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The External Anion Binding Site of the Human Erythrocyte Anion Transporter: DNDS Binding and Competition with Chloride

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Summary. The interaction between chloride and the anion transport inhibitor DNDS (4,4′-dinitro stilbene-2,2′-disulfonate) at the external anion binding site of the human erythrocyte anion transporter was examined by two techniques: a) chloride tracer flux experiments in the presence of varying concentrations of DNDS, and b) DNDS equilibrium binding experiments in the presence of varying concentrations of intracellular and extracellular chloride, Cl_i and Cl_o. DNDS inhibited competitively the Cl_o-stimulated chloride efflux from intact red cells at 0 °C and pH 7.8 with an inhibitor constant of 90 nM. Under the same conditions DNDS bound reversibly to one class of binding sites on intact cells with a capacity of 8.5×10^5 molecules/cell. Cl_o competitively inhibited DNDS binding with an inhibitor constant of 6 mM. In the absence of Cl_o the DNDS binding constant was 84 nM. The competition between chloride and DNDS was also tested in nystatin-treated cells in which Cl_o always equaled Cl_i. Under these conditions the values of the DNDS binding constant and the chloride inhibitor constant were significantly larger. All these data were in quantitative agreement with a single-site, alternating access kinetic scheme with ping-pong-type kinetics that we have previously developed for modeling chloride exchange transport. The data also served to rule out special cases of an alternative two-sited sequential-type kinetic scheme. DNDS binding experiments were also performed at 10 and 20 °C. We found that neither the DNDS binding constant nor the Cl_o inhibitor constant were significantly changed compared to 0 °C.

Key words erythrocyte · anion transport · stilbene · inhibitor binding

Introduction

Stilbene disulfonates are useful tools in studies of erythrocyte anion transport. The derivatives SITS (4-acetamide-4′-isothiocyano stilbene-2,2′-disulfonate) and DIDS (4,4′-diisothiocyano stilbene-2,2′-disulfonate) are potent inhibitors of the anion permeability of the human red cell membrane (Knauf & Rothstein, 1971; Cabantchik & Rothstein, 1972). With intact cells they react covalently and preferentially with one protein of the erythrocyte membrane (Cabantchik & Rothstein, 1972), the band 3 protein (Fairbanks, Steck & Wallach, 1971). In open red cell ghosts they react with band 3 from both the outside and the inside of the cell (Cabantchik & Rothstein, 1974), but anion transport is inhibited only from the outside (Kaplan, Scorah, Fasold & Passow, 1976; Barzilay & Cabantchik, 1979*b*). The main evidence that the band 3 protein is the anion transporter comes from studies which correlated the extent of anion transport inhibition with the extent of labeling of band 3 by DIDS or its analog, H_2DIDS (Cabantchik & Rothstein, 1974; Lepke, Fasold, Pring & Passow, 1976; Ship, Shami, Breuer & Rothstein, 1977). While under certain conditions the two isothiocyano residues of H_2DIDS can cross-link two different sites on the protein, only one probe molecule can bind per transporter unit (Jennings & Passow, 1979). Using this stoichiometry, several laboratories have determined the number of transporter molecules as about 1 million per human red blood cell (Cabantchik & Rothstein, 1974; Halestrap, 1976; Lepke et al., 1976; Funder, Tosteson & Wieth, 1978; Barzilay & Cabantchik, 1979*b*; Fröhlich & Gunn, 1981).

Before the isothiocyano residues of DIDS or SITS react covalently with the anion transporter protein, the stilbenes first undergo a reversible binding step (Shami, Rothstein & Knauf, 1978), presumably through association of an anionic sulfonate residue with the anion binding site. One can therefore study the reversible association of the stilbenes with the transporter by working at low temperatures at which the irreversible reaction takes place only very slowly, or, alternatively, one can use stilbene derivatives which do not possess chemically reactive side groups. By working at 0 °C, Shami et al. (1978) have characterized H_2DIDS as a competitive inhibitor of chloride equilibrium exchange transport. Barzilay and coworkers (Barzilay & Cabantchik,

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1979*a*; Barzilay, Ship & Cabantchik, 1979) investigated a number of reversibly acting stilbene disulfonates for their inhibitory potency. They demonstrated in sulfate equilibrium exchange and inhibitor binding studies with DNDS (4,4′-dinitro stilbene-2,2′-disulfonate) that this inhibitor and sulfate compete with each other for the anion transporter. Dix, Verkman, Solomon and Cantley (1979) have studied another reversibly acting stilbene derivative, DBDS (4,4′-dibenzamide stilbene-2,2′-disulfonate), which they found to bind to red cell ghost preparations in the absence of chloride.

Although the interactions between stilbenes and the transporter appear to be fairly well-characterized in these studies, a very important aspect of anion transport was neglected, namely that in an intact cell system there are two pools of transportable anions: intracellular and extracellular. Most studies so far have been performed on cell preparations in which intracellular and extracellular anion concentrations were varied together. The information obtained in these studies is less than can be obtained by separately varying the anion concentrations. We have previously determined the kinetic properties of the anion transporter in tracer flux experiments under conditions of varying separately the intracellular and extracellular substrate (i.e., chloride and bromide) concentrations. We have shown that these fluxes can be modeled adequately with a simple ping-pong-type kinetic scheme (Gunn & Fröhlich, 1979). In this scheme the anion transporter is assumed to possess a single anion binding site which can alternate in its access to the two sides of the membrane. The tight one-for-one exchange transport of extracellular with intracellular anions is modeled in this scheme by assuming that only the occupied form of the transporter can undergo the translocation step and that the unoccupied binding site cannot alternate its access at a significant rate.

Ultimately, our kinetic model will have to describe all aspects of anion transport which includes self-inhibition by high anion concentrations (Dalmark, 1976), a complex pH dependence that is different for monovalent and divalent anions (Gunn, 1979; Knauf, 1979), a small but important net transporte rate (Hunter, 1971, 1977; Kaplan & Passow, 1974; Knauf, Fuhrmann, Rothstein & Rothstein, 1977), and a temperature dependence of transport which is characterized by two different activation energies, depending on the temperature range studied (Brahm, 1977). In this report we focus our attention on one particular aspect of the anion transporter: the conformation that can reversibly bind the stilbene inhibitors. We will use DNDS binding experiments to probe the kinetic parameters of anion transport. We will demonstrate that the use of DNDS as kinetic probe confirms our data obtained in chloride flux experiments under the same experimental conditions, and that the interactions of DNDS with the anion transporter are well-described by our kinetic scheme. We will show in addition that these binding experiments are a useful tool for obtaining new information on anion transport which is not accessible with our present tracer flux technique. A preliminary report of our findings has been presented elsewhere (Fröhlich & Gunn, 1980, 1981).

Materials and Methods

In this study we performed two different types of experiments on intact human red blood cells. In one series we measured chloride tracer efflux into media of different chloride and DNDS concentrations at pH 7.8 and 0 °C. In the second part we measured the equilibrium binding characteristics of DNDS to intact cells at different intracellular and extracellular chloride concentrations.

Media and Reagents

We used two stock solutions to make up the media for flux and binding experiments: A) 150 mM NaCl, 27 mM glycylglycine, pH 7.8 at 0 °C, and B) 25 mM Na citrate, 200 mM sucrose, 27 mM glycylglycine, pH 7.8 at 0 °C. By mixing these two stock solutions in the appropriate proportions we obtained media of different chloride concentrations in which chloride was substituted isoosmotically and isoionically by citrate/sucrose (Gunn & Fröhlich, 1979). We used citrate as a spectator anion because among the several we tested it appeared to have the least effect on anion transport at pH 7.8 and 0 °C (Gunn & Fröhlich, 1979). We bubbled all media for about 1 hr with nitrogen to remove bicarbonate. Thus we could maintain the chloride gradient during the incubation with DNDS at low chloride concentrations.

