}$§	2.91±0.11	2.91±0.25	NS‖
${}^iM_{Na}^{ouab+furos}$§	2.01±0.07	2.16±0.18	NS
$\Delta^iM_{Na}^{furos}$	0.90±0.06	0.75±0.10	NS
${}^oM_{Na}^{control}$**	3.56±1.04	10.9±1.25	$P < 0.01$
${}^oM_{Na}^{ouab}$‡‡	1.59±0.30	4.63±0.79	$P < 0.02$
$\Delta^oM_{Na}^{ouab}$¶	1.97±0.52	6.25±0.05	$P < 0.01$
${}^oM_{Na}^{ouab+furos}$‡‡	1.10±0.35	3.48±0.67	$P < 0.02$
$\Delta^oM_{Na}^{furos}$¶	0.49±0.17	1.16±0.14	$P < 0.02$

* Na_i was altered through a 14–24 h exposure to TNC (1.0 mM) and varying levels of Na_o. Values are expressed as mean±SEM for 4 exps. All flux values are expressed as millimoles of Na per liter cells per hour.
‡ Student's t test comparing normal and high Na_i cells.
§ Na influx in the presence of the designated inhibitor.
‖ NS, not significant.
¶ Δ or decrement of flux due to the designated inhibitor.
** Na outflux without inhibitors.
‡‡ Na outflux in the presence of the designated inhibitor.

	Normal Na_i	High Na_i	
	mmol/liter cells	*mmol/liter cells*	
Na_i	8.1±1.9	30±4.4	$P < 0.05$
${}^iM_{Na}^{ouab}$	2.5±0.56	1.99±0.08	NS
${}^iM_{Na}^{ouab+furos}$	1.77±0.31	1.43±0.14	NS
$\Delta^iM_{Na}^{furos}$	0.73±0.28	0.56±0.07	NS
${}^oM_{Na}^{control}$	2.99±0.92	6.02±0.59	$P < 0.05$
${}^oM_{Na}^{ouab}$	1.20±0.38	2.75±0.29	$P < 0.02$
$\Delta^oM_{Na}^{ouab}$	1.79±0.55	3.27±0.86	NS
${}^oM_{Na}^{ouab+furos}$	0.78±0.27	1.82±0.33	$P < 0.05$
$\Delta^oM_{Na}^{furos}$	0.43±0.11	0.93±0.06	$P < 0.05$

* Na_i was lowered in outdated bank blood through incubation in medium with glucose, adenine, and inosine; the high Na_i cells were not so incubated. Values are expressed as mean±SEM for three experiments. Other table legends are identical with those of Table I.

differ in their ATP content as well as in their Na_i content. Nonetheless no increase of Na influx could be found after 3–6-fold elevations of Na_i. These data are shown in Table II. In summary, the results with the outdated bank blood were quantitatively similar to the data with the TNC-exposed, fresh RBC.

The data in Tables I and II appear to dissociate the furosemide-inhibited Na fluxes since elevation of Na_i increased (apparently) Na outflux without an effect on Na influx. However, if all the outflux experiments of Tables I and II ($n = 14$) are grouped, the furosemide-inhibited value is 0.77±0.11 mmol/liter cells per h and the comparable value for the furosemide decrement of influx in the 14 studies was 0.75±0.07.

Additional studies ($n = 5$) were done after Na_i was lowered (0.9–2.7 mM) and Na influx was measured. Low Na_i had no discernible effect on Na influx. These studies, in which Na_i is varied, have shortcomings which are elaborated in the Discussion.

