J. Membrane Biol. 77, 57–62 (1984)

The Journal of
Membrane Biology

Thiol-Dependent Passive K/Cl Transport in Sheep Red Cells: IV. Furosemide Inhibition as a Function of External Rb^+, Na^+, and Cl^-

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Summary. The effect of the loop diuretic furosemide (4-chloro-N-furfuryl-5-sulfamoyl-anthranilic acid) on the thiol-dependent, ouabain-insensitive K(Rb)/Cl transport in low K^+ sheep red cells was studied at various concentrations of extracellular Rb^+, Na^+ and Cl^-. In Rb^+-free NaCl media, 2×10^{-3} M furosemide inhibited only one-half of thiol-dependent K^+ efflux. In the presence of 23 mM RbCl, however, the concentration of furosemide to produce 50% K^+ efflux inhibition (IC_{50}) was 5×10^{-5} M. In Rb^+ containing NaCl media, the inhibitory effect of 10^{-3} M furosemide was equal to that caused by NO_3^- replacement of Cl^- in the medium. The apparent synergistic action of furosemide and external Rb^+ on K^+ efflux was also seen in the ouabain-insensitive Rb^+ influx. A preliminary kinetic analysis suggests that furosemide binding alters both maximal $K^+(Rb^+)$ transport and apparent external Rb^+ affinity. In the presence of external Rb^+, Na^+ (as compared to choline) exerted a small but significant augmentation of the furosemide inhibition of $K^+(Rb^+)$ fluxes. There was no effect of Cl^- on the IC_{50} value of furosemide. As there is no evidence for coupled Na^+K^+ cotransport in low K^+ sheep red cells, furosemide may modify thiol-dependent $K^+(Rb^+)$/Cl flux or Rb^+ (and to a slight degree Na^+) modulate the effect of furosemide.

Key Words K/Cl transport · furosemide · SH groups · sheep red cells · N-ethylmaleimide

Introduction

Previously we reported that N-ethylmaleimide (NEM) stimulates a Cl^--dependent, ouabain-insensitive K^+ transport system (K/Cl transport) present at low activity in low K^+ (LK) but absent in high K^+ (HK) sheep red cells [21, 23]. The NEM-activated K/Cl flux is usually 6 to 10 greater than the basal K/Cl flux [21]. As there is also evidence for the presence of this thiol-dependent K/Cl transport system in reticulocytes of both HK and LK sheep [22], we have speculated on its role in the origin of the mature LK steady-state cell [18, 19, 22].

One of the properties of the thiol-dependent K^+ transporter was its inhibition by the loop diuretic furosemide [21] suggesting that K/Cl transport in LK sheep red cells is carried out by a molecule similar in its characteristics to those involved in coupled transport of Na^+, K^+, and Cl^- in a variety of epithelial and nonepithelial cells [1–3, 8, 11–17, 24–28]. Of particular interest was the observation [21], that in LK sheep red cells furosemide exerted its full effect only in the presence of external Rb^+, suggesting a synergism between the loop diuretic and external ions as reported for bumetanide-sensitive Rb^+ influx in duc [14] and turkey red cells [26] and for binding of ^{3}H-bumetanide to kidney membranes [10]. While the cation effects remained a puzzle, others have reported an antagonism between loop diuretic effects or binding and the presence of Cl^- anions [10, 15].

Here I report on studies designed to shed further light on the mechanism of furosemide inhibition of thiol-dependent ouabain-insensitive K/Cl fluxes in LK sheep red cells. The mode by which furosemide inhibition is augmented in the presence of external $K^+(Rb^+)$ and the independence of this process of Cl^- relates to the effects of other 3-amino-5-sulfamoyl benzoic acid derivatives used as loop diuretics and inhibitors of coupled, electroneutral transport [26, 27]. This work was presented in preliminary form elsewhere [20].

Materials and Methods

Furosemide (4-chloro-N-furfuryl-5-sulfamoyl anthranilic acid) with the lot numbers RC 0842 and RC 1412 was a gift of Hoechst-Roussel Pharmaceuticals, Inc., Sommerville, New Jersey. All other materials such as buffers and chemicals were not different from those reported in preceding communications [4, 21, 22]. The cation composition of the LK sheep red cells used in this study was 13 mM/liter cells K^+ and 87 mM Na^+/liter cells. All methods such as augmentation of Cl^- dependent K^+ flux by NEM, cell equilibration in Cl^- or NO_3^--containing media and ouabain-insensitive K^+ effluxes and Rb^+ influxes, the rate equations used for flux calculations and the use of furosemide were

described in detail in the preceding papers [4, 21, 22]. Any experimental deviation from previous methods will be indicated in the Figure legends. For kinetic analysis standard references were consulted [6, 9].