We used nystatin (Mycostatin, Squibb) from stock solutions of 20 mg/ml ethanol-DMSO(1:1). The DNDS was either from ICN-K & K Labs or, as ^{3}H-labeled compound, from ICN (Irvine, Calif.). Since stilbenes isomerize around their central double bond upon illumination (Saltiel, D'Agostino, Megarity, Metts, Neuberger, Wrighton & Zafiriou, 1973) we kept the samples away from light and performed the experiments either in red light or under strongly subdued illumination with the exclusion of daylight. We recrystallized the DNDS samples 2-4 times from NaCl solutions and assayed them spectrophotometrically for isomeric purity. The trans-isomer inhibits anion transport and binds to the red cell membrane with a much higher affinity than the cis-isomer (Fröhlich & Gunn, 1980). From recrystallized samples of the trans-isomer we determined an absorption maximum at 353 nm and an absorption coefficient of $E_{353}=28{,}500$ in distilled water. The cis-isomer had an absorption maximum near 290 nm. The isosbestic point at which both isomers have the same specific absorbance is at 310 nm. We used the ratio of the absorbances at 353 and 310 nm as a measure of the isomeric purity of the DNDS: for the pure trans-isomer the ratio was 2.25–2.30. In our experiments we used only samples with an absorbance ratio of 2.2 or more.

Preparation of Cells

We used fresh, heparinized cells from one donor. Immediately after drawing we centrifuged the blood to remove the white cells

and plasma. We then washed the cells six times with N_2-bubbled stock A to remove bicarbonate. After the final wash we resuspended the cells to a hematocrit of about 80% and stored them on ice until further use in binding experiments. In the tracer flux experiments we preloaded the cells with tracer chloride at a hematocrit of about 40% and packed them by centrifugation in nylon tubes. For details about this procedure *see* Gunn and Fröhlich (1979). All these cells had an intracellular chloride concentration of 110 mmoles/kg cell water.

In one series of DNDS binding experiments we also wanted to vary the intracellular chloride concentration. For this we treated the cells with nystatin (Cass & Dalmark, 1973). We first incubated the washed cells at a hematocrit of 12% in a medium containing 100 mM NaCl, 30 mM sucrose, 20 mM glycylglycine, pH 7.8 at 0 °C and 150 μg/ml nystatin. After 5 min we centrifuged the cells and resuspended them in a medium of the same sucrose and glycylglycine concentrations but lower chloride concentration and 10 μg/ml nystatin. We repeated this procedure several times with media of gradually reduced chloride concentrations. Before each centrifugation we removed an aliquot of cells which we washed once with the same medium as used for their latest incubation (with the omission of nystatin), and put them aside for the DNDS binding experiments. Using this procedure we obtained several batches of cells with different intracellular chloride concentrations. We performed the DNDS binding experiments on these cells in the same media (without nystatin) as used for the final wash.[1]

Chloride Flux Experiments

The procedure of the tracer efflux experiments has been described elsewhere in detail (Gunn & Fröhlich, 1979). Briefly, we injected the tracer-preloaded and packed cells into media of different chloride and DNDS concentrations. At short time intervals we withdrew samples of the mixture through a Millipore filter into syringes. From the rate of appearance of tracer in the efflux medium (i.e. the filtrate) we could calculate the rate of chloride efflux.

Binding Experiments

The first part of the experimental protocol was the same for both cold and ^{3}H-DNDS. We added varying amounts of DNDS stock (1 mM in water) to a series of Corex® glass centrifuge tubes which contained 5.0 ml of medium. One tube received no DNDS as control. For determining the initial DNDS concentration we removed 2.0-ml aliquots from each tube. Then we added equal amounts (in the range of 0.5 to 1.5 ml in different experimental series) of washed cells with a hematocrit of 80%. We mixed thoroughly and then withdrew 0.5-ml aliquots to determine the concentration of cells in the medium by hemoglobinometry or cell counting (Coulter, Model ZB, Hialeah, Fla.). Finally, we centrifuged the tubes for 3 min at 15,000–20,000 × *g* in a Sorvall RC-5 refrigerated centrifuge. In the experiments with cold DNDS we assayed the supernatant after centrifugation for remaining unbound DNDS. In the experiments with ^{3}H-DNDS in which we either centrifuged the cells in the same way or centrifuged them through dibutyl phthalate, we used both supernatant and cell pellets to determine unbound and bound DNDS, respectively. In a typical experiment the time between adding the cells and start of centrifugation was about 1 min. In order to test whether the cells lost any chloride during this period into the media with low chloride concentrations we routinely measured the media and supernatants with a chloride titrator (Radiometer, Model CMT 10). We also found in control experiments that incubation for 30 sec yielded the same binding data as incubation for several minutes.

We measured the optical density of the media and the supernatants at 420, 353 and 310 nm in a spectrophotometer (Gilford, Model 250) to determine the concentration of nonradioactive DNDS. Unfortunately, the supernatant samples always contained small amounts of hemoglobin due to lysis of a few cells. We therefore had to take special care between samples to clean the quartz cuvettes and remove any traces of adsorbed hemoglobin from the cuvette walls, especially at DNDS concentrations below 2 μM. In order to correct for interference from the hemoglobin in solution we measured the absorbance at 420 nm. At 420 nm the absorbance of DNDS is very small. In the control sample without DNDS we measured the ratios of absorbance at 420, 353 and 310 nm of hemoglobin alone, and together with the reading at 420 nm we could calculate the interference by hemoglobin in the DNDS-containing samples and correct for it. We calculated the concentration of free DNDS, D_f, from these corrected readings using an absorption coefficient of $E_{353}=28{,}500$. The concentration of bound DNDS, D_b, is the difference between D_f and the initial DNDS concentration of the medium before the cells were added but corrected for dilution by the 20% extracellular space from these added cells.

In the tracer-DNDS binding experiments we determined D_f by counting the radioactivity of the supernatant after centrifugation. We obtained D_b from the radioactivity in the cell pellet. For this we resuspended the pellet (typically about 400 μl) in 5 ml of isotonic saline containing 100 μM cold DNDS for displacing the radioactivity from the cell membrane. We removed 0.6 ml aliquots for hemoglobin determination and repelleted the cells. We counted 800 μl aliquots of this supernatant in 3 ml Aquasol (in minivials) in a Packard Tri-Carb liquid scintillation counter. Using ^{22}Na we measured the trapped space in the first pellet of a typical experiment and used this to correct for DNDS trapped in this space. This correction was insignificant in the experiments with low concentrations of competing chloride, and at high chloride concentrations (the experiments in Fig. 7) it amounted to at most 5%.

Calculations and Data Presentation

The confidence limits accompanying the values of the flux and binding parameters represent the standard error, obtained by unweighted (binding data) or weighted (flux data) least-squares analysis. In the secondary plots where data with error bars are graphed, we only used the mean of the values to compute the regression line through the points.

Theory

Figure 1 depicts the simple kinetic scheme that we have used to model monovalent exchange transport of the anion X for the anion Y across the red cell membrane (Gunn & Fröhlich, 1979). We modified it here to include reversible binding of DNDS to the outward-facing empty transporter conformation C_o. C_i denotes the conformation of the transporter in

[1] Since these cells were washed only once with nystatin-free medium they were probably not completely restored to their original permeabilities. However, this is of no importance in these binding experiments in which intracellular and extracellular chloride concentrations were supposed to be the same.

