Furosemide Inhibition of Chloride Transport in Human Red Blood Cells

PETER C. BRAZY and ROBERT B. GUNN

From the Department of Physiology and Pharmacology, Duke University Medical Center, Durham, North Carolina 27710, and the Department of Pharmacological and Physiological Sciences, University of Chicago, Pritzker School of Medicine, Chicago, Illinois 60637

ABSTRACT The chloride self-exchange flux across the human red cell membrane is rapidly and reversibly inhibited by 10^{-4} M furosemide, a potent chloruretic agent. Furosemide reduces the chloride flux at all chloride concentrations and increases the cellular chloride concentration at which the flux is half-maximum. Kinetic analysis of the flux measurements made at several furosemide and chloride concentrations yields a pattern of mixed inhibition with a dissociation constant for the inhibitor-transport mechanism complex of 5 \times 10^{-9} M. From this pattern of inhibition and other observations, including that the percent inhibition is independent of pH (range 5.6-8.9), we conclude that the anionic form of furosemide interacts primarily with the chloride transport mechanism at a site separate from both the transport site and the halide-reactive modifier site.

INTRODUCTION

Furosemide (Fig. 1) is a rapidly acting, potent diuretic that is commonly used in clinical medicine. Its administration causes a marked increase in the excretion of sodium, potassium, calcium, magnesium, chloride, phosphate, and water by the kidney in rats, dogs, and man (15). The major site of furosemide's diuretic effect is the ascending limb of Henle's loop (8). Because Hook and Williamson (21) had found that furosemide decreased the ATPase activity in microsomal preparations from rat kidney, and Sachs (26) had found that furosemide decreased the ouabain-sensitive and insensitive sodium permeability in human red blood cells, the mechanism of action of this drug was thought to be an inhibition of cation transport.

More recently, attention has been focused on the inhibitory effects of furosemide on the transport of inorganic anions. Furosemide was one of numerous inhibitors that decreased phosphate tracer efflux across intact human red blood cells (14). The [$^{32}$P]phosphate efflux in solutions with 1 mM total phosphate, pH 7.35, at 37°C was inhibited 50% by 5 \times 10^{-4} M furosemide. This inhibition was reversible and noncompetitive. Recently Burg and Green (3) found that chloride was actively transported out of the lumen of perfused tubules isolated from the rabbit kidney. Furosemide ($10^{-5}$ M) in the lumen of the thick ascending limb of Henle's loop decreased the electrical potential difference from +5 mV (lumen positive) to near zero and decreased the net flux of NaCl from the lumen (4). This observation is consistent with active electrogenic transport of chloride.
which is inhibited by furosemide and passive diffusion of Na from lumen to peritubular fluid. Furosemide can cause a 50% reduction in the short circuit current in frog cornea (6), in which active chloride transport accounts for 90% of the short circuit current (31). In man and dogs systemic furosemide decreased the volume and the bicarbonate concentration of the fluid collected from cannulated pancreatic ducts (25, 16). An anion exchange mechanism has been reported in mammalian pancreatic ducts (24); the observed decrease in bicarbonate concentration of pancreatic fluid caused by furosemide may be a result of an inhibited anion exchange mechanism.

In this paper we report the results of our studies on the effect of furosemide on the chloride transport system in the human red blood cell. The chloride self-exchange transport in erythrocytes shows saturation with increasing chloride concentration, competitive inhibition by other inorganic anions, noncompetitive inhibition by certain organic compounds, a characteristic pH dependence (19), and a high apparent activation energy (13). This anion exchange mechanism appears to depend upon the integrity of an intrinsic erythrocyte membrane protein (5, 20) and can be described by a titratable carrier model (17). We studied the interaction of furosemide with the red cell anion exchange mechanism in an effort to increase our understanding of both the drug's mode of action and the chloride transport mechanism in human red blood cells.

**MATERIALS AND METHODS**

The methods are fully described in Gunn et al. (19); a brief summary is given here. Media with the following millimolar concentrations were prepared from reagent grade chemicals: chloride medium—140 NaCl, 5 KCl, 1.5 CaCl₂, 1.0 MgCl₂, 5 d-glucose, and 27 glycyglycine; acetate medium—140 Na-acetate, 5 K-acetate, 1.5 Ca-(acetate)₂, 1.0 Mg-(acetate)₂, 5 d-glucose, and 27 glycyglycine; nystatin medium—25-600 KCl, 10 NaCl, and 27 sucrose.

Furosemide, a white powder received as a gift from Hoechst Pharmaceuticals, Inc., (Cincinnati, Ohio) was made into a 50 mM stock solution in 0.1 N KOH. Aliquots of the stock solution were added to the appropriate medium before final adjustment of the pH at 0°C. Nystatin (E. R. Squibb and Sons, Inc., Princeton, N. J.) was dissolved in dimethyl sulfoxide to make a stock solution.

Red blood cell suspensions were prepared from freshly drawn heparinized whole
blood from one of the researchers (P. C. B.). The red cells were separated from the
plasma and buffy coat, washed in 170 mM NaCl, titrated to the desired pH with CO₂ and
bicarbonate at 0°C, and then washed several times in cold medium until the chloride was
in steady state between cells and solution. Then ^36Cl (ICN Pharmaceuticals, Inc., Cleve-
land, Ohio, 10 μl of 462 μCi/ml) was added to the suspension and allowed to equilibrate.
This suspension was packed in thin nylon tubes and centrifuged to separate cells from
medium.

