J. Membrane Biol. 103, 217–225 (1988)

The Journal of
Membrane Biology

Effects of pH, Potential, Chloride and Furosemide on Passive Na^+ and K^+ Effluxes from Human Red Blood Cells

A. Martin M. Zade-Oppen,* Norma C. Adragna,** and Daniel C. Tosteson***
Department of Physiology and Biophysics, Harvard Medical School, Boston, Massachusetts, and Department of Physiology and Medical Biophysics, Uppsala University, Uppsala, Sweden

Summary. Ouabain-resistant effluxes from pretreated cells containing $K^+/Na^+ = 1.5$ into K^+ and Na^+ free media were measured. *Furosemide-sensitive* cation effluxes from cells with nearly normal membrane potential and pH were lower in NO_3^- media than in Cl^- media; they were reduced when pH was lowered in Cl^- media. When the membrane potential was positive inside furosemide increased the effluxes of Na^+ and K^+ (7 experiments). With inside-positive membrane potential the *furosemide-insensitive* effluxes were markedly increased, they decreased with decreasing pH at constant internal Cl^- and also when internal Cl^- was reduced at constant pH. The correlation between cation flux and the membrane potential was different for cells with high or low internal chloride concentrations. The data with chloride ≥ 47 mM showed a better fit with the single-barrier model than with the infinite number-of-barriers model. With low chloride no significant correlation between flux and membrane potential was found. The data are not compatible with pure independent diffusion of Na^+ and K^+ in the presence of ouabain and furosemide.

Key Words erythrocyte membrane · furosemide · ion transport · Na-K cotransport · ouabain

Introduction

The concept of a Na^+-K^+ cotransport through the red blood cell membrane was established by Wiley and Cooper [32] though earlier reports described components of the phenomena involved [2, 23, 29]. The transport can be inhibited by furosemide [29, 32] and by other loop diuretics. It was shown [18–20] that in Ehrlich ascites tumor cells this cation transport was associated with a chloride transport [$Na^+ + K^+ + 2\ Cl^-$). Haas et al. [21] showed that this was the case also in duck red blood cells and that these, in contrast to human red blood cells, respond to cell shrinkage by activation of $Na^+ + K^+ + 2\ Cl^-$ transport. A dependence of Na^+-K^+ cotransport on Cl^- ions has also been reported for human red cells [6, 8–10, 14, 30].

The aim was to study a possible effect of membrane potential on the Na^+-K^+ cotransport in human red cells, hoping in this way to further our understanding of the underlying transport mechanism. The transmembrane potential was varied by sucrose substituting for a large part of the $MgCl_2$ in the medium. This results also in an increased pH difference between the cell interior and medium. Therefore conditions both when the internal pH remained constant and when it changed were used. To differentiate between effects due to potential and specific chloride effects, nitrate-containing media were also used. It seemed proper after the results of Wiley and Cooper [32] to define operationally the Na^+-K^+ cotransport as the transport inhibited by furosemide. The unexpected finding that under certain experimental conditions furosemide increased the transport of Na^+ and K^+ suggests that the operational definition of Na^+-K^+ cotransport as furosemide-inhibited transport needs revision. A preliminary report of this work has been presented in abstract form [34].

Materials and Methods

Unless otherwise stated, all chemicals were analytical grade. Solutions were made from quartz-distilled deionized water.

* *Present address:* Department of Physiology and Medical Biophysics, BMC, Box 572, S-751 23 Uppsala, Sweden.

** *Present address:* Department of Pharmacology and Toxicology, Wright State University, School of Medicine. Dayton, OH 45435.

*** *Present address:* Office of the Dean, Harvard Medical School, 25 Shattuck St., Boston, MA 02115.

Experimental Procedures

Venous blood from the same two donors, one male and one female, was used throughout this investigation. The blood was drawn immediately before the experiment into heparinized tubes and centrifuged for 20 min at 17,000 × *g*. This decreased trapped plasma to below 2%. The supernatant and the buffy coat were carefully removed.

Pretreatment

The cells were loaded in the presence of 35 μg/ml nystatin (Sigma Chemical Co., St. Louis, MO) [7] to attain a constant concentration ratio of K^+/Na^+. This was set at 1.5 throughout the investigation. Cells suspended at 40 to 50% hematocrit (Hct) in the loading medium (*see below*) were titrated by an air-CO_2 mixture at ambient temperature to the isoelectric pH, using values given by Dalmark [12]. After centrifugation and resuspension the procedure was repeated until the pH was stable, and the cells were washed twice more. The nystatin-loading medium also contained 54 mM NaCl, 81 mM KCl, 30 mM sucrose [33], and 0.7% methanol. Freshly prepared stock solutions of nystatin in methanol were used in the preparation of loading media. The cells were suspended at or below a 2% Hct in ice-cold loading medium with nystatin for 20 to 30 min. To remove nystatin the cells were twice suspended at or below a 2% Hct in fresh loading medium lacking nystatin but containing 10^{-4} M ouabain at about 30°C [7]. All media used afterwords also contained 10^{-4} M ouabain. The cells were then divided into the same number of batches as the number of experimental conditions and were washed 4 times at 4°C in their respective flux medium (containing ouabain but not furosemide). A suspension of approximately 50% Hct in each flux medium was used to analyze for actual Hct, Hb absorbance, Na^+, K^+, wet and dry weights and for the preparation of the final suspensions for flux measurements.