Results

In the absence of extracellular Rb^+ and in isosmotic, buffered NaCl media, furosemide inhibited ouabain-insensitive K^+ efflux, only up to about 50% when concentrations greater than 10^{-3} M were used. Figure 1 shows an experiment in which 50% of ${}^{o}k_K^{NEM}$, the rate constant of the NEM-stimulated K^+ efflux, was inhibited by 1.8×10^{-3} M furosemide. However, when 23 mM Rb^+ was present in the external medium (replacing equimolar amounts of Na^+), the furosemide concentration required for 50% inhibition (IC_{50}) was 5×10^{-5} M and hence one to two orders of magnitude lower than in the absence of Rb_o^+. In the presence of Rb_o^+, the magnitude of the inhibition of ${}^{o}k_K^{NEM}$ by 10^{-3} M furosemide was the same as Cl^- replacement by NO_3^- in the medium (*see* 23 mM $RbNO_3^-$ point at *y*-axis), a procedure inhibiting ouabain-insensitive K^+ fluxes of control and NEM-stimulated LK cells [21]. In order to understand the apparent synergism between furosemide and external Rb^+ in reducing ${}^{o}k_K^{NEM}$ in LK cells, K^+ effluxes and Rb^+ influxes were carried out simultaneously at various external Rb^+ and furosemide concentrations.

Figure 2 shows the effect of increasing external Rb^+ concentrations, $[Rb^+]_o$, on ${}^{o}k_K^{NEM}$ in controls and in cells exposed to 4 different furosemide concentrations in NaCl media. Figure 2*A* reveals that ${}^{o}k_K^{NEM}$ decreased by about 20- to 30% when cells were exposed to 10^{-3} M furosemide in the absence of external Rb^+ (solid points on *y*-axis). The effect of 10^{-3} M furosemide on ${}^{o}k_K^{NEM}$ became much more pronounced when, in NaCl media, $[Rb^+]_o$ was increased up to 15 mM/liter, where the effect of furosemide on ${}^{o}k_K^{NEM}$ was identical to that of NO_3^- replacement. At lower furosemide concentrations (5×10^{-4} to 10^{-4} M), however, less inhibition of ${}^{o}k_K^{NEM}$ was observed. Figure 2*B* shows the values of $({}^{o}k_K^{NEM})_{\Delta F}$, the furosemide inhibitable K^+ efflux rate constants, calculated as the difference between ${}^{o}k_K^{NEM}$ of furosemide-treated and control LK cells. Note that consistent with earlier findings [21], external Rb^+ exerted a small transeffect on ${}^{o}k_K^{NEM}$ (*see* Fig. 2*A*) compatible with the view of a nonobligatory Cl^--dependent K^+/Rb^+ exchange pathway. From Fig. 2*B* it appeared that the inhibition of ${}^{o}k_K^{NEM}$ involved changes in the maximal transport rate as well as in the apparent affinity ($K_{0.5}^{Rb}$) for external Rb^+. Although shown with the caveat that rate constants cannot with ease be manipulated kinetically, an Eadie-Hofstee plot (Fig. 3) of the furosemide-sensitive ${}^{o}k_K^{NEM}$ versus $({}^{o}k_K^{NEM})_{\Delta F}/[Rb]_o$ seems to confirm this finding as both the maximal rates (*y*-axis) and the apparent $K_{0.5}^{Rb}$ values for extracellular Rb^+ (slopes) and hence their ratios of $({}^{o}k_K^{NEM})_{max}/K_{0.5}^{Rb}$ were altered.