0.25 g of packed cells was added to 25 ml of cold medium at time zero in a thermostated
chamber. The extracellular fluid was sampled by the rapid filtration technique of
Dalmark and Wieth (13). An "infinity" sample was taken from this suspension after the
tracer had reached a steady-state distribution.

A semilog graph of the fraction of tracer remaining in the cells as a function of time
was a straight line whose slope is the rate constant. The product of this rate constant and
the intracellular chloride concentration in milliequivalents per kilogram cell solids is the
self-exchange flux in milliequivalents per (kilogram cell solids·minute). The intracellular
chloride concentration for each flux was calculated from the steady-state ratio of intracel-
lar to extracellular ^36Cl activity (rcl) times the measured extracellular chloride concen-
tration and the kilograms cell water per kilogram cell solids (determined by drying cells to
constant weight). Duplicate flux measurements were performed for each set of condi-
tions. The average range between duplicate measurements was 5% of the flux magni-
tude. The nystatin method of equilibrium dialysis of red cells was used to alter, the
intracellular chloride concentration in one series of experiments, (7, 10).

RESuLTs

Furosemide Inhibition of Chloride Self-Exchange Flux

The self-exchange of chloride isotopes across red blood cells was measured in
the presence of graded concentrations of furosemide at pH 7.80, 0°C. The red
cells were first suspended in chloride medium with a given concentration of
furosemide, loaded with radioactive ^36Cl, and injected into a nonradioactive
chloride medium with the same furosemide concentration. The results are
shown in Fig. 2. The self-exchange flux was inhibited 50% by 2 × 10⁻⁴ M
furosemide. In 5 × 10⁻³ M furosemide the residual insensitive flux was only 12
meq/(kg cell solids·min) or 1.6% of the control flux value. Both the rapidity with
which furosemide inhibited chloride self-exchange and the reversibility of its
effect were shown by the experiments presented in Fig. 3. First the efflux of
chloride from cells prewashed with solutions containing 5 × 10⁻⁴ M furosemide
was compared with the efflux of cells without prior exposure to furosemide.
Both types of cells were injected into chloride media containing 5 × 10⁻⁴ M
furosemide. The efflux rates were identical and the efflux of the cells which had
no prior exposure to furosemide followed the same first-order kinetics. This
indicates that the same level of inhibition was attained before the first sample (at
6 s) by the reaction of the cells with the furosemide in solution. The reversibility
of inhibition was demonstrated by prewashing cells in 10⁻³ M furosemide then
washing an aliquot of cells six times in 4 vol of drug-free medium before
measuring the efflux. The rate constant for the control flux was 0.053 s⁻¹ and for
the flux in 10⁻³ M furosemide 0.0051 s⁻¹ while the efflux rate coefficient of the
pretreated cells washed in furosemide-free medium was 0.050 s⁻¹. This value was
not significantly less than the control.
FIGURE 2. Dose-response curve for furosemide inhibition of the chloride self-exchange flux. The inhibitory effect of furosemide, plotted as the logarithm of the drug's concentration, on the chloride self-exchange flux was measured at pH 7.80, 0°C, in chloride medium. The curve was drawn by eye. The uninhibited chloride flux has a value of 740 meq/(kg cell solids·min). 50% inhibition of the chloride flux occurred at 2 × 10⁻⁴ M furosemide.

FIGURE 3. Effects of furosemide on the efflux of cellular ³⁶Cl. The rate constant for the efflux of chloride tracer was determined from the slope of the appearance of tracer in the extracellular compartment ($1-a_t/a_w$, where $a_t$ is the specific activity at time $t$; and $a_w$ is the specific activity at isotopic steady state) as a function of time in seconds. The plot shows first-order kinetics for the tracer efflux of control cells (○), cells treated with furosemide (10⁻³ M) then washed in drug-free medium (●), cells preincubated and measured in furosemide (5 × 10⁻⁴ M) (□), and drug-free cells that were first exposed to furosemide (5 × 10⁻⁴ M) at the beginning of the efflux measurements (■). The efflux measurements were made in chloride medium, pH 8.00, 0°C. The slope of the graph was determined by linear regression analysis.
The Effect of Cellular Chloride Concentration on Furosemide Inhibition

The chloride self-exchange flux of red cells has been shown to depend upon the cellular chloride concentration (19, 9). Two methods were used to alter the intracellular chloride concentration of erythrocytes. In the first, the red cells were washed repeatedly in Na-acetate medium to which ammonium chloride had been added to achieve the desired chloride concentration. The high permeability of ammonia and carbon dioxide combined with the rapid chloride-bicarbonate exchange permitted the ammonium chloride rapidly to distribute itself across the red cell membrane. Once the chloride had reached steady state between the cells and the medium, the cells were treated with furosemide and the tracer efflux into the same Na-acetate-NH₄Cl medium was measured. Using a second method we looked for an effect of ammonium and acetate ions on either furosemide or the chloride transport mechanism. In this method (7, 10) nystatin was used to reversibly increase red cell permeability while the ionic content was changed (20-600 mM KCl, 10 mM NaCl). Once the cells were in steady state with the new medium, the nystatin was washed off. These cells were then treated with furosemide and the chloride content and tracer efflux measured.