Flux Media

All flux media contained 10 mM glucose, 10 mM buffer and 0.1 mM ouabain, and had an osmolarity of 292–310 mOsm. The "normal" or reference medium (number 1) contained additionally 75 mM $MgCl_2$ and 75 mM sucrose. The pH at the end of the experiments was 7.25. The chloride ratio (inside/outside) was altered by substituting sucrose for $MgCl_2$. This induced changes in membrane potential (E_{Cl}) and increased the difference between internal and external pH. Therefore either the external pH (pH_o) was varied in order to keep the internal pH (pH_i) constant, or the pH_i was allowed to change. $Mg(NO_3)_2$ was used to discriminate between effects of chloride ions and effects of membrane potential and pH assuming NO_3^- to behave similarly to, or at least not very differently, from Cl^- with regard to membrane potential. The cells were always allowed to stand for 10 min or more at about 5°C (including 4 washes) to equilibrate with their respective flux media before coming in contact with furosemide. Stock solutions of the buffer were made by mixing 0.1 M Tris(2,amino, 2,hydroxy,methyl,1-3,propanediol) (Trizma base, Sigma) with 0.1 M 3-(N-morpholino)propanesulfonic acid (MOPS, Sigma) to the desired pH. The flux media contained a 10-fold dilution of this mixture. This buffer was chosen in order not to add permeant ions. 20 mM fresh stock solutions of furosemide were made in Trizma base and diluted to volume with water. The final furosemide concentration in the cell suspensions was 1 mM. Further details of the composition of flux media are given in the Table.

The Flux Incubation

Cell suspensions (Hct ≦ 50%) were further diluted with flux media to a Hct of 1.7 to 4%. At time zero all cell suspensions were transferred from ice baths to 37°C. Triplicate samples were removed from each batch at 30, 60 and 90 min. They were chilled in ice baths and centrifuged at 3°C and 4,400 × *g*. The supernatant fluid was kept for Na^+ and K^+ determinations. The fluid remaining after this measurement was pooled to get a volume large enough for the determination of pH by the use of a standard combined glass-reference electrode (Orion pH meter, Orion Research, Cambridge, MA). The red cells from samples taken at 30 and 90 min (70 μl or less) were quickly washed twice in the cold with 2 ml each of a solution containing 275 mM sucrose, 5 mM $MgSO_4$ and enough HCl to bring the pH to 3.8. This medium was chosen to eliminate extracellular Cl^- without losing that of the cells [35]. 0.6 or 1 ml of a 0.02% acationox (Lancer Div. of Sherwood Medical, St. Louis, MO) solution was added to each cell pellet and the resulting hemolysate was analyzed for Hb absorbance and chloride content.

Analyses

Wet and dry weights were determined by a procedure reported elsewhere [5]. For this, 100 μl of packed cells in pyrex disposable microsampling pipettes (Corning, Corning, NY) were weighed before and after drying to constant weight at 97°C. The results allowed concentrations to be converted from mmol · (liter cells)$^{-1}$ to mmol · (liter water)$^{-1}$.

Hemoglobin was determined as cyanmethemoglobin in a Gilford 300-N spectrophotometer (Gilford Instrument Lab., Oberlin, OH) at 540 nm [25].

Na^+ and K^+ concentrations were determined by atomic absorption spectrophotometry (model 5000, Perkin-Elmer, Norwalk, CT) using an oxygen-acetylene flame. Supernatants from the flux determinations were usually measured without further dilution. The presence of Mg increased the absorbance in the determinations of K^+. The readings were corrected by the use of empirical correction factors that varied with the Mg^{2+} and K^+ concentrations. The effect of the corrections on the calculated flux was less than 10%.

Cl^--determinations of hemolysates were made using a Corning Chloride Meter 920 M (Corning, Corning, NY) with or without previous protein precipitation for which 0.14 ml 10% picric acid in ethanol were added to 0.7 ml of the sample. After centrifugation in the cold the clear supernatant was used for chloridometry. A separate experiment was performed using hemolysates with varying concentrations of both Hb and Cl^- where all samples underwent chloridometry with or without protein precipitation. The result was used to establish correction factors for the cases where Cl^- was determined in the hemolysate without previous protein precipitation.

Osmolarity was measured on the Osmette S automatic osmometer (Precision System, Sudbury MA).

Calculations

Determinations of cellular Na^+, K^+ and Cl^- were made on samples where also the absorbance of Hb had been determined. From the known value of Hb absorbance in the original packed red cells, all concentrations could be expressed in millimoles in the number of red cells originally occupying one liter, for short expressed as mmol · (liter cells)$^{-1}$. Assuming that the original

Table.

Cond. No.	No. of expts.	Experimental conditions									
		pH_o	pH_i	Mean conc. in mmol · dm^{-3} water			Cl_o^- (mM)	$(NO_3^-)_o$ (mM)	Cl_i^-/Cl_o^-	E_{Cl} (mV)	Water fraction
				Na_i^+	K_i^+	Cl_i^-					
1	5	7.25 ± 0.03	7.15 ± 0.04	64 ± 5.9	94 ± 10.7	116 ± 14.9	144	0	0.80 ± 0.104	−6 ± 3.2	0.64 ± 0.010
2	5	7.92 ± 0.05	7.69 ± 0.10	64 ± 7.7	95 ± 13.1	81 ± 8.8	144	0	0.57 ± 0.061	−15 ± 2.7	0.64 ± 0.014
3	7	6.36 ± 0.20	7.16 ± 0.16	70 ± 6.9	106 ± 12.7	90 ± 15.4	15	0	6.2 ± 1.03	+48 ± 4.9	0.62 ± 0.015
4	7	7.31 ± 0.10	7.78 ± 0.12	76 ± 8.7	115 ± 13.0	47 ± 13.3	15	0	3.3 ± 0.84	+31 ± 7.9	0.59 ± 0.011
5	3	6.57 ± 0.08	6.62 ± 0.01	66 ± 10.4	97 ± 17.4	157 ± 20.3	144	0	1.1 ± 0.14	+2 ± 3.3	0.65 ± 0.008
6	3	7.87 ± 0.11	7.82 ± 0.11	68 ± 6.1	114 ± 9.3	12.7 ± 0.15	15	129	0.90 ± 0.047	−3 ± 1.4	0.61 ± 0.018
7	3	7.18 ± 0.09	7.28 ± 0.04	71 ± 3.3	109 ± 6.0	17.6 ± 1.30	15	129	1.3 ± 0.14	+6 ± 3.1	0.61 ± 0.025
8	2	7.05 ± 0.04	7.52 ± 0.01[a]	69 ± 5.3	107 ± 8.7	5 ± 5.0	0	15	—	+29 ± 1.2[a]	0.59
9	2	6.32 ± 0.03	7.13 ± 0.03[b]	70 ± 3.7	110 ± 4.6	8 ± 1.6	0	15	—	+50 ± 0.3[b]	0.62

[a] Calculated on the assumption that the NO_3^- ratio equals the Cl^- ratio of parallel samples in condition 4.

