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A Study of the Relationship between Inhibition of Anion Exchange and Binding to the Red Blood Cell Membrane of 4,4′-Diisothiocyano stilbene-2,2′-disulfonic acid (DIDS) and its Dihydro Derivative (H_2DIDS)

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Summary. DIDS (4,4′-diisothiocyano stilbene-2,2′-disulfonic acid) and H_2DIDS (4,4′-diisothiocyano-1,2-diphenyl ethane-2,2′-disulfonic acid) binding to the human red cell membrane proteins were studied as a function of concentration, temperature and time. Most binding sites were common to both. The common sites were in band 3 of SDS polyacrylamide gel electropherograms (Steck, 1974. *J. Cell Biol.* **62:**1), an unidentified adjacent band, and glycophorin. Reversible and irreversible binding occurred; both inhibited sulfate equilibrium exchange. The time courses of irreversible binding to band 3 and total binding to the membrane as a whole were biphasic. About 20% of H_2DIDS and >60% of DIDS binding were rapid, independent of temperature. Slow H_2DIDS binding was monoexponential, activation enthalpy 23 kcal/mole. The stoichiometry of irreversible H_2DIDS binding to band 3 was 1.1–1.2, concentration-dependent. Under the conditions studied (0–50 μM, hematocrit 10%, 5–37 °C) binding to band 3 was a constant fraction of total binding, 0.7 for H_2DIDS and 0.8 for DIDS.

Inhibition was a linear function of total binding, binding to band 3, and therefore also to nonband 3 sites, with either inhibitor during both phases. H_2DIDS inhibition was complete at 1.9×10^6 or 1.2×10^6 molecules/cell total and band 3 binding respectively. For DIDS the corresponding figures were 1.3×10^6 and 1.1×10^6.

It is shown how reagents of mixed function can react with biphasic kinetics. Binding to multiple contiguous sites may exhibit concentration-dependent stoichiometry. Under such conditions a linear inhibition-binding relationship is neither a necessary nor a sufficient condition for the identification of transport sites.

In an attempt to identify anion permeability controlling sites in the outer surface of the red cell membrane, Cabantchik and Rothstein [4, 3] studied the binding of certain derivatives of stilbene disulfonic acids.

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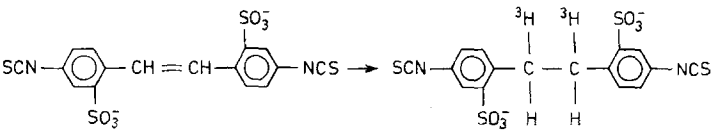
They reported that 4,4′-diisothiocyano stilbene-2,2′-disulfonic acid (DIDS) combines nearly exclusively with the protein in band 3 (nomenclature of Steck [22]) of SDS polyacrylamide gel electropherograms of the red cell membrane [4]. In addition they observed a linear relationship between DIDS binding to that protein and inhibition of anion equilibrium exchange and concluded that the DIDS binding sites on the protein in band 3 are involved in the control of anion exchange. The total number of DIDS binding sites was estimated to be about 300,000 per cell [4].

In most of their experiments Cabantchik and Rothstein [4] used tritiated DIDS to measure binding and untreated DIDS to demonstrate the corresponding inhibition of anion exchange. During the tritiation procedure employed in the formation of the labeled compound the double bond of the stilbene is reduced. The resulting product, $^3H_2$DIDS, is no longer a stilbene, but a 1,2-diphenylethane derivative:



Cabantchik and Rothstein performed control experiments which suggested that at equal concentrations H_2DIDS and DIDS produce identical degrees of inhibition of sulfate exchange. However, a more detailed investigation presented below shows that $^3H_2$DIDS is not a satisfactory label for DIDS. Thus, the former work had to be reproduced by using other materials and techniques. Although we observed a linear relationship between inhibition and binding for both H_2DIDS and DIDS, we found that the linearity observed by the former authors constituted the result of several compensating errors. Our work shows, in addition, that complete inhibition of anion transport is achieved when about 10 times as many molecules of DIDS or H_2DIDS are bound than originally estimated by Cabantchik and Rothstein. An important consequence of this latter finding is that the turnover number for anion transport per H_2DIDS or DIDS binding site is about one order of magnitude lower than previously thought. A detailed consideration leads to the conclusion that for this and other reasons the experiments presented by Cabantchik and Rothstein or in this paper do not prove that the bulk of the binding sites on the protein in band 3 participate in anion

transport. Nevertheless, they are compatible with such a view and show that DIDS and H_2DIDS will continue to be useful tools for the study of anion transport. For this reason, the paper is not confined to a revision of the former work but also deals extensively with the time course and stoichiometry of the binding of the two agents.

Materials and Methods

Synthesis of Labeled H_2DIDS, Determination of Specific Activity, and Assessment of Chemical Reactivity

$^3H_2$DADS [1] was prepared by catalytic hydrogenation. 300 mg of diamino-stilbene disulfonic acid (DADS), purified as previously described [25] were dissolved in 12.5 ml of water at pH 6.0; 50 Ci of tritium gas were allowed to react with the solution using palladium charcoal (Fluka GmbH) as a catalyst. After 45 min the process was terminated by treatment with hydrogen. The solution was then evaporated to dryness; labile protons were thrice allowed to exchange against water. During the third exchange period, 1,000 ml of water containing 700 mg of the cold H_2DADS were used. At the end of this procedure about 30 Ci ^3H remained attached to the substance in the form of stable bonds. Nonradioactive H_2DADS was synthesized by the same technique as the labeled compound except that hydrogen, instead of tritium, was used for the reduction of the double bond.

For the synthesis of the isothiocyanate, samples of 500 mCi of the tritiated H_2DADS were diluted with cold H_2DADS, the solvent was evaporated, and the compound was reprecipitated once from a small volume of solution at pH 7.0 by the addition of the appropriate amount of 6 N hydrochloric acid. The precipitate was filtered off. The purity of the compound was checked by paper electrophoresis in 0.05 M pyridine/acetic acid buffer at pH 6.5. The solution was then treated with a 50-fold excess of freshly distilled thiophosgene at pH 6.0 with continuous stirring for 45 min. The excess of thiophosgene was drawn off, and aliquots of the isothiocyanate solution were evaporated to dryness over sodium hydroxide and P_2O_5. An IR spectrum of the final product served as a further check of its purity. The dry solid was stored in vacuo, or after dissolving in water and separation into small samples in a deep freeze at $-16°$ C. Storage in the frozen state did not excced 8 weeks. During this time there was no measurable degradation of the compound as judged on the basis of its capacity to inhibit anion permeability and the determination of the specific activity by means of the fluorescence technique described below.

The specific activity was determined in 3 different ways: (1) by comparing the inhibition produced by equal concentrations of tritiated and unlabeled H_2DIDS, (2) by measuring the radioactivity of a sample of known weight, and (3) by the fluorescence technique described below. In this latter technique authentic bifunctional H_2DIDS is separated from possible contaminants, including degradation products of the radioactive compound. The technique is as follows:

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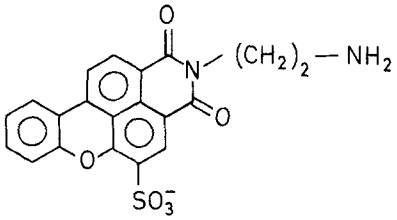
1 Abbreviations used in this paper: H_2DIDS = 4,4′-diisothiocyano dihydrostilbene-2,2′-disulfonic acid; DADS = 4,4′-diamino stilbene-2,2′-disulfonic acid; H_2DADS = 4,4′-diamino dihydrostilbene-2,2′-disulfonic acid; DIDS = 4,4′-diisothiocyano stilbene-2,2′-disulfonic acid; SITS = 4-acetamido-4′-isothiocyano stilbene-2,2′-disulfonic acid; DNFB = 1-fluoro-2,4-dinitrobenzene; V_{max} = maximal rate of transport in a saturable transport system; SDS = sodium dodecyl sulfonate; EDTA = ethylene diamine tetra acetic acid.