Fig. 4a shows the effect of cellular chloride concentration on the self-exchange flux in the presence of 5 × 10⁻⁴ M furosemide at pH 7.80, 0°C, in an Na-acetate-NH₄Cl medium. The solid curve was drawn from a Michaelis-Menten equation. In the absence of furosemide the concentration dependence of the chloride self-exchange under these conditions (Fig. 5 in reference 19) had a shape of a Michaelis-Menten equation at cellular chloride concentrations below 75 mM. At high cellular chloride concentrations the flux decreased. This decrease was not seen in the presence of furosemide, as shown in Fig. 4a. Fig. 4b shows the concentration dependence of the chloride flux in cells prepared by the nystatin method of altering cellular chloride. The curve of the flux values in the absence of furosemide was comparable to that found by Dalmark (9) with a maximum near 150 mM chloride and a decline in the flux value with further increases in the cellular chloride concentration. The curve in the presence of furosemide has the same shape as the curve found in Na-acetate-NH₄Cl medium. A comparison of Fig. 4a and b shows that the magnitude of the chloride self-exchange flux is lower in the Na-acetate-NH₄Cl medium due to the noncompetitive inhibition of the chloride flux by acetate (19). However, the calculated maximum chloride flux (see below) from the data in Fig. 4a is the same as that observed in Fig. 4b. Furthermore, the presence of furosemide (5 × 10⁻⁴ M) when studied by either method prevented the decrease in the self-exchange flux at high cellular chloride concentrations which was observed in the absence of furosemide with both methods (19, 9).

Fig. 5 is a double reciprocal plot showing the data from Fig. 4a (5 × 10⁻⁴ M furosemide) together with similar data from experiments in 10⁻⁴ M furosemide and in the absence of furosemide (only data from cells with 0-75 mM cellular chloride were considered, to minimize the contribution of the self-inhibition apparent at higher chloride concentrations). The kinetic parameters, $V_m$ for the maximum flux and $K_{1/2}$ for the value of cellular chloride concentration when
the flux is half-maximum, were estimated by the statistical method of Wilkinson (30). For the control data \( V_m = 800 \pm 40 \) (SE) meq/(kg cell solids \cdot min) and \( K_{1/2} = 65 \pm 5 \) mM; for the experiments in \( 10^{-4} \) M furosemide \( V_m = 490 \pm 70, K_{1/2} = 106 \pm 30 \); and for those in \( 5 \times 10^{-4} \) M furosemide \( V_m = 213 \pm 6, K_{1/2} = 132 \pm 8 \). By analogy to enzyme kinetic systems this figure indicates that furosemide caused a mixed-type (competitive and noncompetitive) inhibition (28).

In a Dixon plot (Fig. 6), the lines through the data points for each of three chloride concentrations intersect at a point above the abscissa and to the left of the ordinate, again compatible with a mixed type of inhibition (28). The point of intersection of these lines corresponds to the \( K_I \) (dissociation constant of the

---

1 These values from a statistical evaluation of data at only low Cl concentrations (where self-inhibition is not prominent) are different from the observed maximum flux of 480 and concentration of chloride at which the flux is half of 480, namely 35 mM, as reported earlier (19). We believe that the values in the text more accurately reflect the underlying phenomena of the transport system than the simple observables reported earlier.
**Figure 5.** Lineweaver-Burk plot. The reciprocal chloride self-exchange flux is graphed as a function of the reciprocal cellular chloride concentration. All fluxes were performed at 0°C, pH 7.80, and $r_{c1} = 0.75-0.92$ in Na-acetate-NH$_4$Cl medium. The data from the saturation curves of the uninhibited flux and the inhibited flux at two different furosemide concentrations are plotted here. Statistical analysis (30) was used to determine the intercepts (values are given in the text). The pattern of this inhibition is that of a mixed type (competitive-noncompetitive).

**Figure 6.** Dixon plot. The reciprocal of the chloride self-exchange flux is graphed as a function of the furosemide concentration at three cellular chloride concentrations. In this graph the Na-acetate-NH$_4$Cl medium method was used to alter intracellular chloride at pH 7.80, 0°C. The lines were drawn by eye. The pattern of the graph is compatible with mixed-type inhibition and the lines intersect at a point equal to the negative of the dissociation constant ($K_I$) which is $5 \times 10^{-5}$ M for furosemide.
inhibitor-enzyme complex) which was $5 \times 10^{-5}$ M in this case. The deviation from linearity seen at the lowest chloride concentration (26 mM) and the highest furosemide concentration reflects the presence of the furosemide-insensitive chloride flux which becomes a greater portion of the measured flux under these conditions. Because of this furosemide-insensitive flux, the reciprocal of the flux has a maximum value which the lines on the Dixon plot must asymptotically approach at high drug concentrations.

The pH and Temperature Dependence of the Chloride Self-Exchange Flux in the Presence of Furosemide

Two characteristic features of the chloride transport system in red blood cells are the decreasing self-exchange flux at pH values below 7.8 at 0°C (19, 9) and the high apparent activation energy of 30 kcal/mol (12, 13, 9). These parameters were restudied in the presence of $5 \times 10^{-4}$ M furosemide (approximately 75% inhibition of the control flux) to determine if changes in pH or temperature would alter the effectiveness of furosemide inhibition.