[b] Calculated assuming the Cl^- ratio in parallel samples in condition 3 can be used.

The table gives values of Cl^-- and NO_3^--concentrations in the flux media, their pH as measured after 90 min incubation of cells at 37°C, and cellular water fraction and Na^+ and K^+ concentrations prior to the incubation. The Cl_i^--values are means of measured Cl_i^--concentrations in washed cells that had been incubated 30 and 90 min at 37°C. pH_i and E_{Cl} were calculated from the chloride ratio and pH_o. Means are given ± SD.

cells had a density of 1.1 and contained 1.86 g water per g dry wt, allowed concentrations to be expressed in mmol · (liter cell water)$^{-1}$. Effluxes were calculated from the slope of the line relating concentration in the supernatants at 30, 60 and 90 min. The regression coefficient (R) was usually ≧0.99. In a few cases there was a small degree of curvature and the 90 min values were then excluded. By the use of the Hb absorbance of the cell suspensions and the proper dilution factors, the flux values were transformed into mmol · (liter cells)$^{-1}$ · h^{-1}. The membrane potential was assumed to equal the equilibrium potential for chloride (E_{Cl}) and was calculated from the relation

$$E_{Cl} = \frac{RT}{F} \ln \frac{Cl_i^-}{Cl_o^-}.$$

The internal pH was calculated from the external pH assuming

$$H_i^+/H_o^+ = Cl_o^-/Cl_i^-$$

or

$$pH_i = pH_o + \log \frac{Cl_i^-}{Cl_o^-}.$$

Results

Furosemide-Sensitive Effluxes of Na^+ and K^+

In Fig. 1 furosemide-sensitive effluxes are shown under conditions where the membrane potential was approximately constant and near its normal value. Chloride ion concentration was varied by using media containing either $MgCl_2$ or $MgCl_2$ plus $Mg(NO_3)_2$. The pH was also varied. At low Cl^- concentrations no apparent effects of pH were seen but at high Cl^- concentration, where a wider pH range

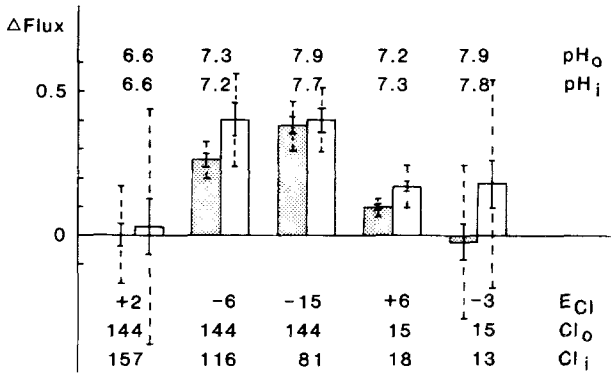

Fig. 1. Mean furosemide-sensitive effluxes (Δ Flux) in mmol · dm^{-3} cells · h^{-1} of Na^+, shaded bars, and K^+, open bars, at 37°C with varying pH and Cl_i^- concentration (mmol · dm^{-3} water) into Na^+ and K^+ free media. These contained 100 mM glucose, 10 mM Tris-MOPS buffer, 0.1 mM ouabain, $MgCl_2$ and/or $Mg(NO_3)_2$, sucrose, ±1 mM furosemide and were made up to 300 mOsm. The low Cl^- was obtained by substitution with NO_3^-. The membrane potential (E_{Cl} mV) calculated from measured Cl^- values, was near normal. The cells in these and following experiments were pretreated with nystatin to contain K^+ and Na^+ in a ratio of 1.5. The number of experiments were (from left to right) n = 3, 5, 5, 3 and 3. *Statistics* in this and following figures: Solid error bars for SEM, dashed error bars for 95% confidence intervals of the means. This includes the uncertainty due to small sample sizes. When comparing results, a difference is significant with 95% confidence or better if there is no overlap between the dashed bars. An overlap does not exclude the possibility of a significant difference. In the text some differences are given with a good significance calculated on paired samples from several experiments. This is not obvious in the figure because only the corresponding means and their statistics are shown

was investigated, the Na^+ fluxes increased when pH was raised from 6.6 to 7.3 and from 7.3 to 7.9 ($0.01 > P > 0.001$) and the K^+ fluxes rose when going from pH 6.6 to 7.3 ($0.02 > P > 0.01$). Effects