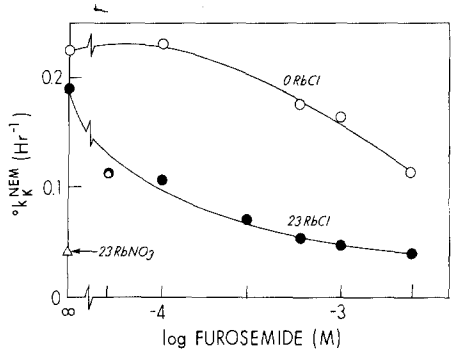

Fig. 1. K^+ efflux rate constants of NEM-treated LK sheep red cells ${}^{o}k_K^{NEM}$, as a function of the furosemide concentration. K^+ efflux was measured into isosmotic Na^+ media + 10^{-4} M ouabain as described previously [21] in the presence (filled circles) and absence (open circles) of 23 mM Rb_o. Media contained no furosemide (0 at *x*-axis) or the concentrations indicated at the *x*-axis. Simultaneously, K^+ efflux was measured into $NaNO_3$ media containing 23 mM $RbNO_3$ but no furosemide (triangle at *y*-axis). ${}^{o}k_K$ values were calculated from 6 experimental time points per data point using the first-order rate equations described earlier [21]

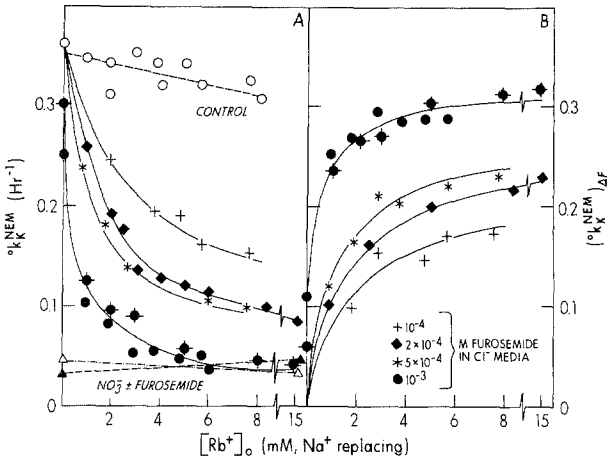

Fig. 2. K^+ efflux rate constants of NEM-treated LK sheep red cells, ${}^{o}k_K^{NEM}$, as a function of various external Rb^+ and varying furosemide concentrations. *A*. Each ${}^{o}k_K^{NEM}$ value was calculated from 6 time points described previously [2] for cells exposed to zero (○), 10^{-4} (+), 2×10^{-4} (◆), 5×10^{-4} (*) and 10^{-3} (●) M furosemide in NaCl media + 10^{-4} M ouabain containing increasing $[Rb]_o$ (replacing Na^+); △, ▲ denote experimental points in presence and absence of 10^{-3} M furosemide and ± 15 mM $[Rb]_o$. *B*. Rate constants for furosemide-sensitive K^+ effluxes $({}^{o}k_K^{NEM})_{\Delta F}$, calculated as the difference between the ${}^{o}k_K^{NEM}$ values of controls and furosemide-treated cells shown in *A*

Previously I have reported furosemide inhibition of both ${}^{o}k_{K}^{NEM}$ and Rb^+ influx, ${}^{i}M_{Rb}^{NEM}$, at a single high extracellular Rb^+ concentration (*cf.* Table 3, ref. 21). Figure 4 analyzes the effect of various furosemide concentrations on ${}^{i}M_{Rb}^{NEM}$ at different $[RbCl]_o$. The raw data, obtained from triplicate determinations of ouabain-insensitive Rb^+ influx in NaCl media (Rb^+/Na^+ replacement), indicate that furosemide exerted complex effects involving reduction of both the maximal Rb^+ influxes at high and of $K_{0.5}^{Rb}$ at low $[Rb^+]_o$ as the concentration of furosemide reached 10^{-3} M. Figure 5 is an Eadie-Hofstee plot of $({}^{i}M_{Rb}^{NEM})_{\Delta F}$, the furosemide-sensitive flux components of Fig. 4, versus $({}^{i}M_{Rb}^{NEM})_{\Delta F}/[Rb]_o$, demonstrating that both the maximum fluxes (*y*-intercepts) fell and the $K_{0.5}^{Rb}$ values (slopes) heightened when the furosemide concentrations were increased from 10^{-3} M (line *b*) to 10^{-4} M (line *d*). Hence the data suggest that furosemide reduced V_{max} of K/Cl transport (about 4.2 mM Rb^+/liter cells × hr in the control) as well as decreased the apparent affinity for the transported Rb^+ causing variable $V_{max}/K_{0.5}^{Rb}$ ratios. Similar data were obtained with 4 different experiments on the same LK sheep. The data speak for mixed-type interactions between furosemide and external Rb^+, the effect on $K_{0.5}^{Rb}$ being much larger than on V_{max}.

