Deoxygenation-Induced Cation Fluxes in Sickle Cells: II. Inhibition by Stilbene Disulfonates

By Clinton H. Joiner

Deoxygenation-induced cation movements in sickle cells were inhibited 80% to 85% by the anion transport inhibitor, 4,4′-disothiocyanato-2,2′-disulfostilbene (DIDS). Morphologic sickling was not altered by DIDS treatment, demonstrating that morphologic sickling was not sufficient to produce cation leaks in sickle cells. DIDS inhibition of deoxygenation-induced cation flux was not affected when I− replaced Cl−, indicating that conductive anion movements did not limit cation flux in deoxygenated cells treated with DIDS. Inhibition was irreversible after preincubation with DIDS at 37°C for 20 minutes, and was not affected by the oxygenation state of cells at the time of drug exposure. Sulfate self-exchange was inhibited at lower DIDS concentrations than deoxygenation-induced flux. Incubation of cells with DIDS at 4°C produced progressive blockade of sulfate exchange, but did not alter deoxygenation-induced cation fluxes. Other stilbene disulfonates, including compounds incapable of covalent reactions, also inhibited deoxygenation-induced cation movements, although several other inhibitors of anion exchange did not. Dissociation of the inhibition of anion exchange and deoxygenation-induced cation flux indicates that the DIDS effect on deoxygenation-induced cation movements does not involve the well-characterized stilbene binding site of the anion exchanger. These data provide evidence for a membrane constituent on the external surface of oxygenated sickle cells capable of interacting with DIDS to prevent the increase in cation permeability associated with sickling. © 1990 by The American Society of Hematology.
unique cation transport mechanism by an agent acting at the external surface of the cell membrane, and may prove helpful in elucidating the mechanism by which deoxygenation alters the permeability of the sickle cell membrane to cations.

MATERIALS AND METHODS

Blood. Sickle cells were obtained from otherwise healthy sickle volunteers by venipuncture into heparinized tubes after informed consent was obtained. All patients had Hb electrophoresis that showed a predominance of Hb S, no Hb A, normal levels of Hb A2, and less than 15% Hb F. Mean corpuscular Hb concentration (MCHC) was calculated from the optical density at 540 nm of Hb from lysed cells, and the hematocrit measured on oxygenated cells in whole blood spun at 13,000g for 5 minutes. Cells were generally used within 4 hours of venipuncture, although on occasion cells were washed and stored overnight in medium containing 15 mmol/L NaCl and 135 mmol/L KCl. Before use in experiments, cells were washed three times in appropriate media.

Incubation media. HEPES-buffered saline (HBS) contained: 140 mmol/L NaCl, 20 mmol/L N-2-hydroxyethylpipperazine-N'-2-ethanesulfonic acid (HEPES) (titrated to 7.45 at 37°C with NaOH), 0.1 mmol/L EDTA, 10 mmol/L glucose. HEPES-buffered Rb medium (HBR) was identical except that NaCl was replaced with RbCl. Phosphate-buffered saline (PBS) contained: 110 mmol/L NaCl, 20 mmol/L Na2HPO4 (pH 7.45), 15 mmol/L tetrakis(hydroxymethyl)ammonium-Cl, 0.1 mmol/L EDTA, 10 mmol/L glucose, 0.1 mmol/L Hb. PBS was present in all incubations at 0.1 mmol/L, added as a 10 mmol/L solution in water. RbCl was obtained from Aldrich Chemical Company (Milwaukee, WI). CsCl from Fisher Scientific (Springfield, NJ). C4 dihydroxybenzene-2,2'-disulfonate (DIDS) was purchased from ICN, K & K Labs (Irvine, CA) and recrystallized as the Na salt from water. Other chemicals were obtained from Sigma Chemical Co (St Louis, MO). Stock solutions (1 to 5 mmol/L) of stilbene disulfonates were prepared fresh daily in appropriate incubation media, and the pH adjusted before further use.