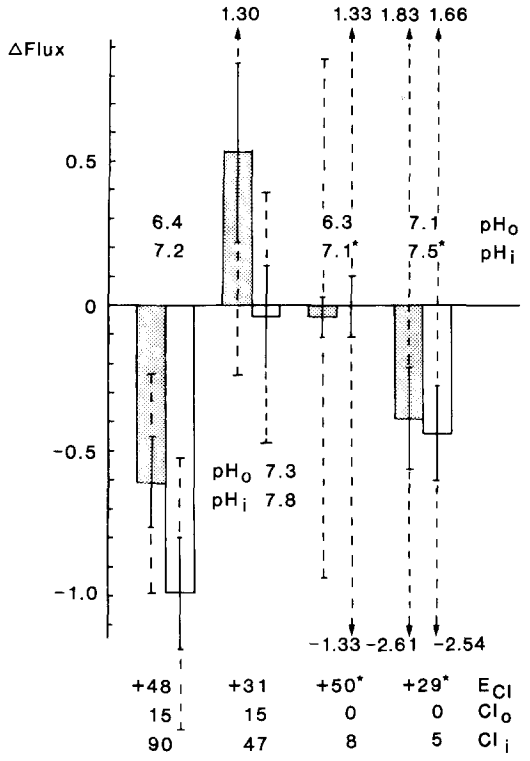

Fig. 2. Mean furosemide-sensitive effluxes (Δ Flux = control flux minus flux in the presence of furosemide) in mmol · dm^{-3} cells · h^{-1} of Na^+, shaded bars, and K^+, open bars, at 37°C with varying pH and Cl^- concentration (mmol · dm^{-3} water). The membrane potential (E_{Cl} mV), calculated from Cl^- ratios, was not normal but inside positive. Media were composed as in Fig. 1 but most of the $MgCl_2$ or $Mg(NO_3)_2$ was replaced by sucrose. The potential in Cl^--free media was assumed to equal the potential in parallel samples containing Cl^-. The assumed E_{Cl} values are marked with a star. The number of experiments were (from left to right) n = 7, 7, 2 and 2. The values of Cl_i = 5 and 8 in this figure and in Figs. 3 and 4 are not significantly different from zero due to the large errors in Cl_i^- determination at very low Cl^- concentrations

of chloride ions can be judged in the same figure. It is clear that both at neutral and alkaline pH the furosemide-sensitive Na^+ and K^+ fluxes are larger when the chloride concentration is high. (At pH_o = 7.9 for K^+ $0.05 > P > 0.02$.) The change in Na^+ flux resulting from a change in Cl^- concentration is more evident at pH_o 7.9 than 7.2 or 7.3.

In the experiment of Fig. 2 sucrose substituted for all but 15 mM Cl^- (7.5 mM $MgCl_2$) or 15 mM NO_3^- in the medium in order to obtain a high positive potential inside and, in consequence, large differences between internal and external pH values. The total fluxes were comparatively large and therefore also the errors of the furosemide-sensitive fluxes. At pH_o = 7.3 and Cl_o = 15 mM the furosemide-sensitive fluxes of Na^+ and K^+ showed large variations between experiments, and the averages from seven experiments were not different from zero. When the cells were equilibrated in an acid Cl^--containing medium the efflux of Na^+ and that of K^+ was larger in the presence of furosemide than in its absence. This is presented with negative values in the figure. This unexpected result is well verified in that the statement is made with $0.01 > P > 0.001$ from the average of seven experiments. Further, the Na^+ fluxes were stimulated ($0.001 > P$) in each of six out of the seven experiments and the furosemide sensitive K^+ fluxes were stimulated ($0.001 > P$) in all seven. When Cl^- was further reduced (from 15 to 0 mM) the furosemide-sensitive fluxes of both cations were not different from zero as observed in only two experiments. However, at pH_o = 7.1 both Na^+ and K^+ effluxes were stimulated by furosemide ($0.001 > P$) in each of two experiments. The effect of reversing the membrane potential therefore, under the right conditions, was to turn a so-called furosemide-sensitive (i.e., a furosemide-inhibited) flux into a furosemide-stimulated flux (*compare* Figs. 1 and 2). Substitution of Cl^- without a concomitant potential change did not show the same effect (Fig. 1).

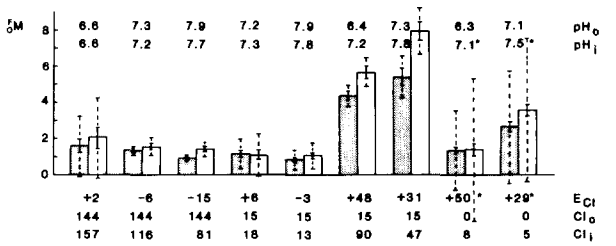

Fig. 3. Mean effluxes in mmol · dm^{-3} cells · h^{-1} of Na^+, shaded bars, and K^+, open bars, in the presence of 1 mM furosemide (E_oM) at 37°C with varying pH, Cl^--concentration (mmol · dm^{-3} water) and membrane potential (mV). Pretreatment and media composition as in Figs. 1 and 2. The number of experiments were (from left to right) n = 3, 5, 5, 3, 3, 7, 7, 2 and 2

Fluxes in the Presence of Ouabain and Furosemide

Data for effluxes of Na^+ and K^+ in the presence of both ouabain and furosemide are given in Fig. 3.

When the membrane potential was near normal no effect of pH was observed. With a membrane potential of +30 to +50 mV protons inhibited K^+ fluxes and this occurred both when Cl_o = 15 or 0 (and $(NO_3)_o$ = 0 or 15) ($0.01 > P > 0.001$). The Na^+ fluxes were inhibited by protons in the low Cl_i^- condition ($0.01 > P > 0.001$), but the difference at higher Cl^- was not significant.

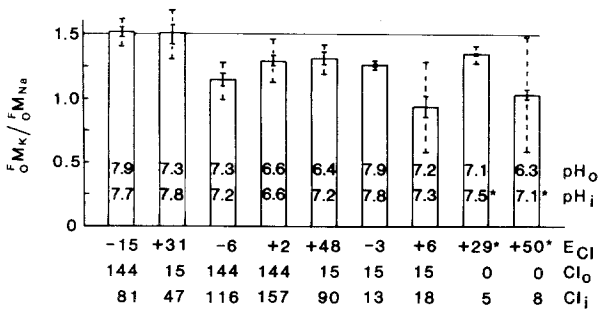

Fig. 4. Mean ratios ($^F_oM_K/^F_oM_{Na}$) of K^+ efflux over Na^+ efflux into media containing furosemide. Red cells had initial concentration ratios $K^+/Na^+ = 1.52 \pm 0.093$ (SD) ($n = 37$), under conditions with varying membrane potential (E_{Cl} mV) and Cl^- concentration (mmol · dm^{-3} water). The media were as stated under Figs. 1 and 2. The lower 95% confidence limit for the K^+/Na^+ concentration ratio was 1.49. In seven out of nine conditions the ratio of fluxes was significantly different from the ratio of concentrations. The number of experiments were (from left to right) $n = 5, 7, 5, 3, 7, 3, 3, 2$ and 2

