J. Membrane Biol. 62, 169–174 (1981)

The Journal of
Membrane Biology

Outward Sodium and Potassium Cotransport in Human Red Cells

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Summary. This paper reports some kinetic properties of Na-K cotransport in human red cells. All fluxes were measured in the presence of 10^{-4} M ouabain. We measured Na and K efflux from cells loaded by the PCMBS method to contain different concentrations of these ions into a medium that contained neither Na nor K ($MgCl_2$-sucrose substitution) in the absence and presence of furosemide. Furosemide inhibited 30–60% of the total efflux depending on the internal ion concentration and the individual subject. We took the furosemide-sensitive fluxes to be a measure of Na-K cotransport. The ratio of Na to K cotransport was 1 over the entire range of internal Na and K concentrations studied. When Na was substituted for K as the only internal cation, cotransport was maximally activated when the Na and K concentrations were between 20 and 90 mmol/liter cells. The concentration of internal Na required to produce half-maximal cotransport was about 13 ± 4 mmol/liter cells ($n=4$), while the comparable concentration of K was somewhat lower. The activation curve was definitely sigmoid in character, suggesting that at least two Na ions are involved in the transport process. The maximum of Na-K cotransport was about 0.5 ± 0.15 mmol/liter cells $\times$ hr ($n=5$); it had a flat maximum in the medium at about pH 7.0, decreasing in both the acid and alkaline sides. Furosemide-resistant effluxes were found to be linear functions of internal Na and K concentrations and to yield rate coefficients of $0.019 \pm 0.002\ hr^{-1}$ and $0.014 \pm 0.002\ hr^{-1}$ ($n=7$), respectively. These values are of the same order of magnitude expected of ions moving across phospholipid bilayers.

Key words: Na^+, K^+, co-transport, erythrocytes, flux, furosemide

Evidence for the existence of a Na-K cotransport system in human red cells has been accumulating for several years. Hoffman and Kregenow (1966) first described ouabain-resistant net uphill Na extrusion from human red cells. They called this pathway *pump II* and suggested that it might be mediated by some form of the ATP-dependent Na-K pump. Sachs (1971) extended their observations and showed that this net outward movement of Na could be inhibited by external K and furosemide. Beauge and Adragna (1971) reported stimulation of ouabain-resistant K influx into human red cells by external Na. Wiley and Cooper (1974) subsequently demonstrated furosemide-sensitive external K-dependent Na influx in human red cells and suggested the presence of cotransport. McManus and Schmidt (1978) have reported the presence of Na-K cotransport in duck red cells, while Geck and Heinz (1980) have described a similar system in mouse ascites tumor cells.

Our interest in Na-K cotransport arises from the recent work of one of us (RPG), suggesting that ion movement through this pathway is *reduced* in the red cells of patients with essential hypertension (Garay & Dagher, 1980; Garay et al., 1980*a*). We have also recently reported that the maximum rate of Na-Li countertransport is *increased* in the red cells of patients with essential hypertension (Canessa et al., 1979, 1980*a*, *b*). The relationship between both findings is under investigation.

We undertook the study reported here as part of an effort to investigate some properties of the Na-K cotransport in the red cells of normal individuals and persons suffering from hypertension. We set out to describe the dependence of Na-K cotransport on the internal concentration of Na and K in human red cells and on pH. Furosemide-inhibited Na and K effluxes into a medium containing neither K nor Na ($MgCl_2$-sucrose substitution) were used as a measure of the Na-K cotransport.

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0022-2631/81/0062-0169 $01.20

Materials and Methods

Preparation of Red Cells

Venous blood, collected from heparinized tubes, was centrifuged at $1750 \times g$ for 10 min, and the plasma and buffy coat were aspirated. Red cells were then washed twice with isosmotic NaCl or KCl, depending on the type of experiment to be performed.

