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# Identification of the Anion Exchange Protein of Ehrlich Cells: A Kinetic Analysis of the Inhibitory Effects of 4,4′-Diisothiocyano-2,2′-Stilbene-Disulfonic Acid (DIDS) and Labeling of Membrane Proteins with $^3$H-DIDS

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**Summary.** In Ehrlich ascites tumor cells 4,4′-diisothiocyano-2,2′-stilbene-disulfonic acid (DIDS) inhibits the chloride exchange both reversibly and irreversibly. The reversible inhibition is practically instantaneous and of a competitive nature with $K_i$ about 2 $\mu$M at zero chloride concentration. This is succeeded by a slow irreversible binding of DIDS to the transporter, with a chloride dependence suggesting binding to the same site as for reversible DIDS binding/inhibition. To identify the membrane protein involved in anion exchange, cells were labeled with $^3$H-DIDS. Incubation of cells for 10 min with 25 $\mu$M DIDS at pH 8.2 leads to more than 95% inhibition of the DIDS-sensitive chloride exchange flux when the chloride concentration is low (15 mM). This condition was used for the $^3$H-DIDS-labeling experiments. After incubation the cells were disrupted, the membranes isolated and solubilized, and the proteins separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The distribution of the $^3$H-activity in the gel showed only one major peak, which could be related to protein with a mol wt of about 30,000 Daltons. The number of transport sites was estimated at about 400,000 per cell, and from the DIDS-sensitive chloride flux under steady-state conditions we calculate a turnover number of 340 ions per sec per site.

**Key Words** anion exchange · DIDS · *Ehrlich* ascites tumor cells · chloride fluxes

## Introduction

Electrically silent anion exchange has been characterized most thoroughly in mammalian red cells, but in recent years membranes of other cells have been found to mediate anion exchange (*see* Wieth & Brahm, 1985). We have previously shown that in the steady state about 95% of the chloride transport in Ehrlich ascites cells is mediated by an electrically silent process (Hoffmann et al., 1979). This electrically silent anion transfer has been found to occur by two separate mechanisms (Hoffman et al., 1981; Sjøholm et al., 1981; Aull, 1982; Hoffmann, 1982). One mechanism is an anion exchange system. The other system is an anion-cation cotransport system which mediates a secondary active $Cl^-$ influx (Hoffman et al., 1983). The two transport systems can be distinguished by their sensitivity to inhibitors, with DIDS (4,4′-diisothiocyano-2,2′-stilbene-disulfonic acid) being an inhibitor of anion exchange, and bumetanide an inhibitor of the anion-cation cotransport (Hoffmann et al., 1983). Furosemide, which is also used as a cotransport inhibitor, is less specific and seems to inhibit also the anion exchange system in red cells (Brazy & Gunn, 1976). Under normal steady-state conditions, chloride flux is dominated by the DIDS-sensitive anion exchange (Hoffman et al., 1983). The anion exchange in Ehrlich cells has many similarities to that found in erythrocytes, e.g. saturation kinetics with "self-inhibition," a pronounced temperature dependence, and competitive interactions of $Br^-$, $NO_3^-$, and $SCN^-$ (Hoffman et al., 1979). The $Cl^-$ flux in Ehrlich cells at 37°C is, however, three orders of magnitude lower than the flux in red cells, and the specificity of the system is less pronounced (Hoffmann et al., 1979).

Stilbene disulfonates are useful tools in studies of erythrocyte anion transport and the derivative DIDS is a very potent inhibitor of the anion permeability of the human red cell membrane (Knauf & Rothstein, 1971; Cabantchik & Rothstein, 1972; Cabantchik et al., 1978; Knauf, 1979). The isothiocyano residues of DIDS react covalently and preferentially with the band 3 protein, an abundant intrinsic membrane protein (Cabantchik & Rothstein, 1972, 1974). Before this irreversible reaction, DIDS undergoes a reversible binding step (Lepke et al., 1976; Ship et al., 1977), presumably through association of the anionic sulfonate residue with the anion binding site. A kinetic analysis of the reversible inhibition indicated that DIDS and chloride compete for a common binding site in the erythrocyte. In the Ehrlich cell 4-acetamide-4′-isothiocyano-2,2′-stilbene-disulfonic acid (SITS) and a reduced form of DIDS ($H_2$DIDS) have been shown to

inhibit sulfate fluxes both reversibly and irreversibly, whereas previous authors have failed to demonstrate irreversible inhibition of chloride transport (Aull et al., 1977; Villereal & Levinson, 1976; Levinson, 1978).

The present study demonstrates that DIDS is a competitive inhibitor of chloride transport in the Ehrlich ascites tumor cell, and that the affinity of DIDS for the exchange diffusion system of Ehrlich cells is much lower than that for the red blood cell (Shami et al., 1978).

It is, moreover, demonstrated that in Ehrlich cells the reversible binding of DIDS is succeeded by a slow irreversible binding of DIDS which is also dependent on the chloride concentration. At low chloride concentration $^3$H-DIDS binds preferentially to one protein of the Ehrlich cell membrane. Therefore, $^3$H-DIDS is used as a marker for the transport site and we have identified the anion exchange protein as a 30,000-dalton band after SDS-PAGE. The number of anion exchange binding sites is estimated at $4 \times 10^5$ sites per cell. Preliminary results of this study have previously been presented (Hoffmann, 1982).

## Materials and Methods

### Cell Suspensions

Ehrlich mouse ascites tumor cells (hyperdiploid strain) were maintained by weekly intraperitoneal transplantation in white female NMRI mice and 8 days after transplantation harvested in a standard Ringer's solution containing heparin (2.5 IU/ml). This solution had the following composition (mM): $Na^+$, 150; $K^+$, 5; $Mg^{2+}$, 1; $Ca^{2+}$, 1; $Cl^-$, 150; sulfate, 1; inorganic phosphate, 1; MOPS (morpholinopropane sulfonic acid ), 3,3; TES (N-Tris-(hydroxymethyl) methyl-2-amino-ethane sulfonic acid), 3.3; and HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid), 5; pH 7.40. The cells were washed by centrifugation (45 sec, 700 $\times$ *g*), first with the standard Ringer's solution and then with the appropriate incubation medium (*see below*). The cell suspensions were incubated at the experimental temperature (37°C) in a shaking bath for 20 to 110 min before the flux experiments. The cytocrit was in all experiments 8% unless otherwise stated. $^3$H-inulin (The Radiochemical Centre, Amersham, England, $5 \times 10^6$ dpm/ml) was added as marker of extracellular space. Throughout the incubation the pH of the suspensions was 7.40 $\pm$ 0.05.

In the incubation media, the chloride concentration of the standard Ringer's solution was varied from 5 to 150 mM by substituting sucrose for NaCl (0.85 mmol sucrose per mmol NaCl was experimentally found to give constant cell volume measured as cell water, *see below*).