Deoxygenation-induced cation flux assay. Rb influx was measured in paired samples of oxygenated and deoxygenated sickle cells treated with ouabain to eliminate ion movements mediated by the Na/K pump. After washing in HBS, cells were suspended at 3% hematocrit; EDTA was present in all incubation media to ensure Ca-free conditions. Deoxygenation was accomplished by flushing the sealed flasks containing suspensions with humidified N2 for 15 minutes at 37°C. In some experiments, alternate vacuum and N2 flushes were used to speed deoxygenation. Oxygen tensions of 5 to 10 mm Hg were obtained and maintained throughout the incubation period. Oxygenated cells remained equilibrated with air. After deoxygenation, flasks were kept sealed, and HBR (pre-equilibrated with either air or N2) was added to give 50 mmol/L Rb final concentration and 2% hematocrit. Samples were taken at time points over the following hour and spun through dibutyl phthalate to separate cells from medium, as previously described in detail.24 Hemolysates were assayed for Hb by optical methods and for Rb by flame emission spectroscopy on a Perkin Elmer atomic absorption instrument (Model 3030B, Norwalk, CT). Cellular Rb was normalized to Hb and converted to millimole per liter cell, using the MCHC calculated on whole blood.4 Thus, calculations were normalized to original cell volume via sample Hb measurements and were therefore independent of cell volume changes during the assay, which were minimal in any case. Rb uptake was linear up to 2 hours, and rarely exceeded 5 mmol/L cells after 1 hour. The rate of change of cellular Rb uptake with time, measured over 1 hour, defined the flux rate, and the deoxygenation-induced flux was calculated as the difference between the fluxes in deoxygenated and oxygenated cells. In some experiments net Na influx and K efflux were measured as the change in cellular concentrations of these ions (measured as for Rb) in cells incubated at 37°C for 3 hours in the oxygenated or deoxygenated state in PBS containing 0.1 mmol/L ouabain.2 The deoxygenation-induced Na or K flux was calculated as described above for Rb. The advantages of Rb influx assays over net Na and K fluxes included a clear-cut initiation of the flux by addition of Rb, short incubation times, and greater precision.

Sulfate influx. Sulfate influx was measured as an assay of anion exchange activity. Sulfate fluxes are mediated by the band 3 anion transport mechanism and their inhibition by 4,4'-diisothiocyanato-2,2'-disulfostilbene (DIDS) correlates precisely with that of chloride self-exchange.7 The practicality of measuring sulfate influx at 37°C under conditions similar to net cation flux measurements makes this assay of anion exchange preferable to chloride self-exchange, which must be measured at low temperature. For measurement of SO42- flux, cells were incubated in SST at 2% to 5% hematocrit for 1 hour at 37°C to allow internal equilibration of SO42-. After addition of appropriate inhibitors, 10 μmol/L Na2SO4 was added to each flask, and samples (20 to 40 μL cells) were taken at 4 minutes and between 19 and 64 minutes, depending on the degree of anion transport inhibition anticipated.

Samples were processed as for cation samples, measuring 35SO42- activity in hemolysates, and normalizing to Hb concentration. Using the 35SO42- specific activity measured in the supernatant of each suspension, SO42- uptake was calculated as millimole per liter original cells. The change in SO42- uptake with time yielded a unidirectional SO42- influx rate. When anion transport was being compared with deoxygenation-induced flux, care was taken to ensure that the two were made under equivalent conditions (drug exposure, pH, temperature, deoxygenation, hematocrit).

Morphologic sickling. Quantitative determination of morphologic sickling was made by counting sickle forms in wet preparations under phase microscopy on samples fixed in 2% glutaraldehyde. One volume of 20 vol % glutaraldehyde stock solution (in HBS), equilibrated with N2, was added to 9 vol of 2% cell suspension. For each sample, a minimum of 100 cells (average 150) were counted in random fields. For scanning electron microscopy, glutaraldehyde-fixed cells were attached to gelatin-coated coverslips, fixed with OsO4 and dehydrated under vacuum, and gold-shadowed.