Loading of Red Cells With Na and K

The internal cation content of the cells was varied, using 2,5-chloro-*p*-mercuribenzene sulfonate by a procedure similar to that described by Garrahan and Rega (1967). Washed red cells were suspended in the adequate salt medium to give a hematocrit of 4%. Depending on the desired internal cation composition, the salt media were prepared by mixing suitable proportions of Na medium and K medium. The Na medium contained (mM): 150, NaCl; 1, $MgCl_2$; 2.5, Na phosphate (pH 7.2 at 4 °C); the K medium contained (mM): 150, KCl; 1, $MgCl_2$; 2.5, K phosphate (pH 7.2 at 4 °C). The osmolarity of the loading solutions was adjusted to 295 ± 5 mOsM. In both media, the PCMBS concentration was 0.02 mM. The cells were incubated in these PCMBS-containing solutions for 20 hr at 4 °C. The suspension media were renewed once after 6 hr of incubation. At the end of the treatment with PCMBS, the cells were spun down at 4 °C for 10 min at $1750 \times g$, and the supernatant was discarded.

Recovery of Red Cells

PCMBS-treated cells were suspended (10% hematocrit) in a recovery medium containing (mM): 145, NaCl, 5, KCl; 5.4, Na phosphate (pH 7.2, 37 °C); 1, $MgCl_2$; 10, glucose; 4, cysteine; 3, inosine; 2, adenine. The final pH was adjusted to 7.4 with Tris base. The Na-depleted cells were resuspended in a recovery solution in which Na was replaced by K; the K-depleted cells were resuspended in a recovery solution in which K was replaced by Na. The cells suspended in recovery solutions were incubated at 37 °C for 1 hr.

Preparation of Cells for Measurement of Fluxes

Fresh cells or PCMBS-treated cells were washed six times with a washing solution (WS) containing (mM): 75, $MgCl_2$; 95, sucrose; 10, Tris-MOPS (pH 7.4, 4 °C); 0.1, ouabain. The osmolarity of this solution was 295 ± 5 mOsM. After the last wash, the cells were suspended in the same washing solution at a hematocrit of 50%. A portion of this suspension was set aside to measure intracellular Na, K, hemoglobin, and hematocrit.

Fifty-percent suspensions of washed red cells were diluted to a hematocrit of 4% in the flux media at 4 °C. The efflux media contained (mM):75, $MgCl_2$; 85, sucrose; 0.1, ouabain; 10 Tris-MOPS (pH 7.4, 37 °C); 10, glucose with and without furosemide (0.5 mM unless otherwise indicated). A stock solution of furosemide (20 mM) was freshly prepared by titration with Tris-base to pH 7.4. Triplicate plastic tubes containing 1.5 ml of flux media were incubated for 20, 40, 60 and 80 min at 37 °C. These media were analyzed and found to contain no more than 5 μM K or Na. To stop the reaction, tubes were transferred to 4 °C for 1 min and then centrifuged for 4 min at $8{,}000 \times g$ at 4 °C. The supernatants were transferred with plastic syringes into plastic tubes for cation analysis in the atomic absorption spectrophotometer.

Na and K effluxes were calculated from the slope relating the external cation concentration of 12 samples with time and the appropriate factors. The furosemide-sensitive component was determined by calculating the difference between the slopes in the absence and presence of the inhibitor.

$$\text{FS efflux} \pm (SD)_3 = \text{total efflux} \pm (SD)_1 - \text{furosemide-resistant} \pm (SD)_2 = \text{FS efflux} \pm \sqrt{(SD)_1^2 + (SD)_2^2}.$$

Na efflux into Na medium was measured in cells loaded with radioactive Na by incubating the cells overnight at 4 °C in a solution containing 150 mM NaCl, 10 mM Tris-MOPS, pH 7.4, and 0.33 μCi/mmol Na^{22}. After six washes in WS, Na outflow was measured as described above. One ml of supernate was mixed with 9 ml of Aquasol (New England Nuclear, Boston, Mass.) and counted in a Packard liquid scintillation counter.

Change in pH

The pH of the flux media was varied by adding different amounts of Tris to an original MOPS solution.

Na and K Determinations

Na and K were measured in appropriate dilutions of media and cells with a Perkin Elmer atomic absorption spectrophotometer (Model 5000). Calibration solutions contained 5–300 μM cations in double distilled water.

Reagents

Ouabain, Tris, MOPS, and PCMBS were purchased from Sigma Chemical Company (St. Louis, Mo.).