The labeled diisothiocyanate (2–10 mg) is dissolved in 4 ml of water and a solution of the xanthene derivative



(30 mg in 2 ml of water, adjusted to pH 8.0–8.5 with 20 mg of sodium bicarbonate) is added. The reaction is allowed to proceed for 4 hr. Aliquots of the solution are applied to 40 × 45 cm sheets of electrophoresis paper in 30 cm wide bands, and electrophoresis is carried out at pH 6.5 in 0.05 M pyridine-acetic acid buffer. The dye and H_2DIDS serve as references. The progress of electrophoresis is followed by observing the strong UV fluorescence of the dye.

The reaction between the diisothiocyanate and the dye leads to the formation of two bands corresponding to the coupling of one or two diisothiocyanate groups to dye molecules. The amount of the product of the reaction with two dye molecules exceeded that of the reaction with one molecule only. This dixanthene derivative moved clearly separated from the unreacted diisothiocyanate. This stilbene-xanthene compound is cut out and eluted with water. The extinctions at 425, 440, 445, 450, 520, 550 nm are measured and compared with the known spectrum of the compound. From the extinction coefficients at 425, 440, 445 nm, concentrations of the stilbene moiety are calculated. Aliquots of the eluate are subjected to counting of radioactivity under the same conditions as the labeled red cell membranes. The specific activity of the labeled H_2DIDS as determined by this method was 187,000 cpm/10^{-10} M as compared to a value of 196,000 cpm/10^{-10} M, which was obtained by measuring the radioactivity in a solution of known concentration prepared by dissolving solid tritiated H_2DIDS. The biological assay (technique 1) yielded values which agreed to within ±15% with the other two estimates (SE $n=4$).

Experimental Procedures

All experiments were performed with oRh$^+$ blood from healthy donors. The blood was stored in acid citrate dextrose buffer until further use, but no longer than 4–6 days.

Before use, the cells were washed 3 times at room temperature in a medium containing 122.5 mM NaCl, 5 mM Na_2SO_4, and 20 mM Na-phosphate pH 7.4. Subsequently, the cells were resuspended in the same medium containing $^{35}SO_4$, and equilibrated at 37 °C for 90 min. At the end of this equilibration period the cell suspension was transferred to the desired temperature and ^{3}H labeled H_2DIDS was added. At this stage, the hematocrit was 10%. After a certain length of time (usually 20 min) H_2DIDS binding to the cells was interrupted by subjecting the cells to a standard washing procedure. This procedure was similar to that employed by Cabantchik and Rothstein [4] and consisted of 2 washes in a solution containing 110 mM NaCl, 5 mM Na_2SO_4 and 50 mM TRIS-Cl pH 7.4, one wash in the same medium containing 0.5% bovine serum albumin (crystalline, Serva), and a last wash in the standard medium described above. Before the last wash, the cell suspension was subdivided into two samples and the sedimented cells from these samples were used for measuring H_2DIDS binding and sulfate equilibrium exchange. The whole washing procedure was performed at 0 °C. In the experiments on the time course of H_2DIDS binding, the reaction between cells and medium was stopped by the addition

of a large excess of ethylene diamine. We assured ourselves that the results obtained by this method agreed with those observed with the method described above.

For measuring SO_4 equilibrium exchange, the cells were resuspended at a temperature between 10° and 37 °C in the standard medium with no radiosulfate present. The hematocrit was 10%. The time course of appearance of $^{35}SO_4$ in the supernatant was followed by liquid scintillation counting. The window settings of the spectrometer were such that traces of radioactive H_2DIDS released from the cells into the medium did not interfere with the counting of $^{35}SO_4$. Rate constants were calculated as described by Zaki *et al.* [25].

For measuring H_2DIDS binding, after the last wash of the standard washing procedure, the cells were hemolyzed by 1:10 dilution in 10 mM KCl solution containing 5.0 mM EDTA pH 7.0 and 0.1% saponin. The membranes were washed in this same medium until white (usually 3–4 times). 0.1 ml of the packed membranes was subsequently mixed with 0.1 ml 5% SDS solution and heated at 100 °C for 3 min. After cooling and addition of 0.8 ml water, the dissolved membranes were frozen and stored at −16 °C until further use. The dissolved membranes were used for measurements of protein concentration by the method of Lowry *et al.* [13], determinations of radioactivity by liquid scintillation counting in Instagel (Packard), and for SDS polyacrylamide gel electrophoresis.

Gel electrophoresis was performed by applying 0.1 ml of the solution of dissolved membranes in SDS to a polyacrylamide gel (5% acrylamide, 0.15% bis-acrylamide). The gels and the electrode buffers contained 0.1 M sodium phosphate pH 7.1 and 0.5% SDS. After 10 min at 1 mamp/tube the current was increased to 5 mamp/tube and maintained at this value until the end of the run (about 5–5$^1/_2$ hours). The gels (10 cm long) were run in duplicates or in triplicates. One gel was cut into about 40–45 slices for radioactivity determinations, the other gels were used for staining with Coomassie blue or the periodic acid Schiff reagent. The radioactivity was counted after dissolving each gel slice in 1.0 ml soluene (Packard) by incubation for 16 hr at 45 °C and subsequent dilution with Instagel. The recovery of the radioactivity on the gels was around 95%.

The experimental procedure for measuring DIDS binding and the inhibition by DIDS of sulfate equilibrium exchange was essentially similar to that described for H_2DIDS, except that DIDS binding was estimated by titration with $^3H_2$DIDS. For titration, the DIDS treated cells were subjected to the standard washing procedure. Subsequently, they were resuspended in the standard medium and exposed to a large excess of $^3H_2$DIDS (25 μM) for 90 min at 37 °C. Difference formation between $^3H_2$DIDS binding to the DIDS treated cells and control cells which had not been exposed to DIDS yielded the amount of DIDS binding. $^3H_2$DIDS binding to the protein in band 3 was determined as described above.

The results of the radioactivity determinations are expressed in molecules/cell. For this purpose the number of H_2DIDS molecules/cell was calculated using the known specific activity of tritiated H_2DIDS and the known numbers of cells per mg of membrane protein. For converting the determined amount of membrane protein into numbers of cells a conversion factor estimated by Dodge *et al.* [6] was used assuming that 1 mg of membrane protein corresponds to 1.41×10^9 ghosts. This conversion factor differs from that used by Cabantchik and Rothstein by the factor 1.29. The concentrations of H_2DIDS or DIDS in figure legends and tables refer to concentrations in the media prior to mixing with the cells. (*See* note added in proof.)

Results

H_2DIDS Distribution Pattern on SDS Polyacrylamide Gel Electropherograms

Fig. 1 shows an electropherogram of ghosts derived from $^3H_2$DIDS labeled red cells. We find that about 70% of the total radioactivity

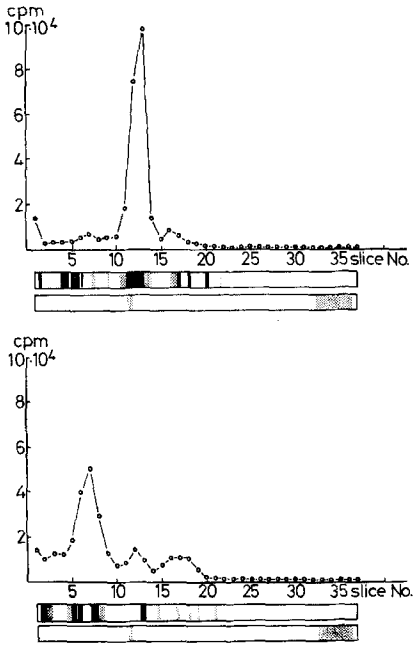


Fig. 1. SDS polyacrylamide gel electropherograms and labeling profiles of ghost proteins isolated from $^3H_2DIDS$ labeled red cells. *Upper curve*: ghosts derived from red cells labeled with 31 μM $^3H_2DIDS$ (20 min 5 °C). *Lower curve*: same ghosts as above, but treated with o-phenanthroline/$CuSO_4$. In each pair of gels, the upper gel represents the Coomassie blue staining pattern, the lower gel the periodic acid Schiff's staining pattern. The Coomassie blue stained band 3 is located underneath the peak of the $^3H_2DIDS$ labeling curve in the upper figure. The periodic acid Schiff's stained band at that location represents glycophorin. Qualitatively similar results are obtained when the cells are exposed to H_2DIDS at 37 °C

in the ghosts is located in band 3 and about 5% in a small adjacent band. The rest of the radioactivity is distributed over the whole gel. The identity of the labeled band adjacent to band 3 in terms of the protein species listed by Steck [22] has not yet been established.

