Kinetics of Residual Chloride Transport in Human Red Blood Cells after Maximum Covalent 4,4'-Diisothiocyanostilbene-2,2'-Disulfonic Acid Binding

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ABSTRACT Irreversible inhibition, 99.8% of control values for chloride transport in human red blood cells, was obtained by well-established methods of maximum covalent binding of 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS). The kinetics of the residual chloride transport (0.2%, 106 pmol/cm²·s⁻¹) at 38°C, pH 7.2) was studied by means of 36Cl⁻ efflux. The outside apparent affinity, expressed by $K_{i/2,c}$, was 34 mM, as determined by substituting external KCl by sucrose. The residual flux was reversibly inhibited by a reexposure to DIDS, and by 4,4'-dinitrostilbene-2,2'-disulfonate (DNDS), phloretin, salicylate, and α-bromo-4-hydroxy-3,5-dinitroacetophenone (Killer III) (Borders, C. L., Jr., D. M. Perez, M. W. Lafferty, A. J. Kondow, J. Brahm, M. B. Fenderson, G. L. Breisford, and V. B. Pett. 1989. Bioorganic Chemistry. 17:96–107), to ~0.001% of control cells, which is a flux as low as in lipid bilayers. The reversible DIDS inhibition of the residual chloride flux depended on the extracellular chloride concentration, but was not purely competitive. The half-inhibition concentrations at [Cl⁻] = 150 mM in control cells ($K_{i,o}$) and covalently DIDS-treated cells ($K_{i,c}$) were: DIDS, $K_{i,c} = 73$ nM; DNDS, $K_{i,c} = 6.3$ μM; phloretin, $K_{i,c} = 19$ μM; salicylate, $K_{i,c} = 4$ mM; Killer III, $K_{i,c} = 8$ mM.

INTRODUCTION

Stilbene derivatives, such as 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), 4,4'-diisothiocyanano-dihydrostilbene-2,2'-disulfonic acid (H₂DIDS), 4,4'-dinitrostilbene-2,2'-disulfonate (DNDS), and 4,4'-dibenzamidostilbene-2,2'-disulfonate (DBDS), are characterized as being specific and efficient inhibitors of anion transport in red blood cells. The specificity of inhibition appears from studies showing that the compounds have little or no effect on other transport processes at concentrations that efficiently inhibit chloride transport. The efficacy is illustrated by the fact that the inhibitors can inhibit transport of monovalent anions such as chloride and bicarbonate ions almost completely (DIDS: Funder, Tosteson, and Wieth, 1978; Brahm and Wimberley, 1989; Gasbjerg and Brahm, 1991; DNDS: Fröhlich, 1982; H₂DIDS:
Cabantchik and Rothstein, 1972; Lepke, Fasold, Pring, and Passow, 1976; DBDS: Dix, Verkman, and Solomon, 1986). It is, however, noteworthy that the maximum inhibition obtained under the conditions used in previous studies is not 100%, but in general ≤ 99.8%. Although the residual chloride permeability appears quantitatively insignificant in comparison with the uninhibited permeability, its magnitude is still far above the net chloride permeability (Hunter, 1977; Knauf, Fuhrmann, Rothstein, and Rothstein, 1977; Bennekou and Stampe, 1988).

A 99.8% inhibition of chloride transport in human red blood cells can be obtained by irreversible binding of DIDS to capnophilin (band 3 or AE1). By exposing these cells to DIDS in the efflux medium a further reduction of chloride transport can be obtained, giving a total inhibition of 99.999%. We will use the term “DIDS-C” when dealing with cells with covalently bound DIDS and the term “DIDS-CR” when dealing with cells with irreversibly bound DIDS exposed to free DIDS in the efflux medium. The term “control cells” is used for cells with no DIDS bound to the cell membrane.

We have studied the kinetics of chloride transport across the red cell membrane in DIDS-C cells. To make a quantitative comparison with chloride transport kinetics in control cells, we have also performed experiments with control cells when data could not be obtained from the literature.

MATERIALS, METHODS, AND CALCULATIONS

All media were prepared from reagent grade chemicals. A standard medium (A) containing 150 mM KCl and 0.5–2 mM KH$_2$PO$_4$ was used for the preparation of the cells and the flux experiments. In experiments where the extracellular KCl concentration was lowered, sucrose was added to the medium to keep osmolarity constant.

Inhibitors

DIDS, prepared as the sodium salt as described previously (Funder et al., 1978), was used for primary stock solutions of 10$^{-2}$ M DIDS in distilled water that were stored frozen until use. DNDS (Pfaltz and Bauer Inc., Waterbury, CT) was kept in the dark until use to avoid photoisomerization and was added from a stock solution of 10 mM in the efflux medium. Phloretin (Sigma Chemical Co., St. Louis, MO) was dissolved in ethanol to obtain a stock solution of 0.1 M. Final ethanol concentration was < 0.1% (vol/vol). $\alpha$-Bromo-4-hydroxy-3,5-dinitroacetophenone (Killer III) was synthesized and kindly supplied by Dr. C. L. Borders, Jr. The compound was dissolved in the efflux medium. Sodium salicylate (pro analysis; E. Merck, Darmstadt, Germany) was dissolved in a 1 M stock solution.

