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Role of the Furosemide-Sensitive Na^+/K^+ Transport System in Determining the Steady-State Na^+ and K^+ Content and Volume of Human Erythrocytes *in Vitro* and *in Vivo*

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Summary. To study the physiological role of the bidirectionally operating, furosemide-sensitive Na^+/K^+ transport system of human erythrocytes, the effect of furosemide on red cell cation and hemoglobin content was determined in cells incubated for 24 hr with ouabain in 145 mM NaCl media containing 0 to 10 mM K^+ or Rb^+. In pure Na^+ media, furosemide accelerated cell Na^+ gain and retarded cellular K^+ loss. External K^+ (5 mM) had an effect similar to furosemide and markedly reduced the action of the drug on cellular cation content. External Rb^+ accelerated the Na^+ gain like K^+, but did not affect the K^+ retention induced by furosemide. The data are interpreted to indicate that the furosemide-sensitive Na^+/K^+ transport system of human erythrocytes mediates an equimolar extrusion of Na^+ and K^+ in Na^+ media (Na^+/K^+ "cotransport"), a 1:1 K^+/K^+ (K^+/Rb^+) and Na^+/Na^+ "exchange" progressively appearing upon increasing external K^+ (Rb^+) concentrations to 5 mM. The effect of furosemide (or external K^+/Rb^+) on cation contents was associated with a prevention of the cell shrinkage seen in pure Na^+ media, or with a cell swelling, indicating that the furosemide-sensitive Na^+/K^+ transport system is involved in the control of cell volume of human erythrocytes. The action of furosemide on cellular volume and cation content tended to disappear at 5 mM external K^+ or Rb^+. The *in vivo* red cell K^+ content was negatively correlated to the rate of furosemide-sensitive K^+ (Rb^+) uptake, and a positive correlation was seen between mean cellular hemoglobin content and furosemide-sensitive transport activity. The transport system possibly functions as a K^+ and water-extruding mechanism under physiological conditions *in vivo*. The red cell Na^+ content showed no correlation to the activity of the furosemide-sensitive transport system.

Key Words Erythrocytes – ouabain-resistant cation transport · Na^+/K^+ cotransport · K^+/K^+ exchange · Na^+/K^+ content · hemoglobin content · volume regulation

Introduction

The so-called *Na^+/K^+ cotransport* system of human erythrocytes mediates bidirectional Na^+ and K^+ movements and can promote downhill as well as uphill net movements of Na^+ and K^+ [3, 27, 38, 42]. It may be operationally defined by *i*) its susceptibility to inhibition by loop diuretics such as ethacrynic acid, furosemide, piretanide and bumetanide [16, 34, 42], *ii*) the mutual acceleration of Na^+ movements by K^+ and of K^+ movements by Na^+ when both ions are present at the same side of the membrane [3, 11, 20, 38, 42], and *iii*) the dependence of the cation transport on chloride [16].

It is still an open question, however, whether the furosemide-sensitive Na^+ and K^+ movements in human erythrocytes are mediated by one system promoting Na^+/K^+ "cotransport," or whether there are separate systems for Na^+-dependent K^+ transport and K^+-dependent Na^+ transport mediating Na^+/Na^+, K^+/K^+ or Na^+K^+/Na^+K^+ "exchange," respectively [15]. The neutral term "furosemide-sensitive Na^+/K^+ transport system" used below is not being supposed to differentiate between these possibilities.

The furosemide-sensitive Na^+ and K^+ transport system of human erythrocytes shows considerable interindividual differences in maximum activity [11, 21, 22] which are thought to be genetically determined [33] and have been brought into relation with essential hypertension [14, 22].

Based on the early observations of Hoffman and Kregenow [26, 27] and on later reports [2, 3, 4, 20, 31, 37, 38, 42] it is often believed that the furosemide-sensitive Na^+/K^+ transport system of human erythrocytes functions as a "second pump" extruding Na^+ from the cells against an electrochemical gradient *in vivo* [20, 21]. This concept neglects the observation of Dunn [17] that furosemide does not alter the net Na^+ gain of human erythrocytes incubated with ouabain over 7 hr in a medium of nearly physiological cation composition (5 mM K^+, 130 mM Na^+). The Na^+ extrusion by the furosemide-sensitive system is blocked by external K^+ [2, 20, 21, 37, 38], and it is by no means clear whether the Na^+ and K^+ extrusion driven by the outwardly directed K^+ gradient ex-

ceeds the inward Na^+ and K^+ movements propelled by the inwardly directed Na^+ gradient under physiological conditions.

In an attempt to ascertain the physiological role of the furosemide-sensitive Na^+/K^+ transport system of human erythrocytes, its well-known dependence on external K^+ concentration [3, 11, 16, 27, 31, 37, 38, 42] is now re-examined, in some experiments K^+ being replaced by its congener Rb^+ which is well accepted by the transport system in the place of K^+ in human [11, 16], duck [24, 25] and sheep [30] erythrocytes. Since the activity of the bidirectionally operating transport system of human erythrocytes is rather low, net changes of cation content and cell volume are studied that are brought about by furosemide in ouabain-poisoned cells during long-term (24 hr) incubations. In addition, the effect of the interindividual differences in activity of furosemide-sensitive transport on red cell Na^+ and K^+ content and volume are examined. Preliminary reports of part of the work have appeared [9, 23].

Materials and Methods

Blood was drawn into heparin from apparently healthy, normotensive individuals. Hematocrit and hemoglobin were determined in fresh blood. After 5 min of centrifugation (4500 × *g*) plasma and buffy coat were removed. The erythrocytes were washed three times in ten volumes of isotonic choline chloride at 4 °C for cation content determination, or at room temperature for the experiments. The erythrocytes were then incubated at 37 °C (hematocrit 0.7 to 1%) in solutions containing 145 mM Na^+, 0 to 10 mM K^+ or Rb^+, 5 mM glucose, 1 mM inorganic phosphate, 1 mM $MgCl_2$, 1 mM ethylene-glycol-bis(2-aminoethylether)N,N′-tetraacetic acid, and 10 mM morpholinopropane sulfonic acid titrated to pH 7.4 at 37 °C with tris-(hydroxymethyl)-amino-methane, the principal anion being chloride (osmolality 305 to 325 mosmol kg water^{-1}). Ouabain, penicillin and streptomycin were dissolved in water, and furosemide in dimethylsulfoxide. The antibiotics and the dimethylsulfoxide added were found not to affect cation movements. The final concentrations were: 0.2 mM ouabain, 0.5 mM furosemide, 100 IU · ml^{-1} penicillin and 30 μg · ml^{-1} steptomycin.