Net sodium fluxes. Controversy exists as to whether the ouabain-uninhibited flux accomplishes net sodium transport. Whereas Hoffman and Kregenow (3) and Sachs (5) have reported net sodium outflux by an ouabain-uninhibited mechanism, Lubowitz and Whittam (6) and Dunn (7) have been unable to document such a net transport step. Hence additional studies were done to clarify this issue. Fig. 2 shows the results of 16-h net fluxes utilizing relatively low Na_o concentrations (22 and 42 mM) in order to minimize Na influx. Na_i was always less than Na_o and hence Na outflux was against a chemical gradient for Na. A single experiment showed that the potential difference across the membrane, as calculated from the distribution ratios of chloride, was approximately 10 mV (interior electronegative) in the KCl solutions and 20–30 mV in
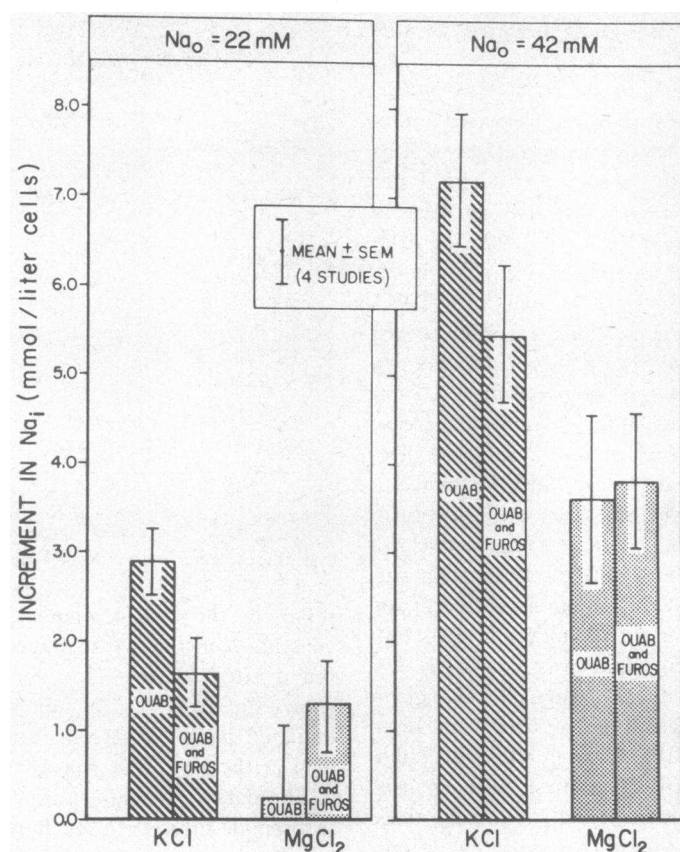
Figure 2 The ordinate shows the increment of intracellular Na (Na$_i$) above control levels (control Na$_i$ = 8.1±0.5 mM) in cells incubated for 16 h in flux medium containing ouabain (0.1 mM) and ouabain plus furosemide (1.0 mM). The solutions contained either 22 or 42 mM NaCl and KCl or MgCl$_2$ to maintain isosmolarity; additionally 1.2 mM sodium phosphate, 27 mM glycylglycine, 4.4 mM MgCO$_3$ (pH 7.4), 10 mM glucose, 3 mM adenine, 10 mM inosine, and 0.1 g albumin/100 ml. Furosemide, added to ouabain-treated cells, did not substantially increase Na$_i$ under any of the circumstances studied and slightly reduced Na$_i$ in high K solutions.

the MgCl$_2$ solutions.[7] Cells incubated in 42 mM Na$_o$ solutions always gained more Na$_i$ than did those in 22 mM Na$_o$ regardless of the counterion used to maintain isotonicity. In addition the K solutions always induced a greater increase of Na$_i$ as compared with the Mg solutions. Two effects of furosemide were noted in ouabain-treated cells: when MgCl$_2$ was the counterion Na$_i$ was changed little whereas when KCl was the counterion Na$_i$ was consistently less in furosemide-exposed cells. Additional net flux data were available for analysis from six of the seven influx studies presented in Tables I and II. In the normal Na$_i$ group (TNC cells and bank blood), cells exposed to ouabain gained 10.9±2.4 mM Na$_i$ whereas cells incubated in ouabain and furosemide gained 9.6±2.4 mM Na$_i$ over 6 h (paired difference,

[7] PD = 61.5 log (Cl$_i$/Cl$_o$); chloride was measured potentiometrically as well as by liquid scintillation (^{36}Cl).

$P < 0.01$).[8] Wiley has recently reported similar data for ouabain-treated cells; i.e., tracer Na influx exceeded net Na influx by roughly 2 to 1 (21). In the high Na$_i$ group no differences of Na$_i$ were seen between the ouabain and ouabain plus furosemide-treated erythrocytes. This tendency of furosemide to diminish slightly the increment of Na$_i$ when added to ouabain solutions was also noted in the experiments of Fig. 2 when K was the counterion. Net Na flux was also determined when Na$_o$ was zero. Fig. 3 shows these data from five experiments. Cells were incubated for 21 h and MgCl$_2$ was used to maintain isotonicity in the absence of Na$_o$. Most of the downhill Na outflux was inhibited by ouabain since the control cells decreased Na$_i$ to 1.1–1.4 mM whereas ouabain-treated cells decreased Na$_i$ to 3.7–4.0 mM from a zero-time Na$_i$ of 6.8±0.1 mM (dif-

[8] Cell H$_2$O does not change under these circumstances (7).

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ference between control and ouabain cells, $P < 0.001$). The concentration of K_o made no difference. Measured K_o was 0.1–0.5 mM in the zero K medium after 21 h incubation. Downhill net Na outflux inhibited by furosemide was small but consistently present (five of five experiments) and Na_i in this group was 4.6 mM or approximately 1 mM higher than the ouabain (alone) solutions (difference, $P < 0.02$). Net K outflux into the K-free solutions (K_o 5.0 mM data were practically identical) reduced intracellular K (K_i) from 99.6±0.4 mM at zero time to 92±1.4 mM at 21 h. The ouabain solutions and the ouabain and furosemide solutions yielded K_i values of 89.9±1.4 and 89.7±2.0 respectively. Hence no ouabain-inhibited or furosemide-inhibited K outflux was detectable under these circumstances.

The interaction of extracellular potassium and furosemide. Hoffman and Kregenow (3) reported in their original paper on pump II that extracellular potassium (K_o) did not affect this mode of Na outflux. Sachs (5) has reported recently that K_o values in excess of 5–6 mM eliminated the furosemide-inhibited Na outflux in the absence of ouabain but peculiarly did not in the presence of ouabain. Fig. 4 shows the results of our experiments which were directed to this question. Five levels of K_o between zero and 19 mM were used in four experiments. K_o did not antagonize the effect of furosemide (alone) upon Na outflux. In addition K_o did not diminish the ethacrynic acid effect (not depicted in Fig. 4). It should also be noted in Fig. 4 that a substantial furosemide-sensitive outflux persists in zero K_o solutions. This makes unlikely any sole effect of furosemide on a Na outflux linked to K influx (such as the classic, ouabain-inhibited, Na-K pump). Fig. 5 depicts the results of an experiment using three lower concentrations of furosemide and titrating K_o up to 19.3 mM in order to evaluate possible competition between K_o and furosemide at minimal inhibitory concentrations of furosemide. No competition between K_o and furosemide was observed. Also we have observed that K_o also does not affect the furosemide-inhibition of outflux when ouabain is present.

