A Furosemide-Sensitive Cotransport of Sodium plus Potassium in the Human Red Cell

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ABSTRACT The influxes of Na^+ and K^+ into the human red cell appear to be interrelated. This relationship was investigated under conditions in which either Na^+ or K^+ concentration outside the cell was varied or one cation was replaced by Mg^{2+}, choline$^+$, or Li^+. The effects of furosemide on Na^+ and K^+ movements were studied in the presence of ouabain.

When ouabain was present, Na^+ influx was higher with K^+ ions externally than with other cations externally. Furosemide inhibited this K^+-stimulated Na^+ influx, but it had little effect when K^+ was absent. Ouabain-insensitive K^+ influx was stimulated two-fold by external Na^+ compared with other cations. Furosemide also inhibited this stimulation, but it had little effect when Mg^{2+} or choline$^+$ replaced external Na^+. Thus it was confirmed that synergism exists between the ouabain-insensitive influxes of Na^+ and K^+ and it was demostrated that furosemide inhibits this cooperative effect. The ouabain-insensitive influx of both K^+ and Na^+ showed a hyperbolic "saturating" dependence on the external concentration of the transported cation. Furosemide therefore eliminates a saturable component of influx of each cation.

The net uptake of Na^+ in the presence of ouabain was stimulated by K^+ ions. A similar effect was observed with red cells, in which Li^+ replaced nearly all the internal Na^+ plus K^+ ions. In these cells, net Na^+ uptake was stimulated by external K^+, and net K^+ uptake was stimulated by external Na^+. Furosemide inhibited this mutual stimulation of net cation entries.

The inhibitory action of furosemide was not limited to inward flux and net movement of Na^+ and K^+. Furosemide also inhibited the efflux of Na^+ into Na^+-free media and the efflux of K^+ into K^+-free media. It appeared, therefore, that the action of furosemide was not explained by inhibition of exchange diffusion.

These data are consistent with an ouabain-insensitive transport process that facilitates the inward cotransport of Na^+ plus K^+-ions, and that can produce a net movement of both ions. Although this process under some conditions mediates an equal bidirectional flux of both Na^+ and K^+, it cannot be defined as exchange diffusion. The cotransport process is inhibited by furosemide.

INTRODUCTION

Many studies have shown that the active movements of Na^+ and K^+ are closely linked under most conditions, since inward K^+ transport occurs only with an outward extrusion of Na^+ ions. An ouabain-sensitive ATPase is known to mediate the active movement of these cations, and this enzyme is stimulated synergistically by internal Na^+ ions and external K^+ ions (1, 2). Less is known about the nature of passive movements of Na^+ and K^+.

In 1956 Glynn (3) demonstrated that the passive movements of Na^+ and K^+ deviated from the values expected of independent leaks of each cation down its electrochemical gradient. This discrepancy has been generally attributed to exchange diffusion[1], and there is some evidence that at least part of the ouabain-insensitive Na^+ movements in red cells results from this process (5). In addition, interpretations of studies of the action of furosemide on Na^+ fluxes maintain that this diuretic drug inhibits Na^+ exchange diffusion in human red cells (6).

While the occurrence of exchange diffusion offers one explanation for the complexity of ouabain-insensitive

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[1] Exchange diffusion is defined as an equal flux of a single ion species in both directions across the membrane mediated by the same carrier that cannot bring about a net ion transport (4).

fluxes, a second possibility is that the ouabain-insensitive fluxes of Na^+ and K^+ are not independent but are coupled, and there is some evidence to support this suggestion. First, the presence of external K^+ ions stimulates Na^+ influx, and concentrations as low as 5 mM K^+ will produce this effect (7–9). Second, the ouabain-insensitive K^+ influx is more than doubled by the presence of external Na^+ ions (5, 10). The present study was undertaken to examine the interdependence of the ouabain-insensitive Na^+ and K^+ movement. The results suggest the existence of a cotransport process, defined as a coupling between the influxes of Na^+ and K^+ for which each ion is the preferred but not the obligatory cosubstrate.

METHODS

Preparation and incubation of red cells. Venous blood was collected into heparin. Red cells were thrice washed at 4°C in the media in which they were later incubated. Glucose at a final concentration of 10 mM was added to the final suspension. When red cells were incubated overnight for 16 h the hematocrit was kept low (0.5—2%), so that glucose was not depleted, and small amounts of sodium penicillin G (50 μg/ml) plus streptomycin sulfate (25 μg/ml) were added to flasks to prevent bacterial growth.

Preparation of low K^+-low Na^+ red cells was achieved by replacing virtually all the internal K^+ and Na^+ with Li^+, with nystatin to increase membrane permeability to cations reversibly (11). Washed red cells were incubated for 20 min at 0°C in a medium containing 150 mM LiCl, 27 mM sucrose, 20 mM imidazole Cl, pH 7.5, plus 40 μg/ml nystatin.[2] Nystatin was then removed by washing the cells four times in the LiCl-sucrose-imidazole medium at room temperature and then another four times in the same medium at 37°C. Before each experiment it was of critical importance to confirm that the Na^+ permeability of the nystatin-treated cells was restored to normal. Sodium influx was measured concurrently both in red cells not treated with nystatin and in the treated cells after the eighth wash, and only when the two influxes were identical were the Li^+-loaded cells used for a subsequent experiment. In two experiments the mean corpuscular volume of the red cells was measured directly by the Coulter Model S Counter (Coulter Electronics, Inc., Fine Particle Group, Hialeah, Fla.) and shown to be unchanged after nystatin treatment. A sample of cells was also dried to constant weight at 95°C and the water content of cells was shown to be unchanged after nystatin treatment.

Inhibitors. Furosemide[3] was dissolved in water by adding a chemically equivalent amount of either NaOH, KOH, or Tris base, and the stock solution adjusted to 100 mM. Furosemide was always added in a final concentration of 1 mM, and the appropriate stock solution was chosen depending on whether Na^+, K^+, or both cations were omitted from the incubation media. Ouabain, 10 mM in water, was added to incubation media to give a final concentration of 0.1 mM.

Cell cation concentration. Cells were rapidly washed four times in cold 110 mM $MgCl_2$ to remove extracellular Na^+ ions. The packed cells were hemolyzed in 0.01 N NH_4OH and the Na^+ and K^+ concentration measured by flame photometry.

Sodium influx was measured from the uptake of radioactivity by cells incubated in media containing $^{22}NaCl$ (approximately 1 μCi/ml), as described by Glynn (3). Samples were taken after 10 and 20 min of incubation at 37°C and the cells were rapidly washed four times in isotonic NaCl at 4°C to remove extracellular radioactivity. Uptake was linear with time over this incubation period, and the mean rate of uptake was taken to be the influx. Duplicate samples at the one time point agreed within ±2%.