In Fig. 1, the integrity of the furosemide-sensitive transport system over prolonged periods of incubation is assessed in cells preincubated with ouabain for 1, 6 and 24 hr in either a 10 mM $Na^+ - 140$ mM K^+ (columns *A*) or a 145 mM $Na^+ - 5$ mM K^+ medium (columns *B*). Obviously, furosemide-sensitive and furosemide-resistant Rb^+ uptake were not markedly altered after the preincubations. It appears safe to conclude, therefore, that the furosemide-sensitive transport system and the cation leak remained unchanged over 24 hr of incubation. It was ascertained in pilot experiments that 0.1 mM bumetanide exerted the same effect as 0.5 mM furosemide. Hemolysis after 24 hr was generally below 1% and never exceeded 2%. The K^+ concentration in the media nominally free of K^+ was 20 to 50 μM, and it increased by 0.1 to 0.3 mM over the 24 hr of incubation.

The cell suspensions (25 ml) were incubated in stoppered 100 ml round bottom polypropylene tubes (diameter 4 cm, air atmosphere) submerged in a water bath under gentle shaking to avoid cell sedimentation. The incubation was stopped by cooling in an ice bath and centrifugation (0 °C). The medium was removed for measurements of K^+ and Rb^+ concentrations. The cells were washed thrice with ice-cold isotonic choline chloride and hemolyzed with a greater than 10-fold excess of 6% 1-butanol (vol/vol). The hemoglobin concentration of the lysates was determined by employing the cyanmethemoglobin method. Cation concentrations were measured by atomic absorption spectrophotometry (atomic absorption spectrophotometer Perkin Elmer 400) after appropriate dilutions with 6% 1-butanol (vol/vol), standards being prepared in the same solvent.

All measurements were done in duplicate with intra-assay variation coefficients of less than ±2% for cations and less than ±0.5% for hemoglobin. Red cell cation contents and transport rates given in results refer to 5.2 μmol hemoglobin tetramer (0.335 g). Statistical analyses were performed by paired (furosemide versus control) and unpaired Student's *t*-test, respectively. *P* values >0.05 were not considered significant. The data shown in Fig. 1 through 5 were obtained with red cells of one donor (donor 1). Similar results were obtained with erythrocytes of three other donors.

Results

Figure 2 shows the time course of net Na^+ gain and net K^+ loss in ouabain-poisoned human erythrocytes incubated over 28 hr without and with furosemide in 145 mM NaCl media containing either 5 mM KCl or 5 mM RbCl. Furosemide did not affect the changes in cation content during the first 6 hr of incubation in the 5 mM K^+ medium. Only

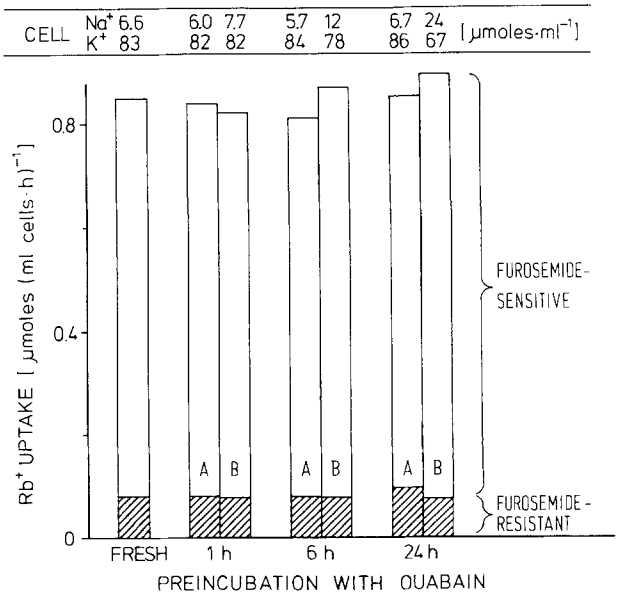

Fig. 1. Stability of the furosemide-sensitive transport system during 24 hr of incubation in a 140 mM $K^+ - 10$ mM Na^+ (*A*) or a 145 mM $Na^+ - 5$ mM K^+ medium (*B*) (0.2 mM ouabain, 37 °C, hematocrit 1%). Furosemide-sensitive and furosemide-resistant Rb^+ uptake was measured in a 145 mM $Na^+ -$ 5 mM Rb^+ medium in fresh erythrocytes and cells preincubated for the time intervals indicated on the abscissa. The cellular Na^+ and K^+ contents are given at the top of the Figure

after 24 hr a significant trend is seen for Na^+ gain being higher and K^+ loss being lower in the presence of furosemide.

In the 5 mM Rb^+ medium, furosemide did not change the Na^+ gain. K^+ loss was reduced by the drug, the effect becoming significant already in 6 hr ($P<0.05$). After 24 hr of incubation, the K^+ loss was lowered by about 10 μmol·ml cells^{-1} Rb^+ uptake was reduced by furosemide to a similar extent, as though the furosemide-sensitive transport system were mediating a 1:1 K^+/Rb^+ (or Na^+K^+/Na^+Rb^+) exchange (Fig. 2, right-hand panels). In the absence of furosemide, the cellular Rb^+ exceeded by far the medium Rb^+ concentration after 24 hr of incubation, Rb^+ thus being accumulated in the cells against an electrochemical gradient (*see also* Fig. 5).

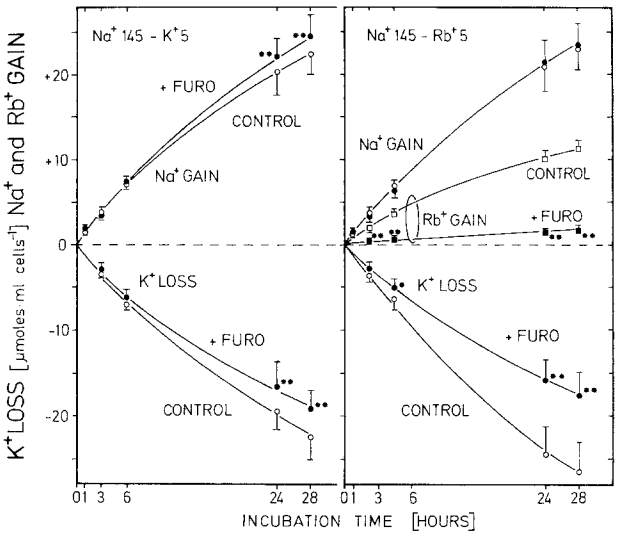

Fig. 2. Effect of furosemide (0.5 mM) on Na^+ gain and K^+ loss of human erythrocytes incubated with ouabain (0.2 mM) in 145 mM NaCl media containing either 5 mM KCl or 5 mM RbCl. Mean values ±SD from 4 paired experiments with red cells of donor 1. $*P<0.95$; $**P<0.005$ (versus control)