Substitution with sucrose reduces both chloride, sodium and ionic strength. Since the saturation curve which we obtain by replacing NaCl with sucrose (*see* Hoffmann et al., 1979), is identical to the one obtained by Levinson (1984), using gluconate to replace chloride, we suggest that the results at high or low external chloride in the present report are only weakly influenced by the changes in $Na^+$ or ionic strength.

### Measurements of Ion Concentrations

Duplicate 1000-$\mu$l samples of the cell suspensions were transferred to preweighed vials for determination of ion content and cell water. The vials were centrifuged (14,000 $\times$ *g*, 60 sec), the supernatant was removed by suction and the samples were weighed. 100 $\mu$l of the supernatant were saved and processed in parallel with the cell pellets for determination of ion content. In one of the samples the packed cells were lysed in 800 $\mu$l distilled water and deproteinized by addition of 100 $\mu$l perchloric acid (70%). In the other sample cell water was determined by drying for 48 hr at 90°C to constant weight. Chloride concentration was determined by coulometric titration (CMT 10 Chloride Titrator, Radiometer, Copenhagen, Denmark).

$^3$H and $^{36}$Cl activities were measured in a liquid scintillation spectrometer (Packard CD 460) using Pico-Fluor 15 (Packard) as scintillation cocktail. The cellular concentrations are given as the concentration in cell water after correction for trapped extracellular medium ($^3$H-inulin space) in the cell pellets. Cells labeled with $^3$H-DIDS were dissolved in Soluene 350 (Packard) and counted in Lipoluma (Lumac), for evaluation of DIDS binding sites.

### $^{36}$Cl-Flux Experiments

The chloride steady-state exchange flux was measured as unidirectional influx (as described by Simonsen & Nielsen, 1971), or as unidirectional efflux (as described by Hoffmann et al., 1979). The tracer exchange experiments were performed under steady-state conditions and the kinetics in the individual experiments were well described by a closed two-compartment model with constant volumes.

The steady-state exchange flux of chloride was calculated as described by Hoffmann et al. (1979) but with the modification that a revised factor converting the fluxes from units of $\mu$mol $\cdot$ g dry wt$^{-1}$ $\cdot$ min$^{-1}$ to pmol $\cdot$ cm$^{-2}$ $\cdot$ sec$^{-1}$ of 0.283 has been used as a result of a better estimation of the area-to-volume ratio (Hoffmann et al., 1984, 1986).

### Synthesis of $^3$H-DIDS

$^3$H-DIDS was synthesized from $^3$H-2-methyl-5-nitro-benzenesulfonic acid. 2-methyl-5-nitro-benzenesulfonic acid (NTS) (Eastmann 5211) was tritiated by the Radiochemical Centre, Amersham. For the oxidation of NTS to the intermediate 4,4'-dinitro-2,2'-stilbene-disulfonic acid (DNDS) *see* Green and Wahl (1897). The $^3$H-DNDS was reduced to [$^3$H]4,4'-diamino-2,2'-stilbene-disulfonic acid ($^3$H-DADS) and $^3$H-DADS was treated with thiophosgene to give $^3$H-DIDS as described by Ship et al. (1977). The purity of the $^3$H-DNDS and the $^3$H-DIDS was verified by UV spectroscopy as described by Fröhlich and Gunn (1981). The ratio $E_{353}/E_{310}$ was higher than 2 showing that the $^3$H-DNDS was the *trans* isomer.

### Membrane Preparation and Solubilization

Homogenization of the cells was performed by nitrogen cavitation using a cell disruption bomb (Parr Instrument Co, no. 4635). The cells were suspended in cold (4°C) $Ca^{2+}$- and $Mg^{2+}$-free standard Ringer's solution (with 1 mM EDTA (ethylene-diamine tetraacetic acid)) to a cytocrit of 8%. The cell suspension was

equilibrated in the cell disruption bomb with 75 atm (1100 psi) of $N_2$ for 15 min at 4°C, with constant gentle stirring. Homogenization occurred by the release of the suspension from the "bomb." Immediately after the release, PMSF (phenylmethylsulfonyl fluoride) was added to a final concentration of 0.1 mM. It has been confirmed by microscope that the nuclei of the cells were not ruptured.

The homogenate was first centrifuged at 200 × *g* for 10 min to remove nuclei and cell debris and at 4,500 × *g* for 10 min to remove a mitochondria-rich fraction. At last a crude membrane fraction containing plasma membranes was pelleted by centrifugation at 120,000 × *g* for 75 min. This membrane fraction was washed in a buffer containing 10 mM Tris, 140 mM NaCl, 1 mM $Na_2$ EDTA and 0.1 mM PMSF, pH = 7.5 and afterwards in 1 mM Tris, 1 mM $Na_2$ EDTA and 0.1 mM PMSF, pH = 7.5.

The washed membrane fraction was solubilized in 4% SDS (sodium dodecyl sulfate), 3% DL-dithiothreitol, 0.06 M Tris, 10% glycerol and 0.002% bromphenolblue adjusted to pH 6.8 with HCl, heated to 90°C for 30 min and centrifuged at 120,000 × *g* for 30 min before electrophoresis.

## SDS-PAGE

The labeling of membrane proteins with $^3$H-DIDS was examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) performed as gradient gel electrophoresis using the discontinuous buffer system described by Laemmli (1970).

The separation slab gel (3 mm thick) was made as a linear gradient of acrylamide (7.5 to 20.5%) by mixing two degassed solutions using a linear gradient mixer. Solution I: 7.5% acrylamide, 0.2% bisacrylamide, 5% sucrose, 0.375 M Tris, 0.1% SDS and 0.04% TEMED; Solution II: 20.5% acrylamide, 0.5% bisacrylamide, 15% sucrose, 0.375 M Tris, 0.1% SDS and 0.02% TEMED. Both solutions were adjusted with HCl to pH 8.8. Polymerization was initiated by making the two solutions 0.01% with respect to ammonium persulfate just before mixing. The mixed solution was covered by isobutanol. A stacking gel was added on the top of the separating gel after washing away the isobutanol with stacking gel solution. The stacking gel contained 4% acrylamide, 0.1% bisacrylamide, 0.125 M Tris, 0.1% SDS, 0.1% TEMED and 0.03% ammonium persulfate to initiate polymerization (pH adjusted to 6.8 with HCl). The electrode buffer (pH 8.8) contained 0.025 M Tris, 0.192 M glycine and 0.1% SDS. The electrophoresis was performed at a constant current (15 mA) whereby a voltage gradient of about 2 V/cm at the start raising to 10 V/cm at the end of the electrophoresis was applied.