Sodium efflux was calculated from the product of efflux rate constant and the Na^+ concentration of cells sampled at the end of the loading period (12). Cells were loaded with $^{22}Na^+$ by incubation at 37°C for 4 h in a Na^+ medium containing 145 mM NaCl, 5 mM KCl, 20 mM imidazole-Cl, pH 7.5, and 10 mM glucose. Cells were washed five times in cold nonradioactive media in which they were later incubated. Portions of washed radioactive cells were then

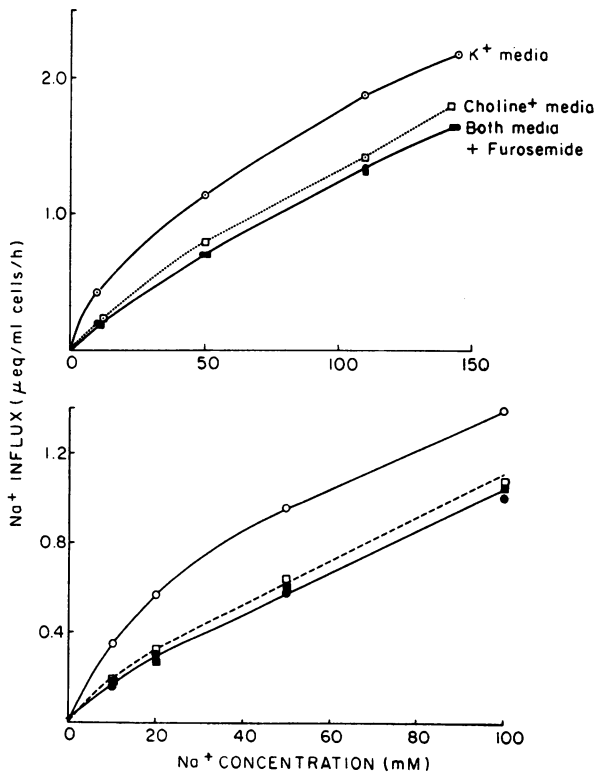

FIGURE 1 (Above) Dependence of Na^+ influx on external Na^+ concentration. Isotonicity of the medium was maintained with either KCl or choline Cl (K^+-free). Ouabain (0.1 mM) was present in all media. The lower curve shows the influx in either medium in the presence of furosemide (1 mM).
(Below) Dependence of Na^+ influx on external Na^+ concentration. K^+ media contained a fixed concentration of KCl (50 mM) and all media were made isotonic with choline Cl. Ouabain (0.1 mM) was present in all media. ○, K^+ media; □, K^+-free media. The lower curve shows the influx in K^+-free (■) or K^+ media (●) in the presence of furosemide (1 mM).

[2] Mycostatin, E. R. Squibb & Sons, Division of Olin Mathieson Chemical Corp., New York.
[3] Hoechst Pharmaceuticals Inc., Cincinnati, Ohio.

added to prewarmed media containing Na^+, as above, or free of Na^+. Na^+-free medium contained 110 mM $MgCl_2$, 20 mM imidazole Cl, pH 7.5, plus 10 mM glucose. Samples were taken at 10, 40, and 70 min incubation, cooled 3 min in iced water and centrifuged. Supernates were removed for counting. A sample of the whole cell suspension was also taken for counting and for measurement of hematocrit, and from these two values the radioactivity initially present inside the cells (N_o) was calculated. The rate constant for the Na^+ efflux was derived from the slope of the line between 10 and 70 min when log $(1 - N_t/N_o)$ was graphed against time, where N_t was the number of counts in the supernate at time t.

Potassium influx and efflux. The method of Glynn (3) was followed. For efflux, washed red cells were loaded with radioactive K^+ by incubation for 4 h at 37°C in buffered saline medium plus glucose (10 mM) and a small volume of isotonic ^{42}KCl. The cells were washed five times in the cold medium in which they were later incubated and the packed cells added to prewarmed media in tubes to give a 5–7% final hematocrit. Tubes were incubated for 0 or 1 h at 37°C and for each medium there were at least two zero-time tubes to determine the percentage of ^{42}K that was extracellular at the beginning of incubation. After 1 h samples were taken to measure the total ^{42}K present. Tubes were then cooled in an ice bath for 3 min, centrifuged, and a sample of the supernate counted to determine the percentage of ^{42}K that was extracellular. This value was corrected for the amount of ^{42}K that was extracellular at zero time and also for the ^{42}K released by hemolysis during incubation (usually less than 0.3% hemolysis). The corrected percentage of ^{42}K that was extracellular at 1 h was multiplied by cell K^+ at the beginning of the final incubation (92–100 μeq/ml cells) to give K^+ efflux.

Acrylamide gel electrophoresis. Stroma were prepared by hypotonic hemolysis of red cells in 5 mM Tris-HCl, pH 7.5, at room temperature before four more washes in the same medium, following the general procedure of Dodge, Mitchell, and Hanahan (13, 14). Stroma were analyzed by polyacrylamide gel electrophoresis in 1% sodium dodecyl sulfate (15).

Statistics. Mean values ±one SD are shown with number of separate experiments in parentheses. Effects of furosemide on cation fluxes were always measured in paired experiments and analyzed by a t test of significance between sample means.

RESULTS

Effect of furosemide on ouabain-insensitive cation influx

Inhibition of K^+-stimulated Na^+ influx by furosemide. Furosemide is known to inhibit Na^+ influx into red cells, and this effect seems more marked when the Na^+ media contain K^+ ions (9). The effect of furosemide on Na^+ influx was therefore measured at various Na^+ concentrations, in media made isotonic with either KCl or choline chloride. Na^+ influx increased from 0.2 to 1.8 μeq/ml cells/h as the Na^+ concentration was raised from 10 to 145 mM in choline (K^+-free) media, in which furosemide has little inhibitory effect (Fig. 1, above). Moreover, the dependence of influx on Na^+ concentration was almost linear in these K^+-free media. In contrast the

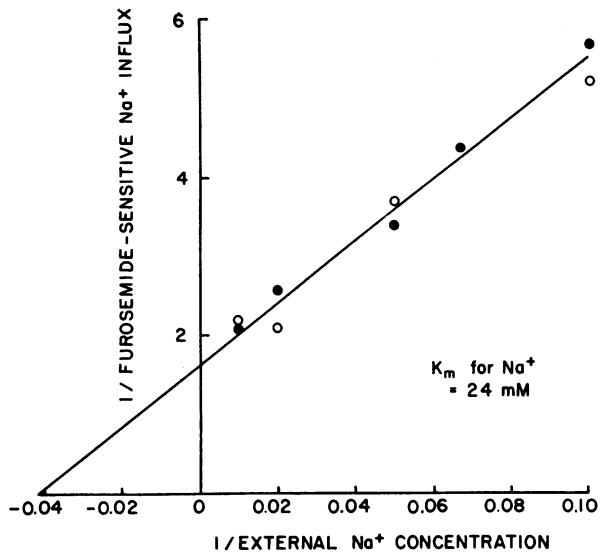

FIGURE 2 Michaelis-Menten plot between reciprocals of the furosemide-sensitive Na^+ influx and the external Na^+ concentration. Results of two experiments on different donors are shown by the open and closed circles. All media contained KCl (50 mM) and ouabain (0.1 mM) and isotonicity was maintained by choline Cl. The line was fitted by the method of least squares ($r = 0.99$).