Dependence of the Action of Furosemide on External K^+ (Rb^+)

The Na^+ gain in ouabain-poisoned cells incubated for 24 hr in a Na^+ medium nominally free of K^+ amounted to 14.6 μmol·ml cells^{-1}. It increased in a curvilinear manner to 19.8 μmol·ml cells^{-1} upon raising external K^+ to 10 mM (Fig. 3). A similar effect was seen when Rb^+ was added to the incubation medium, Rb^+ being slightly more effective than K^+. Furosemide increased the Na^+ gain in the absence of external K^+ or Rb^+ by 5 μmol ml cells^{-1}, and made the Na^+ gain largely independent of external K^+ or Rb^+.

The K^+ loss of about 25 μmol ml cells^{-1} in pure Na^+ media was decreased by about 30% to 18 μmol·ml cells^{-1} when external K^+ was raised to 10 mM. External Rb^+, in contrast, tended to increase the K^+ loss. Furosemide reduced the K^+ loss at low external K^+ concentrations to the value seen at 10 mM external K^+, and made the K^+ loss largely independent fo external K^+ or Rb^+ (Fig. 4).

The furosemide-sensitive movements of Na^+, K^+ and Rb^+ calculated from the data in Fig. 3 and 4 are plotted in Fig. 5. The acceleration in Na^+ gain and the reduction of K^+ loss due to furosemide both fall from about 5 μmol·ml cells^{-1} in 24 hr in media nominally free of K^+ to almost

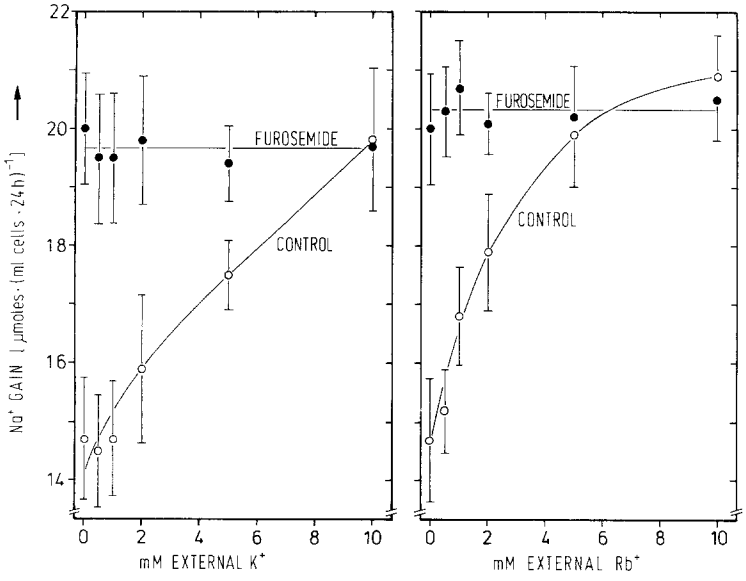

Fig. 3. Effect of furosemide on 24-hr Na^+ gain of ouabain-poisoned human erythrocytes in 145 mM NaCl media as affected by external K^+ and Rb^+. Mean values ±SEM from four paired experiments with red cells of donor 1

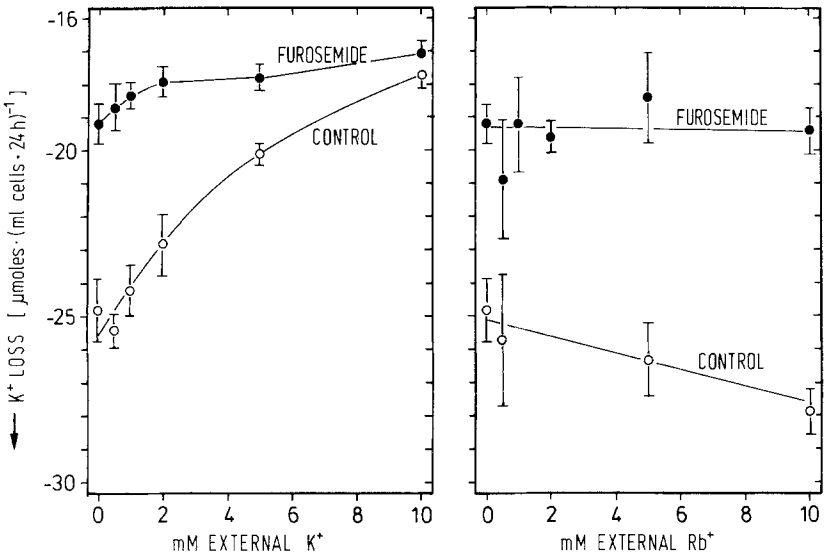

Fig. 4. Effect of furosemide on 24-hr K^+ loss of ouabain-poisoned human erythrocytes in 145 mM NaCl media as affected by external K^+ and Rb^+. Mean values ± SEM of four to six paired experiments with erythrocytes of donor 1

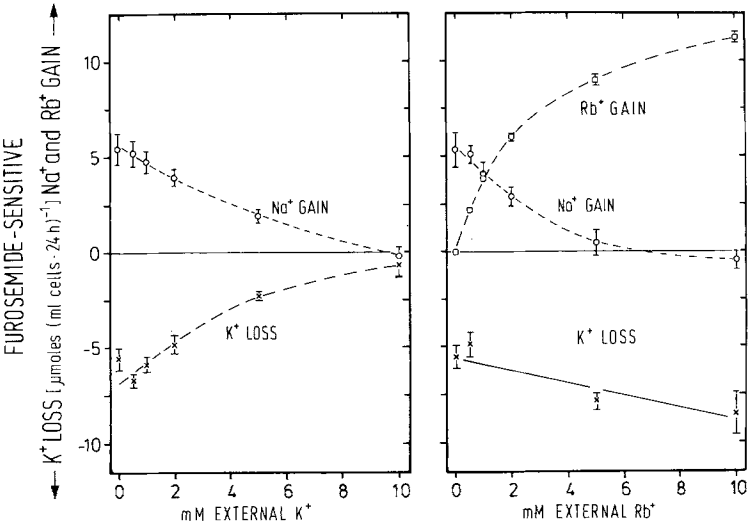

Fig. 5. Dependence of furosemide-sensitive Na^+ and Rb^+ gain and of K^+ loss on external K^+ and Rb^+ in 145 mM NaCl media. The data are taken from Fig. 3 and 4 (mean values ± SEM from four to six experiments). Note that furosemide causes an increase in Na^+ gain, a decrease in Rb^+ gain, and a reduction of K^+ loss

zero at 10 mM external K^+ (left-hand panels in Fig. 5).