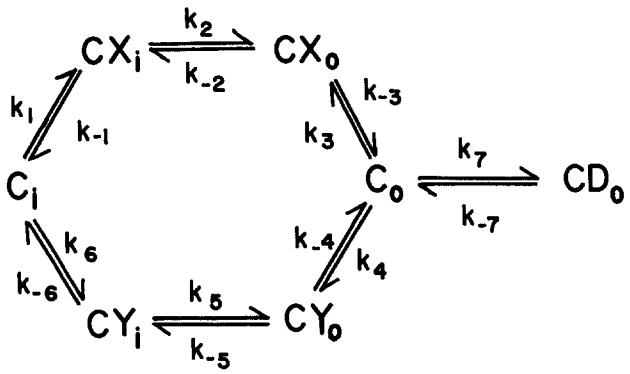

Fig. 1. Kinetic scheme describing the exchange transport of the anions X and Y and binding of extracellular DNDS. k_2, k_{-2}, k_5 and k_{-5} are rate constants describing the conformational change of the loaded transporter. The k_{+i} are the association rate constants of the anions X, Y and DNDS with the conformations C_o or C_i, and the k_{-i} are the dissociation rate constants. In the case of $X={}^{36}Cl$ and $Y={}^{35}Cl$ the corresponding rate constants of the top and bottom half cycle are assumed identical (i.e. $k_1=k_6$, $k_{-1}=k_{-6}$, etc.)

which the empty anion binding site faces the inside of the cell, CX_i and CY_i denote the inward-facing conformation in which the binding site is occupied with X or Y; and CX_o, CY_o and CD_o denote the outward-facing conformations with the site complexed with X, Y or DNDS. With this model it is implied that only one anion can bind at a time to the transporter and that DNDS is a competitive inhibitor of the exchange transport of X or Y (Shami et al., 1978; Barzilay & Catantchik, 1979*b*). We will show that this assumption is correct. Using the scheme in Fig. 1 one can derive expressions for the steady-state concentrations of the different transporter conformations as well as for steady-state anion exchange fluxes in the presence of DNDS (*see* Appendix). In the context of this report we are particularly interested in the species CD_o which we assumed is identical with the bound DNDS, D_b. The expression describing the equilibrium binding of DNDS in the medium is:

$$D_b=\frac{D_b^{\max}\cdot D_f}{D_f+K_D\left(1+\frac{b}{a}\frac{Cl_o}{Cl_i}+\frac{e+f}{a}Cl_o\right)} \tag{1}$$

where K_D is the dissociation equilibrium constant of DNDS binding, $D_b^{\max}$ is the total concentration of binding sites and therefore transport sites, and a, b, e and f are constants which contain only the individual rate constants shown in Fig. 1 (*see* Appendix). Equation (1) has the form of a simple adsorption isotherm or Michaelis-Menten equation and can be rewritten for easier use:

$$D_b=\frac{D_b^{\max}\cdot D_f}{K_D^{app}+D_f} \tag{2}$$

or:

$$\frac{D_f}{D_b}=\frac{1}{D_b^{\max}}(D_f+K_D^{app}). \tag{3}$$

Equation (3) states that a plot of D_f/D_b *vs.* D_f yields the number of binding sites $D_b^{\max}$ from the inverse of the slope, and the negative of the intercept with the abscissa gives K_D^{app}, the apparent binding constant. K_D^{app} depends on both intracellular and extracellular chloride concentrations Cl_i and Cl_o even though DNDS itself is present only on the outside of the cell. When we measure K_D^{app} as function of the chloride concentration, we therefore have to distinguish between two different experimental protocols: 1) Cl_i is kept constant while Cl_o is varied; and 2) $Cl_i=Cl_o$ and both are varied together.

Protocol 1): If Cl_i is kept constant and only Cl_o is varied, the expression for K_D^{app} from Eqs. (1) and (2) is:

$$K_D^{app}=K_D\left[1+\left(\frac{b}{a}\frac{1}{Cl_i}+\frac{e+f}{a}\right)Cl_o\right]. \tag{4}$$

A replot of K_D^{app} *vs.* Cl_o gives then as ordinate intercept the dissociation equilibrium constant of DNDS binding: $K_D^{app}=K_D$, and it gives as the negative of the abscissa intercept the inhibitor constant of extracellular chloride for DNDS binding:

$$K_I=\left(\frac{b}{a}\frac{1}{Cl_i}+\frac{e+f}{a}\right)^{-1}=K_{1/2\text{-out}}. \tag{5}$$

In the scheme of Fig. 1, K_I is identical with $K_{1/2\text{-out}}$, the half-saturation constant of Cl_o-stimulated tracer chloride efflux (Gunn & Fröhlich, 1979; *see also* Fig. 2 and Appendix).

Protocol 2): If both Cl_i and Cl_o are varied together ($Cl_i=Cl_o=Cl$),

$$K_D^{app}=K_D\left[1+\frac{b}{a}+\frac{e+f}{a}Cl\right]. \tag{6}$$

A replot of K_D^{app} *vs.* Cl gives as intercept with the ordinate $K_D^{app}=K_D(1+b/a)=K_D'$, and it gives as the negative of the intercept with the abscissa the inhibitor constant of chloride K_I':

$$K_I'=\frac{a+b}{e+f}=K_{1/2}^{ee}=K_{1/2\text{-out}}^{\max}+K_{1/2\text{-in}}^{\max}. \tag{7}$$

$K_{1/2}^{ee}$ is the half-saturation constant of chloride equilibrium exchange flux, and $K_{1/2\text{-out}}^{\max}$ and $K_{1/2\text{-in}}^{\max}$ are the half-saturation constants of Cl_o- and Cl_i-stimulated chloride exchange flux at saturating trans-concentrations (Gunn & Fröhlich, 1979, and Appendix).

We therefore expect that the experimental values of the DNDS binding constant and the chloride inhibition constant are different in the two protocols. The extrapolated binding constants in the absence of chloride, K_D and K'_D, differ by the factor $(1+b/a)$ where b/a is the asymmetry of the anion transporter given by the asymmetry in the apparent transport affinities: $b/a = K^{max}_{1/2\text{-in}}/K^{max}_{1/2\text{-out}}$. Since we know from previous experiments that this ratio equals about 15 (Gunn & Fröhlich, 1979) we would therefore expect that the value of the extrapolated binding constant is considerably larger in protocol 2) than in protocol 1). In the same way the model predicts that the extrapolated inhibition constant is different in the two protocols. In protocol 1) we expect the same value as $K_{1/2\text{-out}}$, the half-saturation constant of Cl_o-stimulated fluxes. In protocol 2) we expect that K'_I equals the half-saturation constant of chloride equilibrium exchange where both Cl_i and Cl_o are varied together. This $K^{ee}_{1/2}$ which is also identical to the sum of the maximal transport affinities on the inside and the outside [Eq. (7)], is dominated in human red cells by a large value of $K^{max}_{1/2\text{-in}}$ (Gunn & Fröhlich, 1979). We would therefore expect a much larger value for the chloride inhibition constant in protocol 2) than in protocol 1).

Results

Chloride Tracer Flux Experiments

In the chloride tracer flux experiments with intact red cells we determined the effect of DNDS on chloride exchange at 0 °C and pH 7.8. We tested how DNDS affects the half-saturation constant, $K_{1/2\text{-out}}$, and the maximal flux, $V_{max\text{-out}}$, of Cl_o-stimulated tracer chloride efflux. In these experiments we added the DNDS to the efflux medium prior to injection of the packed cells. Unfortunately, the concentration of binding sites for DNDS in the efflux mixture was not negligible compared with the intended concentrations of DNDS.[2] We therefore had to add higher than nominal amounts of DNDS to the efflux medium in order to compensate for binding to the cells and to keep the concentrations of free DNDS the same at all chloride concentrations. We calculated the amount of DNDS needed to compensate for this loss using the efflux volume, the number of red cells to be added and by assuming that chloride and DNDS are competing for 10^6 sites/cell with $K_D = 0.1$ μM and $K_I = 3$ mM (*see also* Theory Section).[3] Depending on the chloride concentrations (at $Cl_o = 0$ the relative loss of DNDS from the medium is the highest) and on the intended concentration of D_f, this correction amounted to 5–60% in addition to the nominally necessary amount.