In 5% polyacrylamide gels, band 3 and a glycoprotein, glycophorin, cannot be separated. For differentiating between H_2DIDS binding to the protein in band 3 and the glycoprotein, we followed Cabantchik and Rothstein and crosslinked the protein in band 3 by subjecting the labeled ghosts to the o-phenanthroline/$CuSO_4$ method of Steck [21]. This leads to a retardation of the protein in band 3 while the migration of glycophorin is not affected. The lower part of Fig. 2 shows that about 15–20% of the total label in the band 3 region has to be attributed to H_2DIDS binding to the glycophorin. After cross linkage the total radioactivity at the location of the "satellite" band is increased. It is

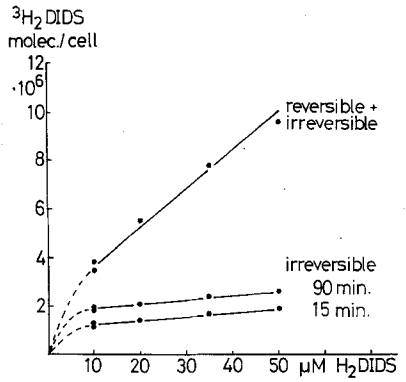


Fig. 2. Total H_2DIDS binding as a function of H_2DIDS concentration in the medium. The upper curve represents reversible plus irreversible binding. Its position varies only insignificantly with time so that the data points obtained after 15 and 90 min of exposure could not be plotted separately. The lower two curves represent irreversible binding as measured after subjecting the cells to the standard washing procedure. The times indicated on the curves represent the times allowed for irreversible fixation. Temperature 30 °C; standard conditions. (*See* note added in proof)

unclear where the additional radioactivity in that band comes from since it cannot be accounted for by cross linkage of proteins with lower molecular weight.

Reversible and Irreversible Binding of H_2DIDS and Effects on Anion Equilibrium Exchange

Previous studies of the effects of isothiocyanate derivatives of stilbene disulfonic acids on anion exchange suggested that irreversible fixation of these compounds to anion permeability controlling sites is preceded by reversible combination [2]. The occurrence of both forms of binding to sites in the red cell surface is directly shown in the experiment represented in Fig. 2. In this experiment the sum of reversible plus irreversible binding was calculated from the decrease of the $^3H_2$DIDS concentration in the medium brought about by the addition of the red cells. Irreversible binding was determined by measuring $^3H_2$DIDS in the ghosts derived from the same cells after interrupting the reaction with $^3H_2$DIDS by the addition of ethylene diamine (final concentration 2.0 mM) and subjecting the cells to the standard washing procedure. While no systematic change with time of the sum of reversible plus irreversible binding could be observed, irreversible fixation continued during the whole length of experimental observation (90 min, 30 °C).

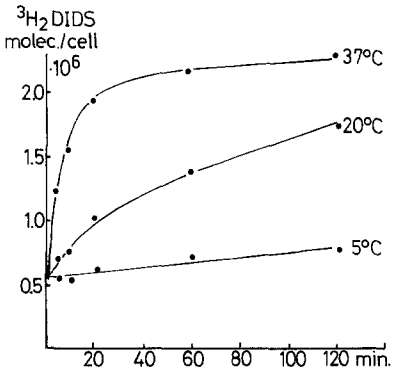


Fig. 3. Time course of total irreversible H_2DIDS binding to red cells as measured at 3 different temperatures. The curves were calculated by means of the equation $y=a_1+a_2(1-\exp(-k_D \cdot t))$. The constants a_1 and a_2 were independent of temperature and assumed to be $0.55 \cdot 10^6$ and $1.69 \cdot 10^6$, respectively. k_D was 0.0013, 0.0116 and 0.109 for 5 °C, 20 °C, and 37 °C, respectively. The intercept on the ordinate represents the fast phase of irreversible binding. H_2DIDS concentration in the medium: 25 μM

The increase of irreversible binding should be associated with some redistribution of the reversibly bound H_2DIDS between cells and medium. However, concentration changes in the medium associated with such redistribution may have escaped detection since the *change* in irreversible binding is small as compared to total binding.

The time course of irreversible H_2DIDS binding to whole cells is shown in more detail in Fig. 3. At temperatures between 5 and 37 °C, the curves can be fitted to the equation:

$$y=a_1+a_2\,(1-e^{-k_D t}) \qquad (1)$$

where a_1, a_2, and k_D represent constants and y the H_2DIDS binding at time t. At $t=0$, $y=a_1$; at $t=\infty$, $y=a_1+a_2$. A representation of the data by this equation implies that binding proceeds in two phases. During an initial rapid phase which is complete before the first data point can be sampled, about 20–25% of the available binding sites are occupied. Thereafter binding continues exponentially with the rate constant k_D. The fraction of rapidly occupied sites $a_1/(a_1+a_2)$ is little if at all dependent on temperature. The rate constant k_D for slow binding varies with temperature. The apparant activation enthalpy is constant over the temperature range studied and amounts to 23 kcal/mole (Fig. 4). These findings suggest that the subdivision of the time course of binding into rapid and slow phases is reasonable.

As will be shown below, irreversible binding to the protein in band 3 amounts to about 70% of the total irreversible binding to the outer

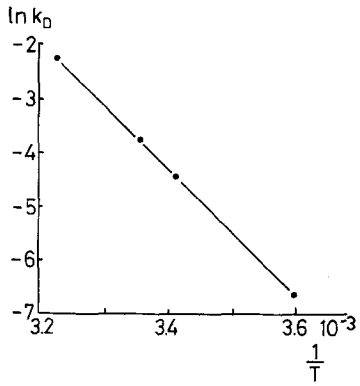


Fig. 4. Arrhenius plot of the rate constants for slow H_2DIDS binding as measured at 4 different temperatures. Standard conditions. H_2DIDS concentration in the medium: 25 μM

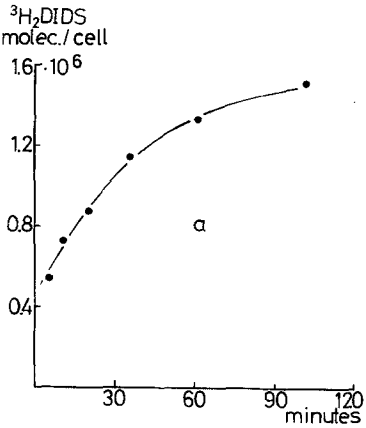


Fig. 5. Time course of transition from reversible to irreversible binding of H_2DIDS. The cells were incubated at 25 °C in the presence of 31 μM H_2DIDS in the medium. At the times indicated on the abscissa the reaction was stopped and irreversible binding was measured as described in the text. *Ordinate*: number of irreversibly bound H_2DIDS molecules on the protein in band 3, per ghost. *Abscissa*: time in min. The curve was calculated by means of Eq. (1), using $a_1 = 0.65 \cdot 10^6$, $a_2 = 1{,}36 \cdot 10^6$, $k_D = 0.0244$. a_1 represents irreversible binding during fast phase

cell surface. Nevertheless, the two phases of binding cannot be related to differences of the time course of H_2DIDS fixation to binding sites on the protein in band 3 and on other membrane proteins. This is shown in Fig. 5 where the time course of irreversible $^3H_2$DIDS fixation to the electrophoretically separated protein in band 3 has been analyzed as described above for total binding. Again two phases of binding are observed. The constants in Eq. (1) are similar to those expected for total irreversible binding at 25 °C.

Anion equilibrium exchange is inhibited by both reversibly and irreversibly bound H_2DIDS molecules. This is illustrated by the experiments shown in Fig. 6 and Table 1. In these experiments the cells were exposed to 25 μM H_2DIDS and sulfate equilibrium exchange was measured either after removal of reversibly bound H_2DIDS by the standard washing procedure or in the presence of H_2DIDS when, in addition to the irreversibly bound H_2DIDS molecules, reversibly bound H_2DIDS molecules are associated with the membrane. Obviously, the inhibition measured in the presence of H_2DIDS in the medium exceeds the inhibition observed after subjecting the cells to the washing procedure. In spite of the diphasic time course of H_2DIDS binding described above, the relationship between irreversible binding and the inhibition of anion exchange observed after various lengths of exposure to H_2DIDS is strictly linear (Fig. 7).