Results

Ouabain-Resistant Na and K Effluxes From Fresh Human Red Cells Into Na- and K-Free Medium

Fresh human red cells, incubated in $MgCl_2$-sucrose medium containing 0.1 mM ouabain, release their internal Na and K linearly with time for at least 80 min (Fig. 1). Both Na and K ouabain-resistant effluxes were calculated from the slope of the function relating the external Na and K concentrations with time. In experiments with fresh cells, the addition of 0.5 mM furosemide inhibited 30–60% of the Na efflux and 10–30% of the K efflux. The dose-response curve for inhibition by furosemide of ouabain-resistant efflux of Na and K into $MgCl_2$-sucrose medium is shown in Fig. 2. Inhibition became maximal between 0.1 and 0.5 mM furosemide. The presence of Na in the external medium does not modify the shape of this curve. This makes unlikely the presence of a competitive effect between external Na sites and furosemide.

The Effect of PCMBS on the Ouabain-Resistant Na and K Efflux

The ouabain-resistant Na and K effluxes from untreated cells were compared with effluxes from cells loaded with different amounts of Na and K using the PCMBS procedure. Data for Na effluxes are

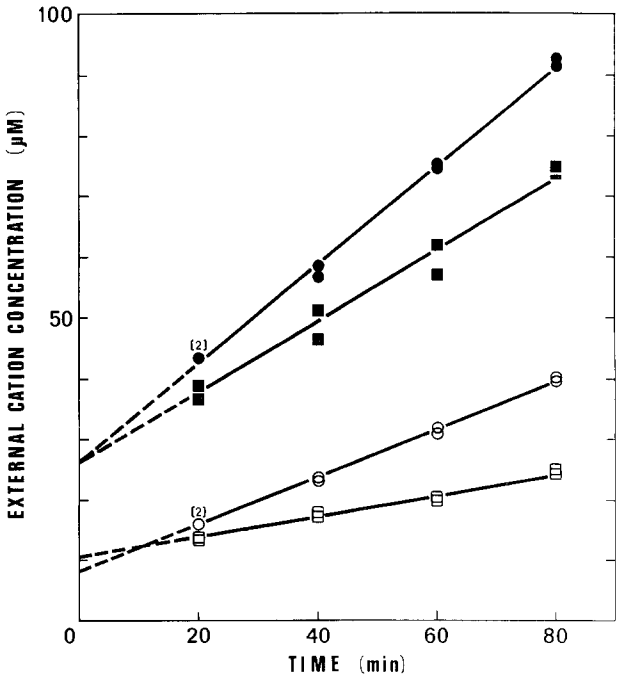

Fig. 1. External cation concentrations are plotted as a function of time after the start of incubation of cells (4.5% vol/vol) in Na- and K-free ($MgCl_2$-sucrose substitution) medium at 37 °C. The fresh cells used in this experiment contained (mmol/liter cells) 88 K and 12 Na. The round symbols (○ for Na, ● for K) indicate observations made in the absence of furosemide. The square symbols (□ for Na, ■ for K) indicate observations made in presence of 0.5 mM furosemide. Effluxes were computed from the slopes of the lines