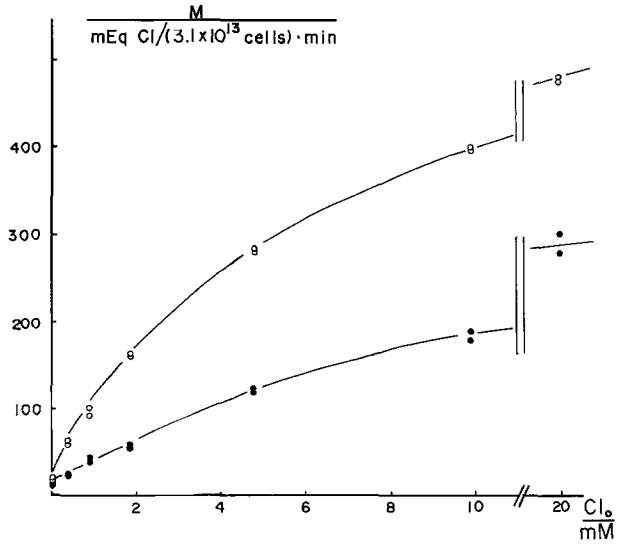

Fig. 2. Tracer chloride efflux at 0 °C and pH 7.8 from intact preloaded cells into media containing different concentrations of cold chloride and DNDS. The concentration of free DNDS, D_f, was kept either at 0.1 μM (open circles) or 0.5 μM (filled circles) by compensating for binding to the cells with additional amounts of DNDS in the efflux media (*see text* for details). The extrapolated maximal flux, $V_{max\text{-out}}$, was 641 ± 12 and 608 ± 44 mmoles/(kg cell solids × min) at $D_f = 0.1$ μM and 0.5 μM, respectively. The half-saturation constants, $K^{app}_{1/2\text{-out}}$, were 7.0 ± 0.5 mM and 24.0 ± 2.9 mM, respectively

Figure 2 shows the results of two experimental series with two different concentrations of free DNDS. A least-squares analysis of the data according to Wilkinson (1961) showed that in both series $V_{max\text{-out}}$ was the same but that at the higher DNDS concentration $K_{1/2\text{-out}}$ was increased. This behavior is indicative of competitive inhibition. Figure 3 shows that $K_{1/2\text{-out}}$ depended linearly on the free DNDS concentration, as expected from a competitive relationship between DNDS and extracellular

[2] This becomes obvious when one considers the binding experiments with cold DNDS. In these experiments we actually measured the amount of DNDS removed by the red cells from the medium, and we calculated the concentration of bound DNDS from the difference between initial and remaining DNDS concentrations. Even at the low hematocrit of 1% which was typical for our flux experiments, the nominal concentration of binding sites was in the range of 0.2 μM.

[3] Although it appears like a circular argument to use this competitive binding scheme for the correction and then to obtain competitive transport kinetics, this procedure is justified because we actually measured the loss of DNDS in our binding experiments. Nevertheless, even without the correction one would expect to see competitive transport kinetics because the extrapolated values of $V_{max\text{-out}}$ are insensitive to the DNDS concentration; only the fluxes at low chloride concentrations – and through this the calculated values of $K_{1/2\text{-out}}$ – are affected by the correction.

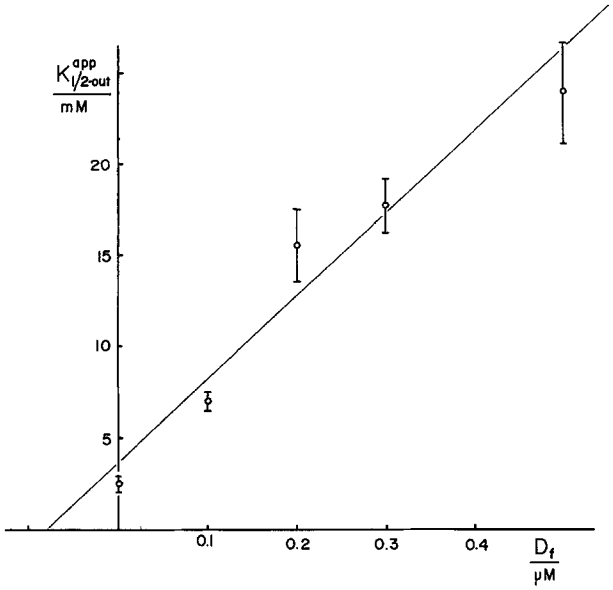

Fig. 3. The half-saturation constants, $K^{app}_{1/2\text{-out}}$, of chloride tracer flux obtained at five different DNDS concentrations, as function of D_f. A least-squares analysis gave a $K_{1/2\text{-out}} = 3.9 \pm 1.6$ mM in the absence of DNDS and a transport inhibitor constant of 91 ± 48 nM

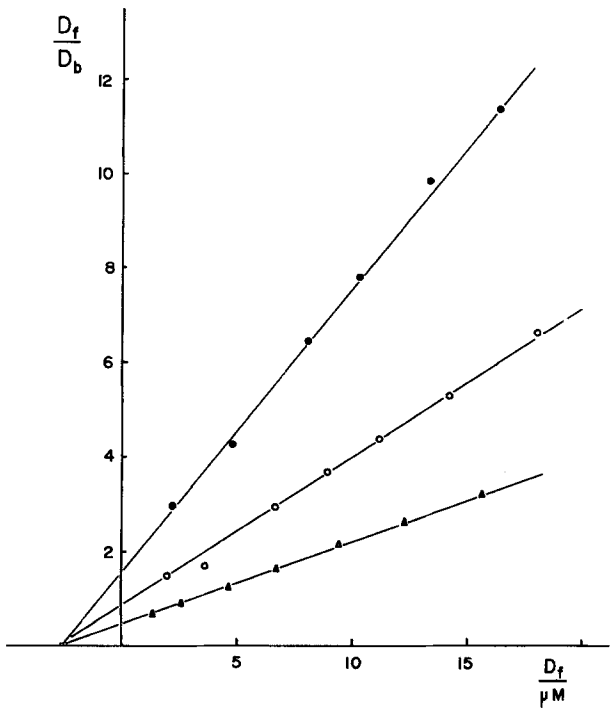

Fig. 4. Binding curves of cold DNDS to intact red blood cells in a medium containing 150 mM chloride at 0 °C and pH 7.8. The three lines were obtained at three different red cell concentrations in the incubation mixtures. D_b^{max}, the concentration of binding sites in the medium, was calculated from the three slopes: (●) 1.65 ± 0.04 μM, (○) 3.16 ± 0.06 μM and (△) 5.62 ± 0.05 μM. When normalized by the red cell concentrations, these three values gave as the number of binding sites $(8.4 \pm 0.2) \times 10^5$, $(8.7 \pm 0.2) \times 10^5$ and $(8.5 \pm 0.1) \times 10^5$ sites/cell, respectively. The values for K_D^{app}, obtained from the abscissa intercepts, were 2.6 ± 0.3 μM, 2.4 ± 0.5 μM and 2.6 ± 0.1 μM, respectively

chloride. The value of the ordinate intercept of this graph is the $K_{1/2\text{-out}}$ value in the absence of DNDS (3.9 ± 1.6 mM), and the negative of the abscissa intercept is the inhibitor constant of DNDS for chloride binding to the outward-facing transporter site (91 ± 48 nM).