Preparation of Cells

Freshly drawn heparinized blood was washed once in medium A, pH 7.2, the buffy coat was removed after centrifugation, and the cells were washed three more times in this medium. To prepare DIDS-C cells the erythrocytes were incubated at 38°C for 45 min at a hematocrit of ~ 50% in a solution with 50 $\mu$M DIDS. The incubation conditions give six to eight times the number of DIDS molecules needed to load the 1–1.1 million sites per cell that are capable of binding DIDS covalently. We obtained the same degree of inhibition whether we performed the DIDS binding procedure once or twice ($f_\text{eff}$ [pmol/cm$^2$·s$^{-1}$] 96.3 [SD ± 8.1] vs. 94.1 [SD ± 11.0], n = 10, P = 0.63). Finally, the cells were incubated with 3.7–18.5 kBq (0.1–0.5 $\mu$Ci) $^{36}$Cl (obtained as KCl; NEN-DuPont, Boston, MA) per ml cell suspension with a hematocrit
of ~50%. The cells were packed for determinations of intracellular radioactivity, cell water content, and efflux experiments as previously described (Dalmark and Wieth, 1972; Brahm, 1977). Experiments with DIDS and Killer III were performed by exposing control cells or DIDS-C cells to the inhibitor of choice added to the efflux medium. The experiments with DNDS, salicylate, and phloretin included washes of the cells, both controls and DIDS-C cells, in medium A containing the inhibitor of choice at the same concentration as added to the medium in which the efflux experiment was performed.

Preparation of Cells for Efflux Experiments in the pH Range 6.0–9.4

The DIDS treatment of the cells was done at pH 7.2, 38°C. The cells were next washed in large volumes of medium A. After each centrifugation the supernatant was sucked off and new medium was added carefully without resuspension of the pellet. Then 0.1 N KOH or HCl was added to the medium and the cells were resuspended. The suspension was then kept at 38°C for 3–4 min to obtain redistribution of ions across the cell membrane. This procedure was repeated until pH was stable at the value at which the flux should be performed. The cells were now loaded with $^{36}$Cl and packed as described above.

Efflux Experiments

The tracer efflux experiments were carried out at 38°C, pH 7.2, unless stated otherwise, by means of the Millipore-Swinnex filtering technique or the continuous flow tube method as described previously (Dalmark and Wieth, 1972; Brahm, 1989). The rate of efflux in both DIDS-C and DIDS-CR cells was well described as a monoexponential time course for solute transport in a closed two-compartment system by:

$$a_t = a_\infty(1 - e^{-kt}) + a_0 e^{-kt}$$

(1)

where $a_\infty$ and $a_0$ are the extracellular radioactivity at time $t$, and at isotopic equilibrium ($t > 6 \cdot T_{1/2}$), respectively, and $a_0$ is the extracellular radioactivity at time zero, caused by trapping of isotope between the cells during the packing procedure. The trapped radioactivity amounts to 2–8%, depending on which efflux method was used. The higher fraction of trapped radioactivity in the flow tube experiments, however, does not contribute to the magnitude of the slope of the tracer efflux curve (Brahm, 1989). The rate coefficient, $k$ (s$^{-1}$), for tracer efflux equals the negative value of the slope of In ($1 - a_t/a_\infty$) vs. time $t$, and was determined by linear regression analysis.

Under equilibrium conditions the extracellular compartment is much larger than the intracellular compartment, and $k$ is the rate coefficient of the unidirectional efflux of tracer. In experiments with low extracellular chloride concentrations the measured rate coefficient ($k'$) was adjusted to the "true" value ($k$) for the unidirectional solute flow as described by Gasbjerg and Brahm (1991).

The unidirectional efflux of chloride, $J_{\text{eff}}$ (mol·cm$^{-2}$·s$^{-1}$), is defined by:

$$J_{\text{eff}} = k \cdot (V_c/A_c) \cdot [\text{Cl}]^{[0]}$$

(2)

where $(V_c/A_c)$ is the ratio of the cell water volume to the cell membrane area. $V_c$ was determined as described previously (Dalmark and Wieth, 1972), and $A_c$ was assumed to be constant, $1.42 \cdot 10^{-7}$ cm$^2$/cell (Wieth, Funder, Gunn, and Brahm, 1974; Brahm, 1982). $[\text{Cl}]^{[0]}$ is the intracellular chloride concentration (moles per cubic centimeter) as calculated from the relation $[\text{Cl}]^{[0]}/[\text{Cl}]^{[0]} = r = [^{36}\text{Cl}]^{[0]}/[^{36}\text{Cl}]^{[0]}$. 

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RESULTS

Inhibition of Chloride Transport by DIDS

DIDS is an efficient inhibitor of anion transport in human red blood cells also at 38°C (pH 7.2) as demonstrated in Table I. The control cells had a chloride self-exchange flux, \( J_{\text{cl}}^{\text{eq}} \), of 52,400 (SD \( \pm \) 6,200) pmol cm\(^{-2}\) s\(^{-1}\) \((n = 17)\). In DIDS-C cells the anion flux was reduced by 99.8% to \( J_{\text{cl}}^{\text{eq}} \) = 106 (SD \( \pm \) 17) pmol cm\(^{-2}\) s\(^{-1}\) \((n = 30)\). The residual chloride flux in DIDS-C cells was further reversibly inhibited by addition of DIDS to the efflux medium. The combined inhibitory effect of irreversible DIDS binding to the cell membrane and addition of 50 \( \mu \)M DIDS to the efflux medium gave a maximum inhibition of 99.999\% \((J_{\text{cl}}^{\text{eq}})_{\text{DIDS}} = 0.60 \text{ (SD } \pm 0.16\text{) pmol cm}^{-2}\text{ s}^{-1} \text{ (} n = 15\text{)}\). Similar results were obtained using resealed red cell ghosts (data not shown).

| Flux | Permeability |
|------|--------------|
| Mean  | SD | N | \( \text{cm}^{2}\text{s}^{-1} \) |
| No inhibition | 52,400 | 6,200 | 17 | 5.0 \( \times \) 10\(^{-4}\) |
| Inhibition by covalent bound DIDS to RBC | 106 | 17 | 30 | 1.0 \( \times \) 10\(^{-6}\) |
| Inhibition as above + 50 \( \mu \)M DIDS in flux medium | 0.60 | 0.16 | 15 | 5.7 \( \times \) 10\(^{-9}\) |