Each sample was run in duplicate. As molecular weight markers the following proteins were used: phosphorylase A(92 kD), bovine serum albumin (67 kD), glutamate dehydrogenase (53 kD), ovalbumin (43 kD) carbonic anhydrase (30 kD), trypsin inhibitor (20 kD) and α-lactalbumin (14 kD). Immediately after electrophoresis, the gel slab was cut into strips. One of the strips from each sample was stained for protein with 0.25% Coomassie Brilliant Blue R-250 in 50% methanol/10% acetic acid for at least 15 hr, and destained in 30% methanol/10% acetic acid. The duplicate strip was washed in 50% methanol/10% acetic acid for ~4 hr to remove lipids, and then cut into slices (2.1 mm) using a manual gel slicer. The slices were transferred to vials and digested with 500 μl Soluene 350 (Packard) for at least 24 hr at room temperature. 8 ml scintillation fluid (Instafluor (Packard)) were added and the $^3$H activity was measured. The recovery of $^3$H activity in the gels was found to be 87 ± 6% ($n$ = 6) of the applied activity (found from strips cut immediately after electrophoresis without the washing step).

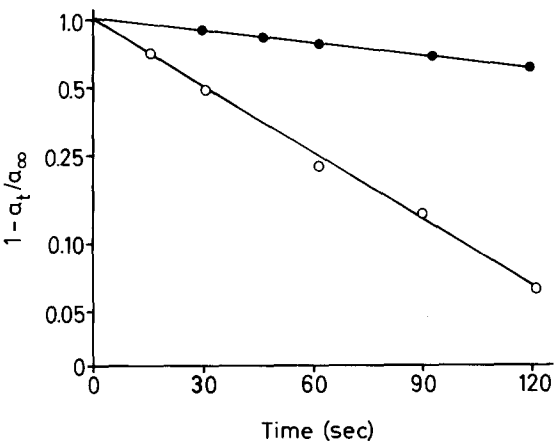


**Fig. 1.** $^{36}Cl^-$ efflux in standard Ringer's solution with (●) or without (○) addition of 100 μM DIDS at time zero. The specific activity at time $t$ ($a_t$) is followed with time and expressed as fraction of the specific activity of the medium at isotopic equilibrium ($a_\infty$). The data plotted ($1 - a_t/a_\infty$) is in a log scale

## Results

### Reversible Inhibition

A typical $^{36}Cl$ efflux experiment (150 mM chloride extracellularly) with and without addition of 100 μM DIDS is shown in Fig. 1. It can be seen that DIDS inhibits the $^{36}Cl$ flux, and that the inhibition is constant already from the time of the first sample (about 30 sec). In a separate experiment we have measured $^{36}Cl$ flux after addition of 100 μM DIDS at time zero or at 5 to 15 min before the start of the flux measurements. The inhibition was identical, confirming that the inhibition is practically instantaneous and also demonstrating that longer inhibition with DIDS does not increase the inhibition further, as long as the reversible inhibition is close to maximum.

The DIDS inhibition is reversible for at least 2 min. To show this, DIDS (100 μM) was added to the cells and 2 min later the cell suspension was washed twice with standard medium to which bovine serum albumin (0.5%) was added in order to remove all reversibly bound DIDS. The flux in these cells was 93 ± 5% of the control flux in cells that were not treated with DIDS before the washing.

In Fig. 2 the $^{36}Cl$ efflux is measured as a function of the DIDS concentration at two different extracellular chloride concentrations (15 and 150 mM). It can be seen that the chloride concentration has a pronounced effect on the inhibition of the chloride flux by DIDS. About 25% of the $^{36}Cl$ efflux is insensitive to DIDS at both chloride concentrations. The DIDS concentration for half-maximal inhibition of the DIDS-sensitive flux (the apparent $K_i$) and the

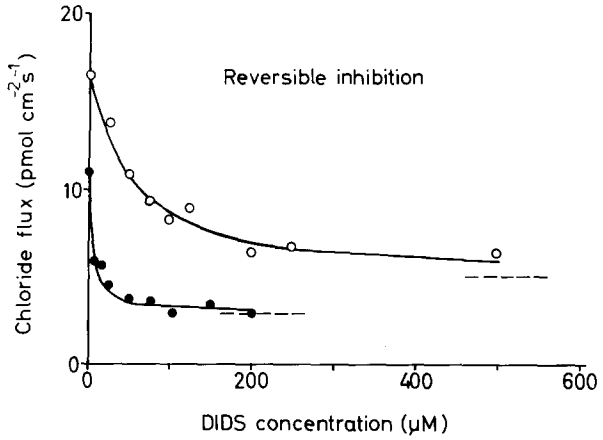


**Fig. 2.** Chloride exchange flux (measured as $^{36}Cl^-$ efflux) as a function of the DIDS concentration at 150 mM chloride (○) and at 15 mM chloride (●). The chloride concentration in the medium was varied by substituting sucrose for sodium chloride. The efflux measurements were determined on five time points taken during the first 2 min after addition of DIDS. During this period of time the irreversible inhibition did not exceed 10%. The DIDS-inhibited part of the chloride flux (control flux minus the measured flux at a given DIDS concentration) versus the DIDS concentration represents an expression of the Michaelis-Menten type (*see* Appendix). The curves shown on the Figure are the curves of this type with the best fit. The dotted lines indicate the DIDS-insensitive flux at infinite DIDS concentration calculated from the fitted curve. The apparent $K_i$ values are found from the fit to 50 ± 9 and 4 ± 1 μM for 150 and 15 mM $Cl^-$, respectively

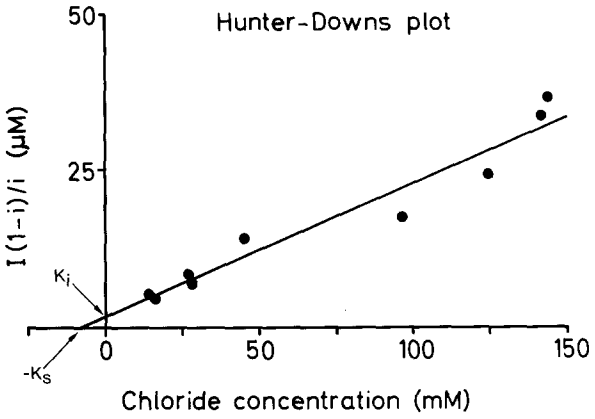


**Fig. 3.** Hunter-Downs plot of DIDS-inhibited chloride flux. The data were obtained from experiments at different chloride concentrations for each of which three parallel fluxes were measured: (1) control flux, (2) DIDS-insensitive flux (DIDS added at a concentration of 300 to 500 μM), and (3) partly inhibited (about 50%) flux. Five of the points (at 14, 16, 125, 141, and 144 mM chloride) are mean values of two to three experiments. After subtraction of the DIDS-insensitive flux the fractional inhibition ($i$) was calculated as $i = 1 - (J_i/J_{i(o)})$ (where $J_i$ and $J_{i(o)}$ represent the respective fluxes in the presence and absence of DIDS (*see* Appendix). The $y$ axis value ($I(1 - i)/i$) is equal to the apparent $K_i$ for DIDS. ($I$) represents the concentration of DIDS

DIDS-insensitive flux were calculated assuming that the fraction of the chloride flux which is inhibited by DIDS at a given DIDS concentration as a function of the DIDS concentration can be described by a saturation curve (*see* Appendix). The saturation curve with the best fit was calculated by a weighted linear regression analysis (using either a Hanes plot or Lineweaver-Burke plot giving the same result). Figure 2 shows that the calculated curves agree closely with the experimental values, indicating that the reversible DIDS inhibition of the chloride flux can be expressed by saturation kinetics.