A membrane potential of +30 to +50 mV means an increased electric driving force for cation efflux compared with a near normal potential. The expected concomitant increase in efflux is obvious in Fig. 3. This effect was not observed in the absence of Cl^- on both sides of the membrane and at low pH_o. At a near normal pH_o, however, the flux at $E_{Cl} = +29$ and $Cl_o = 0$ is larger than when $E_{Cl} = -6$ and $Cl_o = 144$ at $pH_o = 7.3$ for both cations ($0.01 > P > 0.001$). In addition the increase of cation efflux was higher in the presence of intracellular Cl^-. A marked effect of chloride ions was seen when comparing the efflux from cells with a high inside positive potential; the efflux was considerably reduced ($0.05 > P$) when NO_3^- replaced the 15 mM Cl^- in the medium and Cl_i^- was not significantly different from zero (*compare* in Fig. 3 $E_{Cl} = 48$, $pH_o = 6.4$ with $E_{Cl} = 50$, $pH_o = 6.3$, and $E_{Cl} = 31$, $pH_o = 7.3$ with $E_{Cl} = 29$, $pH_o = 7.1$). A similar effect was not observed in cells having a near normal potential (*compare* $E_{Cl} = 6$, $pH_o = 7.2$ with $E_{Cl} = -6$, $pH_o = 7.3$). The low internal Cl^- concentration then was 18 mM.

Assuming that the efflux of Na^+ and K^+ in the presence of both ouabain and furosemide is due to pure electrodiffusion through channels or pores equally permeable to both ions, the ratio of fluxes M_K/M_{Na} should equal the ratio of the concentration of these ions in the cell (the external concentration being zero), assuming no differences in permeability coefficients. In all these experiments the ratio of concentrations K/Na = 1.52 ± 0.093 (SD, $n = 37$). To test this assumption the ratios of the fluxes were calculated and the results are given in Fig. 4. Only in two cases out of nine was the ratio 1.5, in the other cases it was below 1.5 and in two cases not different from unity. No clear relation was found between flux ratio and membrane potential, pH or Cl^--concentration.

Another test of the assumption that the effluxes in the presence of both ouabain and furosemide were due to only simple electrodiffusion (no active and no carrier-mediated transport) was performed in the following treatment. The data were treated in two ways: (i) electrodiffusive flux through a membrane with a single barrier (Fig. 5*A*) and (ii) through a membrane with an infinite number of barriers (Fig. 5*B*). The treatment is given in detail in the Appendix. When the lower flux value out of the pair with and without furosemide was used consistently, the result was that the data did not show a perfect fit with either treatment; instead of a slope of one, the single barrier treatment gave 1.10 ± 0.08 and the infinite number of barriers model gave 1.20 ± 0.09. The differences between these three values are significant ($P < 0.001$).

Discussion

Effects of Furosemide on Efflux of Na^+ and K^+

It was clearly seen, when the membrane potential was near its normal value, that furosemide-sensitive efflux was larger when the chloride concentration was high. This confirms a chloride sensitivity for Na^+-K^+ cotransport in human red blood cells reported previously [3, 8–10, 14, 30]. It lends support to the notion that in human cells this cotransport may be a $Na^+ + K^+ + 2Cl^-$ cotransport like that reported in duck red blood cells [21] and in Ehrlich cells [20].

With a nearly normal membrane potential it was also seen that protons inhibit the furosemide-sensitive efflux of Na^+ and K^+. This, in part, confirms a more complete study of pH effects by Garay et al. [17]. In the light of the present findings it is not possible to conclude whether protons inhibit the Na^+-K^+ cotransport or exert their influence by modulating the furosemide effect. It may be worth noting that a dose-response curve [17] exists for only one pH (7.4, 37°C).

When the membrane potential was positive inside ($Cl_o = 15$ mM) and $pH_o = 6.4$ the efflux of both cations was larger in the presence of furosemide than in its absence. If this unexpected result should be explained as a stimulation of Na^+-K^+ cotransport caused by furosemide, then the consequence would be that furosemide may have simultaneously a stimulating and an inhibiting effect and the rela-