Na^+ influx was higher in K^+-containing media and increased from 0.4 to 2.2 μeq/ml cells/h as the Na^+ concentration was raised from 10 to 145 mM. Furosemide inhibited this influx by between 0.2 and 0.55 μeq/ml cells/h (Fig. 1, above). When the furosemide-sensitive component of Na^+ influx was analyzed by Michaelis-Menten kinetics, a linear relation was obtained between the reciprocals of this flux component and external Na^+ concentration. The K_m for external Na^+ measured graphically was 20 and 22 mM in two experiments in which external Na^+ varied from 10 to 100 mM and K^+ was altered reciprocally. Similar results were obtained when external K^+ was maintained constant at 50 mM and the effect of furosemide on Na^+ influx was measured at Na^+ concentrations between 10 and 100 mM, while isotonicity was maintained with choline Cl (Fig. 1, below). A Michaelis-Menten plot is shown in Fig. 2 for the two of the latter experiments and the points fall closely on a straight line ($r = 0.99$), which extrapolates to give a K_m for Na^+ of 24 mM.

The above results suggested that Na^+ influx was reduced to almost the same value by omitting K^+ as by adding furosemide in the presence of K^+. Various media were then used to replace KCl so their effects on Na^+ influx and its inhibition by furosemide could be tested. Na^+ influx was measured in five separate experiments in media containing 10 mM NaCl, with KCl, choline chloride, $MgCl_2$ or LiCl to maintain isotonicity. Na^+ influx was highest in media containing K^+, and lowest

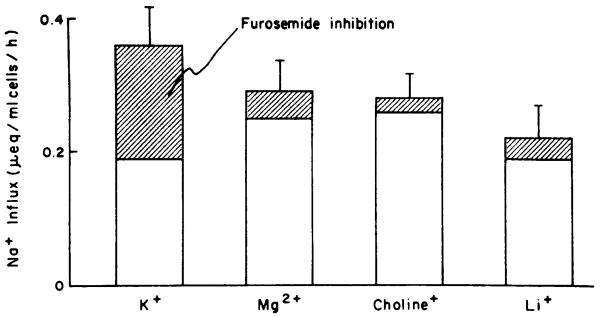

FIGURE 3 Inhibition of Na^+ influx by furosemide in K^+-containing or K^+-free media. Each medium contained 10 mM NaCl plus the indicated cation as the chloride salt. Ouabain was present in all media. Values are the mean for five different donors and one SD is shown by the vertical bar.

in the presence of Li^+. Since Li^+ is shown not to affect the rate of Na^+ entry into red cells (7), it appeared that K^+ ions increased the Na^+ influx by 0.14 μeq/ml cells/h. Furosemide reduced influx by half in the K^+ media (reduction of 0.17 μeq/ml cells/h), but influx was largely unaffected by furosemide in media containing choline, Mg^{2+} or Li^+ ions (Fig. 3). A major effect of furosemide therefore was to inhibit the K^+-stimulated Na^+ influx.

Inhibition of Na^+-stimulated K^+ influx by furosemide. Potassium influx into human red cells is largely an active process, associated with the activity of $(Na^+ + K^+)$-activated ATPase and inhibited by ouabain (16). Nevertheless, some 20–25% of K^+ influx persists in the presence of ouabain, and it is this component of K^+ influx that is stimulated by Na^+ ions in the extracellular fluid (5, 10). The effect of furosemide on ouabain-insensitive K^+ influx was measured for red cells suspended either in NaCl or choline chloride medium containing 0.5–11 mM K^+ ions. In each medium furosemide was added as the KOH-neutralized solution.

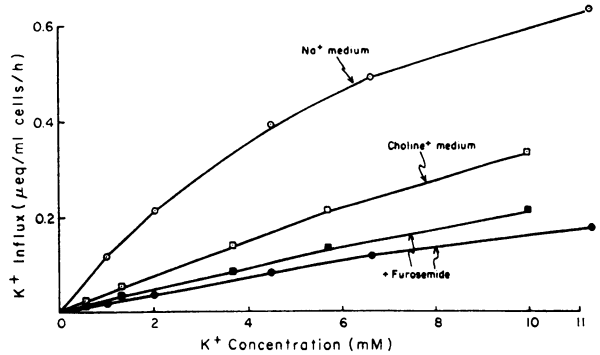

FIGURE 4 Dependence of K^+ influx on external K^+ concentration in the presence of ouabain (0.1 mM). Each medium was made isotonic either with NaCl or choline chloride. The lower two curves show the influx in the presence of furosemide (1 mM).

Ouabain-insensitive K^+ influx was greatest in the medium containing Na^+ ions, and furosemide strongly inhibited this influx (Fig. 4). As external K^+ concentration was raised from 1 to 11 mM, the K^+ influx in the presence of ouabain increased in a hyperbolic relation that tended to form a plateau at higher K^+ concentrations. In contrast, the K^+ influx in the presence of furosemide plus ouabain showed a linear dependence on external K^+ concentration. Although this nonlinear relation between influx and external K^+ concentration has been described before (8, 17), the finding that furosemide can inhibit a K^+ flux is new. Kinetic analysis of the furosemide-sensitive component of K^+ influx gave a linear relationship between the reciprocals of the flux component and external K^+ concentration. The K_m for external K^+ was measured graphically as 6 mM and 8 mM in two separate experiments. Ouabain-insensitive K^+ influx in a choline (Na^+-free) medium was lower than in the Na^+ medium, and in this medium furosemide exerted little inhibitory activity (Fig. 4). Moreover, the relation between the influx and K^+ concentration was linear in the choline medium, whether furosemide was present or not, and the latter result agrees with a recent study (18).