Lipid bilayers:
- Phospholipid* (20°C): 0.1 \( \times \) 10\(^{-9}\)
- Egg lecithin\(^{\text{c}}\) (23°C): 15 \( \times \) 10\(^{-9}\)
- Phospholipid\(^{\text{c}}\) (29°C): 10 \( \times \) 10\(^{-9}\)

*Toyoshima and Thompson (1975)
\(^{\text{c}}\)Gutknecht, Graves, and Tosteson (1978)
\(^{\text{c}}\)Tosteson and Wieth (1979)

The reversible inhibition of chloride transport in DIDS-C cells depended on the concentration of DIDS in the efflux medium as demonstrated in Figs. 1 and 2. The semilogarithmic plot of Fig. 1 depicts the internal fraction of radioactive tracer as a function of time, and illustrates that linear efflux curves were obtained at the different DIDS concentrations in the efflux medium. Hence, the rate of tracer efflux is constant at each DIDS concentration, and the unidirectional chloride efflux could be calculated according to Eqs. 1 and 2 (cf. Materials, Methods, and Calculations).

Fig. 2 A shows the anion flux in DIDS-C cells as a function of the apparent external DIDS concentration (0–1 \( \mu \)M) in the efflux medium. The DIDS concentration is termed apparent because reversible binding of DIDS to the membrane reduces the free DIDS concentration in the solution. However, it is possible (see Appendix) to derive the half-inhibition constant \((K_i, \text{molar})\) under these circumstances by means of an Easson-Stedman plot of \([1/(1-F)]\) vs. \([(\text{DIDS}_7)/F]\), where \( F \) is the fractional inhibi-
Although [DIDS] is the concentration of DIDS molecules in the efflux medium before addition of cells. In these experiments the chloride concentrations and the concentration of DIDS binding sites (i.e., the hematocrit) are kept constant. An Easson-Stedman plot of reversible DIDS inhibition of chloride flux in DIDS-C cells is shown in Fig. 2 B. From these data a $K_{ic}$ of 66 (SE ± 6) nM could be determined (see
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Eq. A18). The number of binding sites per cell was determined to be ~50,000, a value not significantly different from zero (P = 0.63).

External Affinity to Chloride in DIDS-C Cells

The external affinity of the residual chloride transport system to chloride was determined by replacing external KCl with sucrose and keeping the intracellular chloride constant at ~100 mmol/liter cell water. Control experiments with extracellular chloride concentrations <1 mM (data not shown) showed no detectable effect of the ionophore valinomycin (1 μM) on initial 36Cl influx rate; i.e., the residual flux is not affected significantly by changes in membrane potential. Other control experiments with low extracellular chloride (4 mM; data not shown) using nonlinear regression analysis (without the use of an equilibrium sample) gave results similar to those obtained using Eq. 1 and thus demonstrated that even under these extreme conditions the radioactivity in the equilibrium sample was not affected significantly by net efflux of chloride. Fig. 3 shows the unidirectional 36Cl efflux as a function of extracellular chloride concentration. A Michaelis-Menten-like saturation curve was fitted to the data by a nonlinear regression analysis that revealed an external affinity, expressed by a $K_{1/2,c}$ of 34 (SE ± 5) mM (for interpretation of the outside apparent affinity, $K_{1/2}$, see, for example, Eq. 10a in Fröhlich and Gunn, 1986 or Eqs. A4–A6 in Gasbjerg and Brahm, 1991).

![Figure 3](image-url)

**Figure 3.** The dependence of 36Cl efflux in DIDS-C cells (pH 7.2, 38°C) on external chloride concentration. External KCl was replaced by sucrose in order to keep external osmolality constant.

![Figure 4](image-url)

**Figure 4.** The half-inhibition constant of reversible DIDS inhibition of residual chloride transport in DIDS-C cells, $K_{ic}$, as a function of external chloride concentration [Cl\(^{\text{in}}\)] (pH 7.2, 38°C). By linear extrapolation to [Cl\(^{\text{in}}\)] = 0, $K_{ic}$ was determined to be 43 (SE ± 6) nM. Regression line equation: $Y = 0.23$ (SE ± 0.08)X + 43 (SE ± 6).
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\[ K_{i,c} = 6.3 \mu M \]

\[ K_{i,c} = 22 \mu M \]

**FIGURE 5.** (A) Inhibition of chloride self-exchange flux in control cells by DNDS. Because of the slow binding rate of DNDS to the membrane (compared with efflux of \( ^{36} \text{Cl}^- \)) the cells were washed in the efflux medium before the flux experiment. The experiments were performed with the continuous flow tube method at 38°C, pH 7.2. (B) DNDS inhibition of chloride self-exchange flux in DIDS-C cells. The experiments were performed with the Millipore-Swinnex filtering technique at pH 7.2, 38°C.

Dependence of Reversible DIDS Inhibition of Chloride Transport on External Chloride Concentration in DIDS-C Cells

The half-inhibition constant, \( K_{i,c} \), for reversible DIDS inhibition of the residual chloride transport in DIDS-C cells at different external chloride concentrations was determined by replacing external KCl with sucrose. The results are depicted in Fig. 4, showing that \( K_{i,c} \) decreased slightly with a decrease of \([\text{Cl}^-]_o\) (\( P = 0.05 \)).