DNDS Binding Experiments

Figure 4 shows the results of three series of DNDS binding experiments using nonradioactive DNDS. They were all performed under identical conditions at 0 °C and pH 7.8 with $Cl_o = 150$ mM, except that different amounts of red cells were added to the binding media in each series. The data points lie on a straight line in the measured concentration range indicating that we are dealing with a single class of binding sites characterized by a $K_D^{app} = 2.6$ μM. The slopes of the three lines are different because of the different red cell concentrations used. When normalized by the number of cells in the media, however, all slopes yielded identical values for the number of available DNDS binding sites per cell: $D_b^{max} = 8.5 \times 10^5$ sites/cell.

If we keep the concentration of cells constant but change their apparent affinity for DNDS by varying the concentration of competing chloride, we expect series of curves with the same slope but different intercepts. Figure 5 confirms our expectations. In this experiment we performed both series with the same concentrations of cells. The parallel course of the two lines therefore indicates that the number of binding sites was not altered by the change in the extracellular chloride concentration. Lowering Cl_o did, however, lower the value of K_D^{app}. Figure 6 shows that K_D^{app} depends linearly on Cl_o, and this linear relationship extends at least to $Cl_o = 150$ mM (Fröhlich & Gunn, 1981). These observations indicate that extracellular chloride is a competitive inhibitor of DNDS binding. Extrapolation of the data in Fig. 6 showed that in the absence of extracellular chloride DNDS bound to the red cell membrane with an affinity of 84 nM. The inhibitor constant K_I of chloride for DNDS binding was 6 mM.

According to our model, the value of K_D obtained by the experimental protocol of Fig. 5 is the true affinity for DNDS, and the value of K_I is identical with the half-saturation constant, $K_{1/2\text{-out}}$, of the outward-facing transporter. In these experiments we varied only Cl_o while we kept Cl_i constant. We would expect different values for the intercepts in experiments in which we vary Cl_i along with Cl_o because then we would no longer test only the outward-facing configuration. This was verified by the

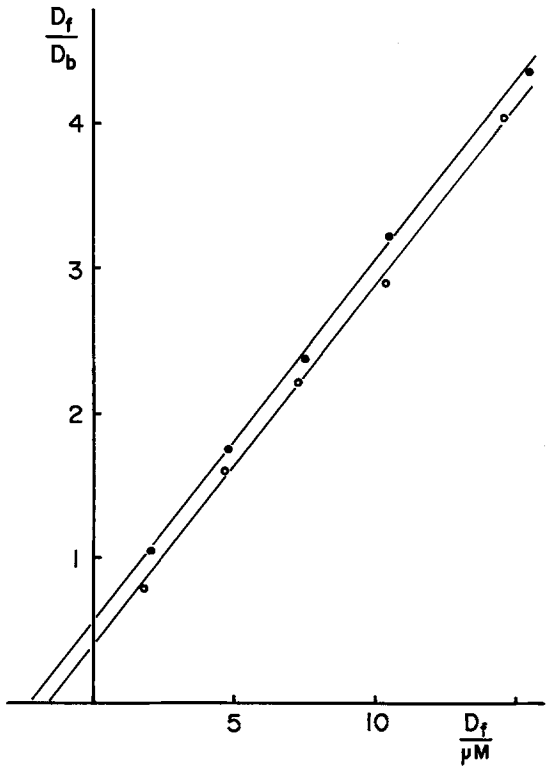

Fig. 5. Binding of cold DNDS to intact cells at different extracellular chloride concentrations. Identical concentrations of red cells were used in the two series. At $Cl_o = 85$ mM (open circles) $K_D^{app} = 1.65 \pm 0.25$ μM and $D_b^{max} = 4.06 \pm 0.07$ μM, and at $Cl_o = 130$ mM (filled circles) $K_D^{app} = 2.20 \pm 0.19$ μM and $D_b^{max} = 4.01 \pm 0.04$ μM

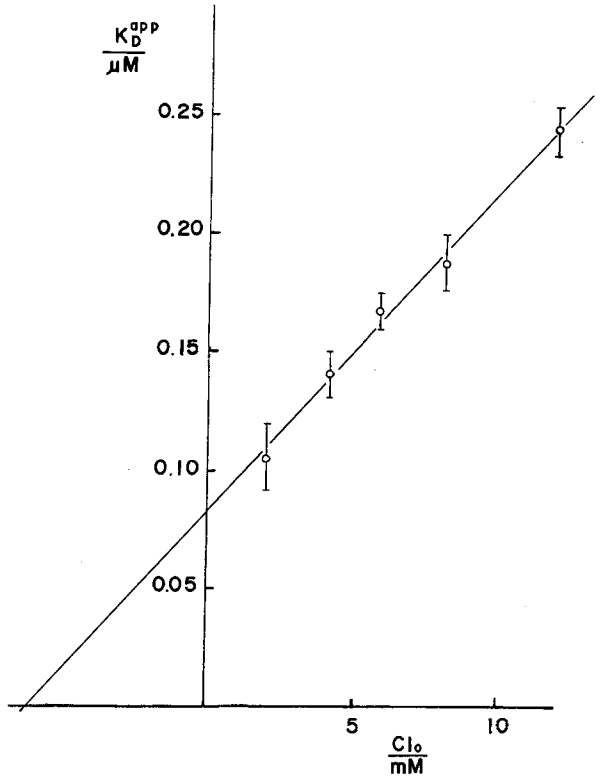

Fig. 6. The apparent binding constant, K_D^{app}, of ^{3}H-DNDS to intact red cells at different extracellular chloride concentrations. The intercepts of the regression line gave $K_D = 84 \pm 4$ nM and $K_I = 6.2 \pm 0.3$ mM

experiments shown in Fig. 7. When the intracellular chloride concentrations of the nystatin-treated cells were the same as the chloride concentrations in the binding media, the extrapolated values of K'_D and K'_I were 590 nM and 39 mM, respectively.

Temperature Studies

The data presented above were obtained at 0 °C. All our previously published data on chloride fluxes were also obtained at this temperature so that we can compare directly the two sets of data. One reason that we collected our flux data at 0 °C is that even at this low temperature chloride fluxes are very rapid with a time constant in the range of 5–20 sec. Given the high activation energy of anion exchange of 120–160 kJ mole^{-1} (Dalmark & Wieth, 1972; Brahm, 1977), it is not possible with our present filtering technique to perform the same kinetic analysis of chloride exchange transport at higher temperatures. It is possible, however, to perform our binding experiments at higher temperatures, as long as the chloride gradient does not collapse during the course of the experiments. We verified this by testing the chloride concentration of the medium before and

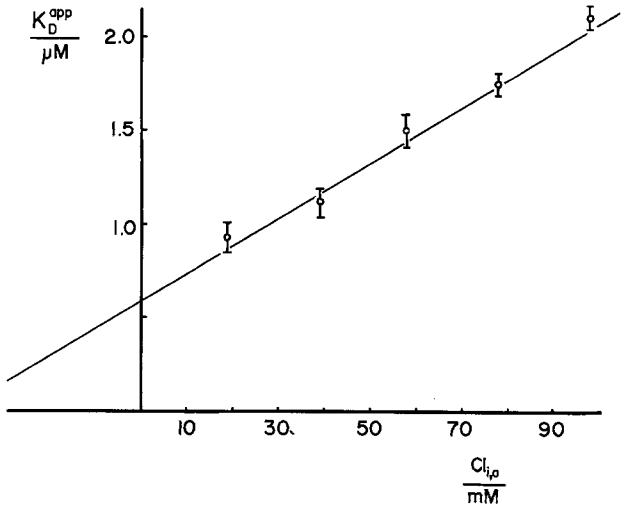

Fig. 7. The apparent binding constant, K_D^{app}, of ^{3}H-DNDS to nystatin-treated red cells in which the intracellular chloride concentrations were the same as the chloride concentrations on the media. The intercepts of the recession line gave $K'_D = 590 \pm$ nM and $K'_I = 39 \pm 4$ mM

after centrifugation of the cells. Figure 8 shows the curves of DNDS binding to intact cells in low chloride media at 0, 10 and 20 °C. Surprisingly, the binding characteristics are not very different at the three temperatures: K_I of chloride remains constant with-