These data suggested that furosemide inhibited a Na^+-dependent component of K^+ influx. This effect of furosemide was confirmed by measuring influx of K^+ at a single K^+ concentration (6 mM) in red cells suspended in NaCl, LiCl, $MgCl_2$, or choline chloride media, all containing ouabain (Fig. 5). In the presence of Na^+ ions, K^+ influx was 0.46±0.10 μeq/ml cells/h ($n = 7$), and was inhibited 67% by furosemide to a value of 0.15±0.01 μeq/ml cells/h ($n = 7$). Ouabain-insensitive K^+ influx in the Mg^{2+} medium was also 0.14±0.01 μeq/

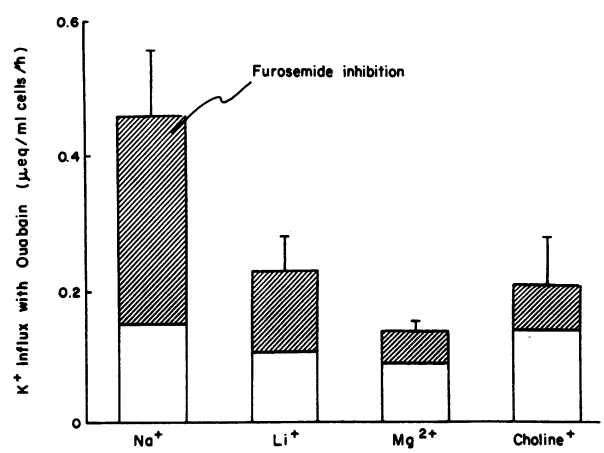

FIGURE 5 Inhibition of ouabain-insensitive K^+ influx by furosemide in Na^+-rich or Na^+-free media. Each medium contained 6 mM KCl plus the indicated cation as the chloride salt. Values are the mean for seven different donors, and one SD is shown by the vertical bar.

TABLE I

Inhibition of Cation Influx by Furosemide

Medium	Na^+ influx	Mean inhibition by furosemide	Ouabain-insensitive K^+ influx	Mean inhibition by furosemide
150 NaCl	1.70±0.20 (6)	0.18±0.12	—	—
145 NaCl 5 KCl	1.96±0.20 (9)	0.39±0.15	0.46±0.10 (7)	0.32±0.10
150 KCl	—	—	1.99±0.03 (3)	0.67±0.05

All fluxes are in μeq/ml cells/h. Data show the mean±1 SD with the number of experiments in parentheses. The mean inhibitions by furosemide were significant at the 1% level (paired experiments). Results in the different media were obtained with different donors in unpaired experiments.

ml cells/h ($n = 7$) and K^+ influx in the choline$^+$ medium was 0.21±0.06 μeq/ml cells/h ($n = 7$) so that the omission of Na^+ ions from the medium reduced K^+ influx to about the same value as in a Na^+ plus furosemide medium. Furosemide caused a small additional decrease in the ouabain-insensitive K^+ influx in Mg^{2+} or choline$^+$ media of 0.05 and 0.07 μeq/ml cells/h, respectively, and both these decrements were significant at the 1% level. Ouabain-insensitive K^+ influx in the Li^+ medium was 0.23±0.05 ($n = 5$) and furosemide inhibited this by 50% (Fig. 5). In summary, K^+ influx was not significantly different in a Li^+, choline$^+$, or Mg^{2+} medium, but the presence of Na^+ caused a highly significant stimulation of K^+ influx. The most striking effect of furosemide in this study was its inhibition of the Na^+-stimulated K^+ influx. However, the diuretic did have slight effects even in Na^+-free media, because it caused small but significant decrements in the K^+ influx in media containing Li^+, Mg^{2+}, or choline$^+$.

Ouabain-insensitive K^+ influx into human red cells was then studied as a function of external K^+ concentration from 1 to 150 mM, both in the presence and absence of furosemide. Isotonicity was maintained with NaCl. Fig. 6 shows the result of a single experiment, in which the K^+ influx in the absence of furosemide rose steeply as the concentration of K^+ was increased but reached a plateau above a K^+ concentration of 90 mM. In the presence of furosemide, the K^+ influx was much slower, and showed a linear dependence on concentration up to 150 mM. Comparison of these two influx curves shows that furosemide abolished a saturable component of ouabain-insensitive K^+ influx, which is consistent with an inhibition by furosemide of a mediated entry of this cation.

An inhibition of cation influx by furosemide was also observed when either K^+ or Na^+ was the sole cation outside the cell. K^+ influx into red cells in a medium containing 150 mM KCl plus ouabain was 1.99 μeq/ml cells/h and furosemide inhibited 0.67 μeq/ml cells/h of this flux (Table I). Na^+ influx was affected by furosemide to a lesser extent, because the Na^+ influx in a 150 mM NaCl medium plus ouabain was 1.70 μeq/ml cells/h and decreased by 0.18 μeq/ml cells/h with furosemide. Clearly furosemide can reduce ouabain-insensitive Na^+ or K^+ influx when either cation is present alone in the external medium, and its effect on K^+ influx is far greater than on Na^+ influx. However, influx of either ion is stimulated by the presence of the other and it is this mutually stimulated component of influx that is especially vulnerable to inhibition by furosemide.

Net Na^+ and K^+ movements in ouabain-treated cells

The results presented so far demonstrate a mutual stimulation or synergism between the unidirectional inward fluxes of Na^+ and K^+ ions. The same effect was also apparent when net inward movements of Na^+ into

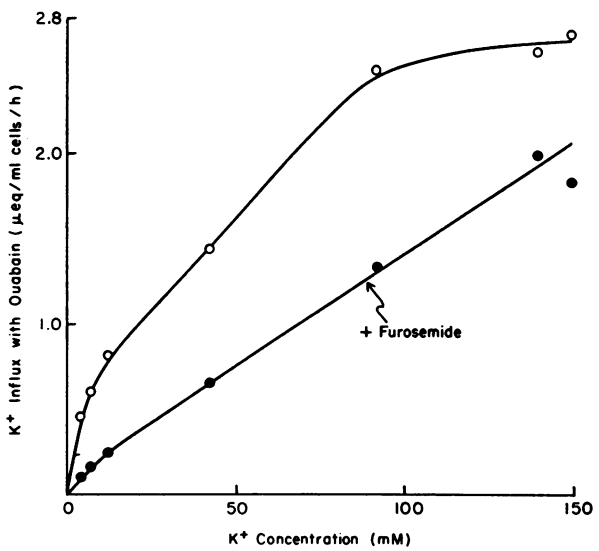

FIGURE 6 Inhibition of K^+ influx by furosemide. NaCl was present to make a NaCl + KCl concentration of 150 mM. Ouabain was added to all media.