RESULTS

DIDS inhibits deoxygenation-induced cation fluxes in sickle cells. Ouabain-insensitive Rb uptake measured in oxygenated and deoxygenated sickle cells in the absence and presence of DIDS is depicted in Fig 1. In oxygenated cells, there was no effect of DIDS on Rb uptake (1.79 ± 0.18 SE v 1.91 ± 0.16, control v DIDS). However, the cation permeability of deoxygenated sickle cells was substantially reduced in the presence of DIDS (7.44 ± 1.10 SE v 2.95 ± 0.30, control v DIDS, P < .001 by unpaired t-test). Panel B illustrates the effect of DIDS on the deoxygenation-induced component of the Rb influx calculated for each of the 12 experiments depicted. Inhibition averaged 81% ± 3% (SE) for these experiments. In experiments in which net sodium and potassium movements were assayed, the average inhibition by DIDS of the deoxygenation-induced movements was 93% ± 3% (SE) for sodium and 99% ± 3% for potassium (see Table 3 below).
The effect of DIDS on the well-defined pathways of Rb influx in normal RBCs is depicted in Table 1. Ouabain-sensitive Rb uptake at 15 mmol/L external Rb (representing Na/K pump activity) was unaffected by DIDS. Likewise, DIDS had no effect in the total ouabain-insensitive component of Rb influx, confirming the experiments in oxygenated sickle cells (Fig 1) where Rb uptake was measured at 50 mmol/L Rb. When ouabain-insensitive Rb influx was divided into bumetanide-sensitive and -insensitive components, a small effect of DIDS was apparent. Ouabain-insensitive, bumetanide-sensitive Rb uptake, taken to represent Na/K/2Cl cotransport, was reduced by 15% on average. However, this effect is probably unrelated to DIDS inhibition of deoxygenation-induced cation flux, since the latter is distinguished from the cotransport system(s) by its insensitivity to Cl replacement by NO₃⁻ and to furosemide. The ouabain- and bumetanide-insensitive component of Rb influx was slightly increased by DIDS treatment, indicating that the reduction of Rb influx in deoxygenated sickle cells by DIDS was not the result of a reduction in membrane permeability by pathways other than the Na/K pump or Na/K/2Cl cotransporter.

**Morphologic sickling is not affected by DIDS.** Because of the association between morphologic sickling and cation leaks in deoxygenated sickle cells, it was important to determine if DIDS had any effect on morphologic sickling, since such an effect might account for the lack of permeability changes with deoxygenation in the presence of drug. Sickle cells were deoxygenated over 1 hour in HBS at 45 μmol/L DIDS, fixed with glutaraldehyde, and the number of sickled cells determined by phase contrast microscopy; oxygenated cells were similarly incubated. In four such experiments, deoxygenated control cells exhibited 79% ± 6% (SD) sickled forms compared with 82% ± 6% (SD) in deoxygenated DIDS-treated cells. Sickles formed included rodlike, holly leaf, and spiculated morphologies, defined as follows: rodlike, length-width ratio exceeding two, without sharp projections or spicules; holly leaf, cells with multiple sharp projections or angular contours, usually but not always somewhat elongated; spiculated, cells with threadlike projections of membrane. Cells exhibiting both holly leaf characteristics and spicules were designated spiculated forms. The majority of sickle forms under these conditions were holly leaf (75% ± 4% of total sickle forms in control and 65% ± 3% in DIDS-treated samples). Spicules were somewhat more common in DIDS-treated cells (25% ± 3% of total sickle forms ± 15% ± 6% in control cells), as is suggested by the scanning electron micrographs presented in Fig 2. Thus, DIDS-treatment of sickle cells in no way attenuated morphologic distortion on deoxygenation.

The electron micrographs also suggest a higher frequency of echinocytes in oxygenated cells treated with 45 μmol/L DIDS. This impression was confirmed by cell counts, in which echinocytes were defined as granular or bumpy cells, without angular projections. Oxygenated DIDS-treated cells had 21% ± 7% echinocytes compared with 4% ± 2% in

### Table 1. Effect of DIDS on Rb Influx Pathways in Normal RBCs

| Rb Influx* (mmol/L, original cells/h) | Ouabain-Sensitive† | Total Ouabain-Insensitive | Ouabain-Insensitive, Bumetanide-Sensitive§ | Ouabain- and Bumetanide-Insensitive
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<tr>
<td>Control</td>
<td>3.07 (0.25)</td>
<td>1.16 (0.54)</td>
<td>0.905 (0.160)</td>
<td>0.385 (0.038)</td>
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<tr>
<td>DIDS†</td>
<td>3.07 (0.25)</td>
<td>1.07 (0.15)</td>
<td>0.600 (0.143)**</td>
<td>0.461 (0.038)**</td>
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<td>Ratio DIDS/Control‡</td>
<td>1.01 (0.04)</td>
<td>0.99 (0.08)</td>
<td>0.85 (0.09)</td>
<td>1.21 (0.06)</td>
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*Five percent suspensions of cells were incubated at 37°C in HBS containing 15 mmol/L Rb and either 0.1 mmol/L ouabain, 0.1 mmol/L ouabain plus 0.10 mmol/L bumetanide or no drugs (uninhibited). Bumetanide was added as a 5 mmol/L stock solution in dimethyl sulfoxide (DMSO) and an equal volume of DMSO was added to other samples. Cellular Rb was measured at 5 and 25 minutes for uninhibited samples, and 5 and 65 for others. Data are reported as mean (SE), N = 9.
†Difference in Rb uptake between uninhibited and ouabain-treated cells.
‡Rb uptake in ouabain treated cells.
§Difference in Rb uptake between ouabain-treated and ouabain-plus-bumetanide-treated cells.
¶Rb uptake in cells treated with ouabain plus bumetanide.
†DIDS, 45 μmol/L, added as a 4.5 mmol/L stock in HBS along with other inhibitors.
§Ratio of flux in the presence of DIDS to that in absence of DIDS, calculated for each experiment and averaged.
**Differences between DIDS-treated cells and control significant at P < .01 by paired t-test.
DIDS inhibits cation fluxes in sickle cells.