The DIDS concentration for half-maximal inhibition is calculated at 4 μM in 15 mM chloride and 50 μM in 150 mM chloride medium, indicating that DIDS is a competitive inhibitor of chloride flux. Thus chloride and DIDS apparently compete for a common binding site.

The DIDS-insensitive flux (indicated by a dotted line) can be calculated as the difference between the control flux and the DIDS-sensitive flux at infinite DIDS concentration. The DIDS-insensitive flux is found to be 2.8 ± 0.2 pmol · $cm^{-2}$ · $sec^{-1}$ in 15 mM chloride and 4.8 ± 0.7 pmol · $cm^{-2}$ · $sec^{-1}$ in 150 mM chloride medium.

We have previously found an apparent substrate inhibition of the chloride flux at chloride concentration above 60 mM (Hoffmann et al., 1979). When this is the case, the reciprocal chloride flux plotted versus the DIDS concentration (Dixon plot) is still linear but the intersection of lines for different substrate concentrations, which normally gives the true $K_i$ (the dissociation constant for the carrier-inhibitor complex) for the inhibitor (Webb, 1963) becomes meaningless (Knauf, 1979). The most valuable information that can be extracted from this plot is thus the $x$ intercept, which corresponds to the apparent $K_i$. From a Dixon plot of the DIDS-sensitive flux (*not shown*) we find that the lines intersect significantly above the $x$ axis as expected for a competitive inhibition and the apparent $K_i$ was calculated at 6 μM in 15 mM chloride and 52 μM in 150 mM chloride which is not significantly different from the values calculated from Fig. 2.

To obtain the true $K_i$ and the dissociation constant for chloride at the site of inhibition the results with reversible inhibition of the chloride exchange flux by DIDS are presented in a Hunter-Downs plot (Webb, 1963) in Fig. 3. The Hunter-Downs plot yields straight lines both in the absence and presence of substrate inhibition as long as the inhibitor only reacts with one of the sites (Knauf et al., 1978). The apparent $K_i$ for the inhibitor is equal to the $y$ axis values ($I(1 - i)/i$) where $i$ is the fractional inhibition and $I$ is the DIDS concentration (Knauf et al., 1978). The apparent $K_i$ for DIDS is clearly dependent on the chloride concentration which is consis-

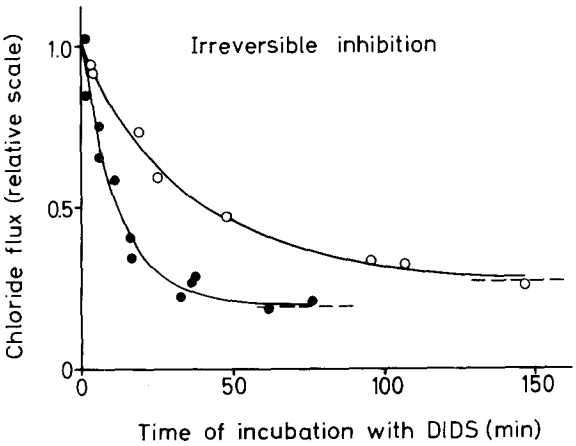


**Fig. 4.** Time course of the irreversible inhibition of the chloride exchange flux by 100 $\mu$M DIDS. Cells were incubated with 100 $\mu$M DIDS for different times (37°C, pH 7.4) in 15 mM chloride medium (●) or 150 mM chloride medium (○). The incubation was terminated by washing 3 times with DIDS-free medium to which was added 0.5% bovine serum albumin. The cells were then suspended in DIDS-free medium, $^{36}Cl^-$ was added to the cell suspension and the chloride flux was determined from the isotope uptake of the cells. The rate constant $\lambda$ for the progression of the irreversible inhibition was 0.08 ± 0.01 $min^{-1}$ in 15 mM $Cl^-$ medium and 0.026 ± 0.003 $min^{-1}$ in 150 mM $Cl^-$ medium. The curve was fitted to a single exponential function by an iterative nonlinear unweighted least-squares analysis. The value for the residual $^{36}Cl^-$ flux at infinite time is indicated as a dotted line

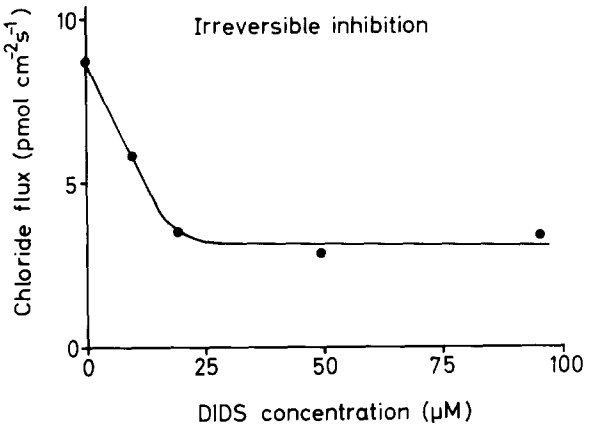


**Fig. 5.** Irreversible inhibition of the chloride exchange flux at 15 mM chloride as a function of the DIDS concentration in the incubation medium. The cells were incubated for 40 min in 15 mM chloride medium in the presence of DIDS in different concentrations (abscissa). For experimental protocol *see* legend to Fig. 4

tent with DIDS operating as a competitive inhibitor of the chloride flux. Mixed inhibition, however, cannot be ruled out. The apparent $K_i$ at 15 mM chloride is determined at 5 $\mu$M which is in good agreement with the 4 $\mu$M found from Fig. 2. The apparent $K_i$ at 150 mM chloride is 35 $\mu$M which is lower than the 50 $\mu$M found from Fig. 2. This could be due to an overestimation of the DIDS-insensitive flux at 150 mM chloride used for the Hunter-Downs plot. For a noncompetitive inhibitor the apparent $K_i$ is independent of the chloride concentration. As an example pyridoxal phosphate is also a reversible inhibitor of chloride exchange flux, the apparent $K_i$ is 1.4 ± 0.3 mM ($n$ = 7) at 15 mM chloride and 1.3 ± 0.3 mM ($n$ = 4) at 150 mM chloride. Thus pyridoxal phosphate is in contrast to DIDS, a noncompetitive inhibitor of the chloride flux.