The pH dependence of the chloride self-exchange flux was determined in red cells equilibrated at 0°C in chloride medium at the desired pH value. Fig. 7 shows the relationship of the self-exchange flux to the pH of the medium for uninhibited cells and cells inhibited by $5 \times 10^{-4}$ M furosemide. The curves for both conditions are concave with a maximum around pH 7.8. The percent inhibition of the control flux at the different pH values was nearly constant (75-80%) over this pH range (5.6-8.9). Thus, the reaction of the transport mechanism with hydrogen ions which causes inhibition did not significantly alter the inhibitory effect of furosemide. This observation is consistent with either a model in which H$_3$O$^+$ and furosemide compete for a common locus or a model in which they have separate loci and independent inhibitory actions. Theoretically, in both of these models the fractional inhibition caused by one inhibitor is dependent upon the concentration of the second inhibitor, because the reactions are reversible and obey the law of mass action (reference 18, p. 322). However, under the experimental conditions of Fig. 7 with a fixed furosemide concentration, very little change in the percent inhibition would occur if either model were operative. Therefore the relationship between the sites of H$_3$O$^+$ inhibition and

2 First consider a single site for noncompetitive inhibition by both H$_3$O$^+$ ($H$) and furosemide ($F$).

$$E + H \rightleftharpoons k_1 HE; E + F \rightleftharpoons k_2 FE; E + Cl \rightleftharpoons k_3 E-Cl; E-Cl + H \rightleftharpoons k_4 HE-Cl; E-Cl + F \rightleftharpoons k_5 FE-Cl$$

are the equations for two noncompetitive inhibitors which can react with the unloaded ($E$) and chloride-loaded ($E-Cl$) carrier. The chloride flux ($v$) equals $k(E-Cl)$. Using conservation of carriers ($\sum E_i = T$) one can calculate the chloride flux in the presence of $H$ only ($v_H$) and in the presence of both $H$ and $F$ ($v_{HF}$) and obtain their ratio.

$$\frac{v_{HF}}{v_H} = \frac{1 + k_1 H}{1 + k_2 H + k_5 F}$$

If $H$ inhibits 80% alone (pH 5.6) and $F$ inhibits 75% alone as in Fig. 7, then $k_2 H = 0.25$ and $k_5 F = 0.34$ and $v_{HF}/v_H = 0.78$. Thus at pH 5.6 the flux in the presence of furosemide is 78% of that in the absence of furosemide. If $k_2 H = 0$ as at pH 8, then $v_{HF}/v_H = 0.75$. The fractional inhibition will be practically independent of pH ranging from 0.75 to 0.78 while H$^+$ inhibition ranges from 0 to 80%.

Second, consider two separate sites for noncompetitive inhibition by $H$ and $F$. $v_{HF}/v_H = (1 +
furosemide inhibition cannot be ascertained by this experiment.

The temperature dependence of the chloride self-exchange flux was determined in chloride medium in which the pH was adjusted to 7.80 against standard buffers at the desired temperature. Again this experiment was made with red cells in the presence or absence of \(5 \times 10^{-4} \text{ M}\) furosemide. Fig. 8 shows this data on an Arrhenius diagram with the log of the chloride flux plotted as a function of the reciprocal temperature in degrees Kelvin. The slope of this graph is proportional to the apparent activation energy. The flux values at the higher temperatures deviated from the line through the values at lower temperatures. This nonlinearity has recently been observed by Brahm (2) who studied the chloride self-exchange between 0°C and 38°C with a flow tube apparatus and may reflect an increase of \(K_{1/2}\) with temperature. Alternatively, we may have introduced a systematic error at the highest temperatures due to the very rapid rate of 

\[
k_2 H / (1 + k_2 H + k_2 F + k_2 k_2 H \cdot F).
\]

If \(k_2\) is small \([k_2 = \text{HFE}/(\text{HE} \cdot F) = (\text{HFE}-\text{CI})/(\text{HE} \cdot \text{CI} \cdot F)]\), then the above calculation holds for a single site; and if not, a reasonable guess is that \(k_2 = k_3\) or that the reaction of \(H\) or \(\text{Cl}\) with their separate sites does not affect the binding affinity for furosemide (F) at its separate independent site. If so, then under the same experimental conditions as above, \(v_{nF}/v_n = 0.75\) at pH 5.6 where \(k_2 H = 0.25, k_F = 0.34\) and \(k_2 k_2 H \cdot F = 0.085\) and \(v_{nF}/v_H = 0.75\) at high pH where \(k_F H = k_2 H \cdot F = 0\).

Thus under these experimental conditions both the single-site and the two-site models predict a pH-independent fractional inhibition by furosemide and cannot be distinguished by these data. Since at very low pH other transport mechanisms come into play, we cannot be sure that at even more extreme conditions these two model mechanisms can be separated by further experiments.

---

**Figure 7.** Effect of extracellular pH on furosemide inhibition of chloride self-exchange flux at 0°C. Chloride self-exchange fluxes were performed at several extracellular pH values with and without furosemide \((5 \times 10^{-4} \text{ M})\) in chloride medium. The \(r_0\) varied linearly with the pH from 1.3 at pH 5.6 to 0.3 at pH 8.9. The curve was drawn by eye through duplicate efflux determinations at each pH value. The percent of inhibition by furosemide was 75% at the low and 80% at the high pH values.
sampling for these data points. In our data the slope of the graph through the lower points yields an apparent activation energy of 29 kcal/mol for both the control and the furosemide-inhibited chloride flux. Thus, there was no unusual temperature sensitivity of the inhibition of the chloride flux by furosemide.