ATPase and furosemide inhibition of Na flux. ATPase activity in RBC and the rate of Na outflux along K_o-stimulated, ouabain-sensitive pathways are highly correlated and intricately associated processes (1). Some previous data suggest that ATPase is not associated with the Na_o-stimulated, ouabain-insensitive Na outflux since this latter component of outflux persisted despite ATP depletion of the cells (3) and analogues of ethacrynic acid which inhibit ouabain-insensitive Na outflux do not interfere with the ATPase re-
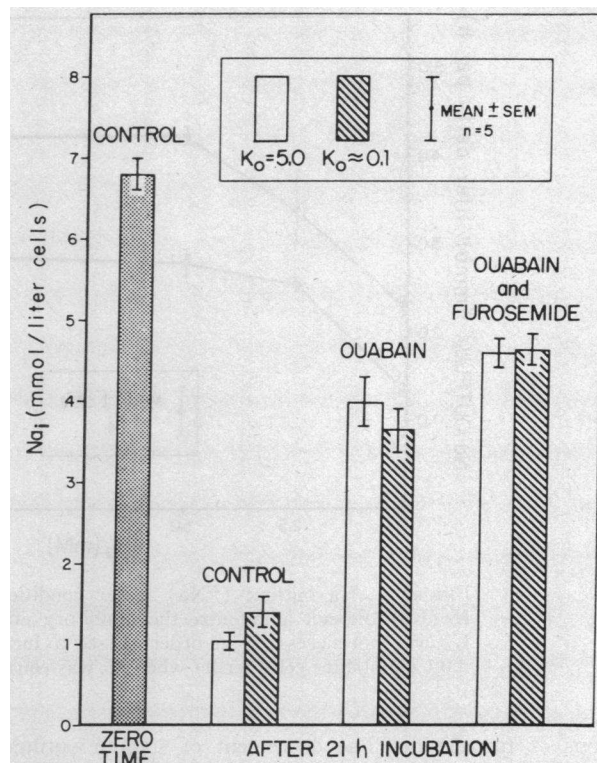

FIGURE 3 These data depict net downhill (i.e., $Na_o = 0$) Na fluxes under the conditions indicated. Na_o was replaced by isosmotic $MgCl_2$. The composition of the medium was otherwise identical with that given in Fig. 1. Most of the decrement of intracellular Na (Na_i) was attributable to the ouabain-inhibited transport system; however, very low values of extracellular K (K_o) did not interfere with the operation of this carrier in the downhill mode. The addition of furosemide inhibited an additional small portion of the downhill Na outflux ($\Delta Na_i = 1$ mM over 21 h; $P < 0.02$).

action (22).[9] Fig. 6 depicts our data from nine experiments concerning the action of furosemide and RBC membrane ATPase. Ouabain (0.1 mM) reduced the ATPase activity 53% and furosemide (1.0 mM) added to ouabain produced an additional 11% decrement (paired $P < 0.01$ and < 0.05 respectively). Furosemide (1.0 mM) without ouabain inhibited total enzyme activity by 43% ($P < 0.02$). The most important data are found in the last two columns with furosemide concentrations of 0.1 and 0.05 mM; these low concentrations did not inhibit the ATPase reaction despite continued inhibition of Na flux. Fig. 7 shows the results of one of three experiments in which sodium outflux and influx were determined on the same cells at lower concentra-

[9] Smith, E. K. M., and L. G. Welt. 1972. Evidence that human erythrocyte "pump II" for Na is independent of membrane ATPase activity. Manuscript submitted for publication.
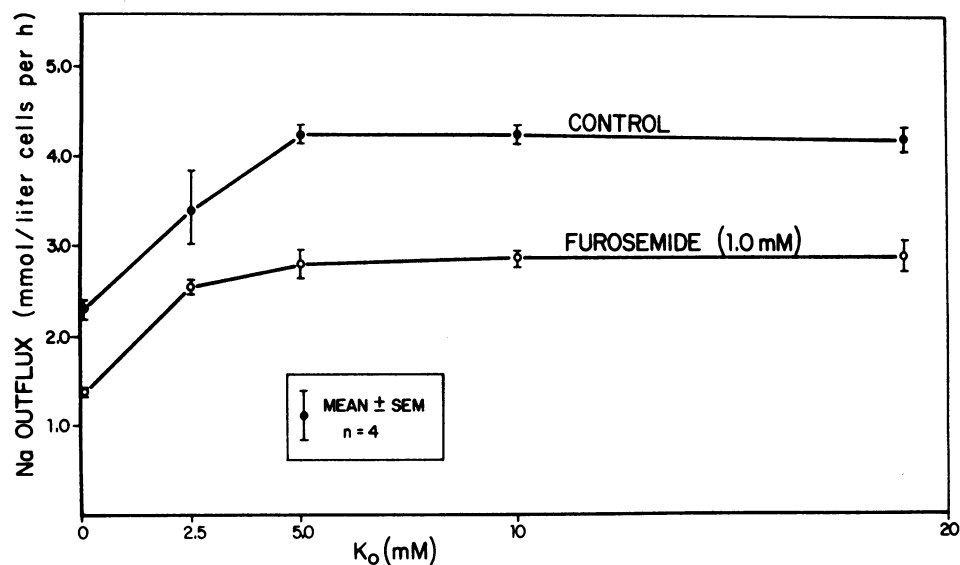
FIGURE 4 Na outflux (^{22}Na) under conditions identical with those of Fig. 1. Extracellular K (K_o) did not antagonize the inhibitory effect of furosemide on Na outflux and conversely K_o was not necessary in order to see a furosemide effect, since outflux was inhibited (0.96 ±0.1 mmol/liter cells per h) when K_o was equal to 0.02 mM.