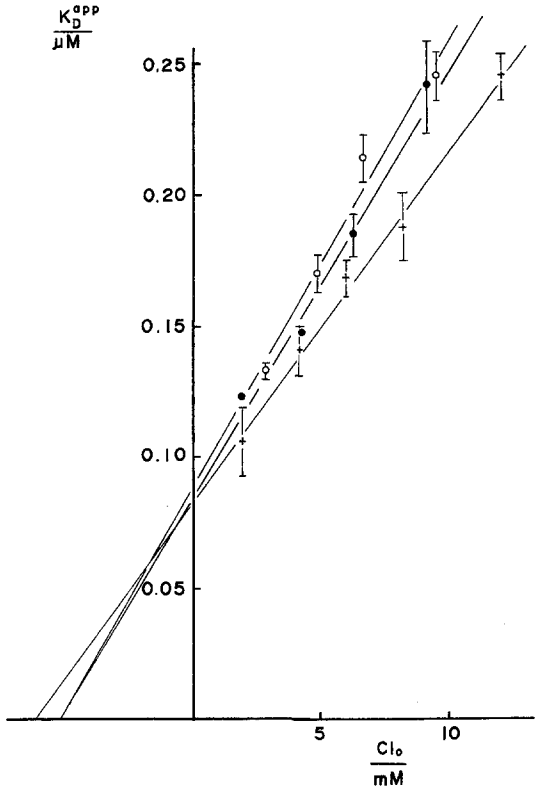

Fig. 8. The apparent binding constant, K_D^{app}, of ^{3}H-DNDS to intact red cells at different chloride concentrations and at three different temperatures. The data at 0 °C (crosses) are the same as in Fig. 6. The intercepts of the regression lines at 10 °C (filled circles) gave $K_D = 86 \pm 8$ mM and $K_I = 5.3 \pm 0.4$ mM, and at 20 °C (open circles) gave $K_D = 90 \pm 1$ nM and $K_I = 5.3 \pm 0.5$ mM

in the confidence limits, and K_D, if it changes at all, shows only a very slight tendency to increase with increasing temperature.

Discussion

With the data presented here we have shown that the anion transport inhibitor DNDS and the transported anion chloride are mutually exclusive in their binding to the anion transporter of the red blood cell from the outside of the cell. We demonstrated in flux experiments that DNDS is a competitive inhibitor of chloride transport, and in binding experiments that extracellular chloride is a competitive inhibitor of DNDS binding. In contrast to the isothiocyano-containing stilbene disulfonate derivatives SITS, DIDS and H_2DIDS, DNDS interacts only reversibly with the transporter. We demonstrated this clearly by the experiments with radioactive DNDS in which we measured D_b as the radioactivity eluted from the cell pellet by excess of cold DNDS. These experiments gave the same results as the experiments with cold DNDS in which we measured D_b indirectly as the difference between initial DNDS concentration and D_f. Judging from the linearity of all binding plots at least up to a concentration of 20 μM, DNDS appeared to bind only to one class of sites on the red cell membrane, and nonspecific binding was not detectable. This is in contrast to the findings of Dix et al. (1979) who found two different classes of sites for DBDS, a high-affinity site of 5–20 nM and a low-affinity site of 2–3 μM, but it is consistent with the experiments of Rao, Martin, Reithmeier and Cantley (1979) who could find only the low-affinity class of sites for DBDS. That the DNDS binding site is somewhere on the anion transporter molecule is evident from the close agreement between flux data and binding data: the inhibitor constant of DNDS for chloride transport has the same value as the binding constant of DNDS to the red cell membrane (91 nM *vs.* 84 nM), and the inhibitor constant of chloride for DNDS binding is similar to the half-saturation constant of chloride transport (6 mM *vs.* 3 mM). The data are therefore entirely consistent with and justify in retrospect our notion we put forward in Fig. 1 that Cl_o and DNDS compete in their binding to the conformation C_o, the unloaded transporter with the anion binding site facing the outside of the cell. They also confirm qualitatively the results of Barzilay and Cabantchik (1979*a*, *b*) who demonstrated the competition between DNDS and the divalent anion sulfate.

Our data give no information whether the mutual exclusiveness between DNDS and Cl_o is for steric or allosteric reasons (Passow, Kampmann, Fasold, Jennings & Lepke, 1980). It is attractive, however, to follow the notion that DNDS and the other stilbene disulfonates actually bind reversibly to the anion binding site with one of the sulfonate residues. This notion of steric interference between stilbene disulfonate inhibitors and transported anions has been discussed and the experimental evidence reviewed extensively by Cabantchik, Knauf and Rothstein (1978). From this it appears that besides the sulfonate residue that interacts with an anion binding site, other factors also determine the tightness of stilbene binding such as the interaction of residues on the stilbene backbone with a hydrophobic area on the protein with electron-donor capacity (Barzilay et al., 1979). In addition, the second sulfonate group needs to be aligned with another site on the protein for maximal binding strength. This is also supported by our finding that the trans-isomer of DNDS is much more tightly bound than the cis-isomer, at least by a factor of a hundred (Fröhlich & Gunn, 1981).

Dix et al. (1979) have concluded from temperature jump relaxation experiments with the fluores-

cent DBDS that binding of the stilbene molecule does not occur in a single step. They suggested a rapid initial binding reaction followed by a slower rearrangement of the complex leading to a more stable conformation. In our equilibrium binding experiments it is not possible to distinguish between binding in a single step or in multiple steps, and our measured equilibrium binding constant could be a combination of the equilibrium constants of binding and subsequent conformational changes. In terms of the steric picture mentioned above, it is possible that the first recognition of the stilbene by the binding site is through the first sulfonate residue and that stabilization of the complex occurs through subsequent interactions involving the noncharged residues and/or the second sulfonate group.