Although there is no readily apparent $Na^+K^+/2Cl$ cotransport in LK sheep red cells [7] and NEM stimulates only Cl^--dependent K^+ transport [4, 18, 21, 23], Na^+ was found to contribute to the effect of furosemide. Figure 6 shows both ${}^{o}k_{K}^{NEM}$ (*A*) and ${}^{i}M_{Rb}^{NEM}$ (*B*) as functions of increasing furosemide concentrations for LK cells suspended in choline-Cl or NaCl media containing 1.5 mM $[Rb^+]_o$ (used as minimum concentration required for activation of the furosemide effect without interfering with the potential action of Na^+). Both K^+ effluxes and Rb^+ influxes were inhibited to a small but significantly

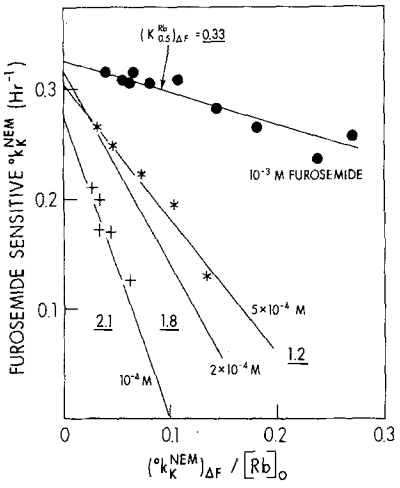

Fig. 3. Eadie-Hofstee plot of furosemide-sensitive K^+ efflux rate constants as function of $({}^{o}k_{K}^{NEM})_{\Delta F}/[Rb]_o$. Symbols are those of data points of Fig. 2. Underlined numbers: $K_{0.5}^{Rb}$ calculated from the slope of the lines (regression lines by least-squares method); other numbers define the furosemide concentrations used

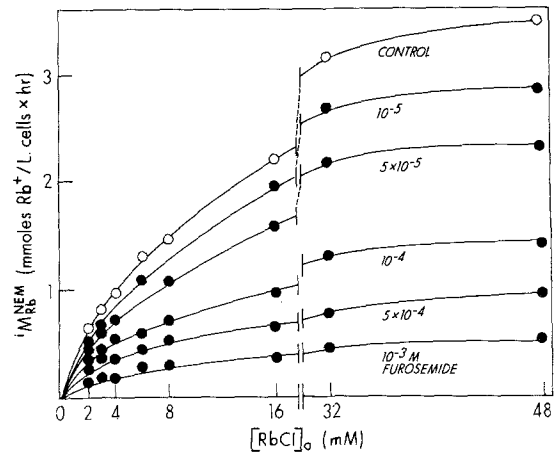

Fig. 4. Ouabain-insensitive Rb^+ influx of NEM-treated cells, ${}^{i}M_{Rb}^{NEM}$, exposed to 5 different furosemide concentrations as a function of varying $[RbCl]_o$. Open circles: controls; filled circles: with furosemide at the indicated concentrations

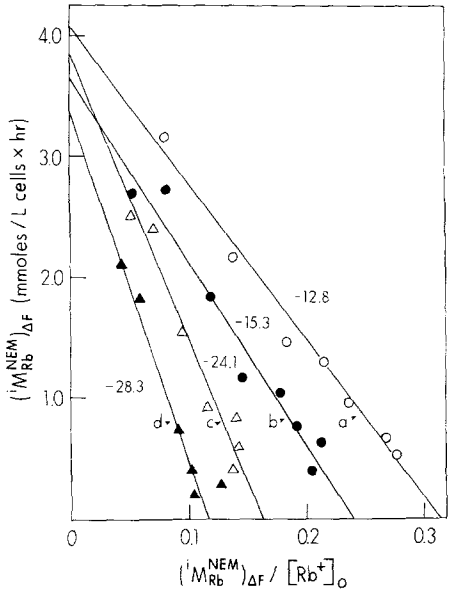

Fig. 5. Eadie-Hofstee plot of ouabain-insensitive, furosemide-sensitive Rb^+ influx $({}^{i}M_{Rb}^{NEM})_{\Delta F}$ of NEM-treated LK red cells versus $({}^{i}M_{Rb}^{NEM})_{\Delta F}/[Rb]_o$. The regression lines (*a–d*) have been calculated from experimental data points of Fig. 4 by least-squares analysis. Furosemide concentrations (M): $b = 10^{-3}$, $c = 5 \times 10^{-4}$; $d = 10^{-4}$; line *a* is control in the absence of furosemide. The negative numbers are the $K_{0.5}^{Rb}$ (mM) values calculated from the slopes of the lines