tions of furosemide. The decrement of sodium outflux and influx was similar at each concentration of inhibitor (as would be expected of a linked transport process) and the lowest concentrations of furosemide were inhibitory. In addition net Na fluxes were determined in the three experiments in which furosemide concentra-

tion was varied and influx and outflux measured. Furosemide plus ouabain did not induce any greater changes of Na_i than did ouabain alone under any of the experimental conditions. These data provide more evidence that furosemide inhibition of cation flux need not depend upon ATPase inhibition, and reinforces the
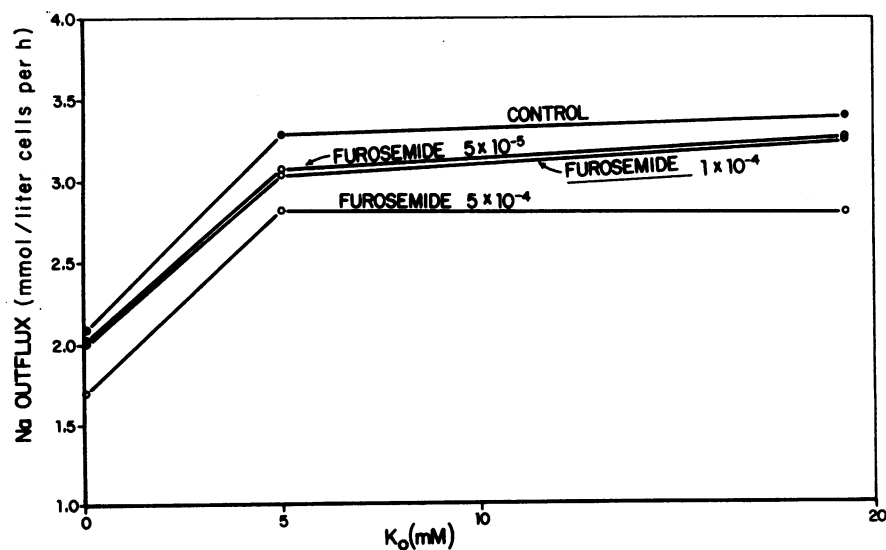

FIGURE 5 The effects of K_o upon the furosemide inhibition of outflux were assessed with furosemide concentrations lower than those used in Fig. 4. Otherwise the circumstances of the experiment were similar to those of Fig. 4. No antagonism or competition between K_o and furosemide was detected.
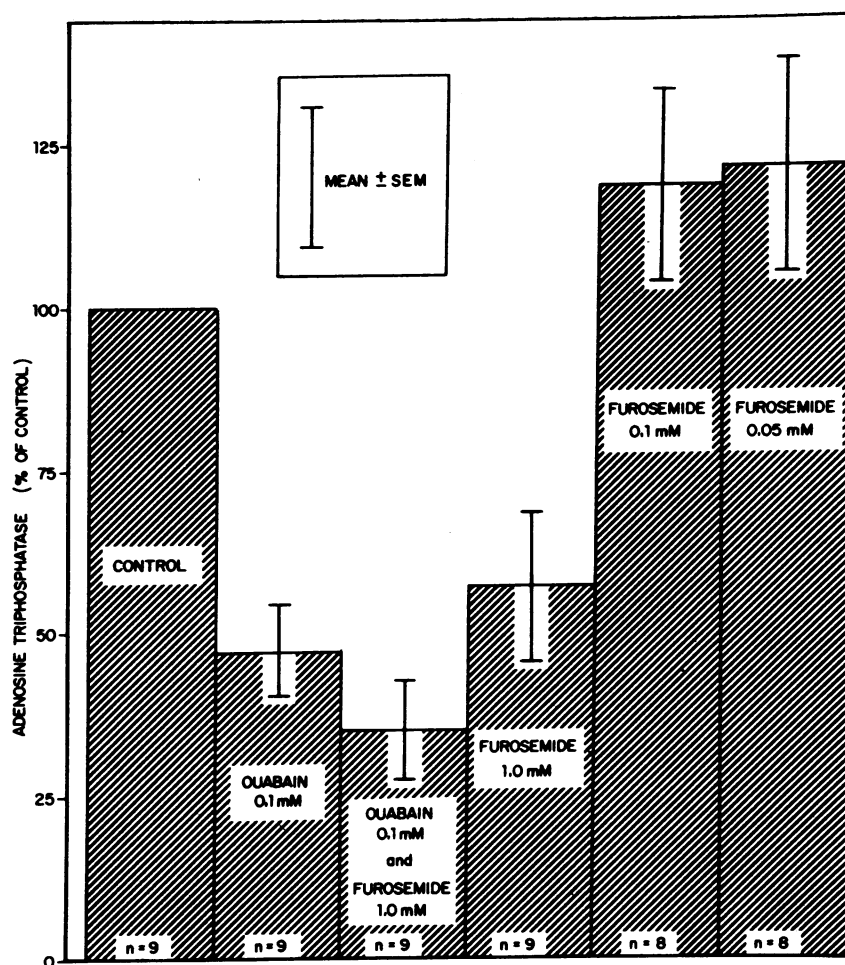
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FIGURE 6 RBC membranes were washed and prepared as described under Methods. The incubation medium contained 80 mM NaCl, 30 mM KCl, 3 mM MgCl₂, 25 mM Tris-HCl, 0.25 mM EDTA, and 2.65 mM ATP. The data are shown as a percent of control activity which was 164±24 nmol inorganic phosphate released/mg membrane protein per h. RBC membrane ATPase was inhibited by furosemide in 1.0 mM concentration but not in 0.1 and 0.05 mM concentrations. In addition 1.0 mM furosemide inhibited 11% of the ouabain-uninhibited ATPase (paired $P < 0.05$).