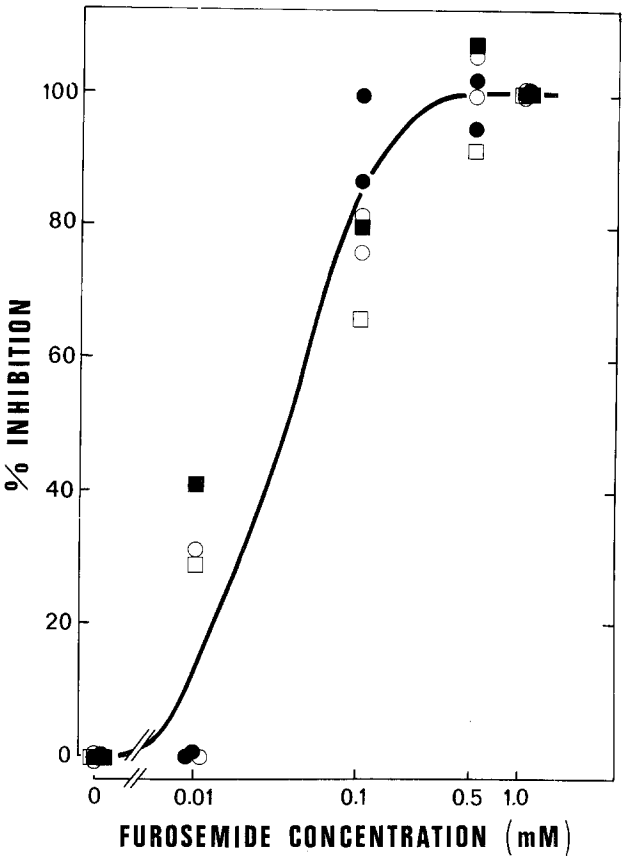

Fig. 2. Dose-response curve for the furosemide inhibition of Na (○) and K (●) efflux into $MgCl_2$-sucrose medium at 37 °C. The replacement of the $MgCl_2$-sucrose medium by 150 mM NaCl medium did not change the dose-response curve for the furosemide inhibition for either Na (□) or K (■) efflux. Na efflux into Na medium was measured using Na^{22} as indicated in Materials and Methods

shown in Fig. 3. The control cells were fresh cells incubated at 4 °C in a medium of similar ionic composition but without PCMBS. Under these conditions, the furosemide-sensitive and resistant Na and K effluxes from fresh cells were not significantly different from those extrapolated from the curve obtained with the PCMBS-treated cells. This result was confirmed in two experiments in addition to that described in Fig. 3. In the absence of furosemide, Na efflux includes a saturable component, while in the presence of the inhibitor the relation between Na efflux and internal Na is linear.

The Effect of the Internal Cation Composition on the Ouabain- and Furosemide-Resistant Na and K Efflux

Ouabain and furosemide-resistant Na and K effluxes from human red cells into $MgCl_2$-sucrose medium were measured as a function of internal cation composition (Fig. 4*A* and *B*). Both Na and K effluxes were linear functions of internal Na and K, respectively. The rate constants were $0.019 \pm 0.002\,hr^{-1}$ and $0.014 \pm 0.002\,hr^{-1}$ for Na and K effluxes, respectively (mean ± SD of 7 experiments). As in these experiments Na^+ replaces K^+, the constant slopes of Fig. 4*A* and *B* seems to indicate that the Na rate constant is independent of the internal K concentration, and the K rate constant is independent of the internal Na concentration. In a more rigorous demonstration of this point, the concentration of one ion species would be held constant at two levels when the other is varied. However, on the basis of our extensive experimentation with PCMBS and nystatin procedures for choline loading, we consider that such experiments will hardly meet the criteria of constant cell volume.

The Effect of Internal Na and K Concentration on the Furosemide-Sensitive Na and K Efflux

The ouabain-resistant, furosemide-sensitive Na and K effluxes from human red cells into $MgCl_2$-sucrose

medium were measured as a function of the internal cation composition (Fig. 5). Both Na and K effluxes seem to be tightly coupled in a 1:1 stoichiometry over the entire range of internal Na and K concentrations used. In addition, the bell-shaped curve shows that the simultaneous presence of both ions is required in order to produce furosemide-sensitive fluxes of either Na or K. The stimulation of the furosemide-sensitive Na efflux by internal Na is clearly sigmoidal (*S*-shaped) (Fig. 6). The maximal stimulation of furosemide-sensitive Na and K efflux was 0.5 ± 0.15 ($n=5$). The $K_{1/2}$ for internal Na was 13

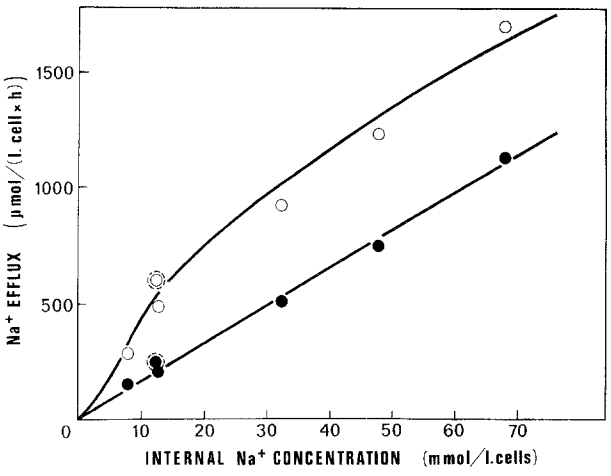

Fig. 3. Ouabain-resistant Na efflux into $MgCl_2$-sucrose medium at 37 °C is plotted as a function of the internal Na concentration. Data were obtained in the absence (○) and presence (●) of 0.5 mM furosemide, using cells loaded by the PCMBS method to contain different concentrations of Na. Observations were also made on fresh cells loaded with PCMBS-free solutions. Na efflux was measured in the absence (◎) and presence (◉) of furosemide