Inhibition of \( J'_o \) and \( J'_c \) by DNDS, Salicylate, Phloretin, and Killer III

To further characterize the residual chloride transport in DIDS-C cells, the \( K_i \) values for DNDS, salicylate, phloretin, and Killer III were also determined. Fig. 5, A and B, shows the inhibition of chloride transport by means of DNDS in control and DIDS-C

| Inhibitor | \( K_{i,o} (0 \text{ DIDS}) \) | \( K_{i,c} (+ \text{ DIDS}) \) |
|-----------|-----------------|-----------------|
| DNDS      | 6 \( \mu M \)   | 22 \( \mu M \)   |
| Phloretin  | 19 \( \mu M \)  | 17 \( \mu M \)  |
| Killer III | 10 \( \mu M \)  | 10 \( \mu M \)  |
| Salicylate | 4 mM            | 8 mM            |
cells as a function of the external DNDS concentration. Because of the relatively low affinity in control and DIDS-C cells, $K_i$ is determined directly from the two plots (Eq. A21). The half-inhibition constants for DNDS in control and DIDS-C cells ($K_{i,o}$ and $K_{i,c}$) were determined to be 6.3 (SE ± 0.6) μM and 22 (SE ± 3) μM, respectively. The same procedure was used to determine $K_{i,o}$ and $K_{i,c}$ for salicylate, phloretin, and Killer III, and the results are summarized in Table II.

**Chloride Flux as a Function of pH**

Fig. 6 shows the residual chloride flux at 38°C in DIDS-C cells in the pH range 6.0–9.4 under conditions of equilibration between the external (pH) and internal (pH). In clear contrast to what was found in control cells in the same pH range (Brahm, 1977), no maximum for chloride efflux was observed in DIDS-C cells. The same observation was made with DIDS-CR cells (data not shown).

**Temperature Dependence of Chloride Transport after Maximum Irreversible DIDS Inhibition**

The temperature dependence of the residual chloride flux in DIDS-C cells was determined in red blood cells in the temperature range 0–38°C at pH 7.2. Fig. 7 is an Arrhenius plot of the temperature dependence of the residual chloride flux. An apparent activation energy of 77 (SE ± 3) kJ·mol⁻¹ was determined by linear regression analysis. The apparent activation energy in DIDS-CR cells was 69 (SE ± 2) kJ·mol⁻¹ (data not shown).

![Figure 6](image1.png)

**Figure 6.** $^{36}$Cl⁻ efflux from DIDS-C cells (38°C) in the pH range 6.0–9.4.

![Figure 7](image2.png)

**Figure 7.** Arrhenius plot of chloride self-exchange flux in DIDS-C cells (pH 7.2). The slope of the straight line gives an apparent activation energy of the transport of 77.2 (SE ± 2.5) kJ·mol⁻¹.
DISCUSSION

Anion transport in human red blood cells is very fast under physiological conditions. The current view is that the transport protein (band 3, capnophilin, AE1) mediates the transport of monovalent anions by a ping-pong mechanism with transport sites that face either the external or internal side of the membrane, and translocate only when an anion is bound to the site. This nonconductive self-exchange flux almost completely overshadows a tiny conductive flow of anions. Studies of the self-exchange flux and conductive net flux of chloride in red cells (Brahm, 1977; Hunter, 1977; Knauf et al., 1977; Lassen, Pape, and Vestergaard-Bogind, 1978; Fröhlich, 1982) have led to the suggestion that the coupling of the obligatory self-exchange once out of 10,000–100,000 cycles may fail and establish the net flow of anions. Fröhlich (1982), who suggested either that the transporter returned empty from one to the other side of the membrane (slippage) or that a chloride ion with low frequency may pass across the membrane without a concomitant conformational change of the transporter (tunneling), concluded that tunneling was probably the origin of the chloride conductance. Conductance studies by Knauf et al. (1977) suggested that 60–70% of the chloride conductance was inhibitable by DIDS, whereas Bennekou and Stampe (1988) showed that the conductive flow of chloride could be inhibited almost 100%.

We were puzzled by the fact that the so-called efficient and specific inhibitor of anion exchange, DIDS, after maximum covalent binding to the red cell membrane inhibited unidirectional chloride efflux by only 99.8%. At 38°C the chloride self-exchange flux is \( \sim 52,400 \text{ pmol-cm}^{-2}\text{s}^{-1} \), and the apparent permeability is \( \sim 5.0 \times 10^{-4} \text{ cm-s}^{-1} \) (Brahm, 1977; this study). A residual flux of 0.2% equals an apparent permeability of \( \sim 1.0 \times 10^{-6} \text{ cm-s}^{-1} \) that is still one to two orders of magnitude larger than the value obtained from the conductance studies (see Bennekou and Stampe, 1988). However, this residual flux may be further reduced to 0.001% of the control value by adding 50 \( \mu \text{M} \) DIDS to the efflux medium in experiments with DIDS-C cells. Our control experiments show that this inhibition is reversible and that the reversibly bound DIDS has a very high affinity for the residual transport system with a \( K_i \) of 73 nM. Our results further indicate that the number of binding sites for reversible DIDS binding is very small compared with the one million binding sites found for reversible DIDS binding in control cells at 0°C (Janas, Bjerrum, Brahm, and Wieth, 1989).

The fact that the Easson-Stedman analysis shows that the number of binding sites is small makes it unlikely that the residual transport of chloride is due to a general slowing down of all transport sites mediating the anion transport. We have considered whether the residual flux is caused by a progressive loss of the ability to bind DIDS irreversibly, e.g., by a glycation of the site for irreversible DIDS binding. If so, the number of functional transporters after irreversible DIDS binding should increase with cell age and a wide range of permeabilities for chloride should be represented in the population of DIDS-C cells. To evaluate this possibility we have made the following theoretical assumptions: (a) the lifetime for an erythrocyte is 120 d; (b) chloride efflux in “newborn” erythrocytes is inhibited by irreversibly bound DIDS down to the level found in DIDS-CR cells; (c) the erythrocytes gradually (linearly) lose the ability to bind DIDS irreversibly with time (e.g., because of glycation of the site for irreversible DIDS binding); (d) the half-time for chloride tracer efflux from this
theoretical, inhomogenous erythrocyte population (obtained from linear regression on a semilog plot) is 29 s (as we find experimentally in DIDS-C cells). With these assumptions we calculated that the rate constant for chloride tracer efflux increases from $1.4 \times 10^{-4} \text{ s}^{-1}$ in “newborn” cells (the rate constant for DIDS-CR cells) to $727 \times 10^{-4} \text{ s}^{-1}$ in cells aged 120 d. We see that: (a) the correlation coefficient for the semilog plot of the theoretically obtained efflux curve is $<0.99$; (b) the intercept is $<0.88$; and (c) the semilog plot is clearly curved with a marked deviation (up to 7%) from the best fit of a monoexponential curve within the time range used. All the points are inconsistent with our experimental results, making it unlikely that increasing cell age abolishes the irreversible binding of DIDS to the cell membrane.