Fig 2. Scanning electron micrographs of oxygenated sickle cells with and without DIDS. Cells were deoxygenated 20 minutes in the absence and presence of DIDS (45 μmol/L), fixed in 2% glutaraldehyde and OsO₄, and gold-shadowed. (A) Control oxygenated cells; (B) control deoxygenated cells; (C) DIDS-treated oxygenated cells; (D) DIDS-treated deoxygenated cells (magnification × 1,500).

controls. However, no difference in echinocytosis was apparent between oxygenated controls and cells treated with 9 μmol/L DIDS, even though deoxygenation-induced cation fluxes were on average 60% inhibited at this DIDS concentration. Therefore, it is unlikely that echinocytosis induced by DIDS related to the inhibition of deoxygenation-induced cation movements.

DIDS inhibits sickling-induced cation fluxes in heterozygote AS cells. To test whether the inhibition of deoxygenation-induced cation fluxes by DIDS might represent the drug’s interaction with an unusual transport system related to cell age or membrane damage in sickle cell populations, the effect of DIDS was assessed on deoxygenation-induced fluxes in heterozygote (AS) sickle cells, which have normal survival times and cation contents. AS cells were induced to sickle by incubation under hypertonic conditions, and under these conditions cation permeability was increased two- to threefold in control AS cells on deoxygenation (Table 2). In DIDS-treated cells, this increase was minimal, as is more clearly illustrated when the deoxygenation-induced flux is considered. The inhibition of the deoxygenation-induced component of Rb influx averaged 83% in this series of experiments, virtually identical to the value obtained in SS cells. Thus, it is unlikely that the DIDS effect on deoxygenation-induced cation fluxes relates to the high proportion of young cells in sickle blood or to chronic membrane damage in sickle cells.

Restriction of net anion movement is not the explanation of DIDS inhibition of deoxygenation-induced cation fluxes. Anion exchange inhibitors have been shown to reduce potassium fluxes induced by valinomycin and calcium by restriction of conductive chloride movement. On the macroscopic level, this is an unlikely explanation of the DIDS effect on deoxygenation-induced cation fluxes, since these fluxes are not electrogenic (sodium influx equals deoxy potassium efflux), and they are approximately one tenth the magnitude of the DIDS-resistant net chloride flux. Nevertheless, deoxygenation-induced cation movements might conceiv-
DIDS inhibition of deoxygenation-induced cation flux can be dissociated from inhibition of anion transport. The finding that DIDS inhibited deoxygenation-induced cation fluxes without alteration of morphologic sickling suggested the hypothesis that inhibition was mediated by DIDS interaction with the anion exchange mechanism. To test this hypothesis, anion transport and deoxygenation-induced cation fluxes were measured in parallel in cells exposed to DIDS under two different experimental conditions: varying drug concentrations during deoxygenation and flux measurements, and preincubating for various times at constant drug concentration.

First, cells were exposed to various concentrations of DIDS during measurements of $SO_4^{2-}/SO_3$ exchange and deoxygenation-induced Rb influx. Results of this type of titration experiment are presented in Fig 3. Anion transport was inhibited at submicromolar concentrations of DIDS, consistent with an estimated band 3 concentration in these suspensions of 0.4 to 0.8 $\mu$mol/L. Anion transport inhibition was measured in both oxygenated and deoxygenated cells to duplicate the conditions of deoxygenation-induced cation flux measurements. Before deoxygenation (ie, in the oxygenated state), cells were exposed to DIDS for 20 minutes, which was found to produce stable levels of irreversible inhibition of both anion exchange and deoxygenation-induced cation flux in pilot experiments. Figure 3 illustrates that subsequent deoxygenation did not substantially alter the relationship between DIDS concentration and inhibition of sulfate exchange, validating this experimental approach to the comparison of inhibition of anion transport to that of deoxygenation-induced cation flux. In contrast to anion exchange, deoxygenation-induced cation fluxes were unaffected below concentrations of 2.5 $\mu$mol/L. Figure 3 clearly illustrates this dissociation of anion transport and deoxygenation-induced