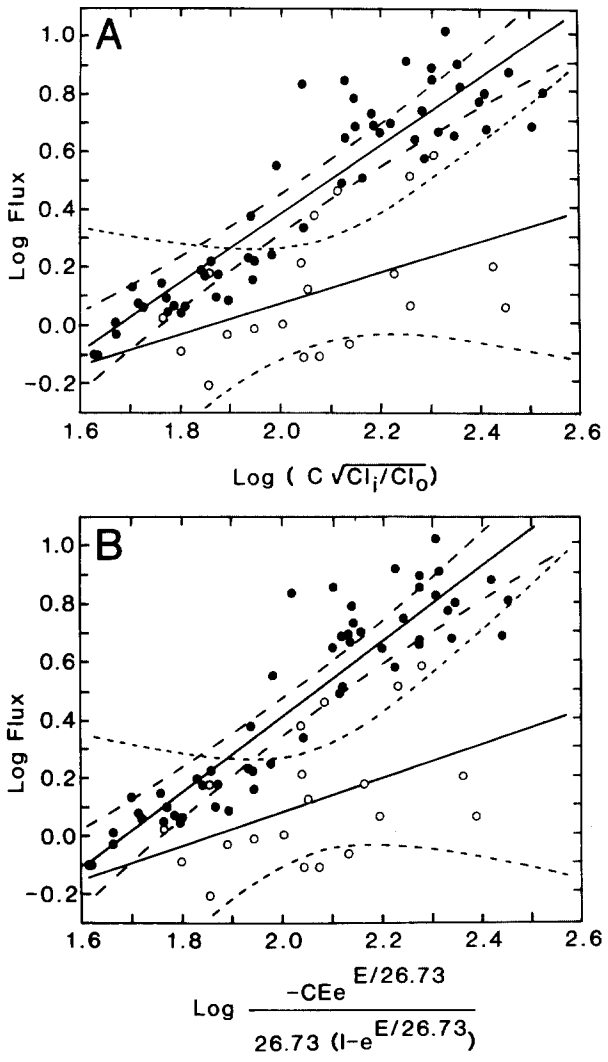

Fig. 5. Effect of the electrochemical driving force on the efflux of Na^+ and K^+ in the presence of furosemide under conditions with varying membrane potential (chloride ratio), pH, and chloride content but constant initial content of Na^+ and K^+. A double logarithmic plot was used to obtain a proper weighting of the points. Fluxes were expressed in mmol · $(dm^3$ cells$)^{-1}$ · h^{-1}. Open circles represent red cells with $Cl_i^- \leqq 18$ mM. Filled circles where $Cl_i^- \geqq 47$ mM. In *A* the driving force was calculated according to the single barrier model and in *B* according to the model with an infinite number of barriers. The full lines, expressed in *A* by Eq. (4) and in *B* by Eq. (7) (*see* Appendix), were obtained by linear regression analysis of results from cells with a high Cl_i^-. From these the permeability coefficients of Na^+ and K^+ were calculated. The lower straight lines similarly belong to cells with a low Cl_i^-. The 99.9% confidence limits of the regression lines are shown as curved broken lines. In both *A* and *B* the slope of the low Cl_i^- regression line is not significant, meaning that the flux may or may not be related to the plotted driving force. The values obtained with cells low in Cl_i^- appear to belong to a statistically different population from that where values were derived from high Cl_i^- cells

tive magnitudes of the two would determine whether the result under varying conditions appears as a stimulation or a partial or a complete inhibition.

The stimulation at $pH_o = 7.1$ cannot have been a cotransport with Cl^- as this anion was virtually absent. Since the publication by Wiley and Cooper [32] Na^+-K^+ cotransport has been synonymous with furosemide-inhibited transport. The results presented here lead to the conclusion that furosemide is not a good test substance to assess the $Na^+ + K^+(+2Cl^-)$ cotransport under all experimental conditions. Further work is required to analyze the mechanism of the observed stimulation of Na^+ and K^+ efflux in the presence of furosemide. It may, however, even if perhaps somewhat far-fetched be of interest in this connection that for a conductance channel an inhibitor has been found to stimulate transport. Eisenman and Sandblom [15] describe this possibility for the blocking effect of tetraethylammonium on cesium transport through gramicidin A channels in glyceryl monooleate bilayers. It has also been shown that amiloride can both stimulate and block Na^+ transport with varying concentrations [27, 31]. The furosemide concentration was constant in this investigation, but varying pH and E_{Cl} could alter the affinities of the binding sites.

Chipperfield and Shennan [11] investigated the effect of piretanide on Na^+ and K^+ efflux from human red blood cells with a reversed membrane potential by gluconate substitution of Cl^- and also varying pH. They did not find any flux-stimulating effects of the drug. It appears therefore that the stimulating effect of furosemide is either more or less specific for this compound or for the experimental conditions used in this investigation.

Fluxes in the Presence of Ouabain and Furosemide

The present investigation was unusual in that the electrical driving force for ion efflux was varied whereas ion concentrations were kept constant [25]. The increased salt permeability of red blood cells in nonelectrolyte media [4, 13] was avoided by the use of a minimum of 7.5 mM $MgCl_2$ or $Mg(NO_3)_2$ in the sucrose media. The opening of voltage-sensitive cation channels should not constitute a problem in the voltage range used here [13]. It was therefore possible to test if the single barrier or the infinite number of barriers model best fitted the experimental data. It can be calculated that they should differ by a factor of 1.3 within the range of varying membrane potentials used in this investigation. A consequence of the smallness of this factor is that the two treatments must be expected to give very similar results. This was obviously also the case as seen when comparing Fig. 5*A* and *B*. In spite of this similarity the models gave statistically significantly different results and the single barrier treatment fitted

more closely to the experimental data from Cl^--containing cells than the infinite number model, although neither model was entirely satisfactory as neither yielded the theoretical slope of unity.

The relation between the flux which may be expected to be a pure diffusive flux through so-called conductance channels and the electrochemical driving force clearly demonstrated (Figs. 3 and 5) a strong dependence on chloride ions, the fluxes being smaller with NO_3^-. This appears to be in contrast to the finding that K^+ moves faster with NO_3^- as counterion than with Cl^- in the Gardos channel [26] and contradictory to the data presented by Funder and Wieth [16] which, however, were obtained under different experimental conditions, It is a new observation which should be the object of further study.

Another unresolved problem is the observation of varying ratios of the K^+ flux/Na^+ flux (Fig. 4) in spite of a constant ratio of their concentrations and varying potentials affecting both ions equally. There is at the moment no explanation available. We can, however, exclude one mechanism. Shrinkage to about 54% water has been reported to increase Na^+ but not K^+ permeability [1]. A less drastic shrinkage was not always avoided in the present investigation (Table 1), but in condition 4 (water fraction = 0.59) the flux ratio was 1.5 and in conditions 1 and 5 (normal water fraction) the ratio was below 1.5. It therefore seems unlikely that the varying flux ratios could be induced by volume alterations in these experiments.

We want to thank Dr. John Sandblom for helpful discussions. Financial support was given by the Swedish Medical Research Council (travel grant K80-14R-5847 to Zade-Oppen).