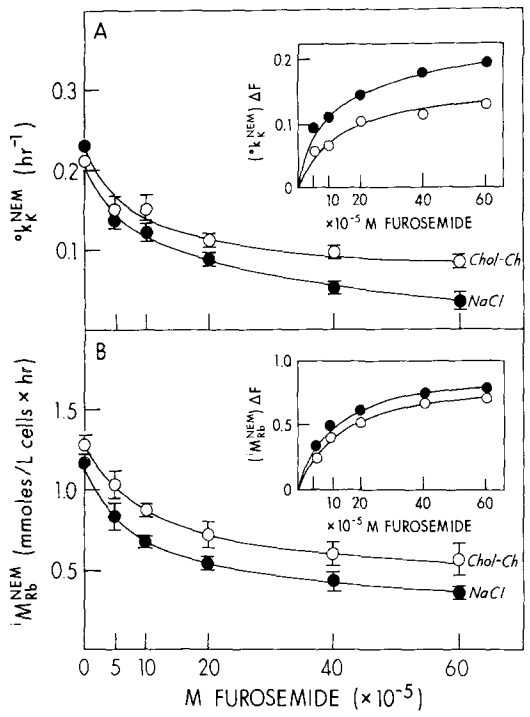

Fig. 6. External Na^+ dependence of the furosemide inhibition of thiol-dependent K^+ effluxes (*A*) and Rb^+ influxes (*B*) in LK sheep red cells. Data are from two independent experiments with bars indicating range of variation. K^+ efflux determinations by 5 time point analysis and derivation of ${}^{o}k_K^{NEM}$, while ${}^{i}M_{Rb}^{NEM}$ was determined at 1 hr in triplicates. Inserts: Furosemide-sensitive ${}^{o}k_K^{NEM}$ versus ${}^{i}M_{Rb}^{NEM}$ of *A* and *B*, respectively

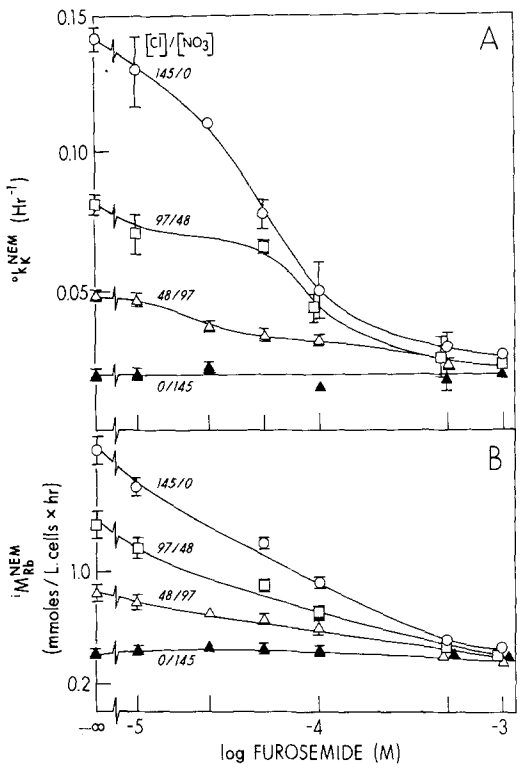

Fig. 7. Effect of Cl^- replacement with NO_3^- on furosemide inhibition of K^+ efflux (*A*) and Rb^+ influx (*B*). Cells were pre-equilibrated in Na^+ media containing 20 mM Rb_o and the indicated Cl^- NO_e^- concentration ratios prior to exposure to various furosemide concentrations. Bars indicate range between 2 independent experiments of simultaneous fluxes, each value obtained by 5 time point analysis of ${}^{o}k_K^{NEM}$ and triplicates of ${}^{i}M_{Rb}^{NEM}$ at 1 hr

greater extent in NaCl than in choline-Cl media. In general, however, the Na_o^+ effects on the furosemide inhibition of the bidirectional fluxes were of much smaller magnitude than the augmentation of the furosemide effect by Rb_o^+. The inserts of Fig. 6 are plots of $({}^{o}k_K^{NEM})_{\Delta F}$ and $({}^{i}M_{Rb}^{NEM})_{\Delta F}$, the furosemide-sensitive flux parameters, as a function of the inhibitor concentrations used. Evidently, the effect of external Na^+ was somewhat greater on K^+ efflux than on Rb^+ influx paralleling the Rb^+-dependent furosemide effects on K^+ efflux as compared to Rb^+ influx (*see* Figs. 2 and 3).