Irreversible H_2DIDS Binding to Whole Cells and to the Protein in Band 3 in Relation to Inhibition of Anion Equilibrium Exchange

Fig. 8 shows the relationship between H_2DIDS concentration in the medium and either H_2DIDS binding or inhibition of sulfate exchange. Binding and inhibition were measured in the same cells after exposure to H_2DIDS for 20 min at the temperatures indicated in the figure and subsequent washings by the standard procedure to remove reversibly bound H_2DIDS.

The relationships between concentration and total binding or binding to the protein in band 3 have the superficial appearance of saturation curves with temperature dependent variations of the numbers of saturable sites (Fig. 8*b* and *c*). However, as can be inferred from the data in Fig. 3 and Table 1, they do not represent saturation of variable numbers of binding sites but varying degrees of completion of reaction.

In order to assess the relationship between H_2DIDS binding and effect on anion permeability, in Fig. 9 the rate of sulfate equilibrium exchange is plotted against the number of irreversibly modified binding sites on the whole cell or on the protein in band 3 of each cell. The data points from all three curves in Fig. 8 and additional data points scatter around a straight line with 100% inhibition at a binding of 2.57×10^6 molecules per whole cell or of 1.74×10^6 molecules per cell on the protein in band 3. (*See* note added in proof.)

The additional data points mentioned above were taken from experiments in which H_2DIDS binding was allowed to take place at concentrations between 0–50 μM, at temperatures between 5 and 37 °C, for times ranging from 5 min to 120 min and in which the flux measurements

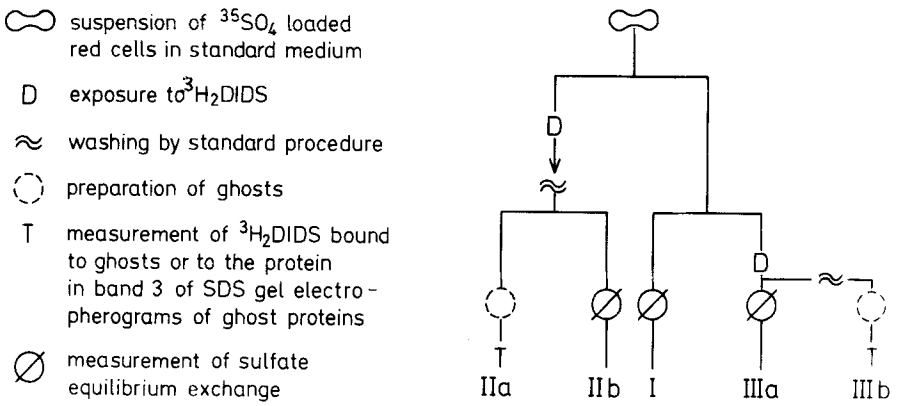
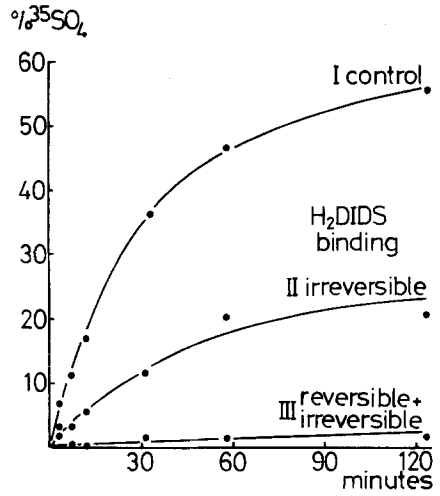


Fig. 6*a*. Effects of reversible and irreversible H_2DIDS binding on sulfate equilibrium exchange. (I) Flux measurements in the absence of H_2DIDS in cells and medium: control. (II*a*) Irreversible H_2DIDS binding as measured after 20 min of exposure to H_2DIDS in standard medium. (II*b*) Flux measurements in the absence of H_2DIDS in the medium: inhibition of sulfate equilibrium exchange by irreversibly bound H_2DIDS only. (III*a*) Flux measurements in the presence of H_2DIDS in the medium: inhibition of sulfate equilibrium exchange by irreversibly plus reversibly bound H_2DIDS. (III*b*) Irreversible H_2DIDS binding as measured 20 min after start of exposure to H_2DIDS: measurement in sample withdrawn from suspension in which the flux measurements are performed. Exposure to H_2DIDS and flux measurements were performed at the same temperature in identical media



	irreversibly bound $^3H_2$DIDS molecules per cell		
	whole cells	band 3	inhibition %
I	0	0	0
II	$1.79 \cdot 10^6$	$1.19 \cdot 10^6$	78.2
III	$1.87 \cdot 10^6$	$1.30 \cdot 10^6$	98.3

Fig. 6*b*. Effect of H_2DIDS on sulfate equilibrium exchange in human red cells. (I) Control, no H_2DIDS present. (II) Cells treated with H_2DIDS prior to the initiation of the flux measurements. Reversibly bound H_2DIDS removed by standard washing procedure. No H_2DIDS present in the medium. (III) H_2DIDS present in the medium during the flux measurements. The details of the experimental procedure are described in the scheme of Fig. 6(*a*). The H_2DIDS concentration was 25 μM, the temperature throughout all stages of the experiment 30 °C, the pH 7.4. The time of exposure to H_2DIDS in sample II was 20 min. *Ordinate*: percent of incorporated $^{35}SO_4$ that appeared in the supernatant. *Abscissa*: time. The table represents irreversible H_2DIDS binding as measured after a reaction time of 20 min, (II) prior to the flux measurements in the absence of H_2DIDS in the medium, (III) during the flux measurements in the presence of H_2DIDS in the medium

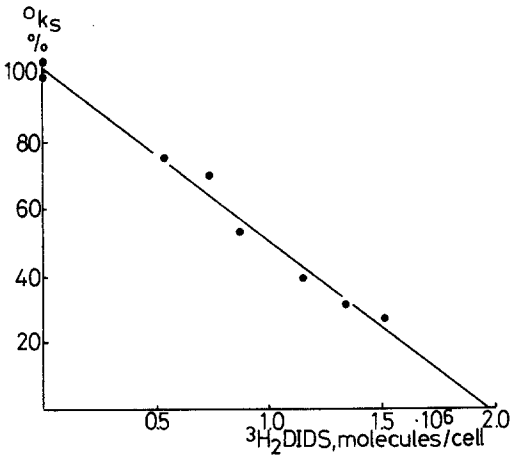


Fig. 7. Rate constant for sulfate equilibrium exchange, ^0k_s, plotted against the number of H_2DIDS molecules irreversibly bound to the protein in band 3. Same experiment as in Fig. 5. Fluxes were measured after interrupting H_2DIDS uptake by ethylene diamine and the standard washing procedure at the times at which the data points in Fig. 5 were obtained. ^0k_s is expressed as percent of the control value obtained in the absence of H_2DIDS

Table 1. Effects of reversible and irreversible H_2DIDS binding on sulfate equilibrium exchange

H_2DIDS μM	Temperature °C	H_2DIDS, molec./cell × 10^6		Inhibition of anion exchange percent	Exposure to H_2DIDS
		Irreversible binding to whole cells	band 3		
5	37	1.61	1.14	75.8	I[a]
5	37	2.19	1.68	91.8	III
20	30	1.79	1.19	76.3	II
20	30	1.87	1.30	100.0	III
20	20	0.938	0.633	42.1	II
20	20	0.942	0.618	97.7	III
20	10	0.360	0.229	16.7	II
20	10	0.368	0.176	92.5	III
(1)	(2)	(3)	(4)	(5)	(6)

[a] The Roman numerals in column (6) refer to experimental procedures described in detail in Fig. 6*a* and designate measurements made after removal of reversibly bound H_2DIDS by the standard washing procedure (II) or during exposure to H_2DIDS (III). In the experiments designated above II and III, irreversible H_2DIDS binding and SO_4 flux were measured as indicated in Fig. 6*a* by methods II*a* and II*b*, or III*a* and III*b*, respectively.