Our value of the inhibitor constant of chloride for DNDS binding, $K_I = 6$ mM, appears at first sight to disagree with the previous reports by Shami et al. (1978) and Barzilay and Cabantchik (1979*a*, *b*) who measured a value of 62 mM for chloride and of 41 mM for sulfate, respectively. In addition, the latter authors reported an affinity of DNDS binding that was nearly ten times weaker than our value of K_D (870 *vs.* 90 nM). With closer inspection of their data and of our kinetic scheme, this difference is resolved. They performed their experiments using a protocol which can test for the type of inhibition of anion transport by the stilbenes, but which does not provide information on the stilbene-binding conformation of the transporter.[4] They varied the concentration of anion on both sides of the membrane simultaneously which, as was shown in the Theory section, is expected to yield different results. When viewed in the proper perspective, their data are well consistent with our kinetic scheme and our experimental data. We demonstrated this in Fig. 7 by measuring the competition between chloride and DNDS under conditions where both Cl_i and Cl_o were varied. The experimental results of this protocol do not reflect solely the property of the outward-facing transport site but a combined property of both the inward- and outward-facing transporter conformations. The inhibitor constant of chloride in this case equals $K_{1/2}^{ee}$, the half-saturation constant of equilibrium exchange transport where $Cl_i = Cl_o$. We have shown previously that in human red cells $K_{1/2}^{ee}$ is dominated by a large value of $K_{1/2\text{-in}}^{\max}$, the transport half-saturation constant by intracellular chloride (Gunn & Fröhlich, 1979). Competition experiments under equilibrium exchange conditions therefore measure primarily the binding of chloride to the inward-facing conformation of the transporter, and the DNDS binding constant determined under these conditions is not the true binding constant of the outward-facing transport site. One can explain this intuitively by considering that the true binding constant is only measured if all binding sites are in the outward-facing conformation. The anion transporter of the red cell has the peculiar property that there is virtually no net transport of anions (Hunter, 1971, 1977; Kaplan & Passow, 1974; Knauf et al., 1977), and that essentially only the loaded transporter can undergo the translocation reaction step characterized $k_{\pm 2}$ or $k_{\pm 5}$ in Fig. 1. The anion binding sites can therefore be preferentially oriented ("recruited," Grinstein, McCullough & Rothstein, 1979; Jennings, 1980) in the outward-facing conformation if Cl_o is zero and Cl_i is nonzero. Under these conditions $K_D^{app} = K_D = 90$ nM. On the other hand, lowering Cl_i while keeping Cl_o constant causes the transporter to be recruited away from an outward- to an inward-facing conformation, and in the extreme case, at $Cl_i = 0$ (with $Cl_o \neq 0$), all transporter molecules would be in the conformation C_i and DNDS could not bind to the red cell membrane. Lowering Cl_i and Cl_o together has therefore two effects: it reduces the competition by extracellular chloride with DNDS for the conformation C_o (thus lowering K_D^{app}), but it also increases the number of sites of the conformation C_i thus reducing the number of transporter molecules to which DNDS could bind.[5] The extrapolated value of K'_D at $Cl_i = Cl_o = 0$ therefore reflects the higher concentration of D_f necessary to recruit the sites back towards the outside of the cell for maximal extent of binding. In our experiments (Fig. 7) this apparent affinity was 590 nM which should be compared with the value of 870 nM reported by Barzilay and Cabantchik (1979*b*). Both values are significantly different from the true affinity of 90 nM.

Even though we have modeled our DNDS binding data with our ping-pong kinetic scheme, in principle the interpretation of the competitive behavior

[4] This approach of studying transport under equilibrium exchange conditions was done for reasons of simplicity and ease of analysis, because it avoided side effects such as anionic diffusion potentials or complications from substitution with other anions (Cabantchik et al., 1978). The drawback of this approach is that even though the competitive nature of the interaction between DNDS and chloride can be determined, the values of the inhibition parameters do not reflect the "true" parameters.

[5] Actually, while the steady-state concentrations of C_i and C_o increase with decreasing Cl_i and Cl_o, their ratio, $C_i/C_o = b/a$ (Fröhlich & Gunn, *unpublished observation*), is independent of the anion concentration when $Cl_i = Cl_o$ (Dalmark, 1975; Knauf et al., 1980). It is this C_i at $Cl_i = Cl_o = 0$ that is not readily available for DNDS binding. Since we are dealing with an extrapolation towards the conditions of $Cl_i = Cl_o = 0$, recruitment due to the very slow conformational changes between C_i and C_o that contributes to net transport does not influence the C_i/C_o ratio here (Passow et al., 1980).

of DNDS and chloride is model independent. The relationship stating that K_I of Cl_o for DNDS binding equals $K_{1/2\text{-out}}$ of chloride transport also holds for different types of kinetic models, for example a two-site sequential-type kinetic scheme which could alternatively be used to model previous chloride transport data (Gunn & Fröhlich, 1979; Knauf, 1979). According to this scheme the strict one-for-one anion exchange found in red cells is achieved by two transport sites, one facing the inside and one facing the outside of the cell, both of which have to be occupied before translocation can occur simultaneously. Like the chloride transport data, the data here can only show quantitative consistency with the simple single-site, alternating access ping-pong scheme but cannot rule out the possibility of a sequential-type mechanism altogether. It is, however, possible again to exclude certain specific sequential-type schemes. Since DNDS is purely competitive with Cl_o, this excludes a scheme in which Cl_i is bound first, followed by binding of Cl_o. What might be even stronger evidence in favor of a ping-pong-type scheme is the fact that the two different binding protocols yielded different values for the binding constant of DNDS and the inhibitor constant of chloride. As outlined above, the difference can be explained readily by the phenomenon of recruitment. In a sequential-type scheme recruitment is less common and only possible in special cases. One would have to assume that only one of the two alternating anion binding sites can bind DNDS and that this site is recruited towards the outside by DNDS and inwards by a chloride concentration gradient. This specialized scheme is constrained further by the observation of Grinstein et al. (1979) that the irreversible reaction of DIDS with the transporter from the outside of the cell blocks NAP-taurine binding to an inward-facing anion binding site, and by the observation of Knauf, Tarshis, Grinstein & Furuya (1980) that the inhibitory potency of H_2DIDS depends on the ratio of Cl_i/Cl_o. These observations are easily explained by the single-site, alternating access (i.e. ping-pong-type) model that was also assumed by these authors.

The data in Fig. 8 show that the affinity of the transporter for DNDS depends only very little on temperature in the range between 0 and 20 °C. This means that the difference in the free energy ΔG, between the inhibitor-loaded (CD_o) and the unloaded state (C_o) of the transporter changes very little with temperature. In our case, $\Delta G = -RT \ln K_D^{-1}$ or $K_D = \exp(\Delta H/RT - \Delta S/R)$ where ΔG, ΔH and ΔS are the difference in the free energy, enthalpy and entropy between the two states, respectively. Also, the temperature-dependent term in the expression for K_D containing ΔH must be small compared with the ΔS-containing term. Assuming that the temperature dependence of K_D from Fig. 8 is actually significant one can calculate that the binding of DNDS is accompanied by a small exothermic enthalpy change of $\Delta H = -0.5$ kcal mol^{-1} (2 kJ mol^{-1}) and an increase of the entropy of $\Delta S = 30$ cal mol^{-1} K^{-1} (125 J mol^{-1} K^{-1}). The reason for this large increase in entropy upon DNDS binding is not clear. It could reflect a change in the hydradion of the DNDS or of the anion binding site, or it could be due to a conformational change of the protein upon DNDS binding.

Recently, Verkman, Dix and Solomon (1981) published a preliminary report on the thermodynamic parameters of DBDS binding to unsealed red cell ghosts. Similar to previous reports (Dix et al., 1979), they observed two types of binding sites in ghosts that were suspended in chloride-free media containing citrate for physiological ionic strength. Both types of binding sites (with apparent dissociation constants of 65 nM and 820 nM) appeared to have the same temperature dependence characterized by a $\Delta H = 1.4$–1.5 kcal mol^{-1} and $\Delta S = 26$–30 cal mol^{-1} K^{-1}. While the value of ΔS of Verkman et al. (1981) agrees well with our value above, there is a slight difference between the two values for ΔH. However, considering the different stilbene derivatives used, the different conditions employed and the experimental error limits involved, this difference might not represent a contradiction between the two studies or not even a significant difference.