Other loop diuretics such as bumetanide appear to compete with Cl^- for a common cationic site on the Na^+K^+ cotransport carrier as shown by inhibition studies in duck red cells (15) and bumetanide binding experiments to isolated membranes (10). Hence, it was of interest to see whether, in the LK sheep red cell, Cl^- replacement by NO_3^- would also increase the action of furosemide. Figure 7 shows ${}^{o}k_K^{NEM}$ and ${}^{i}M_{Rb}^{NEM}$ as a function of different furosemide concentrations in LK cells pre-equilibrated in 145, 97, 48 and 0 mM Cl media (NO_3^- replacement) and then exposed to the furosemide concentrations indicated. In the absence of furosemide (values on *y*-axis) gradual Cl^- replacement by NO_3^- caused the characteristic inhibition of ${}^{o}k_K^{NEM}$ reported earlier [21]. Furosemide inhibition of both ${}^{o}k_K^{NEM}$ and ${}^{i}M_{Rb}^{NEM}$, which was studied in 125 mM Na^+ media containing 20 mM Rb_o^+ to insure its full effect (*see above*), was more evident at all concentrations as long as Cl^- was present. There was no significant furosemide effect in NO_3^- media. A plot of the Cl^--dependent K^+ efflux or Rb^+ influx remaining at various furosemide and Cl^- concentrations (Fig. 8) revealed that the IC_{50} values (5×10^{-5} M) of furosemide were clearly independent of external Cl^-, supporting the hypothesis that furosemide binds to a site independent of Rb^+, Na^+, and also Cl^-.

Discussion

The thiol-dependent K/Cl transporter in LK sheep red cells fulfills the major criteria of a classic carrier

system namely saturation kinetics [21], pronounced temperature dependence [21], and inhibition by anions other than Cl^- or Br^- [18–21]. As previously shown, this transport system is capable of net fluxes which most probably constitute electroneutral movements of K^+ and Cl^- although firm evidence of stoichiometric coupling is still lacking. The data in Fig. 2 of this study suggest that furosemide does bind to the externally empty carrier as about 30% inhibition of ${}^{o}k_K^{NEM}$ was observed in the absence of external Rb^+. The inhibitory effect of furosemide was dramatically augmented in the presence of external Rb^+ (Figs. 1, 2, 4), suggesting that the $Rb^+(K^+)$ carrier complex facilitates the furosemide binding and its effect, or that furosemide binds to the empty carrier and thus modifies the transport characteristics for Rb^+ across the membrane. The apparent affinity of the furosemide binding site can only be approximated from the IC_{50} values which were in the range of 5×10^{-5} M (Figs. 1, 8) to 10^{-5} M (Fig. 6). Analysis by the Dixon method overestimated these values due to nonlinearity of the relation of $(1/M_{Rb}^{NEM})_{\Delta F}$ versus the furosemide concentration. This observation indicates the complexity of the furosemide-carrier interaction. From the Eadie-Hofstee plot (Fig. 5) it is apparent that the relative change in $K_{0.5}^{Rb}$ was greater than in V_{max}. Hence both Rb^+ binding and transport may be modified by the furosemide-carrier association. On the other hand, it is difficult to prove that Rb_o^+ does not bind first and modify the carrier prior to its acceptance of the loop diuretic. Such a model has been proposed for the binding of ^{3}H-bumetanide to dog kidney cortex membranes where Na^+ and K^+ positively affected the forward reaction of bumetanide [10]. The difference between these data and the work reported here then may lie not only in the different experimental approaches (inhibition versus binding studies) but also in the fact that furosemide is structurally different from bumetanide and/or that there exist important structural dissimilarities between K/Cl cotransport in LK sheep red cells and Na/K/2Cl cotransport in other membranes. This aspect is underscored by the observation of an inverse relationship between furosemide-sensitive K^+ fluxes and external K^+ concentrations in ascites tumor cells [2].