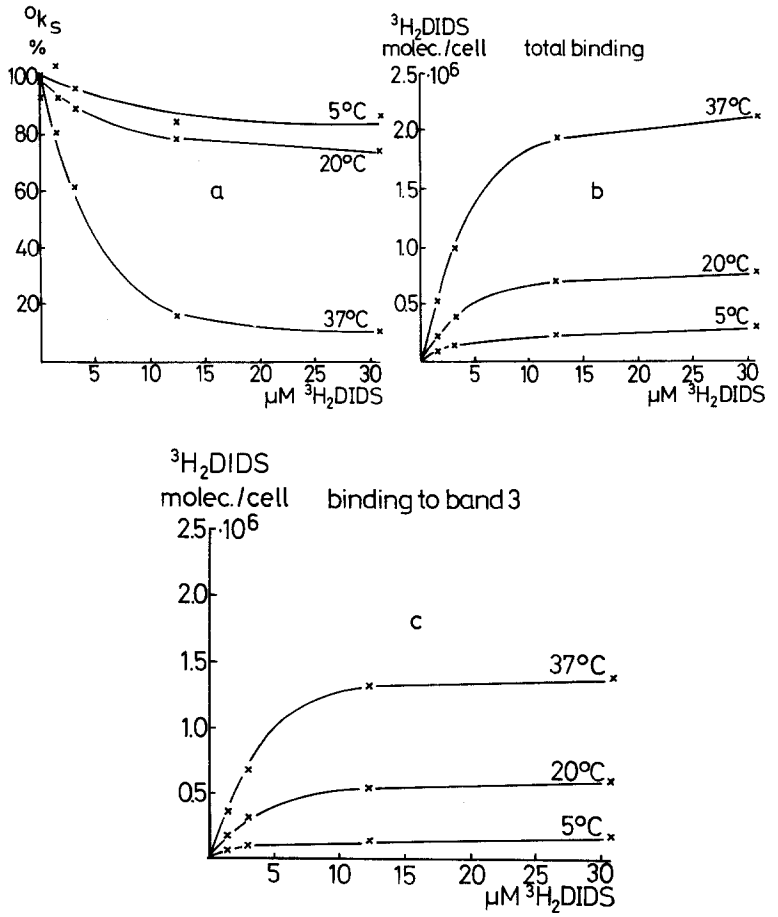


Fig. 8. Effect of varying H_2DIDS concentration in the medium on (*a*) sulfate equilibrium exchange, (*b*) H_2DIDS binding to whole cells and (*c*) H_2DIDS binding to the protein in band 3. Inhibition and binding measured after 20 min of exposure to H_2DIDS and subsequent removal of reversibly bound H_2DIDS by the standard washing procedure. Rate constants, ^0k_s, were measured at 30 °C and are expressed as percent of the control value (no H_2DIDS present)

in the irreversibly modified cells were performed at temperatures between 10 and 37 °C. The incorporation of data based on flux measurements made at varying temperatures is only permissible if at all if temperature inhibition by the various H_2DIDS concentrations reflects the modification of the same step of the transport mechanism. Modification of red cells with dinitrofluorobenzene or pronase lead to considerable changes of the activation enthalpy for anion equilibrium exchange as measured after removal of these modifiers [20]. This indicates that these agents produce two or more different effects on anion transport. In contrast, in H_2DIDS treated cells the activation enthalpy is not significantly differ-

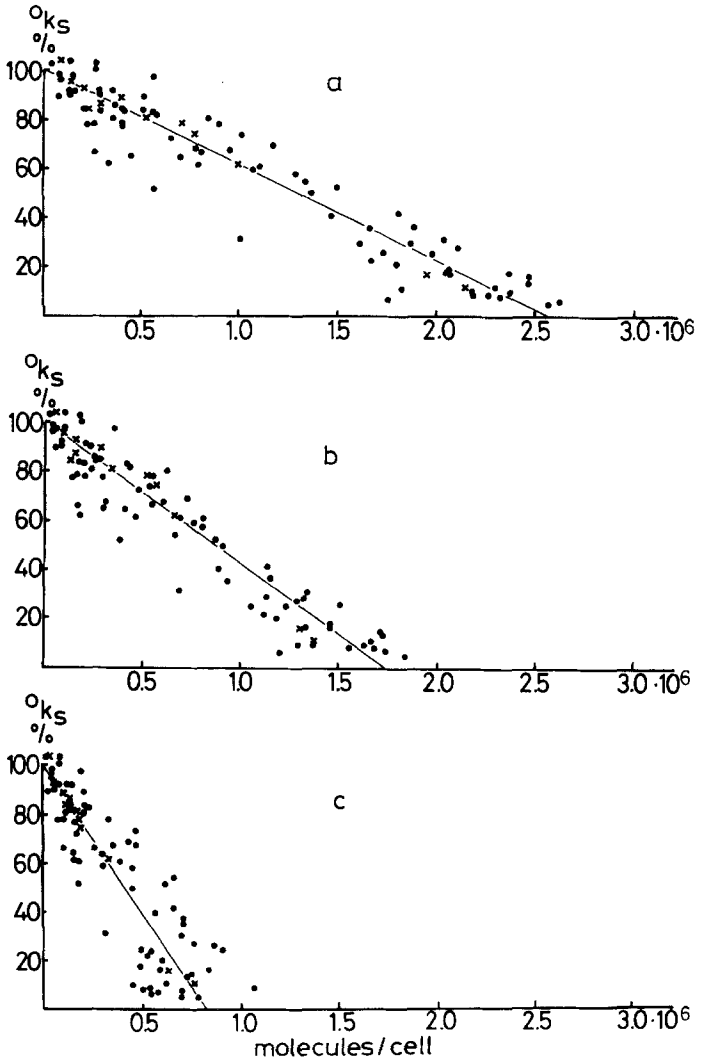


Fig. 9. Sulfate equilibrium exchange as a function of H_2DIDS binding to (*a*) whole cells, (*b*) the protein in band 3, (*c*) binding sites other than those in band 3 (difference between (*a*) and (*b*)). The data points have been obtained in 15 different experiments. There was a tendency for data points from the individual experiments to lie much closer to a straight line than the composite of the data for all experiments. Examples are the data in Fig. 8 which are represented in this figure by crosses and the data shown in Fig. 7 which are also included in this figure but not specifically marked. *Ordinate*: rate constant for sulfate equilibrium exchange in percent of control value measured in the absence of H_2DIDS. *Abscissa*: H_2DIDS binding in molecules/cell. The straight lines in Fig. 9*a* and *b* represent maximum-likelihood estimates as calculated on the assumption that both variables contain error (Acton, [1], p. 132 *ff.*) The result of the calculations was affected to within less than 1% when the correlation between the errors of measured rates of sulfate movements (ordinate) and binding (abscissa) was varied between -1 and $+1$ and when the *ratio* of the variances of ordinate and abscissa was varied between 0.8 and 1.25. Although the curve fitting procedure yields the best fit, it does not allow an estimate of the error of the number of binding sites at maximal inhibition without a hypothesis about the correlation between the errors on ordinate and abscissa. An alternative method for determin-

ent from that of intact cells (32 kcal/mole). This justifies the pooling of the data on inhibition of anion exchange derived from flux measurements with H_2DIDS-treated cells at various temperatures.

Effects of DIDS on H_2DIDS Binding

In the experiment represented in Fig. 10, red cells were mixed with media which contained, in addition to a fixed concentration of $^3H_2$DIDS, increasing concentrations of either DIDS or H_2DIDS. After exposure of the cells to these media for 90 min at 30 °C, the cells were separated from the supernatant and subjected to the standard washing procedure. The figure shows that DIDS is much more effective than H_2DIDS in reducing $^3H_2$DIDS binding to the membrane as a whole and to the protein in band 3. This finding could reflect differences of affinity for common binding sites during initial reversible binding, of the number of binding sites available for subsequent irreversible fixation, of the rates of transition from reversible to irreversible fixation, or any combination of these factors.

Closer inspection of Fig. 10*a* reveals that even in the presence of a large excess of DIDS there is still some residual $^3H_2$DIDS binding which decreases only slightly with a further increase of the DIDS concentration. This residual $^3H_2$DIDS binding amounts to 10–15% of the maximal $^3H_2$DIDS binding as measured in the absence of DIDS. It suggests the presence of binding sites with different relative susceptibilities for DIDS and H_2DIDS. Fig. 10*b* indicates that on the protein in band 3 the differences between binding of H_2DIDS and DIDS are less pronounced or absent.