**DISCUSSION**

Anion transport in human red blood cells was first shown to be furosemide sensitive by Deuticke and Gerlach (14). In their study the furosemide concentration required for 50% inhibition of [³²P]phosphate efflux was $5 \times 10^{-4}$ M. This value is only 2.5 times the concentration required to inhibit by 50% the chloride self-exchange flux reported in this study under quite different experimental conditions. Deuticke and Gerlach measured phosphate self-exchange at 37°C at pH 7.35 in Locke’s solution containing 1 mM phosphate. The chloride and bicarbonate ions in Locke’s solution are inhibitors (probably competitive) of divalent (29) and monovalent inorganic anion transport (19, 11). These inhibitors would decrease the inhibitory effect of furosemide if they reacted with the
anion transport mechanism at a common site. Other differences, e.g., temperature and degree of carrier saturation, may also play a role in the slight difference in the concentration of furosemide required for 50% inhibition in these two systems.

Furosemide interacts with the red cell very rapidly and reversibly. In Fig. 3 one can see that the onset of inhibition by this drug is less than 6 s at 0°C. The rapidity of this effect implies that furosemide acts at a superficial site on the cell. Since furosemide is a large organic acid (pKa = 3.9) one would not expect it to rapidly cross the cell membrane. For comparison parasulfamoyl benzoic acid which is similar to a part of the furosemide molecule (see Fig. 1) does not cross the ox red cell membrane to any significant degree (1). Thus we would not expect the charged form (see below) of the larger furosemide molecule to cross into the red cell within 6 s. Furosemide’s effect on red cell chloride transport was reversed by washing the cells six times with drug-free medium. The relative ease of reversing the inhibition of this drug also suggests that furosemide’s action is on the membrane surface. So while furosemide has been shown to inhibit glycolysis in cell-free preparations (23), it appears to inhibit anion transport in red cells at the level of the cell membrane, and most likely the outer surface of this membrane.

Furosemide has a negatively charged carboxyl group at neutral pH. We measured the effect of altering the pH of the medium on the inhibitory action of furosemide on the chloride self-exchange flux. Over a pH range of 5.6-8.9 (Fig. 7) the percent of inhibition of chloride flux caused by $5 \times 10^{-4}$ M furosemide was nearly constant (75-80%). Since the concentration of uncharged furosemide varied by a factor of 10 while the negatively charged form was nearly constant, this result indicates that the anionic form of the drug is the active inhibitor. We cannot say whether the neutral form of the drug is also an inhibitor.

The kinetic analysis of the experimental data in this paper reveals four points. In the absence of acetate, that is in media containing only chloride and furosemide anions, (a) the dose-response curve shows an apparent stoichiometry of more than one furosemide molecule inhibiting each transport unit, and (b) the curve of the chloride flux graphed against chloride concentration (Fig. 4b) is complex and above a certain concentration, chloride inhibits its own flux. In the presence of acetate anions, (c) the stoichiometry for furosemide inhibition of transport units is 1:1, and (d) furosemide behaves as a mixed-type inhibitor of the chloride self-exchange system (Figs. 5 and 6). The analyses supporting points (a) and (c) are presented below and in Fig. 9.

Dalmark (11) has made a detailed study of the chloride saturation curve (Fig. 4b) with particular attention to the influences of other halide anions on the self-inhibition by chloride. He has proposed that the chloride transport mechanism has two anion binding sites, one for anion translocation and another for non-competitive inhibition of translocation. Dalmark observed chloride self-exchange between 10 and 400 mM chloride in the presence of other halides (10-300 mM) in cells prepared by the nystatin method, and found that halides decreased the calculated maximum flux, increased the $K_{1/2}$, and decreased the chloride self-inhibition. He concluded first that fluoride, bromide, and iodide act both as competitive and noncompetitive inhibitors of the chloride carrier, and are able
to compete with chloride at both of the proposed anion binding sites (transport site = $Cl_1$; modifier site = $Cl_2$), and second, that the binding of another halide on the noncompetitive modifier site ($Cl_2$ site) prevented chloride from binding there and prevented chloride from inhibiting its own flux.

In most of the experiments in our study, chloride concentration was altered in cells by using an Na-acetate-NH$_4$Cl medium. Acetate anions are noncompetitive inhibitors of chloride self-exchange flux (19), and 141 mM acetate almost completely blocks chloride self-inhibition (see Figs. 5 and 6 in reference 19). Acetate, therefore, behaves like the halides as a competitor with chloride at the modifier ($Cl_2$) site which is responsible for self-inhibition. In the presence of acetate, the chloride self-exchange flux follows more closely the equations of Michaelis-Menten kinetics, reflecting mainly the characteristics of the transport ($Cl_1$) site. Thus the action of acetate simplifies the analysis of furosemide inhibition (see Appendix).

The stoichiometry of the reaction between furosemide molecules and the chloride transport mechanism can be surmised by graphing a rearrangement of the Michaelis-Menten kinetic equations in which the slope of the plot indicates the number of molecules of inhibitor acting to inhibit each carrier unit (Fig. 9) (22). The slope in this graph for furosemide inhibition in the absence of acetate (data from Fig. 2) is 1.36, which indicates that the interaction of more than one furosemide with each transport unit is required for inhibition. When one uses the data from Fig. 6 (Dixon plot, $Cl = 146$ mM) where furosemide inhibition is studied in the presence of acetate, the slope of the graph on this plot is 1.03. Thus, furosemide in the presence of acetate requires only one molecule of inhibitor per transport unit, but in the absence of acetate, more than one furosemide molecule binds to each transport unit.