The Na^+ gain due to furosemide was lowered by external Rb^+ more effectively than by external K^+. The reduction of K^+ loss by furosemide, however, was not decreased by external Rb^+, but rather slightly increased.

The furosemide-sensitive Rb^+ uptake rose in a curvilinear manner with the Rb^+ concentration, reaching a value of about 10 µmol · ml cells^{-1} at 5 to 10 mM external Rb^+. This value is nominally similar to that seen for the reduction of K^+ loss due to furosemide (right-hand panels in Fig. 5).

Effect of Furosemide and External K^+ (Rb^+) on Total Cation Content and Cell Volume

In Table 1, red cell Na^+, K^+ and Rb^+ contents are listed before and after 24 hr of incubation with ouabain in Na^+ media without or with addition of 5 mM K^+ or Rb^+, respectively. Cells with a high activity of furosemide-sensitive Rb^+ transport (donor 1) are compared with erythrocytes of low transport activity (donor 2).

In Na^+ media nominally free of K^+, furosemide stimulated Na^+ uptake and inhibited K^+ loss as compared to the ion content changes measured in cells incubated without the drug. The effect was more pronounced in the cells with the higher transport activity. In media containing 5 mM K^+, the change of cellular cation content due to furosemide was reduced by about 60%. In media with 5 mM added Rb^+, the furosemide-sensitive K^+ loss was numerically almost equal to the furosemide-sensitive Rb^+ gain. Apparently, a K^+/Rb^+ exchange with a stoichiometry of close to 1:1 was inhibited, so that the sum of total cations was not altered by the drug. Accordingly, the reduction of the furosemide-effect on red cell Na^+ and K^+ content

Table 1. Effect of furosemide and external $K^+(Rb^+)$ on red cell cation content and volume after 24 hr of incubation of human erythrocytes with ouabain in a 145 mM NaCl medium

		Donor 1			Donor 2		
		Control	Furosemide	Δ Furosemide	Control	Furosemide	Δ Furosemide
Initial	Na^+	5.4±0.1			6.3±0.3		
	K^+	86.4±2.6			97.5±1.7		
	Σ	90.4±2.5			103.7±1.9		
Cell volume		100%			100%		
24 hr	Na^+	20.2±1.1	25.5±0.9	+ 5.3±0.5	23.4±0.7	26.9±1.0	+3.5±0.4
Na^+145	K^+	62.6±0.9	68.1±0.6	+ 5.6±1.5	73.5±1.8	76.1±2.2	+2.5±1.1
	Σ	82.8±3.8	93.6±2.1[a]	+10.8±1.7	96.9±1.5	102.9±1.5[b]	+6.1±1.0
Cell volume		92.8%	101.2%	+ 8.4%	94.1%	98.2%	+4.1%
24 hr	Na^+	22.9±1.6	24.8±1.8	+ 1.9±0.3	25.8±1.9	26.5±1.7	+0.7±0.5
Na^+145-K^+5	K^+	67.2±0.9	69.5±1.0	+ 2.3±0.5	78.0±1.7	78.9±1.4	+1.0±1.2
	Σ	90.3±1.6[c]	94.3±1.6[a]	+ 4.1±0.3	103.7±2.3[c]	105.4±1.3[b,e]	+1.7±1.2
Cell volume		98.6%	101.7%	+ 3.1%	98.7%	99.9%	+1.2%
24 hr	Na^+	25.1±2.6	25.4±2.4	+ 0.4±0.5	28.3±3.9	28.1±3.0	−0.2±1.0
$Na^+145-Rb^+5$	K^+	61.0±2.7	68.9±3.4	+ 7.9±1.2	73.5±2.4	77.8±2.8	+4.3±1.2
	Rb^+	10.6±0.5	1.5±0.4	− 9.1±0.7	6.2±0.4	2.0±0.4	−4.3±0.5
	Σ	96.8±3.5[c]	95.7±4.4	− 1.2±1.5	108.2±3.7[c]	108.7±1.8[e]	−0.2±2.2
Cell volume		103.7%	102.8%	− 0.9%	101.8%	102.1%	+0.3%

The erythrocytes of donors 1 and 2 had a furosemide-sensitive Rb^+ uptake in $Na^+145-Rb^+$ 5 medium of 0.73±0.04 and 0.28±0.03 μmol·(ml cells·hr)$^{-1}$, respectively (mean values ±1 SD, $n=8$ to 10). The cation contents (mean values ±1 SD, from six to nine experiments) are given in μmol per ml cells, normalized to an MCHC of 5.2. The cell volume (v_1) was calculated using the equation $v_1/v_0 = 0.706 \cdot n_1/n_0 + 0.281$ of Dalmark [8], where v_0 is the original cell volume (100%) and n_1 and n_0 are the actual and original cation contents, respectively.

[a] $P<0.0005$ (versus control).
[b] $P<0.005$ (versus control).
[c] $P<0.0005$ (versus Na^+ 145).
[d] $P<0.005$ (versus Na^+ 145).
[e] $P<0.01$ (versus Na^+ 145).

seen with increasing external K^+ concentration (Figs. 3–5, Table 1) is supposed to result from a progressive conversion of coupled Na^+ and K^+ outward transport (at low external K^+) to a 1:1 exchange of both Na^+ for Na^+ and K^+ for K^+ (at normal or elevated external K^+ concentrations).

The relative cell volumes given in Table 1 were calculated from the total cation contents using an equation given by Dalmark [8] which describes the dependence of cell volume on cation content of human erythrocytes. As a result of the cation loss in the absence of furosemide, the cells were shrunken by 6 to 7% after the 24 hr of incubation with ouabain in the pure Na^+ media (Table 1). However, the calculated volume of the control cells from donor 1 rose from 93 to 99 or to 104% upon adding 5 mM K^+ or 5 mM Rb^+, respectively. Furosemide increased the calculated volume of the cells from donor 1 by about 8% in the pure Na^+ media, and by about 3% in the presence of 5 mM K^+, while leaving the volume almost unaltered in the presence of 5 mM Rb^+. Similar but less pronounced effects of external K^+, Rb^+ and furosemide were seen with red cells of donor 2 (Table 1).