impression that furosemide inhibits exchange diffusion of Na in human erythrocytes since the outflux and influx inhibition curves parallel one another.

Energy depletion studies. The energy requirements of the ouabain-uninhibited Na movements are unknown. There is agreement that ATP probably does not subserve this function. Whereas Hoffman and Kregenow (3) found virtual disappearance of their pump II after 14 h depletion of energy, Sachs (5) has reported continued furosemide-sensitive outflux after 24 h of depletion. Table III summarizes the results of four experiments with RBC depleted of energy through preincubation in glucose-free medium for 22 h. The control data were obtained in experiments discussed previously (c.f. Fig. 1). Although not shown in the table, all

ouabain-sensitive outflux had disappeared in the depleted cells. The ouabain-insensitive, furosemide- and ethacrynic acid-sensitive Na outflux diminished to practically nothing in the energy-depleted cells. Whereas the Na₀-dependent Na outflux was significantly reduced in depleted cells exposed to the ouabain medium, no substantial changes were noted in the already small values in the depleted cells incubated in ouabain plus furosemide or ouabain plus ethacrynic acid.

Temperature coefficients and activation energy. Since it is accepted generally that active transport processes have high temperature coefficients and activation energies (i.e., are sensitive to temperature changes) we examined the effects of temperature decrements on Na flux (17). Na outflux was measured at 23, 30, and 37°C
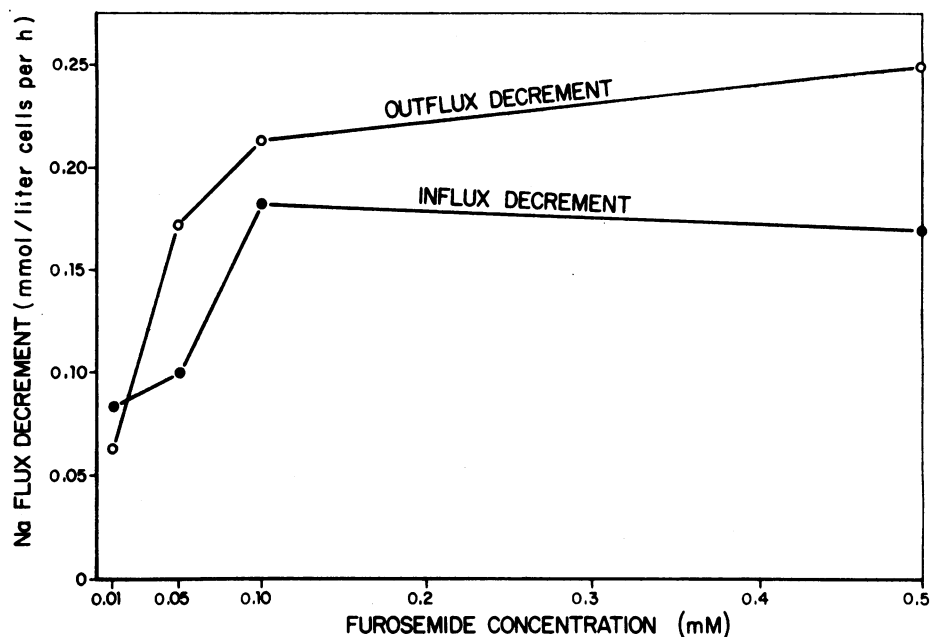
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FIGURE 7 Na outflux and influx (^{22}Na) were measured under circumstances identical with those of Fig. 1. The length of the experiments was 1 h for outflux and 3 h for influx. Increments of furosemide concentration cause parallel decrements of Na outflux and influx. It is important to note the inhibition of flux at concentrations of furosemide which did not inhibit ATPase (Fig. 6).

in four experiments. The temperature coefficients for the ouabain-inhibited and furosemide-inhibited (ouabain-uninhibited) outflux were 4.3±0.9 and 2.0±0.2 respectively (difference, $P < 0.05$). The activation energies, calculated from an Arrhenius plot, were 21,700 and 10,900 cal/mol Na respectively for these fluxes. Again the difference between the classic ouabain-in-

hibited pump and the furosemide-inhibited process was statistically significant with $P < 0.02$.