TABLE II

Effect of External K^+ on Net Na^+ Uptake and K^+ Loss by Red Cells

Experiment	Medium	Cell Na^+			Cell K^+		
		0	16-h	Net gain	0	16-h	Net loss
	mM						
A	150 NaCl	7.7	17.5	+9.8	—	—	—
	145 NaCl 5 KCl	7.7	19.7	+12.0	—	—	—
B	150 NaCl	8.3	19.7	+11.4	—	—	—
	145 NaCl 5 KCl	8.3	22.4	+14.1	—	—	—
C	150 NaCl	7.9	18.2	+10.3	101.5	84.1	−17.4
	145 NaCl 5 KCl	7.9	21.4	+13.5	101.5	92.7	−8.8
D	150 NaCl	8.6	18.1	+9.5	92.3	78.9	−13.4
	145 NaCl 5 KCl	8.6	20.4	+11.8	92.3	86.3	−6.0
Mean values	150 NaCl	8.1	18.4	+10.3	96.9	81.5	−15.4
	145 NaCl 5 KCl	8.1	21.0	+12.9	96.9	89.5	−7.4

Washed red cells were incubated at 0.5% hematocrit in buffered saline media for 16 h at 37°C. Ouabain (0.1 mM) was present in all conditions. Results are in μeq/ml cells.

red cells were measured. The following experiments were all conducted in the presence of ouabain, which inhibits the active cation pump and allows net accumulation of Na^+ by the cell. These net movements are slow and incubations were performed over 16 h so that small differences could be readily detected by flame photometry. In four separate experiments the Na^+ accumulation ranged from 9.5 to 11.4 μeq/ml cells for fresh washed red cells incubated in a 150 mM NaCl medium plus ouabain (K^+ less than 0.05 mM). In every experiment the addition of 5 mM K^+ to the medium increased the net inward Na^+ movement by 2.2 to 2.7 μeq/ml cells, and this effect was highly significant ($P < 0.01$, Table II).

Since active K^+ pumping is blocked, these cells show a net loss of K^+ measured in only two of the above experiments. In both instances K^+ loss was greater in the K^+-free saline (17.4 and 13.4 μeq/ml cells) than in saline media containing 5 mM K^+ (8.8 and 6.0 μeq/ml cells). Subsequent results (see Table IV) show that isotopic K^+ efflux into 150 mM NaCl is not decreased by addition of 5 mM KCl to the medium, so an explanation for the net K^+ changes observed must lie in an effect of external K^+ on net K^+ entry rather than on K^+ exit rates. The results suggest that 5 mM K^+ causes a net K^+ uptake in Na^+-rich media that partially offsets the larger K^+ leak from the cells. Since K^+ movements were measured in only two experiments, the magnitude of this K^+ uptake must be considered uncertain, although the direction of this K^+ effect is in the direction predicted by a mutual stimulation of net inward Na^+ and K^+ movements. The above results do demonstrate that external K^+ ions stimulate net movement of Na^+ into red cells. In order to study the converse effect, i.e. stimulation of net inward K^+ movement by external Na^+ ions, experiments were performed in red cells in which Li^+ replaced virtually all the internal cations.

Net inward cation movements into low K^+-low Na^+ red cells. A major difficulty in interpreting the effect of furosemide on inward net movements of Na^+ arises because this diuretic may inhibit an outward as well as an inward cotransport of cation, which would then result in an apparent lack of effect. This problem was overcome by measuring the net inward movement of cations in cells containing Li^+ as virtually the sole internal cation, so that no net outward movement of Na^+ and K^+ could occur. Moreover when cell K^+ is low, net changes in its concentration can be clearly observed by flame photometry. The results in Table III directly demonstrate that external Na^+ and K^+ mutually stimulate the net inward movement of each other by a process inhibited by furosemide. Red cells were prepared with K^+ and Na^+ concentrations of about 1 and 0.5 μeq/ml cells, respectively, the remainder of the cell cation being Li^+. It was confirmed that the cells did not change their mean corpuscular volume, kept a normal water content, and the Na^+ influx was restored to normal after

the loading ionophore (nystatin) had been washed away (11). The cells were then incubated for 16 h at 2% hematocrit in media containing Na^+, Na^+ plus K^+, or Li^+ plus K^+ ions, both in the absence and presence of furosemide. Ouabain was present in all conditions.

Cells gained 19 μeq Na^+/ml cells in media containing Na^+ as the sole cation, and furosemide did not affect this net movement (Table III). Addition of 5 mM K^+ increased Na^+ uptake by the cells by 3 μeq/ml cells, and furosemide completely inhibited this increment in Na^+ gain caused by K^+ ions. The same cells showed a K^+ gain of 3.0 μeq/ml cells, whereas in the LiCl plus 5 mM KCl, only 1.0 μeq K^+/ml cells entered the cell. Therefore net K^+ uptake was stimulated 2 μeq/ml cells by external Na^+, and furosemide completely inhibited this increment in net K^+ movement caused by Na^+ ions. In the LiCl-KCl media furosemide had little effect on net K^+ entry, which was 1.0 and 0.7 μeq/ml cells, respectively, in the absence and presence of the diuretic. It was concluded that Na^+ and K^+ mutually stimulate the net inward movement of each other by a transport process inhibited by furosemide.

Effect of furosemide on active cation fluxes

Previous studies have differed on whether furosemide affects active Na^+ efflux in human red cells (6, 9), although work with kidney microsome fractions indicate that furosemide can inhibit the $(Na^+ + K^+)$-activated ATPase closely identified with the active cation pump (19). To resolve this discrepancy, K^+ influx and Na^+ efflux were measured in media containing no inhibitor, ouabain (0.1 mM), furosemide (1 mM), or ouabain plus furosemide. The active flux was taken as the component of total flux inhibited by ouabain, and this active component was compared in the absence and presence of furosemide. Although K^+ influx and Na^+ efflux were measured on different days, the cell Na^+ concentrations in the two sets of experiments were similar. Fresh cells were used for measurement of K^+ influx and contained 5.8–8.6 μeq Na^+/ml cells. Fresh cells were also loaded with isotope for measurement of Na^+ efflux and contained 5.0–8.3 μeq Na^+/ml cells at the end of the loading incubation.

TABLE III
Net cation movements into Li^+-loaded red cells

Medium	Furosemide	Cell Na^+			Cell K^+		
		Zero	16 h	Na^+ gain	Zero	16 h	K^+ gain
mM	*mM*	*μeq/ml cells*			*μeq/ml cells*		
150 NaCl	0	0.5	19.6	19.1	—	—	—
150 NaCl	1	0.5	19.5	19.0	—	—	—
145 NaCl 5 KCl	0	0.5	22.7	22.2	1.1	4.1	3.0
145 NaCl 5 KCl	1	0.5	18.1	17.6	1.1	1.9	0.8
145 LiCl 5 KCl	0	—	—	—	1.1	2.1	1.0
145 LiCl 5KCl	1	—	—	—	1.1	1.8	0.7

Red cells were incubated at 2% hematocrit in buffered media for 16 h at 37°C. Ouabain (0.1 mM) was present in all conditions. Values represent the mean of three separate experiments on different donors.