The temperature dependence of K_I of chloride is also very low, and if K_I changes at all, it appears to decrease with increasing temperature. It stands in apparent contrast with the strong positive temperature dependence of the velocity of chloride exchange transport (Dalmark & Wieth, 1972; Brahm, 1977). The difference between the two sets of data is that in their transport experiments Dalmark, Wieth and Brahm measured the activation energy of a single reaction rate constant (of the rate-limiting step in the transport sequence) whereas in our binding experiments we determined an equilibrium constant which is the ratio of two opposing rate constants. If we assume that K_I ($= K_{1/2\text{-out}}$) is a fair representation of the affinity of the outward-facing site for chloride,[6] our data mean that the rates of chloride binding and release have nearly the same activation

[6] This is not necessarily a good assumption because of the influence of the translocation rate asymmetry on the transport affinity (Fröhlich, Milanick & Gunn, *unpublished results*). The only evidence in the literature so far (Gunn & Fröhlich, 1979) indicates that k_2 and k_{-2}, at least for bromide, appear to be similar, in which case this assumption is justified.

energy. We can say nothing, however, about the rate constants of binding and release. Since both K_I and K_D have a very low temperature dependence one might argue that the entropy effect is not confined to the binding of the large DNDS molecule but that it is a behavior of the transporter molecule as a consequence of any anion binding. It could therefore reflect a conformational change of the protein. The interpretation of the K_I data is more complicated, however. The value of K_I (and $K_{1/2\text{-out}}$) is characteristic for the given intracellular chloride concentration Cl_i and depends on Cl_i in a Michaelis-Menten fashion (Gunn & Fröhlich, 1979; *see* Appendix). Since the inside-facing sites are not fully saturated under our experimental conditions ($Cl_i = 100$–110 mM and $K^{max}_{1/2\text{-in}} = 60$ mM), a temperature-dependent change in $K_{1/2\text{-in}}$ could also affect $K_{1/2\text{-out}}$ due to a shift in the degree of saturation of the inward-facing conformation. A possible increase of $K_{1/2\text{-out}}$ with higher temperature could therefore be counteracted by a temperature-induced increase of $K_{1/2\text{-in}}$ (which in turn lowers $K_{1/2\text{-out}}$ at constant Cl_i). The result would be a temperature-independent observable $K_{1/2\text{-out}}$. Judging from the data of Brahm (1977) who measured chloride equilibrium exchange at different chloride concentrations and temperatures it appears that $K^{ee}_{1/2}$ and therefore $K_{1/2\text{-in}}$ does not change appreciably with temperature.[7] It is therefore likely that $K_{1/2\text{-out}}$ of chloride is actually nearly independent of temperature, similar to the binding constant of DNDS.

In summary, we have demonstrated that the reversibly acting stilbene disulfonate DNDS is mutually exclusive with extracellular chloride in its interactions with the anion transporter of the human erythrocyte. In the presence of DNDS chloride was not transported, and in the presence of extracellular chloride DNDS was not bound to the red cell membrane. We could describe the kinetic parameters of chloride transport and of DNDS equilibrium binding by a kinetic scheme originally developed for chloride transport alone. The good agreement between the predictions of this kinetic scheme and the experimental observations provide good evidence in favor of this model. This agreement between chloride transport parameters obtained in chloride transport and in DNDS binding experiments also indicates that DNDS is a useful probe for the kinetic properties of the anion transporter. DNDS binding can be used to study some transport parameters under conditions in which chloride transport experiments are very difficult or impossible. We demonstrated this in binding experiments at elevated temperatures.

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Appendix

1. Flux Equation

If $X_i = {}^{36}Cl_i$, $Y_0 = Cl_0$ and $X_0 = Y_i = 0$, the flux equation for the scheme in Fig. 1 is:

$$\frac{1}{v} = \frac{1}{C_{tot}}\left[\frac{a}{Cl_o}(1 + K_7 D_o) + \frac{b}{Cl_i} + (e+f)\right].$$

v is the rate of chloride tracer exchange, C_{tot} is the number of transporter molecules in the system, Cl_o and Cl_i are the extracellular and intracellular chloride concentration, respectively, and D_o is the concentration of DNDS in the efflux medium. $K_7 = k_7/k_{-7}$ is the equilibrium association constant of DNDS binding (and the inverse of the transport inhibition constant). a, b, e and f are constants which contain only the individual rate constants shown in Fig. 1:

$$a = (k_{-1}k_{-2} + k_{-1}k_{-3} + k_2 k_{-3})/k_{-1}k_{-2}k_3$$

$$b = (k_{-1}k_{-2} + k_{-1}k_{-3} + k_2 k_{-3})/k_1 k_2 k_{-3}$$

$$e + f = (k_{-1} + k_2 + k_{-2})/k_{-1}k_{-2} + (k_{-3} + k_2 + k_{-2})/k_2 k_{-3}.$$

If only Cl_o is varied and Cl_i is kept constant (as we did in the experiments shown in Fig. 2), the maximal Cl_o-stimulated flux, $V_{max\text{-}out}$, is:

$$V_{max\text{-}out} = C_{tot}\left(\frac{b}{Cl_i} + e + f\right)^{-1}$$

and the half-saturation constant, $K^{app}_{1/2\text{-out}}$, is

$$K^{app}_{1/2\text{-out}} = a\left(\frac{b}{Cl_i} + e + f\right)^{-1}(1 + K_7 D_o)$$

$$= K_{1/2\text{-out}}(1 + K_7 D_o)$$

where $K_{1/2\text{-out}}$ is the half-saturation constant in the absence of inhibitor. Note that $K_{1/2\text{-out}}$ is a hyperbolic function of the trans-concentration Cl_i and that it reaches a maximal value at saturating concentrations of Cl_i: $K^{max}_{1/2\text{-out}} = (e+f)/a$.

2. Binding Equation

If both X and Y are chloride, the steady-state concentration of the conformation C_o as fraction of all possible conformations C_{tot}

[7] At first glance, the values quoted by Brahm appear to be strongly temperature dependent. However, these are the values for the chloride concentration at which the flux reached one-half of its peak value. Because of the self-inhibition of transport at higher substrate concentrations these half-maximum constants are smaller than the true values of $K^{ee}_{1/2}$, the Michaelis-Menten constant obtained if self-inhibition is neglected. Brahm's data suggest that the site responsible for self-inhibition shows a strong temperature dependence and thus leaves the impression of an increased value of the half-maximum constant.

of the transporter in the absence of DNDS is

$$\frac{C_o}{C_{tot}} = \left[1 + \frac{Cl_o}{a}\left(\frac{b}{Cl_i} + e + f\right)\right]^{-1}$$
$$= \left[1 + \frac{Cl_o}{K_{1/2\text{-out}}}\right]^{-1}$$

and with extracellular DNDS, D_o, present:

$$\frac{C_o}{C_{tot}} = \left[1 + K_7 D_o + \frac{Cl_o}{a}\left(\frac{b}{Cl_i} + e + f\right)\right]^{-1}.$$

Introducing $K_7 = k_7/k_{-7} = CD_o/C_o \cdot D_0$ one obtains

$$\frac{CD_o}{C_{tot}} = K_7 D_o \left[1 + K_7 D_o + \frac{Cl_o}{a}\left(\frac{b}{Cl_i} + e + f\right)\right]^{-1}$$

and substituting $D_f = D_o$, $D_b = CD_o$, $K_D = K_7^{-1}$ and $D_b^{max} = C_{tot}$ one obtains

$$\frac{D_b}{D_b^{max}} = \frac{D_f}{K_D}\left[1 + \frac{D_f}{K_D} + \frac{Cl_o}{a}\left(\frac{b}{Cl_i} + e + f\right)\right]^{-1}$$

and

$$D_b = \frac{D_b^{max} \cdot D_f}{D_f + K_D\left(1 + \frac{b}{a}\frac{Cl_o}{Cl_i} + \frac{e+f}{a}Cl_o\right)}.$$

As expected, D_b is a saturable function of D_f with D_b^{max} being the total concentration of binding sites and with the second term in the denominator being the apparent binding constant K_D^{app} at the given concentrations of Cl_i and Cl_o.

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