The kinetic analyses in Figs. 5 and 6 indicate that furosemide in the presence of 141 mM acetate behaves as a mixed-type inhibitor of the chloride self-exchange transport system. This result appears to be similar to the halide inhibition of chloride transport reported by Dalmark (11); however, the following comparison of results obtained in the absence and presence of acetate in the experimental medium shows this similarity to be illusory.

The apparent similarity between the action of furosemide and the halides is the observation that in the absence of acetate they both block the self-inhibition by chloride (Fig. 4b in this paper and Figs. 2 and 3 in reference 11). In analogy with the halides and when one considers the two sites proposed by Dalmark, furosemide could compete with chloride at $Cl_1$ and $Cl_2$ to give, respectively, competitive and noncompetitive inhibition as seen with iodide, bromide, and fluoride. The stoichiometry of more than one furosemide molecule per transport unit is consistent with this possibility. In the presence of acetate, however, furosemide also behaves kinetically as a mixed-type inhibitor and requires only one molecule to inhibit each chloride transport unit. Therefore, competition between furosemide and chloride at two sites, $Cl_1$ and $Cl_2$, is untenable. Competition at only $Cl_1$ would not produce mixed-type inhibition but pure competitive inhibition, so exclusive reaction at this site may be removed from consideration. We have argued that acetate competition with chloride occurs at the $Cl_2$ site and it does not seem possible that acetate competition at $Cl_2$ could block
furosemide competition at $Cl_1$ to leave it only acting at $Cl_2$. We therefore must reject the notion that furosemide reacts with $Cl_1$ and $Cl_2$ as Dalmark proposed for halides and we must postulate a third site, $F_1$, which is not the chloride transport site and not the second halide site but which is involved in inhibition by furosemide.

The reaction scheme for mixed-type (competitive and noncompetitive) inhibition by an inhibitor acting at a single site ($F_1$) is diagrammed as follows (27):

\[
\begin{align*}
E + S & \rightleftharpoons K_S ES \xrightarrow{k} E + P \\
E + I & \rightleftharpoons K_I IE + S \xleftarrow{\alpha K_S} IES \\
ES + I & \xleftarrow{\alpha K_S} IES
\end{align*}
\]

The inhibitor, $I$, binds noncompetitively to the enzyme, $E$, and in doing so alters the affinity, $K_S$, of the enzyme for the substrate by a factor $\alpha$ and thus competi-

![Figure 9](image-url)
tively reduces the appearance of the end product, $P$, (in this case the transported anion). Thus furosemide can cause a mixed type of inhibition by binding to the chloride transport unit at a single modifier site, $F_1$, discrete from the transport site. The molecular mechanism by which the affinity of the transport site, $C_{l1}$, is changed is not clear. Two possibilities are that furosemide binding to the $F_1$ site increases local negativity which reduces the chloride concentration at the transport site; and, alternatively, that the binding to $F_1$ decreases the chloride affinity through an allosteric conformational change at the $C_{l1}$ site.

Furosemide apparently can also react with the $C_{l2}$ site. As pointed out earlier, the observation that acetate blocks chloride self-inhibition implies that acetate causes its noncompetitive inhibition at the $C_{l2}$ site. When this conclusion is coupled with the observation that the stoichiometry of furosemide inhibition is reduced from 1.36:1 to 1.03:1 by acetate, one easily and, we believe, correctly is led to conclude that the acetate-inhibitory site and the second site of furosemide binding are the same, namely the $C_{l2}$ site. We therefore believe that furosemide reacts with two nontransport sites on the transport mechanism and that acetate can displace furosemide at one of these sites (see Appendix for this model).

In conclusion, we believe that the negatively charged form of furosemide inhibits the red cell chloride flux by reacting with the transport mechanism at two sites. One ($C_{l2}$) is identical with the site at which chloride can cause self-inhibition. At the second site ($F_1$) furosemide noncompetitively blocks chloride transport and alters the affinity of chloride for the transport site ($C_{l1}$) to produce the pattern of mixed-type inhibition.

**APPENDIX**

From Fig. 9 and for the reasons outlined in the Discussion, it appears that furosemide binds to two nontransport sites on the transport unit. At one of these sites furosemide is displaced by acetate. This is the $C_{l2}$ site which is complexed to the inhibitor when $I$ stands to the right of $E$: $EI$. The $F_1$ site is complexed when $I$ stands to the left of $E$: $IE$.