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<th>Table 2. Effect of DIDS on Deoxygenation-Induced Rb Influx in Heterozygote (AS) Sickle Cells</th>
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<td>&amp; $O_2$ (n = 3) &amp; $N_2$ (n = 6) &amp; Deoxygenation-induced</td>
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<tr>
<td>Control</td>
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<td>DIDS (45 $\mu$mol/L)</td>
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<td>Inhibition by DIDS</td>
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AS cells were washed in HBS made hypertonic with NaCl (375 to 385 mOsm), and incubated in the oxygenated ($O_2$) or deoxygenated ($N_2$) state for 1 hour as described for SS cells. Hypertonic HBR was added after deoxygenation and Rb uptake measured over 1 hour. Data are presented as means (SE) of the number of measurement (n) indicated. Deoxygenation-induced flux was calculated for each measurement by subtracting the flux in oxygenated cells from that in deoxygenated cells measured in the same experiment. Thus, the mean of deoxygenation-induced fluxes does not equal the difference in means of $O_2$ and $N_2$ fluxes. Likewise, the percent inhibition by DIDS of the deoxygenation-induced flux was calculated for each measurement and then averaged.

ably depend on balancing anion movement on the microscopic level, and it is therefore important to eliminate such a known effect of an anion transport inhibitor as a possible explanation of its inhibition on deoxygenation-induced cation fluxes.

It is possible to test this mechanism directly by taking advantage of the fact that conductive iodide movement across the RBC membrane is not mediated solely by the anion exchanger and is not inhibited by DIDS. Thus, if restriction of conductive anion flux by DIDS were responsible for the inhibitory effect on deoxygenation-induced flux, inhibition would be abolished when iodide replaced chloride. Cells were loaded with iodide, which rapidly replaces intracellular chloride via band 3-mediated exchange, and the effect of subsequent DIDS treatment on deoxygenation-induced cation fluxes was then tested. In five such experiments, the blockade of deoxygenation-induced fluxes of sodium and potassium by DIDS was unaffected by iodide substitution (Table 3). These data indicate that restriction of net anion movement is not the mechanism by which DIDS inhibits deoxygenation-induced cation fluxes.

Timing of DIDS exposure relative to deoxygenation does not affect inhibition of deoxygenation-induced cation flux. In five experiments sickle cells were exposed to DIDS (45 $\mu$mol/L at 2% hematocrit) at different times relative to deoxygenation. All samples received identical preincubations, washing, and deoxygenation procedures except for the addition of DIDS; a drug-free sample was used to calculate the uninhibited deoxygenation-induced Rb influx. In this series of experiments, DIDS added before deoxygenation, and therefore present before, during, and after deoxygenation produced 88% (±4% SE) inhibition. Comparable inhibition (83 ± 7%) was observed when cells were pretreated with DIDS at 37°C for 30 minutes followed by washing twice in medium containing 100 mg% bovine serum albumin to remove unbound drug. When pretreated cells were re-exposed to DIDS during deoxygenation, no further inhibition was observed (86 ± 4%). Addition of DIDS 25 minutes after deoxygenation, at which time deoxygenation-induced fluxes were fully activated, also resulted in maximal inhibition (87% ± 3%). Thus, inhibition of deoxygenation-induced fluxes by DIDS was not dependent on the oxygenation state of sickle cells at the time of exposure to drug.

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<th>Table 3. Comparison of DIDS Inhibition of Deoxygenation-Induced Na and K Fluxes in Chloride and Iodide Media</th>
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<tr>
<td>&amp; Na Influx (nmol/L, cell/h) &amp; K Efflux (nmol/L, cell/h)</td>
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<td>Chloride Media</td>
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<td>% Inhibition</td>
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<td>Iodide Media</td>
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<td>+ DIDS</td>
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<td>% Inhibition</td>
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Cells were incubated in PBS (chloride) or iodide media ± 45 $\mu$mol/L DIDS. In iodide media, I⁻ replaced Cl⁻ completely and the concentrations of other ions were equal in the two media. Cells were washed three times in appropriate media before flux measurements, standing 15 minutes at room temperature between washes to allow I⁻ to equilibrate intracellularly. Data are mean (SE), n = 5.
DIDS INHIBITS CATION FLUXES IN SICKLE CELLS