The $y$ intercept of the line in Fig. 3 is 1.8 $\mu$M, which corresponds to $K_i$ at zero chloride concentration. The substrate dissociation constant ($K_s$) is estimated at 8 mM from the $x$ intercept (equal to $-K_s$) of Fig. 3.

## Irreversible Inhibition

The DIDS inhibition becomes irreversible with time. Figure 4 shows the $^{36}Cl$ influx as a function of the incubation period with 100 $\mu$M DIDS, both in 15 mM chloride and in 150 mM chloride medium. The flux measurements were carried out by the influx method (*see* Materials and Methods). It can be seen that the points are in good agreement with the fitted monoexponential function. The rate constant (*see* legend to Fig. 4) for the irreversible inhibition with 100 $\mu$M DIDS is 3 times higher in the low chloride medium than in the high chloride medium. Thus DIDS binding is strongly dependent on the chloride concentration. It is found that 19 ± 4% of the chloride flux in low chloride and 25 ± 3% of the flux in high chloride are insensitive to DIDS. This is in good agreement with the DIDS-insensitive flux after reversible inhibition estimated at 25% (*see* Fig. 2). It corresponds to a DIDS-insensitive flux of 1.3 ± 0.2 pmol · $cm^{-2}$ · $sec^{-1}$ in 15 mM chloride and of 5.9 ± 0.6 pmol · $cm^{-2}$ · $sec^{-1}$ in 150 mM chloride medium.

In a series of experiments we tried to optimize the conditions for specific irreversible binding of $^3H$-DIDS to the anion exchange protein. From Fig. 4 it is seen that incubation in a 15-mM chloride medium with 100 $\mu$M DIDS for 40 min will result in nearly maximal irreversible inhibition of the chloride flux. Since the apparent $K_i$ for DIDS is only 5 $\mu$M in 15 mM chloride medium (*see* Fig. 3), then a DIDS concentration of about 30 $\mu$M in the incubation medium would be expected to give more than 80% reversible inhibition. Higher DIDS concentrations would not accelerate the irreversible binding of DIDS to the chloride transport protein to any significant degree but rather cause more unspecific binding. Figure 5 confirms that incubation with 25 to 100 $\mu$M DIDS for 40 min results in the same irreversible inhibition of chloride exchange flux.

The results shown in Fig. 6 for 15 mM chloride

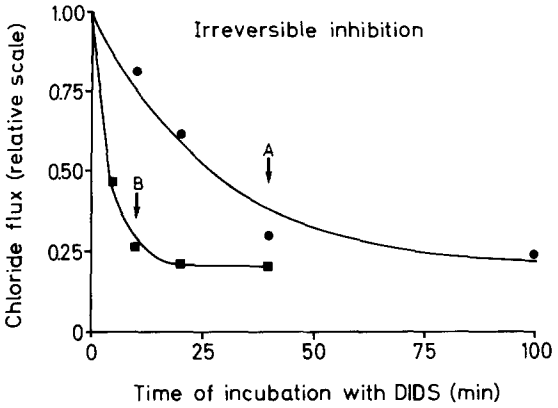


**Fig. 6.** Time course of the irreversible inhibition of the chloride exchange flux by 25 $\mu$M DIDS at two different pH values. Cells were incubated in 15 mM chloride medium (37°C) at pH 7.4 (●) or pH 8.2 (■). For experimental protocol *see* legend to Fig. 4. The $^{36}Cl^-$ influx (ordinate) was measured at pH 7.4 in all experiments. The conditions used for labeling with $^3$H-DIDS in Fig. 7*A* and *B* are indicated by arrows *A* and *B*, respectively. The rate constant $\lambda$ for the progression of the irreversible inhibition was 0.038 ± 0.001 $min^{-1}$ at pH 7.4 and 0.231 ± 0.001 $min^{-1}$ at pH 8.2. The curve was fitted to a single exponential function by an iterative nonlinear unweighted least-squares analysis

at pH 7.4 (circles) confirm that in 15 mM chloride 40 min of incubation with 25 $\mu$M DIDS is enough to give about 80% of the possible irreversible inhibition. Thus incubation for 40 min with 25 $\mu$M $^3$H-DIDS at 15 mM chloride was chosen as the labeling condition in Fig. 7*A*. The rate of the irreversible reaction with DIDS is in red blood cells known to increase with increasing pH (Ship et al., 1977). It is seen from the curve marked with squares in Fig. 6 that this is also the case in Ehrlich cells. The rate constant (*see* legend to Fig. 6) for irreversible binding with DIDS is about 6 times higher at pH 8.2 than at pH 7.4. As incubation for 10 min with 25 $\mu$M DIDS at pH 8.2 gives even higher inhibition of chloride exchange flux than 40-min incubation at pH 7.4, this condition was chosen for the labeling with $^3$H-DIDS in experiments like the one shown in Fig. 7*B*.

To localize the $^3$H-DIDS in the individual membrane proteins, isolated membranes were subjected to SDS-PAGE. Figure 7 shows the distribution of membrane-bound $^3$H-DIDS of a membrane sample from Ehrlich cells pretreated as indicated by the arrows in Fig. 6. The 30,000-Dalton region (gel slices 28 to 31) contains the only clear peak in the gel. Another more diffuse peak is found around 14,000 Dalton (gel slices 40 to 50).

To see whether the radioactive peak around slices 40 to 50 in Fig. 7 is really connected with proteins, the electrophoresis shown in Fig. 8 is run several hours longer. It is seen that the peak around 30,000 Dalton is unchanged whereas the other peak in radioactivity is now located in a region where no proteins can be identified. The smallest protein seen in the gel (around 14,000 Daltons) is about 8 slices separated from the peak. The 30,000-Dalton region is thus the only peak where the $^3$H-DIDS can be related to a membrane protein. At the present we cannot suggest what it is that binds DIDS irreversibly and can be fixated in the gel in front of the proteins. Phospholipids which are known to bind DIDS (Cabantchik & Rothstein, 1974) migrate out of the gel with the buffer front.