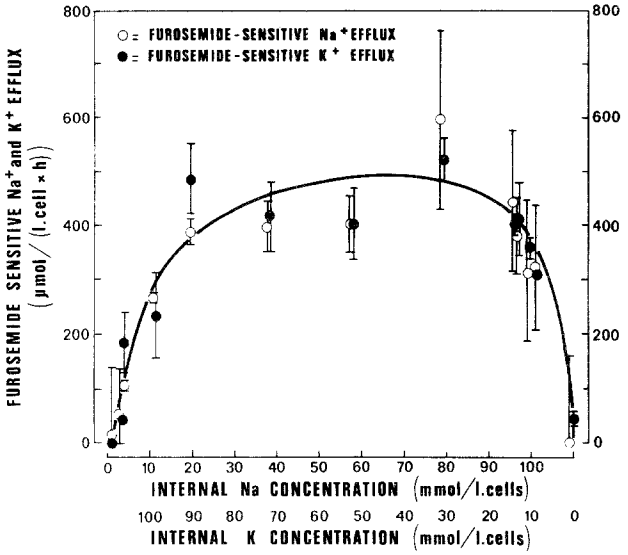

Fig. 5. Ouabain-resistant, furosemide-sensitive Na (○) and K (●) effluxes into $MgCl_2$-sucrose medium are plotted as a function of internal Na and K. Each point represents the mean ±SD for a flux calculated as the difference between fluxes in the absence and presence of furosemide (0.5 mM). Each flux was measured as indicated in Fig. 4. The data shown in this figure were obtained on the red cells from one donor. Similar results were obtained with cells from five other donors. Notice that the standard deviation of the furosemide-sensitive efflux increases at higher cellular concentrations of Na or K