The described observations can be used as a basis for the quantitative determination of irreversible DIDS binding to the red cell membrane. For this purpose red cells are first exposed to DIDS, subsequently subjected to the standard washing procedure, and then exposed to $^3H_2$DIDS. The SDS polyacrylamide gel electropherogram reproduced in Fig. 11 shows that under the described conditions DIDS is capable of nearly completely preventing the binding of $^3H_2$DIDS to the protein in band 3

ing the maximal numbers of binding sites consists of plotting the relationship between rate constant and binding for each individual experiment by eye, and then calculating the mean of all extrapolated values and the corresponding standard error of this mean. The result of this procedure was $2.6 \cdot 10^6 \pm 0.13$ sites/cell and $1.7 \cdot 10^6 \pm 0.08$ sites/cell for total binding and binding to band 3, respectively. These figures are based on the conversion factor given by Dodge *et al.*, [6]. *See* text and note added in proof

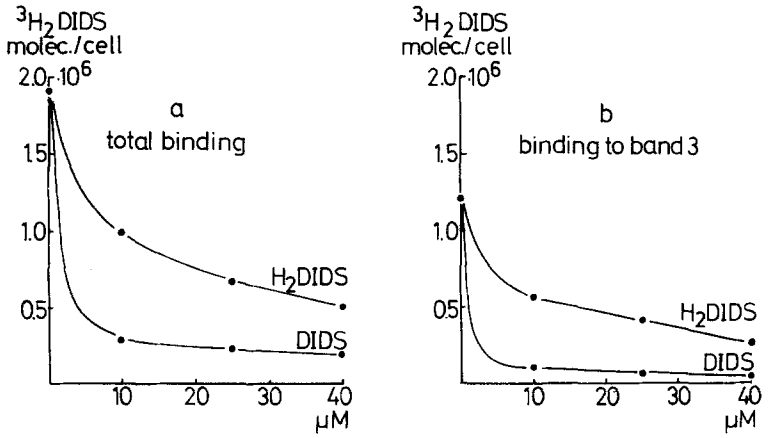


Fig. 10. $^3H_2$DIDS binding to the cell membrane in the presence of increasing concentrations of H_2DIDS or DIDS. Concentration of $^3H_2$DIDS, 10 μM. Time of exposure, 90 min. Temperature, 30 °C. *Abscissa*: concentration of nonradioactive H_2DIDS or DIDS, respectively

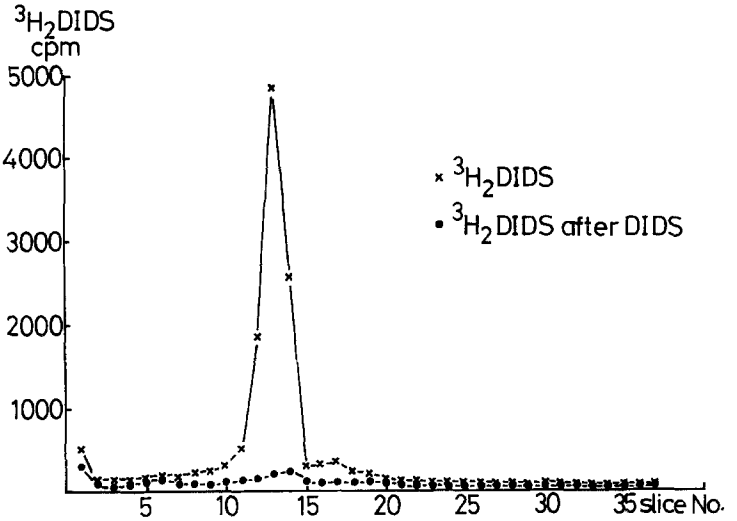


Fig. 11. Effect of treating red cells with DIDS prior to the exposure to $^3H_2$DIDS on SDS polyacrylamide gel electropherograms of membrane proteins. The cells were first incubated in the presence of 12 μM DIDS for 20 min at 37 °C. After removal of unreacted DIDS by washing, they were incubated in the presence of 25 μM $^3H_2$DIDS for 90 min at 37 °C. The curve marked *x* represents $^3H_2$DIDS binding to cells which had not been preexposed to DIDS

and to the adjacent "satellite" band. This allows the estimate of DIDS binding by forming the difference between $^3H_2$DIDS binding to untreated and DIDS-treated red cells. The difference formation renders the determination of binding of DIDS less accurate than that of $^3H_2$DIDS. More-

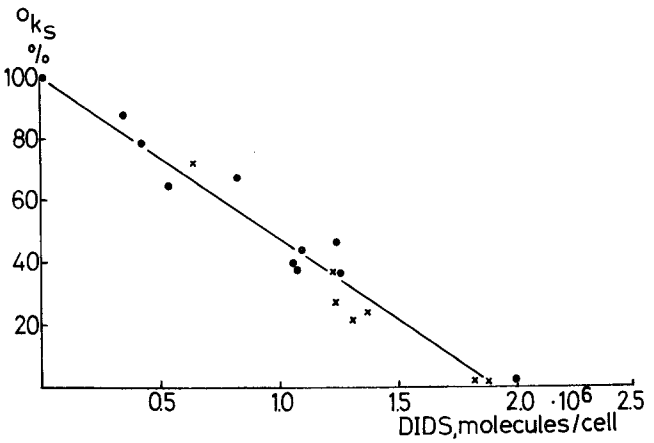


Fig. 12. Rate constant for SO_4 equilibrium exchange in percent of control with no DIDS as a function of total DIDS binding. Time of exposure to the inhibitors, 20 min. Standard conditions. Rate constants, ^0k_s, were measured at 30 °C and are expressed as percent of the control value (no DIDS present)

over, DIDS binding to sites which are unable to react with H_2DIDS are not detected. Therefore, the estimate of DIDS binding may be biased in favor of an underestimate.

Irreversible DIDS Binding to Whole Cells and to the Protein in Band 3 in Relation to Inhibition of Anion Equilibrium Exchange

In a number of experiments whose design was basically similar to that of the experiments on the relationship between H_2DIDS binding and inhibition of anion exchange we find that there exists a linear relationship between inhibition and binding of DIDS to the whole cell (Fig. 12). As in the experiments with H_2DIDS, binding to the protein in band 3 is a constant fraction of total binding. However, DIDS binding to the protein in band 3 amounts to 80% of the total binding instead of 70% as observed with H_2DIDS. Anion transport was completely inhibited when DIDS binding amounted to 1.9×10^6 molecules per whole cell, or 1.5×10^6 molecules per cell on the protein in band 3.

It was shown above that the time course of H_2DIDS binding to whole cells and to the protein in band 3 could be subdivided into two phases. The rapid phase was limited to about 20% of total binding with H_2DIDS, whereas with DIDS it amounted to about 60% of total binding (Fig. 13), and for the reasons given above this figure may represent an underestimate. Still, as with H_2DIDS the relationship be-

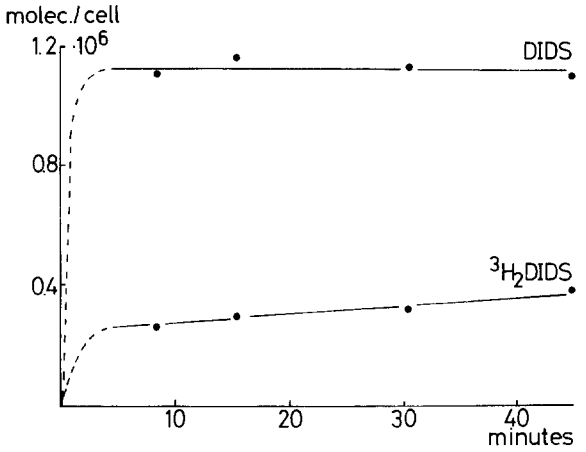


Fig. 13. Time course of binding of DIDS and H_2DIDS to whole cells. Temperature 5 °C. Concentrations of DIDS and H_2DIDS 10 μM. The reaction was interrupted by the addition of sufficient ethylene diamine to give a final concentration in the medium of 2.0 mM. The total number of sites available for the binding of H_2DIDS (90 min 37 °C) was $2.6 \cdot 10^6$/cell