To verify the cell volume changes predicted from the cation content data in Table 1, the mean cellular hemoglobin content (MCHC) was measured in the red cells of the two donors after 24 hr of incubation. MCHC and cell volume are inversely related to each other in a given cell specimen. As shown in Table 2, both furosemide and external K^+ reduced the MCHC, indicating a swelling of the cells. The effects of furosemide and external K^+ (Rb^+) were not additive. In the cells of donor 1, K^+ lowered the volume effect of furosemide

Table 2. Effect of furosemide and external K^+ on MCHC after 24 hr of incubation of human erythrocytes with ouabain in a 145 mM NaCl medium

	Donor 1			Donor 2		
	Control	Furosemide	Δ Furosemide	Control	Furosemide	Δ Furosemide
Initial	5.45±0.05			5.07±0.04		
24 hr						
Na^+ 145	5.59±0.04	5.22±0.06[a]	−6.6%	5.23±0.12	5.00±0.03[a]	−4.5%
Na^+ 145 − K^+ 5	5.37±0.09[c]	5.29±0.05[b,d]	−1.5%	5.06±0.09	5.01±0.12	−0.1%

Donors 1 and 2 were the same as in Table 1. Mean values (μmol hemoglobin tetramer · ml cells^{-1}) ± 1 SD from six experiments.

[a] $P<0.0005$ (versus control).
[b] $P<0.025$ (versus control).
[c] $P<0.0025$ (versus Na^+ 145).
[d] $P<0.05$ (versus Na^+ 145).

from 6.6 to 1.5%, and furosemide lowered the effect of external K^+. The alterations of MCHC were smaller in the erythrocytes of donor 2. On a percentage basis, the measured MCHC changes were numerically similar to the volume changes calculated from the cation contents in Table 1. Accordingly, it is concluded that both furosemide and external K^+ induce a cell swelling due to a cation accumulation (or prevent a cell shrinkage due to a cation retention) in ouabain-poisoned cells, the effect of furosemide being reduced in the presence of external K^+, and vice versa.

Relation between Furosemide-Sensitive Cation Transport, MCHC and Red Cell K^+ Content *in Vivo*

The results presented in the previous paragraph demonstrate that the furosemide-sensitive Na^+/K^+ transport system is involved in the control of total cation content and cell volume of ouabain-poisoned human erythrocytes *in vitro*, especially at low external K^+ concentrations. It was thus examined whether the interindividual differences in the maximum activity of the furosemide-sensitive transport system contribute in determining the MCHC and the individual red cell Na^+ and K^+ contents in man *in vivo*.

A highly significant, negative correlation is seen between the rate of furosemide-sensitive Rb^+ uptake and red cell K^+ content among 74 donors (Fig. 6). If the relationship shown in Fig. 6 were to be causal, this could indicate that the furosemide-sensitive transport system functions as a K^+-extruding mechanism *in vivo*, the steady-state red cell K^+ content being lower the faster the furosemide-sensitive transport system operates.

Mean cellular hemoglobin content *in vivo* was positively correlated to the furosemide-sensitive

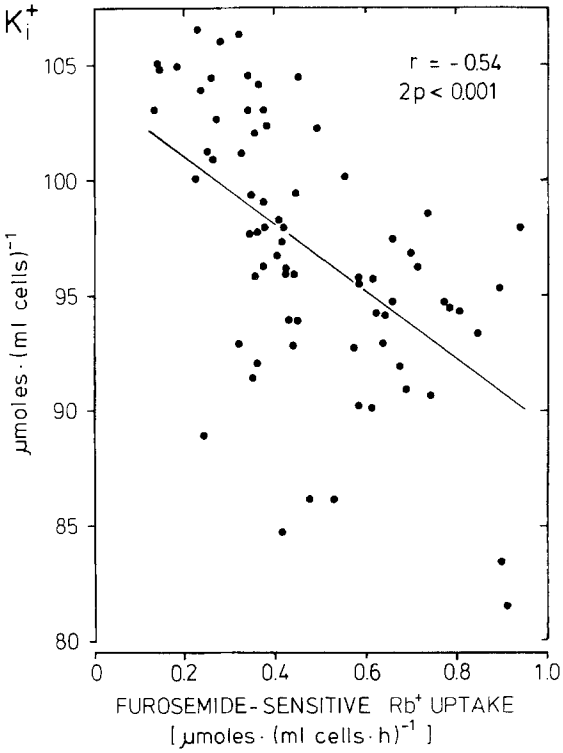

Fig. 6. Relation between red cell K^+ content and the activity of the furosemide-sensitive transport system of human erythrocytes. Furosemide-sensitive Rb^+ uptake was measured in 145 mM Na^+ − 5 mM Rb^+ media on red cells of 74 apparently healthy donors. The red cell K^+ contents and transport rates are normalized to an MCHC of 5.2. A similar relationship was obtained when the actual values of furosemide-sensitive transport and K^+ content were analyzed.

Rb^+ transport (Fig. 7). Apparently, a low water content (i.e., a high hemoglobin content) is osmotically induced by the low K^+ content of erythrocytes with a high activity of the transport system. Red cell K^+ content (or red cell Na^+ plus K^+ content) and MCHC were negatively correlated to

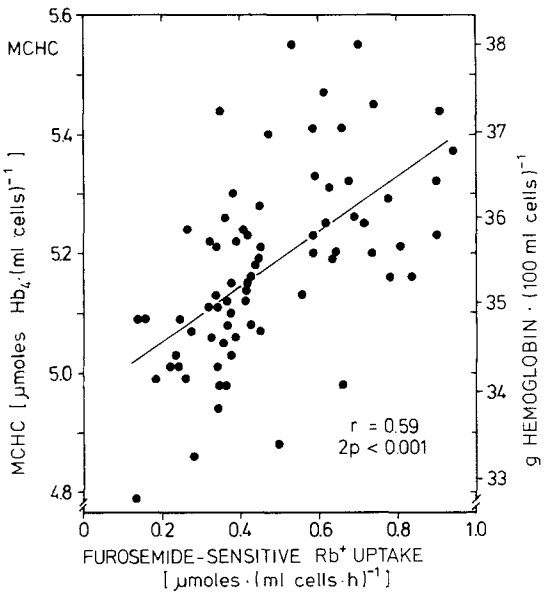

Fig. 7. Relation between MCHC and furosemide-sensitive Rb^+ uptake in human erythrocytes. The 74 donors are the same as in Fig. 6

each other, as to be expected ($2p<0.01$, *data not shown*).