DISCUSSION

The major question addressed in this paper is whether a second Na pump (i.e., ouabain-uninhibited) is present in human erythrocytes. No substantial evidence could

TABLE III

Effects of Energy Depletion on Ouabain-Uninhibited Sodium Outflux ($^{o}M_{Na}$)

		Control (Na$_i$ 8±0.3)	Energy depleted (22 h) (Na$_i$ 7.7±1)	
		mmol/liter cells per h	*mmol/liter cells per h*	
$\Delta^{o}M_{Na}^{furos}$*	(Na$_o$ 130 mM)	0.52±0.06 (4)	0.18±0.02 (4)	$P < 0.01$‡
$\Delta^{o}M_{Na}^{furos}$*	(Na$_o$ 0.1 mM)§	0.21±0.04 (4)	0.06±0.006 (4)	$P < 0.001$
$\Delta^{o}M_{Na}^{etha}$*	(Na$_o$ 130 mM)	0.58±0.08 (6)	0.09±0.04 (4)	$P < 0.001$
$\Delta^{o}M_{Na}^{etha}$*	(Na$_o$ 0.1 mM)	0.16±0.05 (6)	↑0.20±0.03 (4)‖	$P < 0.01$
Removal of Na$_o$ decrement:¶				
Ouabain medium		0.58±0.05 (8)	0.36±0.02 (4)	$P < 0.001$
Ouabain and furosemide		0.27±0.04 (4)	0.24±0.03 (4)	NS
Ouabain and ethacrynic		0.13±0.02 (6)	0.08±0.07 (4)	NS

* The decrement (Δ) of outflux due to the inhibitor in the presence of ouabain 0.1 mM. All data expressed as mean±SEM (no. studies).

‡ Nonpaired t test comparing cells depleted of energy with control cells.

§ MgCl$_2$ replaced NaCl.

‖ Outflux increased rather than decreased.

¶ The total decrement of outflux consequent to the removal of Na$_o$ in the specified medium.

be adduced which supported this hypothesis. On the contrary most but not all of the data support the conclusion that exchange diffusion of Na accounts for much of the ouabain-uninhibited Na flux in human erythrocytes. Table IV lists seven criteria or predictions which should be met if the aforementioned conclusions are correct. The Discussion will deal with these predictions in the sequence listed.

Ouabain and other cardiac glycosides inhibit the Na outflux-K influx transport system commonly referred to as the Na-K pump. However, approximately 1.0 mmol/liter cells per h of Na outflux, persists in the presence of ouabain (c.f. Fig. 1). This ouabain-uninhibited Na outflux was reduced to 0.5 mmol when Na_o was removed and replaced by Mg. This Na_o-dependent outflux has been observed by others (3, 5, 6, 19) and suggests an exchange diffusion process since Na is apparently required on both the *cis* (interior) and *trans* (exterior) side of the membrane. Garrahan and Glynn have reported that the Na efflux, stimulated by Na_o, is not observed if choline chloride rather than $MgCl_2$ serves as the counterion which replaces Na_o (18) and it has been suggested that extracellular Mg may inhibit a ouabain-insensitive active efflux of Na (23). In unpublished studies we have replaced Na_o with chloride salts of seven different counterions (choline, tetraethylammonium, tetramethylamine, Li, Ce, K. Rb) and have observed a ouabain-insensitive, Na_o-dependent Na efflux under every circumstance. The kinetics of this Na_o-dependent outflux, as shown in Fig. 1, appear to be linear between Na_o of 0 and 135 mM. If this process is carrier mediated, and if a limited number of saturable sites exist for the transported ion, then the familiar hyperbolic kinetics of a saturable process might have been observed. It is possible that this transport pathway shows zero-order kinetics at higher levels of Na_o and that it is a first-order process at least up to 135 mM Na_o. Garrahan and Glynn observed a similar linear relationship between Na_o and Na fluxes (Na-Na exchange) in zero K_o solutions. It seems well established that diuretics such as ethacrynic acid (3, 6, 7), furosemide (5,7), and triflocin (24) reduce the Na outflux in ouabain medium. This decrement of outflux, produced by these diuretics, is about 0.5 mmol/liter cells per h, a value which approximates closely the magnitude of the Na_o-dependent outflux. We have concluded previously that these are similar if not synonymous processes (7, 24). If this is true, the diuretics should not be inhibitory in the absence of Na_o and the removal of Na_o should have a negligible effect on Na outflux in the presence of the diuretics. The data shown in Fig. 1 generally substantiate these conclusions; however, small inhibitory effects due to ethacrynic acid or furosemide persisted when Na_o was zero and a small, Na_o-de-

TABLE IV
Assumptions and Predictions Concerning Ouabain-Uninhibited Exchange Diffusion of Na in Human Erythrocytes