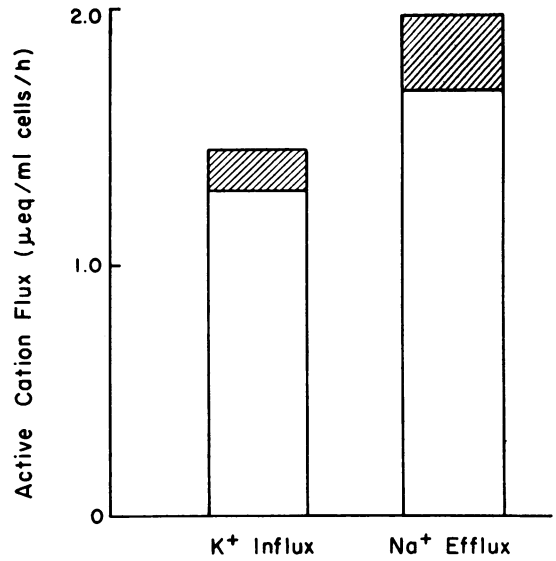

FIGURE 7 Inhibition of ouabain-sensitive (active) fluxes by furosemide. Fluxes were measured in fresh red cells suspended in 145 mM NaCl, 6 mM KCl plus 20 mM imidazole, pH 7.5. Values for the inhibition by furosemide are shown by the shaded areas and represent the means from four (K^+ influx) and ten (Na^+ efflux) different donors.

Inhibition of active K^+ influx. Active K^+ influx was measured at various external K^+ concentrations between 1 and 12 mM, and at every concentration furosemide inhibited the flux by 10–15%. The mean active K^+ influx into fresh cells at a K^+ concentration of 6 mM was 1.46±0.20 μeq/ml cells/h ($n = 4$) and furosemide caused a reduction of 0.14, which was significant ($P = 0.02$) (Fig. 7). This clear effect of furosemide on an active flux was supported by measurements of Na^+ efflux.

Inhibition of active Na^+ efflux. The major part of Na^+ efflux from human red cells is an active process and is abolished by ouabain (16). Active Na^+ efflux was 2.08±0.23 μeq/ml cells/h in fresh red cells from 10 normal donors, and furosemide inhibited this active flux by 0.30±0.05 ($n = 10$), the decrement being statistically very significant ($P < 0.001$). As shown in Fig. 7, furosemide retards active cation transport in fresh red cells by 10–15% when judged either by K^+ influx or Na^+ efflux.

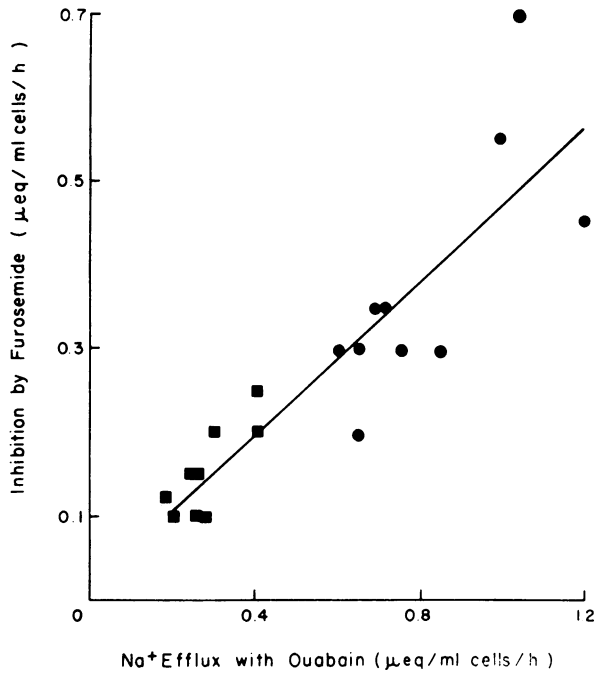

FIGURE 8 Correlation between the total ouabain-insensitive Na^+ efflux and its inhibition by furosemide. For each donor the efflux was measured both in Na^+-rich (145 mM NaCl, 5 mM KCl) or in Na^+-free (110 mM $MgCl_2$ with no KCl) media. The regression line has been fitted by the method of least squares. ●, Na medium; ■, Mg^{2+} medium.

Effect of furosemide on ouabain-insensitive cation efflux

Inhibition of ouabain-insensitive Na^+ efflux. The Na^+ efflux that persisted in the presence of ouabain ranged from 0.6 to 1.2 μeq/ml cells/h (mean 0.81±0.20 μeq/ml cells/h) for fresh cells suspended in a Na^+ medium. Furosemide inhibited this flux by 0.38±0.14 μeq/ml cells/h ($n = 10$), confirming the studies of Dunn and of Sachs (6, 9). Moreover, the absolute inhibition of efflux by furosemide was proportional to the magnitude of the efflux (Fig. 8). In relative terms it appeared that furosemide exerted a far greater effect on ouabain-insensitive Na^+ efflux (50% reduction) than on active Na^+ efflux (15% reduction).

Na^+ efflux into Na^+-free solutions was also measured for fresh red cells in a $MgCl_2$ medium containing ouabain. Efflux from these cells ranged from 0.2 to 0.4 μeq/ml cells/h ($n = 9$) with a mean of 0.30±0.09 μeq/ml cells/h, and again furosemide reduced each individual efflux by half. Furosemide inhibited Na^+ efflux by a mean of 54±10% for cells in a $MgCl_2$ medium and this effect did not differ significantly from the inhibition of Na^+ efflux into a high Na^+ medium (46±11%). A recent paper by Dunn (20) reports similar results, as the ouabain-insensitive Na^+ efflux was reduced about 50% by furosemide whether cells were suspended in a high-Na^+ or Na^+-free medium. When the ouabain-insensitive Na^+ effluxes into either Na^+ or Mg^{2+} media were compared with the inhibition of each efflux by furosemide, a strong correlation was obtained, with $r = 0.89$ and $P < 0.01$ (Fig. 7). The relation was of the form $y = 0.46\ x$, and indicated that furosemide inhibited 46% of the ouabain-insensitive Na^+ efflux when the latter ranged between 0.2 and 1.2 μeq/ml cells/h. In summary, the ouabain-insensitive Na^+ efflux was stimulated by external Na^+ ions, which confirms previous observations (5, 10, 21). However, the ouabain-insensitive Na^+ efflux into both Na^+-rich and Na^+-free media showed a similar sensitivity to furosemide, so it appeared that external Na^+ ions are not essential for this inhibitory action of furosemide.