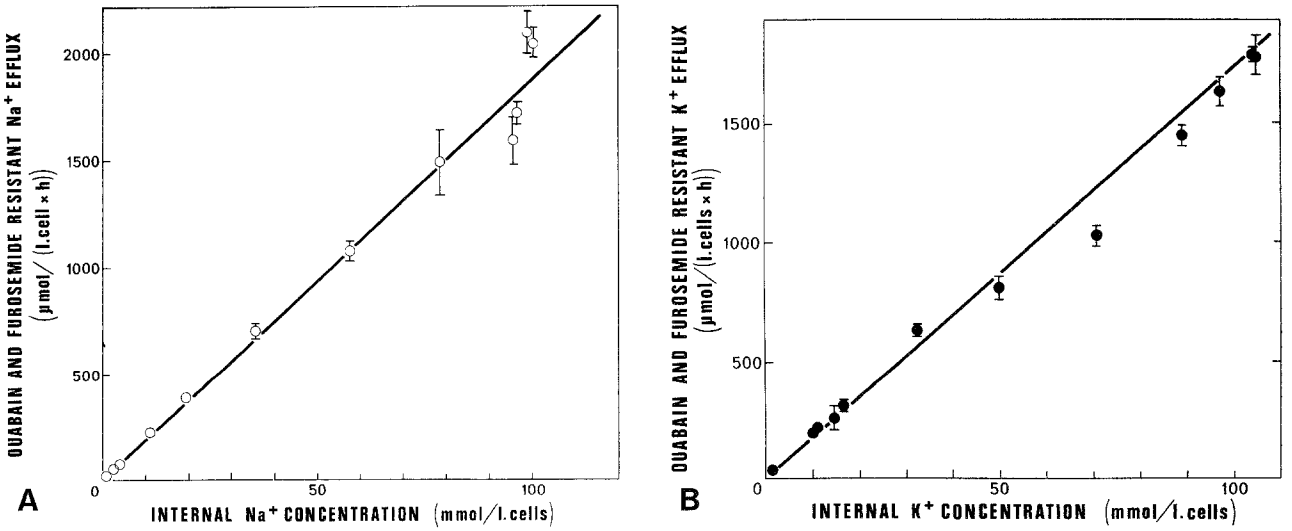

Fig. 4. Ouabain-resistant, furosemide-resistant Na (○) (*A*) and *K* (●) (*B*) effluxes into $MgCl_2$-sucrose medium are plotted as a function of the internal Na or K concentrations. Each point represents the mean ±SD of a flux calculated from cation analyses made in triplicate samples taken at 20, 40, 60 and 80 min. The rate constants computed from the slopes of this experiment were 0.018 hr^{-1} for Na and 0.017 hr^{-1} for K

± 4 mM ($n=4$). The $K_{1/2}$ for internal K was 4 mM ($n=1$). A Hill plot for the dependence of (FS)-Na efflux on internal Na gave a value of 1.6 ± 0.2 ($n=5$).

We also studied the effect of external concentration of the co-ion on the furosemide-sensitive and furosemide-resistant effluxes of Na and K. External co-ion concentration did not alter furosemide-resistant effluxes of Na or K. However, 30 mM external K reduced furosemide-sensitive Na efflux by 32 and 57% in two different donors. Conversely, external Na (Na_o) did not alter furosemide-sensitive K efflux. The independence of (FS)-K efflux from Na_o excludes simple models in which the internal sites of the cotransport system oscillate during cation translocation and may be trapped by Na_o.

Figure 7 shows the effect of pH on the maximal rate of outward Na-K cotransport; i.e., the furosemide-sensitive effluxes of Na and K into $MgCl_2$-sucrose medium when the internal concentrations of both Na and K were high enough to fully activate the system. The maximum fluxes displayed a flat maximum around pH 7.0.

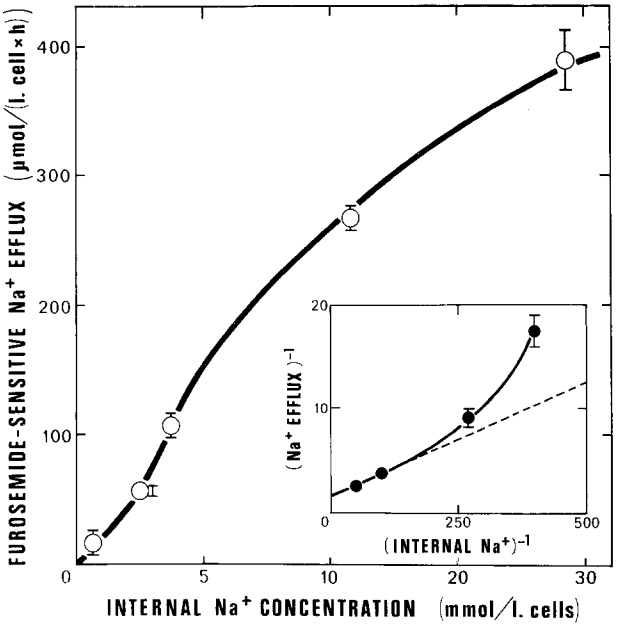

Fig. 6. Ouabain-resistant, furosemide-sensitive Na efflux into $MgCl_2$-sucrose medium at 37 °C is plotted as a function of internal Na concentration. Each point represents the mean ± SD of a flux calculated as described in Fig. 4. The inset is a double reciprocal plot of the data shown in the larger figure. Cellular Na concentration is in $(\mu mol/liter\ cells)^{-1}$ and the Na efflux in $(mmol/liter\ cells \times hr)^{-1}$. The data shown were obtained with the red cells from one donor. Similar results were obtained with cells from five other subjects

Discussion

Furosemide-Resistant Na and K Efflux

The furosemide-resistant fraction of the Na and K efflux into $MgCl_2$-sucrose-ouabain medium has the properties of a membrane leak. Under these conditions, the effluxes show no saturation with the increase in internal concentration of the relevant ion. The rate constants are of an order of magnitude close to the ground permeability of an artificial bilayer (Beauge & Lew, 1977). In the presence of external Na, the ouabain-resistant, furosemide-resistant Na efflux does not behave entirely as a leak but also appears to include a component of Na-Na exchange (Sachs, 1971; Pandey et al., 1977).