No significant correlation was seen between furosemide-sensitive Rb^+ transport and red cell Na^+ content ($2p>0.4$, $n=138$, *data not shown*). The activity of the furosemide-sensitive system was negatively correlated to the plasma K^+ concentration ($n=65$, $r=-0.27$, $2p<0.05$).

Discussion

Furosemide-Sensitive Cotransport versus Exchange

In Na^+ media nominally free of K^+, the furosemide-sensitive, chloride-dependent transport system for Na^+ and K^+ of ouabain-poisoned human erythrocytes mediates a Na^+ and K^+ net extrusion with a Na^+/K^+ "cotransport" stoichiometry of close to 1:1. Upon adding K^+ to the incubation medium, the cation net outward transport by the furosemide-sensitive system progressively decreases in magnitude and becomes zero at about 10 mM external K^+ (Figs. 3 to 5). In Na^+ media with 10 mM K^+, the unidirectional inward and outward movements of both Na^+ and K^+ proceed with an inward/outward "exchange" stoichiometry of 1:1. Accordingly, addition of furosemide to Na^+ media containing 10 mM K^+ exerts no effect on red cell cation content (Figs. 3 and 4). This finding is in accordance with the earlier observation of Dunn [17] that furosemide does not alter the net Na^+ gain of human erythrocytes incubated with ouabain in media of nearly physiological Na^+ and K^+ content.

The net Na^+ and K^+ movements mediated by the furosemide-sensitive transport system reflect the balance between unidirectional inward and outward transport. Accordingly, the net Na^+ and K^+ loss mediated by the system at external K^+ concentrations lower than 10 mM results either from a reduction of unidirectional inward transport or from an acceleration of unidirectional outward transport, or from both.

Using Rb^+ as an analog replacing K^+ at the external aspect of the transport system, it was shown that lowering the external Rb^+ concentration slightly decreases furosemide-sensitive K^+ outward transport (Figs. 4 and 5). Accordingly, the net K^+ loss at external K^+ concentrations below 10 mM is not due to an accelerated K^+ extrusion. It rather results from the decrease of inward K^+ transport by the furosemide-sensitive system that occurs upon lowering the external K^+ concentration [3, 11, 38, 42].

The effect of furosemide and external K^+ (Rb^+) on Na^+ net movements is more difficult to interpret since no tracer for Na^+ was used in the present experiments. Application of Li^+ [5] or radioactive Na^+ was not intended because Li^+ and Na^+ are also transported by the ouabain-resistant Na^+/Li^+ (Na^+/Na^+) exchange system [10, 39]. Furthermore, the Na^+/K^+ stoichiometry of furosemide-sensitive inward transport is uncertain, values ranging between 0.2:1 [16] and 1:1 having been reported [42]. There is also a furosemide-sensitive, chloride-dependent K^+ (Rb^+) inward transport occurring in Na^+-free choline or Mg^{2+} media [11, 41]. This Na^+-independent transport contributes about one-third to the furosemide-sensitive Rb^+ uptake by human erythrocytes suspended in Na^+ media containing 5 mM Rb^+. It varies in maximum activity strictly proportional to the Na^+-dependent Rb^+ uptake among different donors and is not altered by a $\pm 10\%$ change in cell volume (Duhm & Göbel, *unpublished results*), in contrast to the Na^+-independent K^+/K^+ exchange observed in bird erythrocytes [25].

In interpreting the effect of furosemide and external K^+ (Rb^+) on Na^+ net movements it has to be considered *i*) that external K^+ (Rb^+) is known to accelerate unidirectional inward Na^+ transport [3, 16, 42] and *ii*) that external K^+ (Rb^+) reduces outward Na^+ transport [2, 20, 21, 37, 38] (but *see* [18, 27]). Accordingly, the acceleration of Na^+ gain at low external K^+ (Rb^+) concentrations caused by furosemide must largely result from an

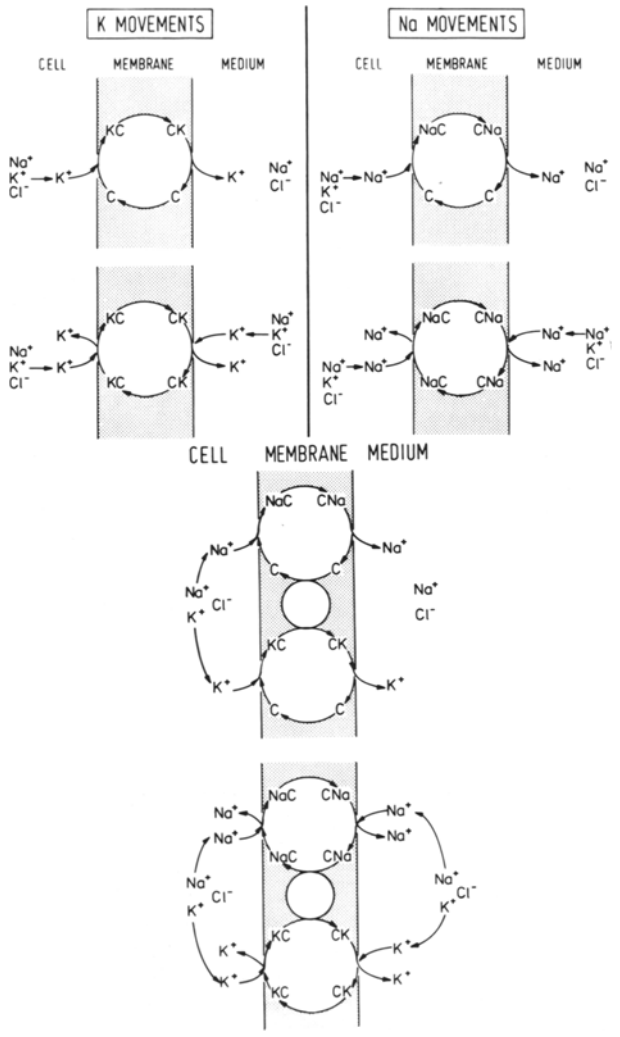

Fig. 8. Scheme of furosemide-sensitive Na^+ and K^+ movements in human erythrocytes. The respective upper and lower panels differ in that external K^+ is present in the lower panels, thus allowing for inward K^+ and Na^+ transport. For further explanation *see* text. The inhibitory effect of external K^+ on Na^+ extrusion [2, 20, 21, 37, 38] is not included in the scheme. No implications are made with respect to the charge of the transport system (*C*)

inhibition of Na^+ outward transport exceeding the simultaneously inhibited inward transport in magnitude. The acceleration of Na^+ gain seen upon increasing the external K^+ (Rb^+) concentration is thought to result from both an inhibition of Na^+ outward and an acceleration of Na^+ inward transport. External Rb^+ is more effective than K^+ at the outer aspect of the furosemide-sensitive transport system, as indicated by the data in Figs. 3 and 5: the unidirectional inward and outward Na^+ movements through the furosemide-sensitive system were numerically identical at 6 mM external Rb^+, as compared to 10 mM external K^+.