## The Numbers of Anion Exchange Sites

The irreversible binding of DIDS allows an estimate of numbers of binding sites. 25 $\mu$M $^3$H-DIDS (specific activity 1.6 × $10^7$ cpm/$\mu$mol) was added to cells in a low chloride medium with a cytocrit of 4%. After 40 min $^3$H-DIDS was washed away by three washes with 0.5% bovine serum albumin in a 150 mM chloride medium solution. The cells were collected by centrifugation (14,000 × *g*, 1 min), dissolved and the $^3$H activity was determined. From the measured radioactivity per ml cells we calculate the number of DIDS molecules per cell at (4 ± 1) × $10^7$ ($n$ = 10) using a cell volume of 0.9 × $10^{-9}$ ml. Provided all DIDS was on the membrane and assuming a surface area of 1.3 × $10^{-5}$ $cm^2$/cell (Hoffmann et al., 1986), Ehrlich cells have 3.1 × $10^{12}$ DIDS binding sites per $cm^2$.

The yield of the membrane fraction (purified 10 times) was estimated at 54 ± 7% ($n$ = 3) from the recovery of a membrane marker enzyme (either 5'-nucleotidase or alkaline phosphodiesterase, both of which gave the same result). Using this value for the yield of the membrane fraction we have estimated the number of specific bound DIDS molecules using two different approaches: (1) The membrane fraction contained 15 ± 1% ($n$ = 5) of the total cellular bound $^3$H-DIDS and as the yield was 54% this means that 28% of the cellular $^3$H-DIDS label is tightly associated with the cell membrane. The residual 72% is probably associated with the glycocalyx (Levinson, 1980), which is likely to be released during the homogenization and lost during the differential centrifugation. Of the membrane-bound $^3$H-DIDS only 30 ± 3% ($n$ = 4) is fixed in the gel; the remaining are probably associated with lipids (*see* Cabantchik & Rothstein, 1974). The part of the 30,000-Dalton peak above the mean cpm value in slice 10 to 30 and slice 36 to 44 contains 8.7 ± 1.8% ($n$ = 4) of the counts fixed in the gel; thus only 0.7% of the $^3$H-DIDS irreversibly bound to the cells is associated with the 30,000-Dalton protein. Using the value 4 × $10^7$ DIDS binding sites per cell and assuming that 1 DIDS molecule binds per 30,000-

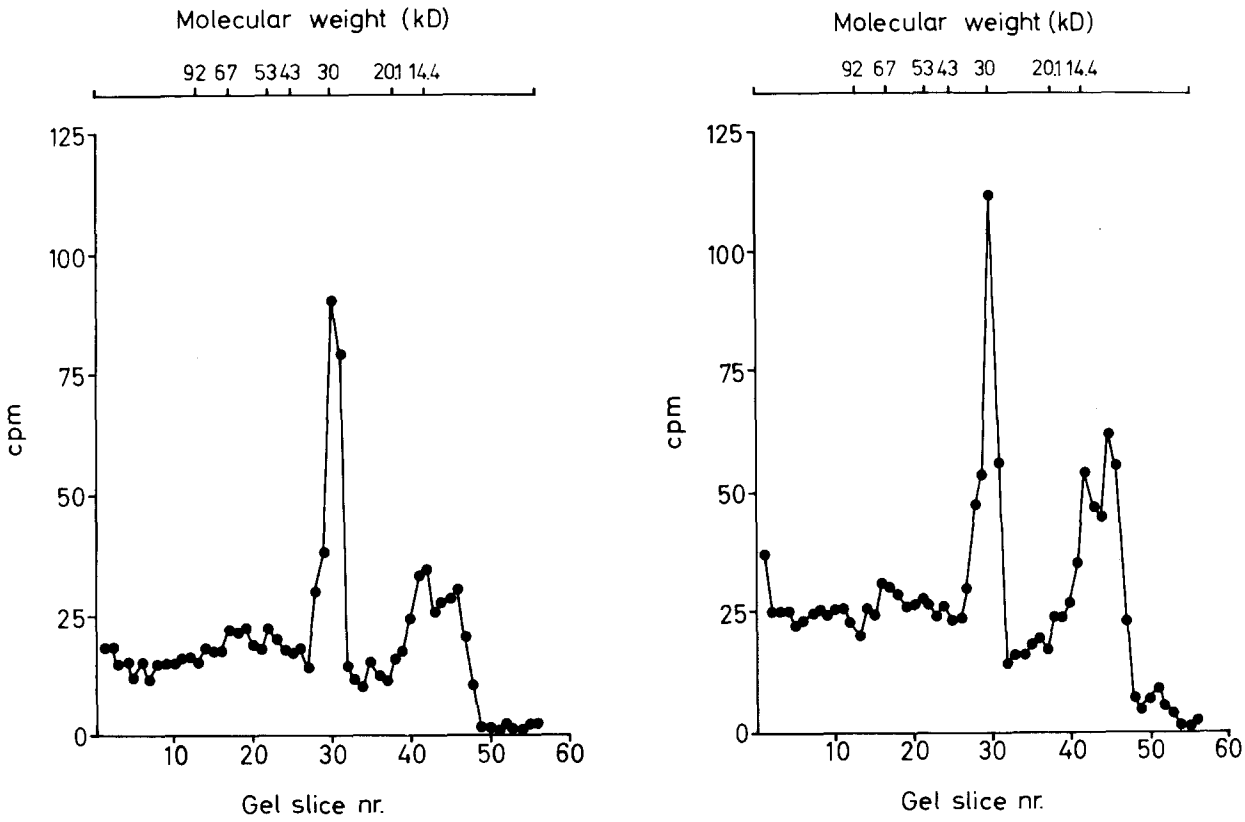


**Fig. 7.** Distribution of $^3$H-DIDS in Ehrlich cell membrane proteins after labeling with 25 $\mu$M $^3$H-DIDS in 15 mM chloride medium at *A*: pH 7.4 for 40 min and *B*: pH 8.2 for 10 min. The irreversible binding of $^3$H-DIDS (specific activity $1.6 \times 10^7$ cpm/$\mu$mol) was carried out as described for DIDS in Figs. 6 and 4. Membrane proteins (300 $\mu$g) were separated by electrophoresis on a 7.5 to 20.5% SDS-polyacrylamide gradient gel. The electrophoresis was stopped when the buffer front was about 1 cm from the bottom of the gel. For isolation and solubilization of membranes and for the electrophoresis, *see* Materials and Methods. The positions of the marker proteins are indicated on the Figure by their molecular weight (kD). Figures 7*A* and *B* are representative of 3 and 2 independent experiments, respectively

Dalton protein this gives a number of copies of the 30,000-Dalton protein of $0.28 \times 10^6$/cell or $2.2 \times 10^{10}$ sites/cm$^2$. (2) One ml of cells gave 7 ± 1 mg protein ($n = 5$) in the membrane fraction, and using the yield of 54 ± 7% this corresponds to 13 mg membrane protein per ml of cells. From the experiment illustrated in Fig. 8 and three similar ones we find that after SDS-PAGE of 300 $\mu$g membrane protein the 30,000-Dalton peak contains 332 ± 56 cpm above the mean value in slice 10 to 30 and slice 36 to 44. This corresponds to 14,386 cpm per ml of cells or $0.49 \times 10^6$ sites/cell (using the specific activity of $1.6 \times 10^7$ cpm/$\mu$mol DIDS and the number $1.1 \times 10^9$ cells per ml of cells (Hoffmann et al., 1984)), or $3.8 \times 10^{10}$ sites/cm$^2$.