References

1. Adragna, N.C., Tosteson, D.C. 1984. Effect of volume changes on ouabain-insensitive net outward cation movements in human red cells. *J. Membrane Biol.* **78:**43–52
2. Beaugé, L.A., Adragna, N. 1971. The kinetics of ouabain inhibition and the partition of rubidium influx in human red blood cells. *J. Gen. Physiol.* **57:**576–592
3. Beaugé, L., Lew, V.L. 1977. Passive fluxes of sodium and potassium across red cell membranes. *In:* Membrane Transport in Red Cells. J.C. Ellory and V.L. Lew, editors. pp. 39–51. Academic, London
4. Bolingbroke, V., Maizels, M. 1959. Calcium ions and the permeability of human erythrocytes. *J. Physiol. (London)* **149:**563–585
5. Canessa, M., Adragna, N., Solomon, H.S., Connolly, T.M., Tosteson, D.C. 1980. Increased sodium-lithium countertransport in red cells of patients with essential hypertension. *N. Eng. J. Med.* **302:**772–776
6. Canessa, M., Brugnara, C., Cusi, D., Tosteson, D.C. 1986. Modes of operation and variable stoichiometry of the furosemide-sensitive Na and K fluxes in human red cells. *J. Gen. Physiol.* **87:**113–142
7. Cass, A., Dalmark, M. 1973. Equilibrium dialysis of ions in nystatin-treated red cells. *Nature New Biol.* **244:**47–49
8. Chipperfield, A.R. 1980. An effect of chloride on (Na + K) co-transport in human red blood cells. *Nature (London)* **286:**281–282
9. Chipperfield, A.R. 1981. Chloride dependence of frusemide- and phloretin-sensitive passive sodium and potassium fluxes in human red cells. *J. Physiol. (London)* **312:**435–444
10. Chipperfield, A.R., Shennan, D.B. 1983. Chloride dependence of diuretic-sensitive passive Na-efflux from human red blood corpuscles. *J. Physiol. (London)* **342:**16–17P
11. Chipperfield, A.R., Shennan, D.B. 1984. The effect of changing pH and Em on ouabain-insensitive Na and K efflux from human erythrocytes. *J. Physiol. (London)* **353:**134P
12. Dalmark, M. 1975. Chloride and water distribution in human red cells. *J. Physiol. (London)* **250:**65–84
13. Donlon, J.A., Rothstein, A. 1969. The cation permeability of erythrocytes in low ionic strength media of various tonicities. *J. Membrane Biol.* **1:**37–52
14. Dunham, P.B., Stewart, G.W., Ellory, J.C. 1980. Chloride-activated passive potassium transport in human erythrocytes. *Proc. Natl. Acad. Sci. USA* **77(3):**1711–1715
15. Eisenman, G., Sandblom, J.P. 1983. Energy barriers in ionic channels: Data for gramicidin A interpreted using a single-file (3B4S″) model having 3 barriers separating 4 sites. *In:* Physical Chemistry of Transmembrane Ion Motions. G. Spach, editor. pp. 329–347. Elsevier, Amsterdam
16. Funder, J., Wieth, J.O. 1967. Effects of some monovalent anions on fluxes of Na and K, and on glucose metabolism of ouabain treated human red cells. *Acta Physiol. Scand.* **71:**168–185
17. Garay, R., Adragna, N., Canessa, M., Tosteson, D. 1981. Outward sodium and potassium cotransport in human red cells. *J. Membrane Biol.* **62:**169–174
18. Geck, P. 1980. Sodium-potassium-chloride cotransport: A target system for inhibition by high-ceiling diuretics. *Kidney Int.* **17:**405 (*Abstr.*)
19. Geck, P., Heinz, E. 1980. Coupling of ion flows in cell suspension systems. *Ann. N.Y. Acad. Sci.* **341:**57–66
20. Geck, P., Pietrzyk, C., Burckhardt, B.-C., Pfeiffer, B., Heinz, E. 1980. Electrically silent cotransport of Na^+, K^+, and Cl^- in Ehrlich cells. *Biochim. Biophys. Acta* **600:**432–447
21. Haas, M., Schmidt, W.F., McManus, T.J. 1982. Catecholamine-stimulated ion transport in duck red cells: Gradient effects in electrically neutral (Na + K + 2Cl) co-transport. *J. Gen. Physiol.* **80:**125–147
22. Hodgkin, A.L., Katz, B. 1949. The effect of sodium ions on the electrical activity of the giant axon of the squid. *J. Physiol. (London)* **108:**37–77
23. Hoffman, J.F., Kregenow, F.M. 1966. The characterization of new energy dependent cation transport processes in red blood cells. *Ann. N.Y. Acad. Sci.* **137:**566–576
24. Kampen, E.J. van, Zijlstra, W.G. 1961. Standardization of hemoglobinometry: II. The hemiglobincyanide method. *Clin. Chim. Acta* **6:**538–544
25. Kessler, M., Semenza, G. 1983. The small-intestinal Na^+, D-glucose cotransporter: An asymmetric gated channel (or pore) responsive to $\Delta\Psi$. *J. Membrane Biol.* **76:**27–56
26. Lauf, P.K. 1985. Induced ouabain-insensitive K^+ fluxes in biological membranes: A review of trigger mechanisms, operational modes and linkage with cellular metabolism. *In:*

Prostaglandins and Membrane Ion Transport. Advances in Ion Transport Regulation. P. Braquet, R.P. Garay, J.C. Frölich, and S. Nicosia, editors. pp. 379–390. Raven, New York
27. Li, J.H.-Y., Lindemann, B. 1983. Chemical stimulation of Na transport through amiloride-blockable channels of frog skin epithelium. *J. Membrane Biol.* **75**:179–192
28. Parlin, R.B., Eyring, H. 1954. Membrane permeability and electrical potential. *In:* Ion Transport across Membranes. H.T. Clarke, editor. pp. 103–118. Academic, New York
29. Sachs, J.R. 1971. Ouabain-insensitive sodium movements in the human red blood cell. *J. Gen. Physiol.* **57**:259–282
30. Shennan, D.B. 1983. The influence of chloride replacement on K efflux from human RBC. *J. Physiol. (London)* **346**:89P
31. Thurman, C.L., Higgins, J.T. 1982. Amiloride stimulation of sodium transport in the presence of calcium and a divalent cation chelator. *Biochim. Biophys. Acta* **689**:561–566
32. Wiley, J.S., Cooper, R.A. 1974. A furosemide-sensitive co-transport of sodium plus potassium in the human red cell. *J. Clin. Invest.* **53**:745–755
33. Zade-Oppen, A.M.M. 1980. A note on osmotic pressure measured with ionophore treated red blood cells. *Biophys. Chem.* **12**:57–61
34. Zade-Oppen, A.M.M., Adragna, N., Tosteson, D.C. 1982. Passive efflux of Na^+ and K^+ from red blood cells and effect of furosemide. *Acta Physiol. Scand. Suppl.* **508**:64 (*Abstr.*)
35. Zade-Oppen, A.M.M., Schooler, J.M., Cook, P., Tosteson, D.C. 1979. Effect of membrane potential and internal pH on active sodium-potassium transport and on ATP content in high-potassium sheep erythrocytes. *Biochim. Biophys. Acta* **555**:285–298

Received 7 April 1988

Appendix

The Relation Between Flux and Electrochemical Driving Force

The data were treated as flux through a membrane with a single barrier in **I** and with an infinite number of barriers in **II**.