The PCMBS technique (Garrahan & Rega, 1967) for changing the internal cation composition yields cells which recover their normal permeability. We used low concentrations of PCMBS (0.02%) and a substrate solution for the sealing-incubation step which permitted recovery of Na and K permeability close to that of fresh cells. However, the rate constants for ouabain- and furosemide-resistant Na and K efflux reported here from fresh and PCMBS-treated erythrocytes into the Mg^{2+}-sucrose media (0.011-

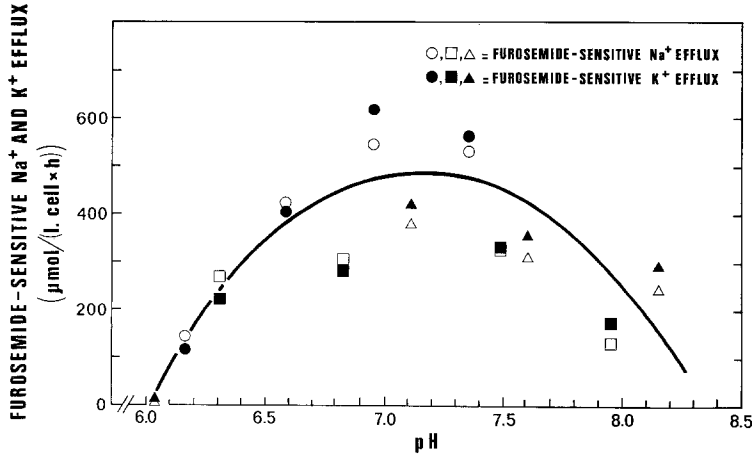

Fig. 7. Ouabain-resistant, furosemide-sensitive (0.5 mM) effluxes of Na (○, □, △) and K (●, ■, ▲) into $MgCl_2$-sucrose medium at 37 °C are plotted as a function of external pH. External pH was varied with Tris-MOPS (10 mM) buffer just in the efflux media. Data were gathered on three batches of red cells (each denoted by a symbol of different shape; i.e., ○, □, △) taken from three different donors. The cells used in these experiments contained (mmol/liter cells) 25–35 of both Na and K. Isotonicity was maintained with internal choline

$0.024\,hr^{-1}$) are still 2–3 times higher than those estimated from the linear component of curves of influx as a function of external Na^+ or K^+ concentration in fresh human red cells (Glynn, 1956). These differences have been previously observed in a pure bilayer with an asymmetric lipid composition. The surface charge and the potential energy profile that these lipid distributions generate depends on the ionic composition across the cell membrane (Latorre & Hall, 1978). Furthermore, we have observed interindividual differences in the recovery of normal cell volume and passive cation permeability after PCMBS treatment.

The Activation of the Na-K Cotransport System by Internal Na and K in Human Red Cells

When the furosemide-sensitive Na and K effluxes are plotted against the internal Na and K concentrations, two bell-shaped superimposable curves are obtained as expected for a 1:1 cotransport mechanism (Fig. 5). The simplest model which accounts for these observations assumes the existence of a carrier with one site which is selective for Na and other selective for K. Only the carrier loaded with both ions may translocate across the red cell membrane. This simple model may be excluded as the stimulation for the cotransport of internal Na has a sigmoidal shape (Fig. 6). As the Hill's coefficient of the Na stimulation varies between 1 and 2, there must be at least two sites for internal Na. The sigmoidal activation of the outward Na-K cotransport by internal Na differs from the hyperbolic behavior of the inward Na-K cotransport described by Wiley and Cooper (1974). Our data do not give a clear answer to the question of whether the stimulation of internal K is also sigmoidal. Preparing cells with a sufficiently low internal K concentration with normal cell volume to solve this problem proved difficult using the PCMBS technique. In order to frame a satisfactory model, more kinetic information is certainly needed.

This work was supported by NIH Grant GM-25686 and partly supported by Harvard Medical School and a CNRS (France) Program.

A preliminary report of this work was presented at the 24th Annual Meeting of the American Biophysical Society (Garay et al., 1980*a*, *b*).

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Received 10 December 1980; revised 26 February 1981