Inhibition of K^+ efflux. Furosemide reduced K^+ efflux into saline media containing 5 mM KCl by 0.33±0.13 μeq/ml cells/h ($n = 8$) and this value did not differ significantly from the mean reduction of ouabain-insensitive K^+ influx under the same conditions (0.32 μeq/ml cells/h). Although this suggested that furosemide inhibited an exchange diffusion component of K^+ fluxes, the results described below made this interpretation less likely, because the K^+ efflux from red cells into K^+-free media was inhibited by furosemide to the same extent.

The efflux of K^+ was measured into media containing Na^+, K^+, Na^+ plus K^+, Mg^{2+} plus K^+, Li^+, Li^+ plus K^+, choline$^+$, or choline$^+$ plus K^+ to determine any possible external cation dependency for the inhibitory action of furosemide. K^+ efflux was in the range of 1.31 — 1.65

TABLE IV
Effect of Furosemide on K^+ Efflux

Cation composition of medium	Ouabain	$^{42}K^+$ Efflux	Mean decrease in flux with furosemide
mM		*μeq/ml cells/h*	
145 Na^+, 5 K^+	−	1.65±0.13	0.34
145 Na^+, 5 K^+	+	1.50±0.08	0.32
150 Na^+	+	1.31±0.06	0.23
150 K^+	+	1.57±0.10	0.43
110 Mg^{2+}	+	1.00±0.12	0.30
110 Mg^{2+}, 5 K^+	+	0.93±0.11	0.18
150 Li^+	+	1.44±0.15	0.39
145 Li^+, 5 K^+	+	1.50±0.14	0.49
150 choline $^+$	+	1.50±0.15	0.55
145 choline$^+$, 5 K^+	+	1.56±0.08	0.44

Data show mean efflux± 1 SD of five separate experiments with different male donors. The decrements in efflux due to furosemide were significant at the 1% level.

μeq/ml cells/h in all the above media except the isotonic $MgCl_2$ media, in which efflux was very significantly reduced to 0.93 — 1.00 μeq/ml cells/h. Furosemide reduced K^+ efflux by 0.2 — 0.5 μeq/ml cells/h in every one of the above media, and this effect was statistically significant ($P < 0.01$; Table IV). Thus there did not appear to be a clear requirement for a single external cation for furosemide to inhibit K^+ efflux.

Two subsidiary results were of interest. It is known that some 15% of isotopic K^+ efflux is inhibited by ouabain and presumably occurs through the active pump mechanism (18). The effect of furosemide on K^+ efflux was therefore studied in saline media both with and without ouabain. Furosemide inhibited K^+ efflux by 0.3 μeq/ml cells/h, and the decrement was the same in the presence and absence of ouabain (Table IV). The efflux was also inhibited by ouabain by 0.15 μeq/ml cells/h; but since both inhibitions were additive, it seemed that furosemide inhibited the movement of K^+ through a pathway other than the active pump.

The second result was that the presence of K^+ in a high Na^+ medium increased K^+ efflux in the presence of ouabain. This effect of external K^+ in stimulating K^+ efflux into Na^+-rich media is well known (3, 18) but furosemide inhibited K^+ efflux whether or not K^+ was present in the medium outside the cell.

Gel electrophoresis of stroma. There were no differences detected between the band patterns obtained on gel electrophoresis of stroma from cells incubated either with or without furosemide.

DISCUSSION

Several findings suggest that Na^+ and K^+ may be cotransported into the red cell by the same mediated process. First, Na^+ influx is stimulated by the presence of K^+ ions in the medium and this increment in Na^+ influx is prevented by furosemide. Similarly, the K^+ influx, which persists in the presence of ouabain, is doubled or trebled when Na^+ ions are added to the medium, and this increment of K^+ influx is abolished by furosemide. The synergism observed between the inward movements of Na^+ and K^+ in the presence of ouabain suggests that each ion facilitates the inward transport of the other. Moreover, the inhibition of K^+-stimulated Na^+ influx as well as Na^+-stimulated K^+ influx by furosemide indicates that these mutually stimulated cation fluxes occur through the same pathway, which is sensitive to furosemide. Results of the net movements of Na^+ and K^+ in ouabain-treated red cells confirmed that each ion stimulates the net inward movement of the other and that the increment in the entry of each cation is prevented by furosemide. Thus, external K^+ increased Na^+ uptake by cells by 3 μeq/ml cells while external Na^+ stimulated net K^+ uptake by about 2 μeq/ml cells, and both increments were completely inhibited by furosemide (Table III). These data on net cation movements complement the conclusions based on Na^+ and K^+ fluxes and indicate that the furosemide-sensitive transport mechanism can produce net movement of Na^+ plus K^+ into the cell. Since the magnitudes of the furosemide-sensitive influxes of Na^+ and K^+ are not significantly different (0.39 and 0.32 μeq/ml cells/h respectively) the data are consistent with an inwardly directed cotransport mechanism that may be defined as a stoichiometric coupling between the inward movements of Na^+ and K^+, and for which each ion is the preferred but not the obligatory cosubstrate.

K^+ influx in the presence of ouabain does not increase in direct proportion to the external concentration of K^+ but tends to reach a limiting value, as the concentration is increased as observed previously (8, 17). However, in the presence of furosemide, the ouabain-insensitive K^+ influx shows a linear dependence on external K^+ concentration. Thus, furosemide eliminates a component of K^+ influx that shows the saturation kinetics typical of a facilitated diffusion process (22). Omission of Na^+ from the medium likewise eliminates a saturable component of K^+ influx (18), and the magnitude of the Na^+-sensitive component was very nearly equal to the component inhibited by furosemide. The furosemide-sensitive component of K^+ influx in the presence of ouabain was analyzed by Michaelis-Menten kinetics, and a K_m for K^+ of 7 mM was obtained. Na^+ influx also shows a nonlinear dependence on external Na^+ concentration provided that K^+ ions are present in the medium (7, 12, 21), and in the presence of furosemide the influx becomes an almost linear function of concentration. Again it appeared that furosemide inhibits a component of Na^+ influx that entered the cell by a process of facilitated diffusion. The decrement in Na^+ influx caused by furosemide was related to the external Na^+ concentration in a way that could be well described by Michaelis-Menten kinetics (Fig. 2). Analysis of the double reciprocal plot gave a K_m for Na^+ ions of 24 mM in media containing high K^+ concentrations. It is of interest that this K_m value of 24 mM for external Na^+ is similar to the K_m value for internal Na^+ (20 mM) in stimulating active cation transport in intact red cells (2, 21, 23, 24).