Although these sites may only be on the outside surface of the cell, the use of an asymmetric model and inclusion of the distribution of carriers between the inside and outside would introduce unwarranted complications at the present level of our knowledge. The analysis has, therefore, been restricted to a single set of reactions and the resulting parameters are, of course, only apparent dissociation constants. The kinetic model and equations for this situation are given below.

\[
\begin{align*}
E + S & \overset{K_S}{\rightleftharpoons} ES & \overset{k}{\rightarrow} E + P \\
+ & \quad + & \quad + \\
I & \quad I & \quad I \\
\downarrow K_I & \quad \downarrow \alpha K_I & \quad \downarrow \beta K_I \\
IE + S & \overset{\alpha K_S}{\rightleftharpoons} IES & \overset{\beta K_I}{\leftleftharpoons} IEIS
\end{align*}
\]

$K_S$ = dissociation constant for carrier and substrate,

$K_I$ = dissociation constant for carrier and inhibitor,

$\alpha$ and $\beta$ are coefficients both greater than 1.0.
BRAZY AND GUNN  Furosemide Inhibition of Chloride Transport

Total carrier $E_i = E + ES + IE + IES + IEI + IEIS$

$$= \frac{[ES]K_S}{[S]} + \frac{[ES][I]}{[S]K_f} + \frac{[ES][P]K_S}{\alpha K_I} + \frac{[ES][P]}{\alpha K_f K_I^2}.$$

The appearance of product is as follows:

$$v_o = k [ES] = \frac{k [E_S][S]}{(K_S + [S])}$$

for the uninhibited reaction, and

$$v_i = \frac{k [E_S][S]}{K_S + [S] + \frac{K_S [I]}{K_f} + \frac{[I] [S]}{\alpha} + \frac{[P] [S]}{\alpha} + \frac{[P]}{\alpha K_f [I]}}$$

for the inhibited reaction. Let $V_{max} = k [E_S]$. Then the reciprocal plot will be

$$\frac{1}{v_i} + \frac{1}{V_{max}[S]} \left( K_S + [S] + \frac{[I]}{K_f} \left( \frac{[S]}{\alpha} + K_S \right) + \frac{[P]}{\alpha K_f} \left( \frac{[S]}{\alpha} + K_S \right) \right),$$

and the Dixon plot will not be linear. The presence of acetate ions reduces the Dixon plot (Fig. 6) to a linear form and this implies that acetate ions cause a marked increase in the value of $\beta$.

The double logarithmic plot (Fig. 9) has the following equation:

$$\frac{v_o}{v_i} = \frac{1}{1 + \frac{[S]}{[S] + K_S \left( \frac{[I]}{\alpha K_I} + \frac{[P]}{\alpha K_f [I]} \right)}}$$

$$\frac{v_o - 1}{v_i} = \frac{1}{1 + \frac{[S]}{[S] + K_S \left( \frac{[I]}{\alpha K_I} + \frac{[P]}{\alpha K_f [I]} \right)}}$$

The slope of the double logarithmic plot will be 1.0 when $\beta$ is large and $[I]$ is small and 2.0 when $[I]$ is large. Acetate again causes $\beta$ to be a larger number and makes the slope = 1.0. Without acetate over the concentration range studied the slope = 1.3.

From the kinetic data and graphs (Figs. 5 and 6) values for the constants were determined. The values for $V_{max}$ and $K_S$ (called $K_{1/2}$) are given in Results. $K_I$ was determined to be $5 \times 10^{-9}$ M from the Dixon plot; a comparable value of $7.7 \times 10^{-9}$ M was obtained from the Lineweaver-Burk plot. The coefficient $\alpha$ has a value of 2.44 from the data on the Lineweaver-Burk plot and 2.64 from the data on the Dixon plot. The coefficient $\beta$ even in the absence of acetate must have a value of at least 100 for the $I^2$ term in the above equations to be less than 10% of the linear term in $I$. This will permit the graph in Fig. 9 of the data obtained in the absence of acetate to appear linear. Furthermore, by using $\beta = 100$ the slope of the calculated line will be 1.25. Thus both the observed linearity ($I^2$ term small) and slope greater than unity (very close to the 1.96 and 1.30 values found by experiment) can be met having $\beta = 100$. If one uses a larger value of $\beta = 1,000$ for the experiments in the presence of acetate, one calculates a slope of 1.03, the same as that found by experiment. In addition, this larger value of $\beta$ is required for the Dixon plot (Fig. 6) to be linear up to furosemide concentrations of $10^{-3}$ M.

We have neglected the form $EI$ (inhibitor bound only to the nontransport $C_{12}$ site) in the above model since $\beta$ is estimated to be so much greater than $\alpha$. If $\beta = 100$, $EI$ will be only 1% of the free $E$ form at $5 \times 10^{-4}$ M furosemide, while the amount of $IE$ (inhibitor bound only to the $F_1$ site) will be equal to that of the free $E$ form. If $\beta = 1,000$ the $EI$ complex is only 0.1% of the free $E$ form and contributes even less. The inclusion of the $EI$ complex would therefore complicate the equations without illuminating the mechanisms. Practically speaking, the $C_{12}$ site is never complexed with furosemide unless the $F_1$ site is already complexed with another furosemide.
This work was supported by grants HL-12157 and HL-18069 from the National Heart and Lung Institute. Peter C. Brazy was a recipient of a National Kidney Foundation Research Fellowship during this work. Robert B. Gunn is the recipient of a Research Career Development Award from the National Heart and Lung Institute (K4-HL-00208).

This work was presented in part at the fall meeting of the American Physiological Society, 1975. The abstract was published in *The Physiologist*. 18:151.

Received for publication 2 June 1975.