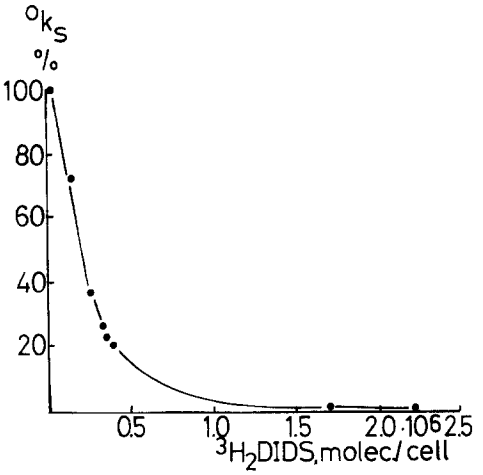


Fig. 14. Inhibition of sulfate exchange by DIDS plotted against binding of $^3H_2$DIDS. Binding and inhibition were measured in separate experiments at identical concentrations of DIDS and $^3H_2$DIDS. Same procedure as used by Cabantchik and Rothstein (*see* text)

tween binding and inhibition follows a straight line over the whole range between 0 and 100% inhibition (Fig. 12). This indicates that the effect on anion exchange cannot be associated with binding to sites which can be identified by their rate of reaction with the inhibitor.

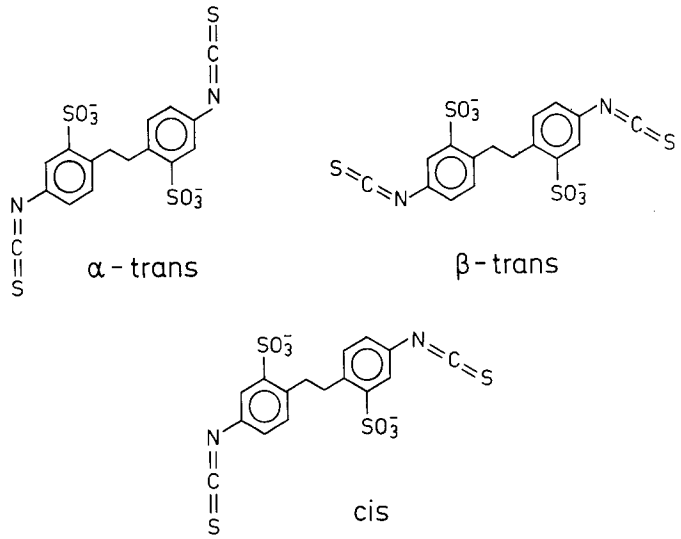


Fig. 15. The three isomers of the H_2DIDS molecule

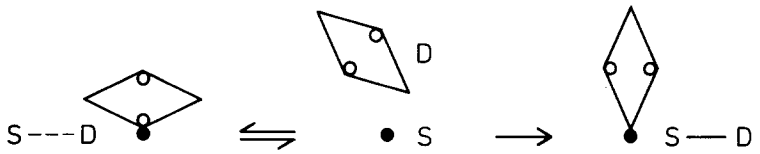


Fig. 16. Possible reactions between H_2DIDS and a single site. The H_2DIDS molecule is approximated by a rhombus of angle 60 °C with the sulfonic acid groups at the ends of the shorter diagonals (*o*) and the isothiocyanate groups at the apices. *D* and *S* denote H_2DIDS and the binding site in the membrane, respectively. *D*---*S* represents binding by Coulombic forces, *D*—*S* by covalent linkage. The choice between covalent and Coulombic reaction will depend on the protonation equilibria of the membrane site involved

Use of $^3H_2$DIDS as a label for DIDS

As has already been mentioned in the introduction, Cabantchik and Rothstein [4] measured in separate experiments total binding with $^3H_2$DIDS, and inhibition of anion exchange with DIDS. Assuming that the red cell does not discriminate between H_2DIDS and DIDS, they plotted the results obtained at equal concentrations of the two inhibitors against one another. Fig. 14 shows what happens when we follow this procedure using data obtained under essentially similar conditions as those employed by Cabantchik and Rothstein (exposure to DIDS or $^3H_2$DIDS for 20 min at 5 °C). Obviously, only the initial portion of the curve is linear and the number of binding sites attributed to a given degree of inhibition is much lower than estimated from plots of DIDS

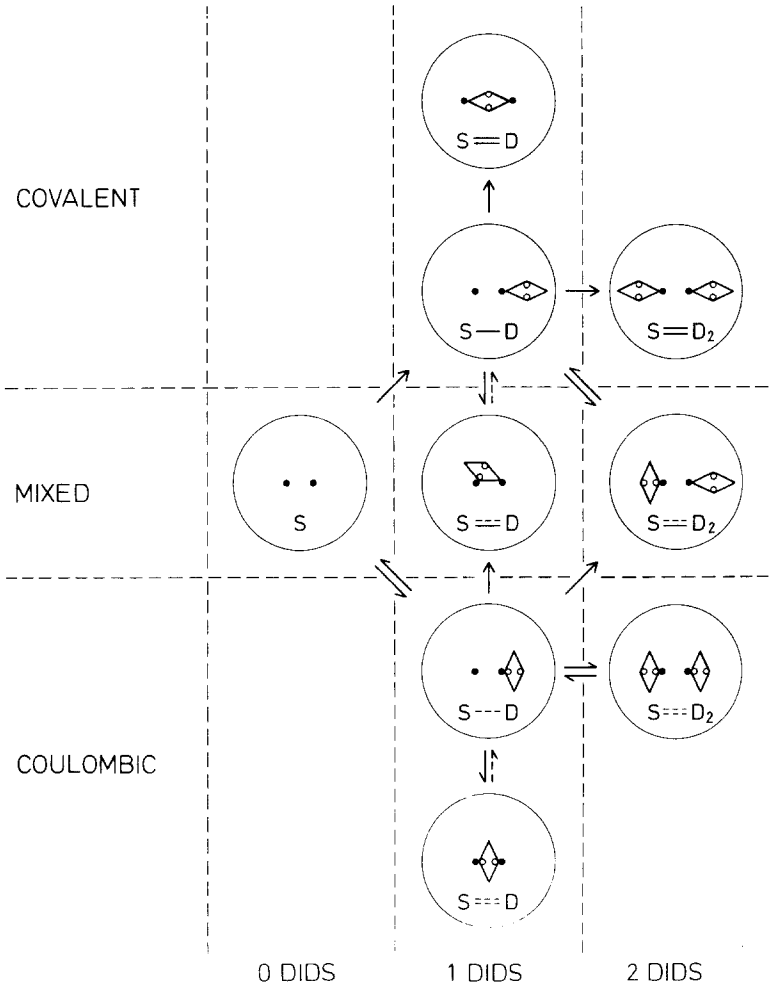


Fig. 17. Possible reactions between pairs of identical sites and H_2DIDS. *D* and *S* denote H_2DIDS and a pair of binding sites in the membrane, respectively. --- and —— represent binding by Coulombic forces or covalent linkage, respectively. The existence of the forms *S---D* and *S---D* and that of the form *S═D* are mutually exclusive if identical intersite distances are assumed. Any joint occurence would depend on the mobility of the sites

binding against the effect of DIDS or of H_2DIDS binding against the effect of H_2DIDS. These findings can be explained by the fact that at equal concentrations, after 20 min at 5 °C, DIDS binding exceeds $^3H_2$DIDS binding (*see* Fig. 13). Under the specified experimental conditions, the rate of irreversible $^3H_2$DIDS binding slows down much further below saturation of its binding sites than DIDS (*cf.* Fig. 13). Although these findings seem to explain most of the differences between the results

obtained in Frankfurt and Toronto, the question remained open why Cabantchik and Rothstein found, in two control experiments, that at equal concentrations in the medium $^3H_2$DIDS and DIDS produced the same degree of inhibition (*see* Fig. 2 of their paper [4]) and why they obtained a linear relationship when they plotted H_2DIDS binding against inhibition by DIDS. A detailed discussion with Dr. Aser Rothstein revealed that after the tritiation of that batch of 4,4′-diamino stilbene-2,2′-disulfonic acid, which was used in Toronto for the synthesis of the diisothiocyanate derivative, the manufacturer did not perform the usual exhaustive hydrogenation of the labeled product. As a consequence, this product still contained a large fraction of the original stilbene which, after treatment with thiophosgene was converted to DIDS. Thus, the batch of the tritiated diisothiocyanate derivatives used for the published work of Cabantchik and Rothstein probably contained large quantities of DIDS besides relatively small amounts of $^3H_2$DIDS. This explains why the tritiated and the untreated DIDS produced the same degree of inhibition. It remained unresolved why the estimate of the number of binding sites in the red blood cell was not even lower than actually reported and which compensating factors led to the observed linear relationship between binding of $^3H_2$DIDS and the effect of DIDS on anion transport.