(a) Outflux is a function of Na_o
(b) Influx is a function of Na_i
(c) Influx and outflux linked
(d) No net Na flux
(e) Outflux independent of K_o
(f) Outflux unrelated to ATPase
(g) Activation energy lower than for the classic pump

pendent Na outflux was seen in the presence of ouabain and ethacrynic acid or furosemide respectively. Since the measured Na_o was never zero because of downhill leak of Na out of the cells, exchange diffusion could persist because of an unstirred layer effect. On the other hand the situation may be more complex and the residual effects of diuretics in zero Na_o solutions, and of Na_o in diuretic medium, could indicate several different ouabain-insensitive Na transport processes. Sachs (5) has reported data very similar to ours in that he observed a furosemide effect in ouabain medium when $Na_o = 0.35$ mM; however, others have found no effect of ethacrynic acid in ouabain medium if Na_o is zero (3, 6).

^{22}Na influx should increase as Na_i is increased if there is Na exchange diffusion. Lubowitz and Whittam have reported such results (6, 21). They found a 0.5 mmol increment of Na influx when Na_i was raised from 8.0 to 37. 0 mM. In our experiments, listed in Tables I and II, similar results could not be obtained. Initially we used TNC to alter intracellular cation content since this allowed the use of fresh cells and since the change of permeability induced by TNC was reversible as judged by normal control rates of Na influx and of ouabain-inhibited active Na outflux. Nonetheless, no increment of Na influx was seen despite a mean increase of Na_i from 10.1 ± 3.0 to 43.1 ± 9.1 mM (range, 5.5–68.3 mM). Since these experiments did not duplicate the results of Lubowitz and Whittam (6), we utilized their methodology to alter Na_i (see Table II) although this method has intrinsic disadvantages (see Methods). Despite a change of Na_i from 8.1 ± 1.9 to 30 ± 4.4 mM, no increase of ^{22}Na influx could be documented. The explanation for these contrasting results is unknown. It is possibly important that we measured influx at 2 h intervals over a 6 h period whereas Lubowitz and Whittam utilized a 1 h incubation. Because of the possibility that the Na sites on the interior of the membrane were saturated when Na_i was normal (which seemed unlikely since Na_i appeared to stimulate furosemide-inhibited efflux), influx of Na

was measured after decreasing Na$_i$. These maneuvers did not reduce Na influx. Studies utilizing cells with altered Na$_i$ have significant disadvantages: (a) K$_i$ changes reciprocally with Na$_i$; (b) cellular size may be altered, and recent work suggests that Na and K influx may be altered when RBC change volume (25); (c) Na$_i$ progressively rose 3-fold during the low Na$_i$ experiments (since Na$_o$ was 140 mM) and hence steady-state conditions were not present.

If the furosemide-inhibited portion of ouabain-uninhibited Na outflux is linked to a comparable Na influx additional requirements of exchange diffusion are fulfilled. We have previously presented data on this aspect of the problem (7). When all of the outflux and influx studies of Tables I and II were analyzed, we found that the furosemide decrement of the ouabain-uninhibited flux was 0.77±0.11 for outflux and 0.75 ±0.07 for influx (c.f. Fig. 7 also). These data confirm our original conclusions that furosemide affected a linked outflux and influx of Na.

A critical feature differentiating active transport from exchange diffusion is the absence of net uphill cation transport along the pathway in question if the process is exchange diffusion. It is most helpful to use furosemide for these studies since ethacrynic acid increases membrane permeability and thereby alters net transport through leak changes (7). The data depicted in Fig. 2 are interpreted to support the conclusion that no uphill net transport occurs along the furosemide-sensitive pathways in these experiments. In addition net Na fluxes were calculated for six of the seven fluxes listed in Tables I and II. Again ouabain- and furosemide-exposed cells did not gain more Na, and in fact gained somewhat less Na when Na$_i$ was normal, than ouabain-exposed cells regardless of the initial Na$_i$. These results confirm and extend previous data concerning the absence of any net uphill flux along the furosemide-inhibited pathway (7). Since furosemide added to ouabain under certain circumstances caused less increment of Na$_i$ than ouabain alone, the possibility should be raised that furosemide reduced slightly the downhill entry of Na into the cells. Lubowitz and Whittam (6) reached conclusions similar to ours concerning the absence of a ouabain-uninhibited net uphill Na transport step. Sachs (5) reached different conclusions based upon experiments which are not exactly comparable to ours. He has reported a small ouabain-insensitive net Na outflux against an electrochemical gradient when cells are incubated in 13–23 mM Na$_o$ or zero K$_o$ solutions with MgCl$_2$-sucrose or choline chloride as counterions. The experiments performed by Sachs maintained a constant extracellular chloride when Mg$_o$ replaced Na$_o$. Transmembrane potential difference was therefore greater in

our experiments with MgCl$_2$ (but not with KCl) as compared with those of Sachs (5).