The effect of Na^+ may be explained in terms of its action at the furosemide-carrier complex: independent of Na_o^+, furosemide-inhibited K^+ efflux and Rb^+ influx with IC_{50} values of about 10^{-5} M furosemide (Fig. 6). A clear synergism between loop diuretic inhibition, and $K^+(Rb^+)$ and Na^+ has been shown for bumetanide for Na/K/2Cl cotransport in duck [14, 16] and turkey [26, 27] red cells. In LK sheep red cells one would have to postulate that

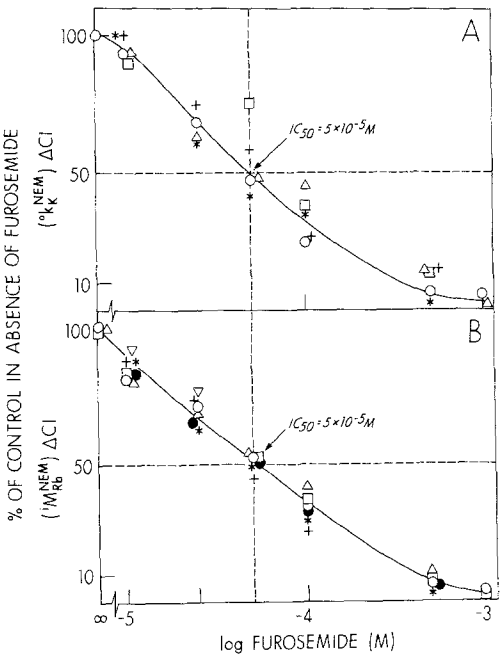

Fig. 8. Chloride-sensitive ${}^{o}k_K^{NEM}$ and ${}^{i}M_{Rb}^{NEM}$ values remaining after exposure to 6 different Cl^- concentrations (from 36 to 145 mM using NO_3^- replacement including data from Fig. 10) as a function of the logarithm of the furosemide concentration

Na^+ facilitates furosemide inhibition by merely binding to a functionally inoperative site since thus far there is no evidence for coupled Na/K/2Cl transport. Alternatively, Na^+ may compete for the $Rb^+(K^+)$ site of the K/Cl transporter.

Furthermore, there was no change in the IC_{50} values when Cl^- was partially replaced from 145 to 48 mM by NO_3^-. Hence the relationship between furosemide inhibition and Cl^- is different from that described for Na/K/2Cl cotransport in other membranes [10, 15] and may be an important molecular distinction between the two types of cotransporters. However, the kinetically mixed type transport inhibition is somewhat similar to that of Cl/Cl self-exchange [5].

The interaction of the K/Cl transporter with Rb^+, Na^+ and Cl^- is certainly much more complex than discussed here. Not taken into account was the observation that the loop diuretic affected ${}^{o}k_K^{NEM}$ to a relatively greater extent than ${}^{i}M_{Rb}^{NEM}$ which may be due to some built-in asymmetry of the K/Cl transport system or other factors such as permeation into the cell. Furthermore, to compare correctly the K/Cl transport system stimulated by NEM in LK cells with any one of the other species, one needs to consider the absence of a transmembraneous Na^+ gradient. Consequently, the mode by which furosemide appears to inhibit thiol-dependent K/Cl

transport in LK sheep red cells makes this system very attractive as a model for the study of the molecular mechanism of action of those loop diuretics whose interaction with cations and anions has not yet been fully ascertained. The pathophysiologic consequences are also of interest, as this study shows that inhibition by furosemide of neutral salt transport is not solely dependent on the drug concentration but also on the type and concentration of cations around the site of pharmacological action.

For the elucidation of the mechanism of the HK-LK transition in sheep red cells this study is of paramount significance: First it further defines the K/Cl transporter, which may play a crucial role in generating the LK steady-state cells. Second, the here-described inhibitory characteristics may be assumed to exist for 3-amino-5-sulfamoylbenzoic acid derivatives similar to furosemide but with much higher binding affinities suitable for a final biochemical attack on the molecule that differentiates the passive K^+ permeability of an LK from that of an HK sheep red cell.

This work was supported by NIH grant AM 28236/HEM and carried out with the technical assistance of James Franklin who translated the equations used into a computer program for calculation of the rate equations used (*cf.* ref. 2), and with skillful word processing by Steffani Webb.

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Received 22 March 1983; revised 27 June 1983