The molecular basis of the furosemide-sensitive transport system is still unknown and one can only speculate whether there is one transport unit or a complex of subunits performing Cl^--dependent transport of Na^+ and K^+ [15, 24]. A proposal for the mechanism of the furosemide-sensitive transport system is outlined in the scheme of Fig. 8. Two separate units for Na^+ and K^+ are tentatively assumed. In the scheme, *C* denotes such a unit which binds, translocates and releases ions at the two sides of the membrane.

The K^+ system mediates an outward transport which depends on internal Na^+ and Cl^-. As external K^+ (Rb^+) is increased, a unidirectional K^+ (Rb^+) inward movement additionally develops until at 10 (6) mM external K^+ (Rb^+) the two unidirectional fluxes become equal (i.e., an apparent 1:1 K^+/K^+ or K^+/Rb^+ "exchange" develops), provided Na^+ and Cl^- are also present in the medium. The fraction of Na^+-independent, furosemide-sensitive K^+ inward transport mentioned above is not considered in the scheme.

The Rb^+ inward transport even occurs against an electrochemical Rb^+ gradient (Figs. 2 and 5), as if the furosemide-sensitive transport system would function as a "second K^+ pump" [6].

The Na^+ system promotes a Na^+ extrusion when K^+ and Cl^- are present at the internal side. The Na^+ extrusion can even occur against an electrochemical Na^+ gradient, the system thus functioning as a "second Na^+ pump" in the sence of Hoffman and Kregenow [27]. External K^+ and Cl^- allow for an additional inward Na^+ movement so that at 10 mM external K^+ (6 mM Rb^+) an apparent 1:1 Na^+/Na^+ "exchange" develops.

In this concept, two separate systems for K^+ and Na^+ are mutually dependent on the presence of the other cation (and Cl^-). However, uphill movements against an electrochemical gradient of either Na^+ (Fig. 3) or K^+ (Rb^+, Fig. 2) were observed which are probably driven by the downhill gradient of the cation partner. Accordingly, there must exist either a coupling mechanism between the two systems (lower panel in Fig. 8), or alternatively, the Na^+ and K^+ movements ascribed here to two systems are mediated by one and the same component. The degree of coupling of Na^+ and K^+ movements may be variable as evidenced by the Na^+-independent inward K^+ transport described above and by the reduced Na^+/K^+ stoichiometry of outward transport reported for swollen human erythrocytes [1].

In summary, a concept is introduced where furosemide-sensitive transport phenomena of unidirectional 1:1 Na^+/K^+ "cotransport" in the in-

ward or the outward direction and 1:1 "exchange diffusion" of Na^+ and K^+ are considered complementary rather than mutually exclusive, as previously considered [2, 4, 17, 18, 31, 38, 42]. No implications are made with respect to Cl^- moving itself. Accordingly, the inward and outward "cotransport" of Na^+ plus K^+ in the sense of Wiley and Cooper [42] and of Garay et al. [20] is only one aspect of the simultaneously ongoing Na^+ and K^+ movements mediated by the furosemide-sensitive system of human erythrocytes.

Driving Forces

The driving forces for net Na^+ and K^+ movements mediated by the bidirectionally operating furosemide-sensitive transport mechanism(s) can be calculated according to the equation

$$\mu_{net} = RT \ln \frac{[Na_e^+]\cdot[K_e^+]\cdot[Cl_e^-]^2}{[Na_i^+]\cdot[K_i^+]\cdot[Cl_i^-]^2} \quad (1)$$

which has been derived to describe the electroneutral 1 Na^+:1 K^+:2 Cl^- "cotransport" system of bird erythrocytes [25, 32]. In this equation, μ_{net} is the net chemical potential, and $[Na^+]$, $[K^+]$ and $[Cl^-]$ are the respective internal (i) and external (e) concentrations.

The net driving force calculated from the above equation is plotted against the external K^+ concentration at various internal Na^+ concentrations in Fig. 9. A constant Donnan ratio $r_{Cl^-} = Cl_i/Cl_e^-$ of 0.66 [19], a constant sum of internal Na^+ plus K^+ of 160 mM, and an external Na^+ concentration of 145 mM are assumed.

The calculated net driving force is zero at 4.5 mM external K^+ in erythrocytes with a red cell Na^+ concentration of 10 mM in cell water (i.e., a Na^+ content of 6.5 μmol ml^{-1}). With falling external K^+ an outwardly directed driving force is generated of progressively increasing magnitude. Upon raising external K^+ an inwardly directed driving force develops. The relationship is shifted downward along the ordinate when red cell Na^+ is elevated, the point of zero driving force thus shifting to the right on the K^+ axis. A 50% decrease of plasma K^+ and a 100% increase in red cell Na^+ are equally effective.

The filled circles in Fig. 9 represent the furosemide-sensitive Na^+ movements measured in the range between 1 and 10 mM external K^+ (Fig. 5). The data can be fitted reasonably well to the curve calculated for 20 mM internal Na^+ which is close to the mean value of the cell Na^+ concentration over the 24 hr of incubation with ouabain. A reasonable fit can also be obtained with the furosemide-sensitive K^+ movements shown in Fig. 5. The data thus indicate that the furosemide-sensitive Na^+/K^+ transport system of human erythrocytes can be adequately described by Eq. (1), assuming an obligatory coupling of 1 Na^+:1 K^+:2 Cl^- moving across the membrane. The fits were equally good, however, when the Cl^- terms were omitted from Eq. (1). It is to be noted that such a parallelism between driving force and net transport rate is only to be expected under conditions where the change in driving force results from a change in the concentration of a reaction partner in a range where the system is not fully saturated with respect to that partner. This requirement is fulfilled for the Na^+ and K^+ concentration ranges in Fig. 9, since the system is half saturated at external K^+ and internal Na^+ concentrations of about 5 [11, 16, 42] and 20 mM [20], respectively.