The aforementioned studies examined net Na transport against an electrochemical gradient. We also examined downhill net Na transport to assess the meaning of the small inhibitory effect of the diuretics when Na$_o$ approximated zero. Under these circumstances furosemide raised Na$_i$ 1 mM when added to ouabain (Fig. 3). It is interesting to note the net downhill Na outflux which is sensitive to ouabain. Whereas control cells dropped the Na$_i$ to almost 1 mM after 21 h of incubation in Na-free solutions, ouabain-treated cells decreased the Na$_i$ to only 4 mM (zero time was 7 mM). This observation of the Na-K carrier operating as a downhill flux pathway has been made by others (Maizels [26] showed net flux; Garrahan and Glynn [18, 27] and Sachs [19] showed tracer flux). Post, Albright, and Dayani (28) have obtained contrasting results since they could not demonstrate a ouabain-sensitive net Na outflux in Na- and K-free solutions. Even though this carrier is thought to require extracellular K in order to achieve net Na outflux (regardless of Na gradients), our data and those of others (18, 19, 27) document the ouabain-inhibited, downhill outflux in the relative absence of K$_o$. It is possible that an unstirred layer of K ions, due to K leak out of the cells, accounts partially for these findings (27).

The fifth requirement listed in Table IV is that Na outflux should be independent of K$_o$. The classic, ouabain-inhibited Na pump depends upon K$_o$ since K influx and Na outflux are linked. Hoffman and Kregenow reported that pump II was not dependent upon K$_o$ (3, 4). However, Sachs (5) has recently presented data showing a progressive competition between K$_o$ and furosemide inhibition of Na outflux so that, when K$_o$ exceeded 5–6 mM, furosemide no longer inhibited outflux. The experiments shown in Figs. 4 and 5 were conducted to examine the role of K$_o$ on the furosemide-sensitive Na outflux. Our data show conclusively that the effect of furosemide upon Na outflux is not affected when K$_o$ is varied from 0 to 19 mM. When minimally inhibitory doses of furosemide were used no competition between K$_o$ and furosemide could be demonstrated. The reasons for the differences between our results and Sachs's are not known. Additional observations also show no effect of increasing K$_o$ upon the furosemide-inhibited outflux in the presence of ouabain. Since this furosemide-sensitive component of outflux neither depends upon K$_o$ nor is inhibited by K$_o$, we conclude that the process is independent of the Na-K pump and may be exchange diffusion.

Whatever is the explanation for ouabain-uninhibited Na outflux in human RBC, there is general agreement that ATP is not the substrate for the reaction (3–5,

22, footnote 9). This conclusion has been reached through energy depletion studies (3–5) and through the use of analogues of ethacrynic acid which inhibit the outflux but do not inhibit ATPase (22, footnote 9). In confirmatory experiments we showed that furosemide, 1.0 mM, inhibits both ouabain-sensitive and ouabain-insensitive ATPase. In this regard it resembles ethacrynic acid (10, 29, footnote 9). However, 0.1 and 0.05 mM concentrations of furosemide are clearly not inhibitory to the ATPase whereas these doses continue to reduce both Na outflux and influx (c.f. Fig. 7). Despite the inhibitory action of 1.0 mM furosemide on the membrane ATPase, it seems likely that the effects of furosemide on outflux, at least at low concentrations, are not dependent upon ATPase inhibition.

The energy source for the ouabain-uninhibited Na movements is unknown. Hoffman and Kregenow (3) suggested that it was not ATP since 14 h of energy depletion eliminated the ouabain-sensitive flux (ATP-dependent) but did not affect the ouabain-insensitive flux. After 27 h of depletion pump II disappeared. Sachs (5) has reported recently that 24 h of energy depletion did not reduce either the furosemide-sensitive or Na$_o$-dependent components of the ouabain-insensitive Na outflux. Our results, shown in Table III, agree more closely with Hoffman and Kregenow's original work. After 22 h of energy depletion the effects of furosemide, ethacrynic acid, or the removal of Na$_o$ upon the ouabain-uninhibited Na outflux were significantly reduced or eliminated. At this point no ouabain-sensitive flux remained. Although these experiments do not answer the question of what is the energy source for this component(s) of outflux, the studies do show a metabolic dependence of the flux. This is entirely consistent with the explanation of exchange diffusion since a carrier-mediated process undoubtedly requires some energy even though no useful work in the form of net transport is performed. On the other hand it seems reasonable that less energy would be needed by the process if it were exchange diffusion and not a second pump. Essig summarizes the pitfalls of categorizing a flux on the basis of energetic considerations as well as the theoretical problems concerning a definition of exchange diffusion based upon linked fluxes and transstimulation of the flux (30).

Temperature coefficients and activation energies are a measure of the sensitivity of the flux to temperature changes. Active processes generally have higher temperature coefficients and activation energies than do passive processes (1, 17). Since some passive fluxes do have high temperature coefficients, a failure to discriminate between the ouabain-inhibited and the furosemide-inhibited fluxes would be impossible to interpret. On the other hand we observed significantly lower temperature coefficients and activation energies for the ouabain-uninhibited and furosemide-inhibited Na outflux than for the ouabain-inhibited outflux. This suggests but does not prove that the former process is more likely to be exchange diffusion.

In conclusion, it can be stated that ouabain-insensitive, diuretic-sensitive Na transport in human RBC is a complex and perhaps not a single process. Whereas much of the evidence cited in the present paper supports the explanation of Na-Na exchange, some of the data are inconsistent with this single conclusion (e.g., no increase of Na influx after increased Na$_i$, small but significant effects of diuretic in zero Na$_o$ solutions, and a minimal but persistent Na$_o$-dependent efflux in the presence of diuretic inhibitors).

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