We may therefore conclude that in the population of DIDS-C cells there is also a narrow range of chloride permeabilities; i.e., the number of functional transport sites per cell varies insignificantly in the population of DIDS-C cells. The residual flux is mediated by a small fraction of transport sites that perform at the optimum activity after maximal irreversible DIDS binding, or a somewhat larger fraction of transport sites mediates the anion transport with a somewhat slower rate. The fraction of transport sites still performing anion transport is, under all circumstances, small compared with the total amount of transport sites.

It would be of interest to compare the $K_i$ value of 73 nM for DIDS inhibition of the residual transport system at 38°C with the corresponding $K_i$ value for reversible DIDS inhibition of chloride transport in control cells. The reversible DIDS inhibition in control cells is believed to be caused by mixed hydrophobic and ionic interactions between band 3 protein ligands and the two sulfonic groups in DIDS. The irreversible binding occurs as a second step between the reversibly bound DIDS and an adjacent amino group of a lysine residue (see Janas et al., 1989). Because the irreversible binding takes a considerable time at 38°C (time constant $1/k \approx 2.2$ min; Janas et al., 1989) compared with the efflux half-time of $\sim 50$ ms, the kinetics of a reversible inhibition of chloride transport in control cells should be obtainable by means of the rapid continuous flow tube method at this temperature. However, at 38°C the rate constant for reversible DIDS binding to the cell membrane appears to be too slow compared with the rate constant for $^{36}$Cl efflux. We found (data not shown) that at the lower inhibitor concentrations the reversible inhibition was not at its maximum before most of the intracellular radioactivity was lost to the efflux medium, making the determinations of rate constant too uncertain. The half-time for irreversible

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1 Assuming a diffusion coefficient for DIDS in water in the range of $10^{-6}$–$10^{-3} \text{ cm s}^{-1}$, the maximum (diffusion limited) bimolecular rate constant for reversible DIDS binding is in the range of $10^9$–$10^{10} \text{ M}^{-1} \text{s}^{-1}$. If the DIDS concentration is 1 nM, then the rate constant for DIDS binding is in the range of 1–10 s$^{-1}$. The rate constant for chloride transport in control cells is $\sim 10$ s$^{-1}$; hence, at very low DIDS concentrations the reversible DIDS binding appears to be diffusion limited. However, we also observed increasing DIDS inhibition with time in the lower micromolar range, where the maximum rate constant for DIDS binding is expected to be in the range of $10^9$–$10^{10} \text{ s}^{-1}$ (i.e., much faster than the chloride exchange rate). The explanation for this slow rate of reversible DIDS binding is discussed by Janas et al. (1989), who also observed a surprisingly slow rate of reversible DIDS binding at 0°C. The localization of the DIDS binding site 3.0–4.5 nm from the internal membrane surface suggested by Rao, Reithmeier, and Cantley (1979) supports the idea that steric hindrance could be the cause of the slow binding rate. However, this explanation is at variance with the observation that $\text{H}_2\text{DIDS}$, which is believed to bind to the DIDS binding site, binds much faster than DIDS at 0°C (Shami, Rothstein, and Knauf, 1978).
DIDS binding, on the other hand, is too short to allow a prewash of the cells in the DIDS-containing efflux medium before the efflux experiment, as was done at 0°C (Janas et al., 1989). However, at 0°C the half-time for irreversible DIDS binding is ~3 h and reversible inhibition of 36Cl efflux with DIDS has been studied in prewashed control cells by means of the Millipore-Swinnex method (Janas et al., 1989). The affinity of reversible DIDS binding in control cells at 0°C was also found to be very high, with a $K_i$ of 30 nM.

Our observation that covalently bound DIDS reduces the number of binding sites for reversible DIDS binding is compatible with the concept that the binding sites for reversible and irreversible DIDS binding in control cells are very close to each other. We cannot exclude that the difference between the $K_i$ values obtained with control cells at 0°C (30 nM) and with DIDS-C cells at 38°C (73 nM) is due to temperature effects, indicating that the reversible DIDS inhibition in DIDS-C cells is of the same kind as the reversible DIDS binding in control cells, the only difference being that the residual transporters cannot bind DIDS irreversibly.

Our data obtained at 38°C indicate that $K_i$ depends on extracellular chloride (Fig. 4). However, our data do not seem to fit a ping-pong model where DIDS only binds to the outward-facing conformation of the transport protein and competes with extracellular chloride (Eq. A20). From the data shown in Fig. 4, a $K_i^{1/2,c}$ of 189 (SE ± 72) mM is required to explain the data if the inhibition is of the competitive type. However, a value of 34 (SE ± 5) mM was obtained from the data shown in Fig. 3. The discrepancy can be explained by assuming that the inhibition is only partly competitive, or that the population of residual transporters is not homogenous but consists of subpopulations with different affinities for competitively inhibiting DIDS. The apparent outside affinity, expressed by the $K_i^{1/2,c}$ value of 34 mM in DIDS-C cells, should be compared with the $K_i^{1/2}$ value of 2.8 mM obtained in control cells (Knauf and Brahm, 1986).