Fig 3. Inhibition of anion exchange and deoxygenation-induced cation flux at various DIDS concentrations. Sulfate influx was measured as described in Materials and Methods in oxygenated (C) and deoxygenated (D) sickle cells at 3% hematocrit; the concentrations of DIDS represent the nominal initial concentrations of drug based on weight and the purity of the preparation stated by the supplier. The fraction of SO$_4^{2-}$ exchange was calculated for the DIDS-inhibitable component, which was 99% of the SO$_4^{2-}$ influx. For measurement of deoxygenation-induced influx (III), 3% suspensions were deoxygenated in the presence of the stated nominal DIDS concentrations for 20 minutes before the addition of Rb buffer. Pilot experiments indicated that irreversible inhibition was maximal after 20 minutes' preincubation; ie, longer incubation did not result in greater inhibition of deoxygenation-induced flux, even at concentrations producing partial inhibition. The fraction of total deoxygenation-induced influx is depicted, rather than the DIDS-sensitive component. However, even if the DIDS-resistant component were subtracted from the total deoxygenation-induced flux the titration curve remains distinct from that of anion exchange. Data points are mean (SE) of five experiments.

Fig 4. Time course of inhibition of anion and deoxygenation-induced cation flux by preincubation with DIDS at 4°C. Cells were preincubated in HBS at 2% hematocrit and 45 μmol/L DIDS at 4°C. At the indicated times, suspensions were diluted into 5 vol of ice-cold HBS containing 100 mg% bovine serum albumin. After three washes with HBS plus albumin, cells were divided and washed three times with either HBS or SST. Deoxygenation-induced cation fluxes were assayed as described in Materials and Methods. Cells for anion flux measurement were resuspended in SST, warmed to 37°C, and $^{35}$SO$_4$ uptake measured as described. Because cells were not preequilibrated with SO$_4^-$, this assay represented Cl:$^{35}$SO$_4$ exchange. Data are from a single experiment, representative of three.

DIDS: at 1.5 μmol/L DIDS, anion transport was greater than 90% inhibited, whereas deoxygenation-induced cation fluxes exhibited only 15% inhibition. Maximal inhibition of cation fluxes (85%) was achieved around 50 μmol/L DIDS.

DIDS inhibition of anion and deoxygenation-induced cation transport was also compared in cells pre-exposed at 4°C for various times to 45 μmol/L DIDS and washed before flux assay. Under these conditions, DIDS concentration was approximately 100 times that of band 3 monomer and 1,000 times higher than the apparent $K_i$.$^{31}$ The progressive irreversible inhibition of anion exchange, illustrated in Fig 4, reflects the rate of the covalent reaction between the isothiocyanate groups of DIDS and the lysine amino group located at the stilbene binding site of the anion exchanger.$^{32}$ Deoxygenation-induced cation fluxes were not irreversibly inhibited by 4°C preincubation with DIDS, again dissociating the effects of DIDS on this system from that on anion transport.

Inhibition of deoxygenation-induced cation fluxes by other compounds. Figure 5 shows the effects of several stilbene derivatives and related compounds on deoxygenation-induced fluxes in sickle cells. In this series of experiments DIDS produced 85% inhibition, and the closely related molecule, H$_2$-DIDS, yielded 91% inhibition. The nonfunctional disulfostilbene, 4-isothiocyanato-4'-acetoxy-stilbene-2,2'-disulfonate (SITS), inhibited the deoxygenation-induced flux by 50%, indicating that crosslinking by the bifunctional DIDS molecule is not responsible for its inhibitory activity. Consistent, albeit submaximal, inhibition was also found with DNDS, an analogue of DIDS that has high affinity for the anion exchanger but is incapable of covalent binding.$^{33}$ Likewise, similar inhibition of deoxygenation-induced fluxes was obtained with the hydrophobic aromatic compound dipiridamole, a highly potent but relatively nonspecific inhibitor of anion exchange that interacts noncovalently with the stilbene binding site on band 3.$^{34}$ Sulfophenyliothiocyanate (SPICT) reacts covalently with external amino groups on the RBC membrane, including the lysine of the stilbene binding site on band 3, to produce inhibition of anion exchange.$^{35}$ This compound did not inhibit deoxygenation-induced fluxes (Fig 5) and, in fact, produced a slight (20%) increase in these ion movements. Phloretin, a lipid soluble compound that inhibits anion exchange via a site distinct from the stilbene receptor,$^{16}$ was also without effect on deoxygenation-induced cation fluxes. In addition to phloretin, which inhibits Na:Li exchange as well as anion transport,$^{37}$ several other cation transport inhibitors were tested for effects on deoxygenation-induced cation fluxes: phlorizin (0.20 mmol/L), amiloride (1 mmol/L), cetiedil (0.20 mmol/L), and bumetanide (0.05 mmol/L) were not inhibitory (data not shown).