I. For the application to the case with a single barrier where the outside concentration is approximately zero, the relation between cation flux and electro-chemical forces is

$$J = PCe^{FE/2RT} \tag{1}$$

which is obtained if in Eq. (15) of Parlin and Eyring [28] the external concentration $C_n = 0$ and the signs adjusted so that the membrane potential (E) is the inside potential minus the outside potential where J = efflux [mmol · (liter cells)$^{-1}$ · h^{-1}], P = permeability coefficient [(liter water) · (liter cells)$^{-1}$ · h^{-1}], C = cation concentration (mmol · liter^{-1}), and F, R, and T have their usual meanings.
According to Nernst the membrane potential is

$$E = \frac{RT}{F} \ln \frac{Cl_i}{Cl_o} \tag{2}$$

where Cl_i and Cl_o denote the inside and outside chloride concentrations. Combining Eqs. (1) and (2) gives

$$J = PC \sqrt{\frac{Cl_i}{Cl_o}}. \tag{3}$$

In order to determine P, linear regression analysis of the interdependence of log J and log P + log ($C\sqrt{Cl_i/Cl_o}$) was used rather than the nonlogarithmic expression in order to obtain a proper weighting of the individual points.

Analysis of the entire material gave a correlation coefficient (R) of only 0.74 (n = 74). This is one expression of the great scatter of the points (filled and open circles) seen in Fig. 5*A*. The material was divided into two groups which were treated separately. The first group contained the flux values from conditions 1–5 (Table 1), i.e., all conditions where Cl_i was relatively high. The second group contained values from conditions 6–9, in which Cl_i was at or below 18 mmol · (liter water)$^{-1}$.

In the first group the values for $P_{Na} = 0.009$ and $P_K = 0.007$ were calculated and these were not significantly different. Therefore Na^+ and K^+ flux values were treated in one group. Linear regression analysis gave the equation

$$\text{Log } J = -1.99(\pm 0.15) + 1.19(\pm 0.07) \log \left(C \sqrt{\frac{Cl_i}{Cl_o}} \right) \tag{4}$$

$n = 54$, $R = 0.92$, $P = 0.010\ ^{+0.005}_{-0.003}$ (liter water) · (liter cells)$^{-1}$ · h^{-1}, which, assuming for the average red cell a surface area of 142 μm^2 and a volume of 87 μm^3, gives

$$P = 1.74 \cdot 10^{-10}\ ^{+0.87\times10^{-10}}_{-0.52\times10^{-10}} \text{ cm} \cdot \text{sec}^{-1}.$$

The flux values from conditions where Cl_i^- was low showed a large scatter (open circles in Fig. 5*A*) and linear regression analysis gave $R = 0.47$ ($n = 20$). Much of the area between the 99.9% confidence lines of the linear regression line of this population is well outside the corresponding area for the points obtained for cells with a high Cl_i^-, suggesting that it is justified to treat the results as consisting of two separate populations.

II. The diffusive flux over a membrane with an infinite number of barriers is expressed by the Goldman equation [22]. Adjusting the equation to the present sign convention and to fluxes in mmol · liter^{-1} · h^{-1} we obtain

$$J = PX \tag{5}$$

where

$$X = -CEe^{E/26.73}/26.73(1 - e^{E/26.73}) \tag{6}$$

if E is expressed in mV.

In order to determine P the logarithmic relationship was used again so as to obtain a proper weighting of the experimental points.

In spite of the mathematically different approach, the results are quite similar to those above. The values for P_{Na} (0.005) and P_K (0.004) in the first group (high Cl_i) were not significantly different as seen before. The combined material gave the following equation after linear regression analysis:

$$\text{Log } J = -2.18(\pm 0.16) + 1.29(\pm 0.08)\log X \quad (7)$$

$$n = 54,\ R = 0.92.$$

This gives $P = 0.007\ ^{+0.003}_{-0.002}$ (liter water) · (liter cells)$^{-1}$ · h^{-1} which, with the same assumption for red cell surface to volume ratio as above, equals

$$P = 1.13 \cdot 10^{-10}\ ^{+0.49\times10^{-10}}_{-0.37\times10^{-10}}\ \text{cm} \cdot \text{sec}^{-1}.$$

Again the flux values from conditions where Cl_i was low showed a large scatter (open circles in Fig. 5*B*) and linear regression analysis of the points gave $R = 0.47$ ($n = 20$). Again results obtained with cells having a high and a low Cl_i, respectively, appear to belong to statistically different populations as judged from the 99.9% confidence limits.

In order to test the fit between experimental results and theory (treatments I and II) the slope coefficient in Eqs. (4) and (7), respectively, should be compared with the theoretical value of 1. In both treatments the slopes were larger than one. However, the material included values from condition 3, where furosemide increased the fluxes. When the values from this condition were replaced by the corresponding control values for the same condition, so that consequently the lower flux out of the value pairs of control and furosemide were used, then the slope coefficient in Eq. (4) became 1.10 ± 0.08. The slope coefficient in Eq. (7) became 1.20 ± 0.09. Both values are significantly different from 1 and different from each other ($P < 0.001$).