The inhibition experiments with the reversibly binding inhibitor DNDS did not allow a determination of DNDS binding sites by the Easson-Stedman method because DNDS has a relatively low affinity to the transporter. In control cells we found a $K_i$ of 6.3 (SE ± 0.6) μM at [Cl] = 150 mM. Assuming competitive inhibition (Fröhlich, 1982) and a $K_i^{1/2}$ of 2.8 mM (Knauf and Brahm, 1986), a true $K_i$ (dissociation constant) of 115 nM is obtained by use of Eq. A20. This result extends the weak temperature dependence of DNDS binding observed by Fröhlich (1982), who obtained dissociation constants of 84, 86, and 90 nM at 0, 10, and 20°C, respectively.

In DIDS-C cells we found a significantly higher $K_i$ of 22 (±3) μM for DNDS.

The Arrhenius plot of the residual chloride flux in DIDS-C cells shows a constant apparent activation energy of 77 (SE ± 3) kJ·mol⁻¹ in the temperature range 0–38°C. Brahm (1977) found a nonlinear temperature dependence for chloride efflux in control cells in the same temperature range with apparent activation energies of 125 (SE ± 4) kJ·mol⁻¹ in the range 0–15°C and 84 (SE ± 4) kJ·mol⁻¹ in the range 15–38°C.

In DIDS-CR cells we found an Arrhenius activation energy of 69 (SE ± 2) kJ·mol⁻¹. This value is not very different from the values found in DIDS-C cells. Toyoshima and Thompson (1975) determined the Arrhenius activation energy for chloride transport across an artificial lipid bilayer membrane using egg phosphatidylcholine vesicles and obtained a value of 19 ± 2 kcal·mol⁻¹ (80 ± 8 kJ·mol⁻¹) that is not significantly
different from the value obtained in DIDS-CR cells. Hence, the activation energy for chloride transport in DIDS-CR cells does not allow us to determine whether a phospholipid or a nonphospholipid transport mode is responsible for chloride transport in DIDS-CR cells.

It is of interest to compare our results obtained by use of tracer methods with inhibition data obtained by studies of net chloride efflux. However, it should be noted that the apparent constant field permeabilities that can be obtained from conductance values (e.g., by Eq. 262 in Sten-Knudsen, 1978) may not be equivalent to the permeabilities obtained in tracer studies even in DIDS-CR cells (see, for example, Bennekou and Stampe, 1988). If we assume that they are equivalent, we can conclude that the combined effect of reversibly and irreversibly bound DIDS inhibits exchange more efficiently than conductance. The conductance study of Bennekou and Stampe (1988) at 37°C in control cells and in cells exposed to 16 µM DIDS in the efflux medium gave apparent constant field permeabilities of 7.6·10^{-8} and 0.27·10^{-8} cm·s^{-1}, respectively. The values should be compared with our respective values of 50,000·10^{-8} and 0.57·10^{-8} cm·s^{-1} for 36Cl efflux (see Table I). Combining the data, it can be calculated that the exchange component of the chloride efflux is reduced by 99.9994% (or 170,000 times), whereas the conductive component is reduced by only 96.4% (or 28 times). It is not likely that the residual conductive transport is caused solely by conductive chloride efflux across the lipid bilayer. According to Toyoshima and Thompson (1975), the permeability for chloride transport in phosphatidylcholine vesicles (Table I) is due principally to a carrier-mediated exchange process involving a phospholipid-HCl complex.

The permeability data for chloride transport across artificial lipid membranes are somewhat scattered and values from 0.01 to 1.5·10^{-8} cm·s^{-1} are obtained in the temperature range 20–23°C (Table I). At 20°C we measured a permeability coefficient of 0.1·10^{-8} cm·s^{-1} in DIDS-CR cells (data not shown). From the red blood cell conductance data and the data obtained with artificial lipid bilayer membranes we suggest that the residual permeability in DIDS-CR cells is due to chloride efflux through a residual conductive pathway as well as phospholipid-mediated chloride exchange across the lipid bilayer of the red cell membrane. The scatter of permeability data on artificial lipid bilayer membranes makes it impossible for us to conclude whether nonphospholipid, nonconductive chloride transport (protein-mediated exchange) contributes to the residual permeability in DIDS-CR cells. Hence, it is possible that covalent and reversibly bound DIDS in combination inhibit nonphospholipid, nonconductive chloride transport completely.

**Conclusion**

Our comparative study of unidirectional chloride efflux in control cells and in cells treated irreversibly with DIDS shows that the kinetics of chloride transport in control and DIDS-treated cells differ with respect to the outside apparent affinity to chloride and DNDS, to pH, and to temperature dependence. The number of residual transporters after maximum irreversible DIDS binding is very low compared with control cells in accordance with the notion that the function of a transporter is blocked by irreversible DIDS binding that also prevents a further reversible binding of DIDS to the transporter. It is not possible from our data to conclude whether the
population of residual transporters is a subpopulation of slightly modified band 3 molecules or a transport system outside band 3 that cannot bind DIDS covalently.

It is important to note that maximum irreversible DIDS binding does not inhibit the mediated chloride transport completely. A further reversible DIDS binding is necessary to reach an apparent chloride permeability as low as values reported for artificial lipid bilayer membranes.

APPENDIX

In experiments where the anion exchange is determined at low concentrations of the high affinity inhibitor DIDS, the concentration of free DIDS molecules in the suspension may be significantly lower than the DIDS concentration in the efflux medium ([DIDS]_{t}) before addition of the packed red blood cells. Because of the high affinity of the inhibitor, a significant degree of inhibition of anion exchange is attained at inhibitor concentrations that are of the same order of magnitude as the concentration of inhibitor binding sites at the hematocrit of 0.5-1% used in our efflux experiments. Therefore, the concentration of DIDS molecules in the efflux medium may be significantly reduced by resuspension of packed red blood cells. If one uses [DIDS]_{t} in calculations of the half-inhibition constant (K_{i}), for example, this value is overestimated because the free DIDS concentration that half-inhibits the anion efflux is lower than [DIDS]_{t}.