In summary, several anion transport inhibitors with structural similarities to DIDS blocked the deoxygenation-induced change in cation permeability in sickle cells, although inhibition of the two transport processes was
membrane constituent involved in activating or mediating fluxes are consistent with an interaction with an external and increase Hb-oxygen affinity. In contrast, the characteristics of DIDS inhibition of deoxygenation-induced cation fluxes differs from that of several anti-sickling compounds that affect cation permeability of deoxygenated sickle cells. Cetiedil increases cation permeability of oxygenated cells, leading to cell swelling that inhibits sickling. Both of these compounds are homobifunctional crosslinking agents, and in the case of PMD, there was evidence that due to crosslinking by this homobifunctional reagent.

**DISCUSSION**

This study has demonstrated that a substantial portion of the deoxygenation-induced change in cation permeability in sickle cells can be blocked by DIDS and other stilbene disulfonates. These compounds did not inhibit morphologic sickling, and DIDS inhibition of deoxygenation-induced fluxes was not dependent on crosslinking. Furthermore, because the stilbene disulfonates are membrane-impermeant and presumably act only at the external surface of the cell, direct effects on Hb can be eliminated as an explanation of the inhibition of deoxygenation-induced fluxes. In these characteristics, the DIDS effect on deoxygenation-induced cation fluxes differs from that of several anti-sickling compounds that affect cation permeability of deoxygenated sickle cells. Cetiedil increases cation permeability of oxygenated cells, leading to cell swelling that inhibits sickling. The anti-sickling compounds dimethyladipimidate (DMA) and N,N'-phenylldimaleimide (PMD) have been shown to block deoxygenation-induced K loss along with morphologic sickling. Both of these compounds are homobifunctional crosslinking agents, and in the case of PMD, there was evidence that the anti-sickling activity and the effect on cation fluxes resulted from crosslinking rather than chemical reactivity. In addition, both DMA and PMD permeate the cell membrane and increase Hb-oxygen affinity. In contrast, the characteristics of DIDS inhibition of deoxygenation-induced cation fluxes are consistent with an interaction with an external membrane constituent involved in activating or mediating this permeability change.

The dissociation between morphologic sickling and the deoxygenation-induced cation flux in DIDS-treated cells suggests that the relationship between membrane distortion and cation leak may be more complex than it has appeared. Morphologic sickling was quantitatively and qualitatively normal in DIDS-treated cells, while deoxygenation-induced flux was 10% to 15% of control. In other words, DIDS-treated sickle cells exhibited morphologic sickling, including spicule formation, with a minimal increase in cation permeability compared with control cells. These results are explained most simply by a model in which deoxygenation-induced cation fluxes are activated by spicule formation and the activated pathway inhibited by DIDS (analogous to ouabain inhibition of the Na pump). Alternatively, DIDS might block the activation of the pathway by morphologic sickling. However, to accommodate the fact that DIDS was equally inhibitory when added after sickling was complete, this model would require that activation of the pathway be ongoing and reversible, in contrast to the static nature of the morphologic changes associated with sickling. In either case, the characteristics of DIDS inhibition of deoxygenation-induced cation fluxes are inconsistent with a model whereby membrane mechanical "stretch" leads directly to cation leak. Rather, the data suggest that morphologic distortion triggers a sequence of physicochemical events in the membrane that result in permeability changes, with DIDS acting at one of the steps in that process. Additional data are required before the details of such a mechanism can be specified.

The capacity of DIDS to inhibit deoxygenation-induced cation fluxes irreversibly (Table 2) demonstrates the presence of a nucleophilic group in the vicinity of the binding site associated with inhibition of these fluxes. This binding site was present on the surface of oxygenated cells, indicating that this site was not unmasked in the course of deoxygenation. While covalent binding of DIDS produced irreversible flux inhibition, the covalent reaction per se was not involved in inhibition, as evidenced by the ability of DNDS and dipyridamole to inhibit, and by the lack of effect of SPICT. As mentioned earlier, these findings, as well as the inhibitory capacity of SITS, also indicate that the DIDS effect was not due to crosslinking by this homobifunctional reagent.