One implication of this linkage observed between inward movements of Na^+ and K^+ is that a minimum of two transport sites are present on the outward-facing aspect of the furosemide-sensitive pathway. While these two sites show specificity for Na^+ and K^+, respectively, it is likely from the characteristics of other cotransport systems that the specificities are not absolute and that each ion is not an obligatory cosubstrate (25). Thus the furosemide-sensitive mechanism may transport not only $Na^+ - K^+$ pairs but also $K^+ - K^+$ pairs, if Na^+ is unavailable for combination with its transport site. This

possibility may explain the inhibition of ouabain-insensitive K^+ influx by furosemide for cells incubated in 150 mM KCl plus ouabain (Table I). Furosemide inhibits K^+ influx by 0.67 μeq/ml cells/h under these conditions and this effect may be due either to an inhibition of passive K^+ leak or to a blockage in $K^+ - K^+$ pair movement through the cotransport mechanism. However, cells suspended in 150 mM KCl show little or no net K^+ movement, because the internal concentration of K^+ is about 145 μeq/ml cell water, so it is difficult to attribute the effect of furosemide entirely to inhibition of passive K^+ leak. Although these data might suggest $K^+ - K^+$ pair movement through the cotransport mechanism, direct evidence for this type of transport is still lacking. $K^+ - K^+$ pair movement through a transport mechanism is not unique in cation transport because kinetic evidence exists for two or more external binding sites for K^+ on the active cation pump (17, 24, 26, 27). Moreover, the stoichiometry of active cation movements in the red cell suggests that two K^+ ions are transported inwards for each turnover cycle of the active cation pump (23 28).

The action of furosemide is not confined to inhibition of cation cotransport, because the diuretic also inhibits active cation fluxes by 10–15% as measured either by active K^+ influx or active Na^+ efflux. This effect of furosemide on active cation transport supports the results of Dunn (6), and moreover Sachs (9) has shown that furosemide inhibits the $Na^+ - Na^+$ exchange that occurs through the active pump mechanism in K^+-free solutions. The effect of furosemide on active as well as passive fluxes of Na^+ and K^+ shows that its action is less specific than that of other transport inhibitors, such as ouabain. Moreover, the present data do not exclude a small effect of furosemide on simple downhill passive leaks of cations, since the diuretic inhibits Na^+ influx into cells suspended in K^+-free media. Potassium-free conditions inhibit the activity of the inward cotransport mechanism, so it is possible that the very small effect of furosemide here may be on a passive leak. Could the entire effect of furosemide on Na^+ influx be inhibition of a diffusional leak? Several findings argue against this possibility. First K^+ ions must be present for furosemide to inhibit Na^+ influx maximally and moreover, furosemide abolishes the specific stimulation of Na^+ influx by Na^+ ions in the presence of K^+. It appears that one major effect of furosemide is to block the synergism between Na^+ and K^+ in stimulating Na^+ entry, a finding that cannot totally be explained by an effect of furosemide on a simple leak.

Cotransport mechanisms are now recognized in a variety of tissues in which a coupled movement of Na^+ ions together with amino acids or sugar can lead to net movement of these solutes. Such movements can occur even against a concentration gradient and its seems likely that the energy for uphill solute movement is derived from the coupled movement of Na^+ down its gradient (25). An analogous cotransport of Na^+ plus K^+ ions should likewise result in inward K^+ movement against the concentration gradient of this ion. Uphill K^+ movement was not consistently achieved during the inward cotransport of Na^+ plus K^+ ions into Li^+-loaded red cells. The mean cell K^+ from three different donors after a 16-hr incubation was 4.1 μeq/ml cells, i.e., 6.2 μeq/ml cell water (Table III), not very much greater than the K^+ concentration externally (5 μeq/ml). However, in one of the three experiments cell K^+ reached 8.2 μeq/ml cell water. Perhaps the slowness of the K^+ gain precluded a greater uphill K^+ movement. The mean rates of furosemide-sensitive Na^+ and K^+ movements into the Li^+-loaded red cells were only about 30–60% of the values for the corresponding flux components into fresh red cells. The reasons for this difference are not clear but may be related to the duration of incubation used for measurement of net movements (16 h) and influxes (20 or 60 min). The results of net Na^+ movements in the presence of ouabain were comparable for both fresh and Li^+-loaded red cells. Net gain of Na^+ in fresh cells was stimulated 2.6 μeq/ml cells by the presence of external K^+ ions, while the comparable figure for Li^+ loaded red cells was 3 μeq/ml cells. Recent data from Dunn (20) also indicate that external K^+ increases the net movement of Na^+ into ouabain-treated red cells. These results indicate that the Na^+ plus K^+ cotransport mechanism can accomplish net inward movements of both cations, although data for net uphill K^+ movements are inconclusive.

It is interesting to compare the relative effects of furosemide on both influx and efflux of cation. Ouabain-insensitive Na^+ efflux is inhibited 0.38 μeq/ml cells/h by furosemide and the decrement in efflux equals the decrement in Na^+ influx caused by furosemide (0.39 μeq/ml cells/h) for cells incubated in Na^+-rich media. This effect of furosemide was also described by Dunn (6) and attributed to an inhibition of exchange diffusion of Na^+ ions. However, this interpretation is not consistent with the present results, which show that furosemide inhibits Na^+ efflux into Na^+-free media, in which exchange diffusion could not occur. A similar argument has been advanced by Sachs (9) against a contribution by exchange diffusion to the ouabain-insensitive fluxes of Na^+. The present data show that furosemide has a similar effect, in relative terms, in both Na^+-rich and Na^+-free media, since in both conditions furosemide inhibits the ouabain-insensitive Na^+ efflux by half. This suggests that no one external cation is required for furosemide to inhibit the ouabain-insensitive Na^+ fluxes. Ouabain-insensitive K^+ influx is inhibited 0.32 μeq/ml cells/h by furosemide, while K^+ efflux is reduced by 0.33 μeq/ml cells/h, and the two values are not signif-

icantly different. However, furosemide reduces K^+ efflux by the same amount, whether external K^+ is present or not, so that an inhibition of $K^+ - K^+$ exchange could not explain the effect of furosemide on K^+ fluxes. Moreover, furosemide inhibits K^+ efflux by about the same amount, whatever the nature of the external cation. Thus the present study demonstrates that furosemide inhibits the ouabain-insensitive efflux of both Na^+ and K^+ by an action that is not simply due to an inhibition of exchange diffusion.

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