Problems of this kind are well known in enzyme kinetics, and a suitable method for determination of inhibition constants has been developed (the Easson and Stedman method; Webb, 1963). Although this method has been used in studies of inhibition of chloride efflux from red cells (Janas et al., 1989), it was not shown that it is valid for the proposed ping-pong mechanism for anion exchange in these cells.

The principle of the method is as follows: In a plot of 1/(1 - F) vs. [I]/F, where F is the fractional inhibition and [I] is the total concentration of the inhibitor, a straight line should be obtained for systems with 1:1 probe-ligand interactions. K_{i} is determined as the reciprocal of the slope of the straight line, and the intercept with the ordinate equals minus the concentration of binding sites divided by K_{i}.

The kinetics of the anion exchange mechanism (the ping-pong model) has been described thoroughly by Fröhlich and Gunn (1986). For a shorter description of ping-pong kinetics under self-exchange conditions, refer to Gasbjerg and Brahm (1991). The reaction scheme for the ping-pong mechanism is:

\[
\begin{align*}
\text{Cl}^{(0)} + E^{(0)} & \rightleftharpoons \text{CIE}^{(0)} \rightleftharpoons \text{CIE}^{(0)} \rightleftharpoons \text{CIE}^{(0)} + E^{(0)}
\end{align*}
\]

Intracellular chloride, Cl^{(0)}, is transported across the cell membrane by combining with the inward-facing site of the anion transporter, E^{(0)}. The inward-facing complex, CIE^{(0)}, may undergo a conformational change into an outward-facing complex, CIE^{(0)}. Finally, CIE^{(0)} may dissociate into the outward-facing conformation of the transporter, E^{(0)}, and extracellular chloride, Cl^{(0)}, whereby a reaction half cycle is completed. The equilibrium constants in the ping-pong model are defined as:

\[
K_{1} = \frac{k_{1}}{k_{-1}} = \frac{\left[\text{CIE}^{(0)}\right]}{\left[\text{Cl}^{(0)}\right] \cdot \left[E^{(0)}\right]}
\]

(A2a)

\[
K_{2} = \frac{k_{2}}{k_{-2}} = \frac{\left[\text{CIE}^{(0)}\right]}{\left[\text{Cl}^{(0)}\right]}
\]

(A2b)
It can be shown that the unidirectional efflux of chloride from the intra- to the extracellular compartment, $J_{\text{eff}}$, is given by:

$$J_{\text{eff}} = \frac{k_1 k_2 k_3}{k_2 k_3 + k_1 k_3 + k_1 k_2} \cdot [Cl^{00}] \cdot [E^{0}] \quad (A3)$$

(see, for example, Britton, 1964, part II, pp. 15-16). $[E^{0}]$ is expressed in moles per square centimeter, $[Cl^{00}]$ in molar, and the composite rate constant in molar$^{-1}$ per second, and consequently the unidirectional efflux will be expressed in units of (moles per square centimeter) per second. The fractional inhibition is defined as:

$$F = 1 - \frac{J_{\text{eff}}}{J_{\text{uf}}} \quad (A4)$$

where $J_{\text{uf}}$ and $J_{\text{eff}}$ are the unidirectional chloride efflux in the presence and in the absence of the inhibitor, respectively. It is assumed that the inhibitor acts only by reducing the number of active transporters; i.e., in Eq. A3 only the concentration of $E^{0}$ is changed by introduction of an inhibitor. By inserting Eq. A3 into Eq. A4:

$$F = 1 - \frac{[E^{00}]}{[E^{0}]} \quad (A5)$$

where $[E^{00}]$ and $[E^{0}]$ are the concentration of free inward-facing binding sites in the presence and in the absence of the inhibitor, respectively. From Eqs. A2a-c it follows that the concentration of inward-facing binding sites, $[E^{0}]$, is proportional to the sum of the concentration of all free forms of the transporter in the absence or in the presence of the inhibitor ($[Cl^{00}]$ and $[Cl^{0}]$ are kept constant):

$$[E^{0}] = \frac{[E^{0}] + [CIE^{0}] + [CIE^{00}] + [E^{00}]}{1 + [Cl^{0}] K_1 + [Cl^{0}] K_1 K_2 + \frac{[Cl^{00}]}{[Cl^{00}]} K_1 K_2 K_3} \quad (A6)$$

By introducing

$$[E] = [E^{00}] + [CIE^{0}] + [CIE^{00}] + [E^{0}] \quad (A7)$$

in Eq. 6 and substituting Eq. A6 in Eq. A5:

$$F = 1 - \frac{[E]}{[E]} = 1 - \frac{[E]}{[E]} \quad (A8)$$

is obtained, where $[E]$ denotes the total concentration of all inhibitor free forms of the transporter when the inhibitor is present, and $[E]$ = $T$ denotes the total concentration of all forms. Rearrangement of Eq. A8 yields:

$$\frac{1}{1 - F} = \frac{T}{[E]} \quad (A9)$$

An apparent inhibitor constant, $K_{\text{app}}$, is defined as:

$$K_{\text{app}} = \frac{[E] [I]}{[E]} = \frac{([E^{0}] + [CIE^{0}] + [CIE^{00}] + [E^{00}])[I]}{[E^{0}] + [CIE^{0}] + [CIE^{00}] + [E^{0}] \quad (A10)}$$
$K_{app}$ may also be expressed by means of the equilibrium constants in Eq. A1, the fixed chloride concentrations, and the dissociation constants for inhibitor binding to the four different forms of the transporter, which are defined as:

$$K_{i1} = \frac{[E^{(0)}][I]}{[E^{(0)}]}$$

(A11a)

$$K_{i2} = \frac{[CIE^{(0)}][I]}{[CIE^{(0)}]}$$

(A11b)

$$K_{i3} = \frac{[CIE^{(0)}][I]}{[CIE^{(0)}]}$$

(A11c)

$$K_{i4} = \frac{[E^{(0)}][I]}{[E^{(0)}]}$$

(A11d)