## Discussion

The experiments show that DIDS, for a short period after addition acts as a reversible inhibitor. The reversible inhibition with DIDS is practically instantaneous (Fig. 1), indicating that DIDS acts on the outside of the cell membrane, as it does in the human red blood cell (*see* Deuticke, 1977). The permeability to 4,4′-diisothiocyano-1,2-diphenylethane-2,2′-disulfonic acid ($H_2$DIDS) has been shown to be low in the Ehrlich cell (Levinson et al., 1979).

The irreversible binding of DIDS is low within the first 2 min. After 2 min the residual flux was 93 ± 5% of the control. In the human red blood cell the irreversible binding is much faster (Ship et al., 1977). The DIDS inhibition in Ehrlich cells becomes irreversible with time, with a maximal inhibition of 75% in 150 mM chloride (Fig. 4). This is different from what is found with SITS and $H_2$DIDS. SITS inhibition of the chloride flux in Ehrlich cells was found to be completely reversible (Villereal & Levinson, 1976; Aull et al., 1977) and irreversibly bound $H_2$DIDS was found to inhibit sulfate, but not chloride transport (Levinson, 1978). In these experiments Levinson used up to 100 $\mu$M $H_2$DIDS for 30 min at 37°C. Figure 4 shows that with DIDS this period of incubation gives an inhibition of 40%.

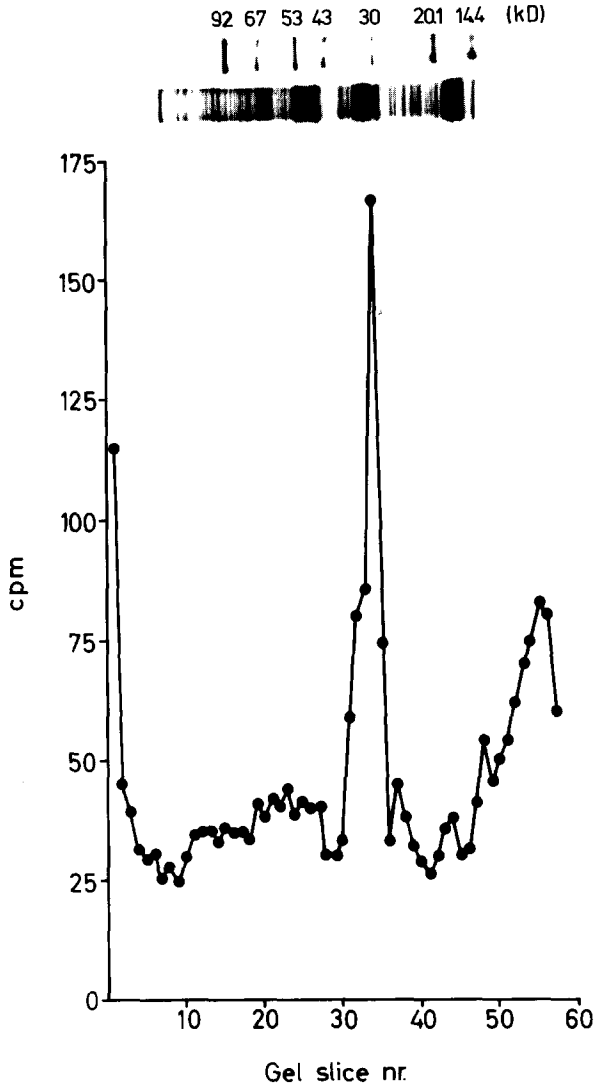


**Fig. 8.** Distribution of $^3$H-DIDS in Ehrlich ascites cell membrane proteins after labeling with 25 $\mu$M $^3$H-DIDS in 15 mM chloride medium at pH 8.2 for 10 min. The experiment is identical to Fig. 7*B* except that the electrophoresis was continued for several hours after the buffer front had reached the bottom of the gel in order to get a sharper separation and to see whether the activity between slice 40 and 50 in Fig. 7 had relation to any proteins. The protein band closest to the front in the present gel is in slice 45. The activity between slice 50 and 60 is not related to any stainable proteins. The stained gel and the calibrating gel described in Materials and Methods are shown in the upper part of the Figure. The molecular weight of the markers is given in kD. The Figure is representative of five independent experiments

Also in the red blood cell there are differences between the effect of $H_2$DIDS and DIDS. Ship et al. (1977) found that at 5°C $H_2$DIDS binds five times slower than DIDS to the red blood cells. Since we find that 150 min are needed for maximum irreversible DIDS effect, it is possible that the time necessary for a detectable effect of $H_2$DIDS could be more than the 30 min used for the Ehrlich cells (Levinson, 1978).

The rate of irreversible inhibition at 15 mM chloride is three times faster than the rate at 150 mM chloride (*see* legend to Fig. 4). Part of this difference is explained by the competitive interaction between chloride and DIDS. The apparent $K_i$ for DIDS at 150 mM chloride is 35 $\mu$M (*see* Fig. 3) or 50 $\mu$M (*see* Fig. 2). Since we use 100 $\mu$M DIDS in Fig. 4, it follows from the expression: $K_i = I(1 - i)/i$ (*see* legends to Fig. 3) that the fractional inhibition ($i$) is equal to 0.66–0.74. Thus about 70% of the transport sites at 150 mM chloride are occupied by DIDS. At 15 mM chloride the apparent $K_i$ is 4 to 5 $\mu$M and about 95% of the transport sites are occupied by DIDS. If we correct for this competitive effect of chloride, the new rate constant for irreversible inhibition is then about 0.08 $min^{-1}$ (as calculated from experiments at 15 mM chloride) and about 0.04 $min^{-1}$ (as calculated from experiments at 150 mM chloride). The remaining difference between the rate constants might be explained by the fact that a decrease in chloride concentration causes an increase of the interfacial surface potential (Wieth & Bjerrum, 1982). A more positive surface potential will decrease the local hydrogen ion concentration. DIDS is shown to bind to the $\varepsilon$ amino group in a lysine residue in the anion transport protein from the human red blood cell (Ramjeesingh et al., 1981). We suggest, as a working hypothesis, that DIDS binds to an uncharged amino group in the anion transport protein in the Ehrlich cell. A decrease in the hydrogen ion concentration will increase the number of uncharged amino groups and thereby increase the rate of DIDS binding. This can explain the difference between the rate of DIDS binding in low and high chloride (Fig. 4). This is also supported by the fact that the rate of irreversible inhibition is about 5 times faster at pH 8.2 than at pH 7.4 (*see* Fig. 6).