REFERENCES

1. Aubert, L., and R. Motais. 1975. Molecular features of organic anion permeability in ox red blood cell. *J. Physiol. (Lond.)* 246:159-179.
2. Brahm, J. 1975. Chloride permeability in human red cells at 0-38°C. 5th International Biophysics Congress. (Abstr.).
3. Burg, M., and N. Green. 1973. Function of thick ascending limb of Henle’s loop. *Am. J. Physiol.* 224:659-668.
4. Burg, M., L. Stoner, J. Cardinal, and N. Green. 1973. Furosemide effect on isolated perfused tubules. *Am. J. Physiol.* 225:119-124.
5. Cabantchik, Z., and A. Rothstein. 1974. Membrane proteins related to anion permeability of human red blood cells. 1. Localization of disulfonic stilbene sites in proteins involved in permeation. *J. Membr. Biol.* 15:207-226.
6. Candida, O. 1973. Short circuit current related to active transport of chloride in frog cornea: effects of furosemide and ethacrynic acid. *Biochim. Biophys. Acta.* 298:1011-1014.
7. Cass, A., and M. Dalmark. 1973. Equilibrium dialysis of ions in nystatin treated red cells. *Nat. New Biol.* 244:47-49.
8. Clapp, J., and R. Robinson. 1968. Distal sites of action of diuretic drugs in the dog nephron. *Am. J. Physiol.* 215:228-235.
9. Dalmark, M. 1975. Chloride transport in human red cells. *J. Physiol. (Lond.)* 250:39-64.
10. Dalmark, M. 1975. Chloride and water distribution in human red cells. *J. Physiol. (Lond.)* 250:65-84.
11. Dalmark, M. 1976. Effects of halides and bicarbonate on chloride transport in human red cells. *J. Gen. Physiol.* 67:223-234.
12. Dalmark, M., and J. Wieth. 1970. Chloride and sodium permeabilities of human red cells. *Biochim. Biophys. Acta.* 219:525-527.
13. Dalmark, M., and J. Wieth. 1972. Temperature dependence of chloride, bromide, iodide, thiocyanate, and salicylate transport in human red cells. *J. Physiol. (Lond.)* 224:583-610.
14. Deuticke, B., and E. Gerlach. 1967. Beeinflussung von Form und Phosphat-permeabilität menschlicher Erythrocyten durch Hämolysine, Benzol-derivate und pharmakologisch aktive Substanzen. *Klin. Wochenschr.* 45:977-983.
15. Goldberg, M. 1973. Handbook of Physiology. J. Orloff and R. Berliner, editors. American Physiological Society, Washington, D. C. Sect. 8. 1003-1031.
16. Guelrud, M., J. Rudick, and H. Janowitz. 1972. Effects of some inhibitors of sodium transport (ATPase inhibitors) on pancreatic secretion. *Gastroenterology.* 62:540-546.
17. Gunn, R. 1973. A titratable carrier for monovalent and divalent inorganic anions in red blood cells. In *Erythrocytes, Thrombocytes, Leukocytes.* E. Gerlach, K. Moser, E. Deutsch, and W. Wilmanns, editors. Georg Thieme Verlag, Stuttgart. 77-82.
BRAZY AND GUNN  Furosemide Inhibition of Chloride Transport

18. GUNN, R., and J. COOPER. 1975. Effect of local anesthetics on chloride transport in erythrocytes. J. Membr. Biol. 25:311–326.

19. GUNN, R., M. DALMARK, D. TO_mERSON, and J. WIETH. 1973. Characteristics of chloride transport in human red blood cells. J. Gen. Physiol. 61:185–206.

20. Ho, M., and G. GUIDOTTI. 1975. A membrane protein from human erythrocytes involved in anion exchange. J. Biol. Chem. 250:675–683.

21. Hook, J., and H. WILLIAMSON. 1965. Lack of correlation between natriuretic activity and inhibition of renal Na-K-activated ATPase. Proc. Soc. Exp. Biol. Med. 120:358–360.

22. Johnson, F., H. Eyryng, and R. Williams. 1942. The nature of enzyme inhibitors in bacterial luminescence: sulfanilamide, urethane, temperature and pressure. J. Cell. Comp. Physiol. 20:247–268.

23. Klahr, S., J. Yates, and J. Bourgoignie. 1971. Inhibition of glycolysis by ethacrynic acid and furosemide. Am. J. Physiol. 221:1038–1043.

24. Mangos, J., S. McSherry, S., Nousia Arvantakis, and R. Schilling. 1974. Transdustral fluxes of anions in the rat pancreas. Proc. Soc. Exp. Biol. Med. 146:321–328.

25. Nakano, S. 1968. Physiological observations on the exocrine pancreas. Effects of some agents on pancreatic secretion. Nagoya J. Med. Sci. 31:79–116.

26. Sachs, J. 1971. Ouabain-insensitive sodium movements in the human red blood cell. J. Gen. Physiol. 57:259–282.

27. Segel, I. 1975. Enzyme Kinetics. Behavior and Analysis of Rapid Equilibrium and Steady State Enzyme Systems. John Wiley & Sons, New York. 161–202.

28. Webb, J. 1963. Enzyme and Metabolic Inhibitors. Vol. 1. Academic Press, Inc., New York. 149–165.

29. Wieth, J. 1970. Effect of some monovalent anions on chloride and sulfate permeability of human red cells. J. Physiol. (Lond.). 207:581–609.

30. Wilkinson, G. 1961. Statistical estimations in enzyme kinetics. Biochem. J. 80:324–332.

31. Zadunaisky, J. 1966. Active transport of chloride in frog cornea. Am. J. Physiol. 211:506–512.