Insertion of Eqs. A11a–d in Eq. A10 yields:

$$K_i,app = \frac{[E^{(0)}] + [CIE^{(0)}] + [CIE^{(0)}] + [E^{(0)}]}{K_{i1} + K_{i2} + K_{i3} + K_{i4}}$$

(A12)

Elimination of $[E^{(0)}]$, $[CIE^{(0)}]$, $[CIE^{(0)}]$, and $[E^{(0)}]$ in Eq. A12 by use of Eqs. A2a–c yields:

$$K_{i,app} = \frac{1}{K_{i1}} + \frac{1}{K_{i2}} + \frac{K_{i2}K_{i3}}{K_{i1}K_{i2}}$$

(A13)

($K_{i,app}$ is equivalent to ID$_{50}$ (Eq. 45) in Fröhlich and Gunn, 1986.) Insertion of Eq. A10 in Eq. A9 yields:

$$\frac{1}{1 - F} = \frac{T[I]}{[EI]K_{i,app}}$$

(A14)

The concentration of inhibitor in the efflux medium before addition of cells ([I$_r$], the nominal inhibitor concentration) is equal to the sum of the concentrations of bound and free inhibitor after addition of cells:

$$[I_r] = [I] + [EI]$$

(A15)

Here $[EI]$ is expressed in molar, whereas in Eq. A14 it is expressed in moles per square centimeter. However, we might as well have chosen to express the concentration of binding sites in molar because the hematocrit is kept constant in the different experiments. This would not have changed Eq. A14, which is dimensionless. Insertion of Eq. A15 into Eq. A14 yields:

$$\frac{1}{1 - F} = \frac{1}{K_{i,app}} \left( \frac{T[I_r]}{[EI]} - T \right)$$

(A16)

The concentration of all forms of the transporter, $T$, equals the sum of the concentration of free and inhibited forms:

$$T = [E] + [EI]$$

(A17)
Eqs. A17 and A8 are inserted in Eq. A16 to eliminate [EI]:

$$\frac{1}{1 - F} = \frac{1}{K_{i,app}} \left( \frac{T - [E_i]}{[F]} \right) = \frac{1}{K_{i,app}} \left( \frac{[I]}{F} - T \right)$$

(A18)

Eq. A18 shows that the Easson-Stedman method may also be used in studies of inhibition of anion transport mediated by a ping-pong mechanism. If $1/(1 - F)$ is plotted against $[I]/F$, a straight line is obtained with a slope equal to the reciprocal apparent inhibition constant and an intersection with the ordinate equal to minus the ratio of the concentration of binding sites to the apparent inhibitor constant.

In this derivation it has implicitly been assumed that the inhibitor could bind to all four forms of the transporter with different affinities (dissociation constants); i.e., the inhibition is of the mixed type. From Eq. A18 it is seen that knowledge about the type of inhibition (mixed, noncompetitive, uncompetitive, or competitive) is not necessary for determination of the concentration of binding sites. In principle, there are 15 possible combinations of inhibition types (i.e., the inhibitor may or may not bind to each of the four forms of the transporter, giving 16 combinations, of which one (no binding at all) is the control situation. If the inhibitor binds to all four forms of the inhibitor, the inhibition is of the mixed type, and the interpretation of the inhibition constant is expressed by Eq. A13. In the special case, where the inhibitor affinities to all four forms are equal (noncompetitive inhibition): $K_{i1} = K_{i2} = K_{i3} = K_{i4} = K_i$, Eq. A13 reduces to:

$$K_{i,app} = K_i$$

(A19)

If the inhibitor binds only to the outward-facing conformation of the transporter (competitive inhibition), Eq. A13 reduces to:

$$K_{i,app} = K_{i4} \left( \frac{[Cl^{\infty}]}{K_{1/2}} + 1 \right)$$

(A20)

where $K_{1/2}^{i,4}$ is the extracellular chloride concentration at which the unidirectional efflux is half of its theoretical maximum value in experiments where $[Cl^{\infty}]$ is varied and $[Cl^{\infty}]$ is kept constant (see Gasbjerg and Brahm, 1991).

In experiments where the inhibitor concentration by far exceeds the concentration of inhibitor binding sites, we used a less elaborate method for determination of half-inhibition constants. Irrespective of the type of inhibition, the unidirectional efflux in the presence of an inhibitor, $J_{i,eff}$, may always be expressed by:

$$J_{i,eff} = \frac{K_{i,app}}{K_{i,app} + [I]}$$

(A21)

which may be obtained by combining Eqs. A4, A8, A17, and A10. In these experiments $K_{i,app}$ and $J_{i,eff}$ were obtained by nonlinear fitting of Eq. A21 to the experimental data.

P. Gasbjerg was the recipient of a research grant from the Faculty of Natural Sciences, University of Copenhagen. J. Brahm was the recipient of a Senior Research Fellowship from The Alfred Benzon Foundation. The financial support from NOVO’s Fond and The Danish Medical Research Council is gratefully acknowledged. We thank Dr. Poul Bennekou for discussions about his conductance data, Dr. Michael Hansen for comments, and Mrs. Kirsten Heerup, Mrs. Birgitte Dolberg Olsen, and Mrs. Lise Mikkelsen for their expert technical assistance.

Original version received 19 May 1992 and accepted version received 8 February 1993.
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