A fraction of the chloride flux is insensitive to DIDS (Figs. 2 and 4). We have previously shown (Hoffmann et al., 1979) that 6% of the chloride flux is a conductive flux amounting to 1 pmol · $cm^{-2}$ · $sec^{-1}$ out of the DIDS-insensitive flux which is 5 or 6 pmol · $cm^{-2}$ · $sec^{-1}$ in Figs. 2 and 4, respectively. It has, moreover, been shown that the Ehrlich ascites cell has an electroneutral anion-cation cotransport system (Geck et al., 1980). The cotransport system is insensitive to DIDS, and very sensitive to furosemide (Hoffmann et al., 1983). In the 15 mM chloride medium we have demonstrated a DIDS-insensitive, furosemide-sensitive flux which might represent cotransport. In 150 mM chloride medium, however, we concluded, based on experiments with bumetanide (Hoffmann et al., 1983), that the co-

transport seemed to be negligible under steady-state conditions. Aull (1982) has demonstrated a DIDS-insensitive, furosemide-sensitive flux also in a high chloride medium. Since the DIDS-insensitive flux found in the present paper is significantly higher than the conductive flux, the relation of this flux to the different transport systems cannot be defined with certainty.

Since the 30,000-Dalton region in Figs. 7 and 8 is the only peak where the $^3$H-DIDS can be related to a membrane protein, it is our working hypothesis that this represents the anion exchange protein. Correlation between the size of the peak and inhibition under several different experimental conditions is under investigation. We have, by two approaches with different uncertainties, found the number of copies of the 30,000-Dalton protein to be 280,000 and 490,000 per cell or $2.2 \times 10^{10}$ and $3.8 \times 10^{10}$ per $cm^2$ cell surface. If this is compared with the number of band 3 or Capnophorin (Wieth & Bjerrum, 1983) molecules which is $9 \times 10^5$ per cell (Bjerrum et al., 1983) or $6.3 \times 10^{11}$ per $cm^2$ (using a surface area of 142 $\mu m^2$) (Brahm, 1977), we find that Ehrlich cells have a 20 times lower density of anion exchange protein than the human red blood cell. The turnover number in red blood cells is $5 \times 10^4$ ions/sec (Brahm, 1977). The turnover number in Ehrlich cells can be calculated at 340 ions/sec, (using a chloride exchange flux of 17 pmol per $cm^2$ per sec (Fig. 2)) or 150 times slower than in red blood cells. It thus seems that Capnophorin in red blood cells is specialized in an extraordinary high turnover rate for anions. Capnophorin in red cells has a molecular weight of 95,000 Dalton but after cleavage with intensive chymotrypsin treatment a purified preparation of lipid vesicles containing only traces of other peptides than a 15,000- and a 9,000-Dalton segment has been reported to display specific anion transport (Rothstein et al., 1980). It is thus not unlikely that the 30,000-Dalton protein identified in Ehrlich cells can be responsible for specific anion exchange.

The kinetic analysis of the reversible inhibitory effect of DIDS on chloride exchange (Fig. 3) agrees with DIDS operating as a competitive inhibitor of chloride transport with a $K_i$ of 1.8 $\mu$M. This value is 20 times the apparent $K_i$ at zero chloride concentration for $H_2$DIDS in red blood cells. Thus the affinity for DIDS in Ehrlich cells is lower than the affinity for $H_2$DIDS in red blood cells. $K_s$ for the site where DIDS is competing with chloride is found to be 8 mM. In Hoffmann et al. (1979) we found a $K_{1/2}$ (the chloride concentration for half-maximal flux) of 15 mM from a saturation curve, where sucrose substituted for NaCl. The fluxes in these experiments were a sum of the exchange fluxes and the fluxes through the cotransport system (Levinson, 1984) so $K_{1/2}$ might overestimate $K_s$ of the exchange system. It thus seems likely that the competition between DIDS and chloride is on the substrate site.

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## Appendix

The chloride flux consists of a DIDS-sensitive part and a DIDS-insensitive part. The flux measured at zero DIDS concentration ($J_o$) is equal to the sum of the DIDS-sensitive flux ($J_{i(o)}$) and the DIDS-insensitive flux ($J_R$). The flux measured at a given DIDS concentration ($J$) is equal to the uninhibited part ($J_i$) of the DIDS-sensitive flux plus $J_R$. $J_{i(o)}$, $J_i$ and $J_R$ depend on the chloride concentration. We measure $J_o$ and $J$:

$$J = J_i + J_R$$

and

$$J_o = J_{i(o)} + J_R$$

then

$$J_o - J = J_{i(o)} - J_i,$$

Since

and $J_{i(o)} - J_i$ can be expressed by a saturation curve of the Michaelis-Menten type. From equations used in enzyme kinetics to describe the inhibition of substrate-inhibited enzymes by inhibitors different from the substrate, the following equations can be derived for $J_{i(o)} - J_i$. The nomenclature is slightly changed from Webb (1963):

For competitive inhibition:

$$J_i = J_{max}/(A + B(1 + I/K_i)/S + S/C);$$

for noncompetitive inhibition:

$$J_i = J_{max}/((1 + I/K_i)(A + B/S + S/C))$$

(Webb, 1963, p. 134).

Where $J_{max}$ is a hypothetical flux obtained at infinite chloride concentration if self-inhibition were absent, $S$ is the chloride concentration, $I$ the DIDS concentration, $K_i$ the inhibitor dissociation constant and $A$, $B$, and $C$ are constants. If the DIDS concentration is zero both equations reduce to:

$$J_{i(o)} = J_{max}/(A + B/S + S/C).$$

By subtraction and rearrangement one obtains the following expressions for the DIDS-inhibited part of the flux $J_{i(o)} - J_i$:

competitive inhibition:

$$J_{i(o)} - J_i = \frac{J_{max}/(A + B/S + S/C)}{1 + \left(\frac{K_i S}{B(A + B/S + S/C)}\right) \times \frac{M}{I}};$$

$$M = (A^2 + 2AB/S + 2AS/C + 2B/C + B^2/S^2 + S^2/C^2)$$

noncompetitive inhibition:

$$J_{i(o)} - J_i = J_{max}/((A + B/S + S/C) \times (K_i/I + 1)).$$

Both equations are of the Michaelis-Menten type with respect to $J_{i(o)} - J_i$ as a function of $I$. The value of the function for infinite DIDS concentration is the total DIDS-sensitive flux ($J_{i(o)}$).