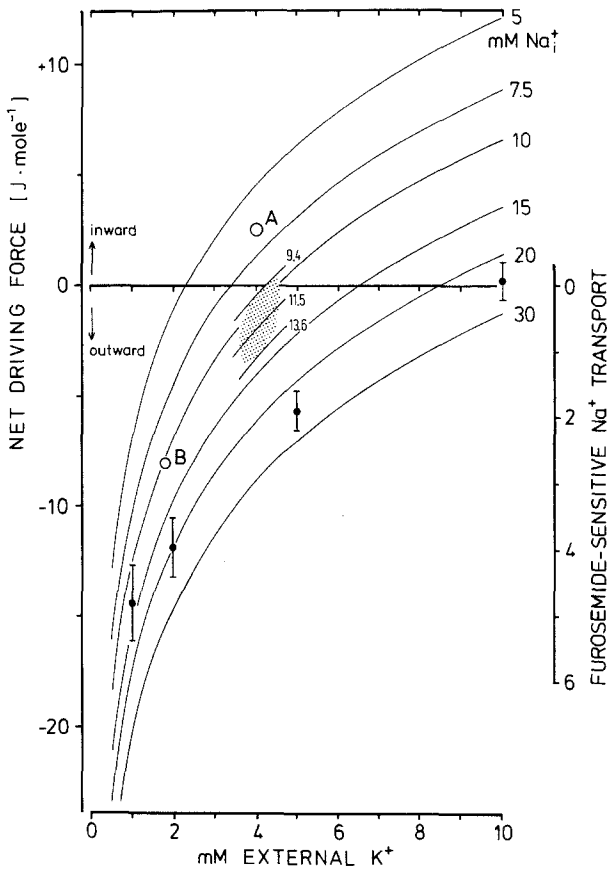

Fig. 9. Net driving force of Na^+/K^+ cotransport as a function of external K^+ and internal Na^+ concentration. The curves are calculated from Eq. (1) under the assumptions stated in the text. The dotted area indicates the working region of normal human erythrocytes *in vivo*. For explanation of the closed circles (furosemide-sensitive Na^+ transport in μmol·(ml cells·24 hr)$^{-1}$) and the open circles *see* text.

The Furosemide-Sensitive Transport System Can Alter the Cell Volume

The relationships in Fig. 9 predict that the furosemide-sensitive transport system can serve as a Na^+-, K^+- (and Cl^-?)-extruding mechanism at low external K^+ concentrations. The salt extrusion must be accompanied by an osmotically obliged water loss, the transport system thus being involved in the control of cell volume of human erythrocytes. At low external K^+ concentrations, the system should thus contribute in preventing colloidosmotic hemolysis, in addition to the Na^+/K^+-pump [7]. Such a volume effect of the furosemide-sensitive transport system is demonstrated in Tables 1 and 2. Furosemide (and external K^+ or Rb^+) inhibits a net loss of cations and water in ouabain-poisoned human erythrocytes suspended in pure Na^+ media. The volume effect was more pronounced in red cells with a higher transport activity.

The furosemide-sensitive volume effect described here for human erythrocytes is probably not related to the regulation of cell volume by linked cation fluxes as proposed by Poznansky and Solomon [35, 36]. Furthermore, the volume effect does not need to be initiated by previous cell swelling or catecholamines. It thus differs from the regulatory volume decrease and the response towards catecholamines found in bird erythrocytes [29, 32, 34] and from the "uncoupling" of Na^+ from K^+ movements seen in swollen human erythrocytes [1]. Finally, furosemide-sensitive K^+ transport is not [11] or only slightly [1] volume dependent in human erythrocytes, in contrast to the system found in bird [25, 29, 32, 40] and rat erythrocytes [12, 13].

Role of the Furosemide-Sensitive Transport System *in Vivo*

The question arises as to the net driving force for furosemide-sensitive Na^+ and K^+ transport in human erythrocytes *in vivo*. Among 130 individuals we found a mean red cell Na^+ of 7.5 ± 1.4 μmol·ml cells^{-1} (range 4.9 to 12.2), corresponding to a mean Na^+ concentration of 11.5 mM in cell water, and a mean plasma K^+ concentration of 4.1 ± 0.52 mM (range 3.25 to 5.48). Accordingly, in almost all donors there exists a small outwardly directed net driving force for the transport system *in vivo* (*see* dotted area in Fig. 9).

The *in vivo* red cell K^+ content was negatively correlated, and the *in vivo* mean cellular hemoglobin content (MCHC) was positively correlated to the individual activity of furosemide-sensitive transport in human erythrocytes, as though the transport system were continuously extruding K^+ and water *in vivo* (Figs. 6 and 7). These correlations are to be predicted from a continuous outward operation of the furosemide-sensitive transport system *in vivo* (*see* Fig. 9). A role of the furosemide-sensitive transport system in K^+ homeostasis is also suggested by the finding of a negative correlation between plasma K^+ and furosemide-sensitive Rb^+ transport (*see* Results). In this context the observation is of interest that a mutant mouse fibroblastic cell line deficient in the furosemide-sensitive transport system can maintain cell K^+ and volume at normal values even at such low external K^+ concentrations where normal fibroblasts lose substantial amounts of K^+ and eventually die [28]. These findings are in contrast to the suggestion of Canessa et al. [6] "that the Na–K cotransport reaction operates normally in the direction of K accumulation."

It seems premature to conclude that the correlations shown in Fig. 6 and 7 represent causal relationships. There are a great number of other factors that participate in determining the red cell K^+ and hemoglobin content (e.g., Na^+/K^+-pump activity, plasma K^+, leak permeability, membrane lipids, cell age, hemoglobin synthesis, etc.). Furthermore, no correlation was seen between red cell Na^+ content and the individual rate of furosemide-sensitive transport, although a negative correlation is to be expected from a continuous Na^+ outward movement by the transport system. On the other hand, the transport system certainly participates in extruding Na^+, K^+ and water from human erythrocytes in electrolyte disturbances with elevated red cell Na^+ and reduced plasma K^+ concentrations. The two open circles in Fig. 9 are the calculated working points of the furosemide-sensitive transport system in erythrocytes of normal (*A*) and K^+-depleted rats (*B*) [12]. The furosemide-sensitive transport system of rat erythrocytes is similar to that of human erythrocytes, except that the maximum transport rates in the rat increase more than 10-fold upon cell shrinkage [12, 13], a phenomenon not seen with human erythrocytes [11]. Due to the low stationary Na^+ concentration in normal rat erythrocytes the furosemide-sensitive transport system should work in the inward direction. The fall in plasma K^+ and the increase in red cell Na^+ in rats depleted of K^+ due to dietary K^+ restriction [12] should favor outward net transport, indicating that the net effect of the bidirec-

tionally operating transport system may drastically change under severe electrolyte disturbances. With respect to furosemide-sensitive Na^+ movements, the above predictions were found to be fulfilled in rat erythrocytes [12].

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