![Fig 5. Effect of stilbenes and related compounds on deoxygenation-induced cation flux. Data represent means and SE of five experiments. Deoxygenation-induced Rb influx was measured in the presence of the indicated concentrations of all compounds except SPICT. Cells were preincubated with 5 mmol/L SPICT in HBS for 30 minutes at 37°C followed by washing. After preincubation, cells were washed free of SPICT before flux assay. Cation permeability in oxygenated cells was not altered by treatment with SPICT. 32SO₄²⁻ influx was measured in cells in SST medium exposed to the indicated concentrations of DIDS, SITS, DNDS, and dipyridamole, and inhibition was >95%, 99%, 98%, and 90%, respectively. Anion transport inhibition by H₂-DIDS, phloretin, and SPICT was not measured. Based on reports in the literature, >98% inhibition would be expected for H₂-DIDS and phloretin, and 90% for SPICT under the conditions used.](image-url)
Deoxygenation-induced cation fluxes and anion exchange were clearly differentiated by their concentration dependence of DIDS inhibition (Fig 3). Inferences from these data about the relative affinities of the transport systems must be made with caution because of the irreversible nature of DIDS binding. At least in the case of the anion exchange, DIDS concentration is the limiting factor in transport inhibition under the conditions used, since both drug and band 3 receptor concentrations exceed the estimated $K_i$ of 0.04 μmol/L for the DIDS/receptor interaction. Therefore, the higher DIDS concentrations required for inhibition of deoxygenation-induced fluxes could theoretically reflect a lower receptor affinity, a larger number of receptors, or both. However, regardless of the numbers of receptors involved, if the two transport functions had DIDS receptors of equivalent affinity, superimposable titration curves would have been expected with this experimental protocol, given the following conditions: (1) the presence on oxygenated cells of the receptor associated with deoxygenation-induced fluxes; (2) the absence of significant effects of deoxygenation on the anion exchange titration curve, and (3) the fact that inhibition of both processes was complete before flux assay measurement, signifying completion of the receptor/isothiocyanate reaction (see Fig 3 legend). Because these conditions were all met, it can be concluded that a lower affinity of the receptor associated deoxygenation-induced cation flux inhibition must contribute to the difference in dependence on DIDS concentration of that system compared with the anion exchange mechanism. On the basis of the present data, no inference can be made regarding the number of DIDS receptors associated with inhibition of deoxygenation-induced cation fluxes.

In addition to this difference in apparent receptor affinity, deoxygenation-induced cation transport was differentiated from anion exchange by its response to exposure to DIDS at 4°C: anion transport was progressively inhibited, while deoxygenation-induced flux was not affected. The lack of inhibition of deoxygenation-induced cation flux by preincubation with DIDS at 4°C is not inconsistent with the irreversible inhibition obtained by preincubation at 37°C, but rather indicates that the covalent reaction between the DIDS isothiocyanate and the membrane nucleophile associated with deoxygenation-induced flux inhibition is more temperature-dependent than that between DIDS and the lysine amino group associated with covalent stilbene binding to band 3.

Further evidence distinguishing anion exchange from the mechanism mediating deoxygenation-induced fluxes is found in the response of the two systems to other stilbene derivatives and related compounds. SITS, DNDS, and dipryridamole yielded incomplete inhibition of deoxygenation-induced fluxes at concentrations that produced greater than 90% inhibition of anion exchange. Furthermore, two other anion exchange inhibitors, phloretin and SPICT, had no effect on deoxygenation-induced fluxes. It should be noted that furosemide, which has no effect on deoxygenation-induced fluxes, is also a potent inhibitor of anion exchange.

These three types of differences in response to inhibitors between anion transport and the deoxygenation-induced permeability change (distinct DIDS titration curves, different covalent reactivity toward DIDS at 4°C and different sensitivity toward inhibitors) are sufficient to disprove the working hypothesis that the DIDS effect on deoxygenation-induced fluxes was mediated via the well-characterized stilbene binding site of the anion exchange protein on these cells. Furthermore, the presence of deoxygenation-induced fluxes in AS cells and their inhibition by DIDS argues against the involvement of altered band 3 molecule with a lower reactivity toward DIDS. Identification of the membrane locus for this drug effect promises to shed light on the specific membrane alterations associated with sickling that result in the deoxygenation-induced change in membrane cation permeability unique to sickle cells.

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REFERENCES

9. Joiner CH, Dew A, Ge DL: Deoxygenation-induced fluxes in...
Deoxygenation-induced cation fluxes in sickle cells: II. Inhibition by stilbene disulfonates

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