Discussion

The Protein in Band 3 and Anion Transport

Our results show that $^3H_2$DIDS is not a suitable label for DIDS and that previous estimates of DIDS binding by labelling with $^3H_2$DIDS need to be revised. We find that both DIDS and H_2DIDS have most, but possibly not all, of their binding sites in the cell membrane in common but that DIDS is more effective than H_2DIDS in competing for these common sites. Inhibition of anion transport is complete when 2.6×10^6 H_2DIDS molecules per cell or 2.0×10^6 DIDS molecules per cell are bound to the erythrocyte membrane. These figures were calculated on the assumption that 1 mg of membrane protein corresponds to 1.43×10^9 cells (Dodge et al., [6]). If we recalculate these values using Cabantchik and Rothstein's assumption that 1 mg of protein corresponds to 1.11×10^9 cells [4] we find 3.3×10^6 and 2.6×10^6 molecules/cell for H_2DIDS and DIDS, respectively. Our own recent estimate of the conver-

sion factor yielded 1.95×10^9 cells/mg membrane protein. This would correspond to a total binding of 1.88×10^6 molecules/cell of H_2DIDS and of 1.23×10^6 molecules/cell of DIDS (*see* note added in proof). Thus, our data show that the binding of H_2DIDS or DIDS is at least 4 times as high as was originally thought.

Work with DIDS and other stilbene disulfonic acids has lead to the suggestion of an involvement of the protein in band 3 in anion transport. Do our results affect the validity of this suggestion? To provide an answer, the various pieces of evidence for such involvement are discussed separately below.

1. The only common binding sites for two different inhibitors of anion exchange, dinitrofluorobenzene (DNFB) and stilbene disulfonic acid derivatives, are located in band 3 [15, 25, 26].[2]

2. On SDS polyacrylamide gel electropherograms no DIDS binding sites could be detected at distinct locations outsite the location of band 3 [4, 17].

3. There exists a linear relationship between inhibition of anion exchange by DIDS and H_2DIDS binding to the whole cell. Assuming that the protein in band 3 carries the only specific binding sites it is inferred that this is equivalent to a linear relationship between inhibition and the number of modified sites on the protein in band 3 [4].

4. On the assumption that the turnover number per anion transport site cannot exceed the turnover number for the fastest known enzyme reaction, the number of transport sites/cell has been calculated (Ho and Guidotti [9]). This value agrees with Cabantchik and Rothstein's estimate of the number of DIDS binding sites.

An involvement of the bulk of the protein in band 3 in anion transport was simultaneously suggested by Rothstein *et al.* [17] and Zaki and Passow [26]. However, while the group in Toronto went ahead with the publication *in extenso* of their findings [4] we hesitated until recently [15, 25] since we felt that small numbers of binding sites on membrane constituents other than the protein in band 3 could have remained undetected. In the gel electropherograms of $^3H_2$DIDS treated red cell membranes we find that in fact only about 70% of the total radioactivity resides in band 3. This agrees with an evaluation of the gel electropherograms published by Cabantchik and Rothstein. However, while in Cabantchik and Rothstein's experiments that fraction of the radioactivity

2 Following our example, Rothstein and his associates have recently shown (*Fed. Proc.* **35**:3, 1975) that common binding sites for another pair of inhibitors, H_2DIDS and pyridoxal phosphate, are also confined to the protein in band 3.

which does not reside at the location of band 3 is more or less equally distributed over the whole gel, we always discover a small distinct band adjacent to band 3, which could not yet be related to any of the known membrane proteins. The number of $^3H_2$DIDS molecules in this band amounted to about 12% of those in band 3, i.e., to about 210,000–270,000 sites/cell, as calculated by means of the conversion factors of Dodge *et al.* [6] and Cabantchik and Rothstein [4], respectively. Perhaps in Cabantchik and Rothstein's gels, this "satellite" band is hidden in band 3 as suggested by the occasional appearance of a shoulder of band 3 (*see* Fig. 7 in [4]) and Fig. 8 in [3]). In addition to the satellite band, glycophorin also binds $^3H_2$DIDS. The number of binding sites amounts to about 16% of those in band 3 or 270,000–350,000 sites/cell, where the former value was calculated assuming the conversion factor of Dodge *et al.* [6], the latter using that of Cabantchik and Rothstein [4]. These numbers of binding sites on the two clearly identifiable locations of the gels are of the same order of magnitude as those previously assumed for band 3 [4]. There are good reasons to assume that glycophorin is not involved in anion transport (Tanner, *unpublished work*, *see* also [4]). However, the participation of the protein in the satellite band or still smaller numbers of membrane constituents which have so far escaped detection may also play some role. Obviously the distribution of $^3H_2$DIDS on SDS gel electropherograms is compatible with the assumption that the protein in band 3 is involved but does not prove the case.

The demonstration of a linear relationship between inhibition and DIDS binding to the protein in band 3 could, under certain conditions (*see* below), represent stronger evidence than that listed under (1) and (2), but only if it could be shown that binding to other membrane constituents does not follow a similar relationship. The possible reasons why Cabantchik and Rothstein observed a straight line relationship when they plotted inhibition by DIDS against binding of $^3H_2$DIDS (which should not yield a straight line) are discussed on p. 164. In the present context it may suffice to state that such a linear relationship does nevertheless exist even if inhibition by DIDS or H_2DIDS is plotted against binding of DIDS or H_2DIDS, respectively (Figs. 9 and 12) However, as shown in Fig. 9, both inhibition as plotted against binding to the whole cell and against binding to the protein in band 3 yields a straight line (Cabantchik and Rothstein only measured binding to the whole cell), the slopes of the two straight lines being different. This implies that a plot of sulfate exchange against binding to sites other than those on band 3 should also yield a straight line. Although the difference

formation leads to a considerable increase in the scatter of the data points, it is obvious that our results are compatible with such a straight line. Hence, although the results would support the assumption of a participation of the protein in band 3 in anion transport, they do not prove the case, since it cannot be ruled out that some of the 800,000 binding sites which do not reside on band 3 are involved.[3]

Since it is difficult to eliminate H_2DIDS binding to the satellite band or to some less abundant membrane constituent as a cause of the observed inhibition of anion exchange, an estimate of the minimum number of sites which are required to accomplish anion transport at the observed rate would be useful. Such an estimate has been attempted by Ho and Guidotti [9]. Assuming that the turnover number at an anion permeability controlling site cannot exceed the turnover number of one of the fastest acting enzymes, catalase, Ho and Guidotti arrived at a value of about 10^5 anion transport sites/cell. Our new estimate of the number of H_2DIDS binding sites raises the number of sites on the protein in band 3 to 1.7×10^6/cell, which is far above this limit, and the number of binding sites on glycophorin or the satellite band to the order of magnitude calculated by Ho and Guidotti.

Ion transport through lipid bilayers can be mediated by carriers or channels. Hence it would be useful to extend the approach of Ho and Guidotti to transport via carriers and channels. The turnover number of the carrier transport by valinomycin has been estimated to be about 10^4/sec [11], that for gramicidin A channels to be about 10^7/sec [12] (the figures in [11] and [12] pertain to 25 °C). Using as the turnover number for the anion transport system a value of 3.3×10^{10} ions/cell/sec[4] one arrives at numbers of transport sites per cell ranging from 3.3×10^6/cell to 3.3×10^3/cell. Since the bulk of the anion movements does not contribute to the conductance of the red blood cell membrane [8, 10,

3 In an attempt to label the anion transport protein, Ho and Guidotti [9] recently used, instead of the isothiocyanate of a stilbene disulfonic acid, the isothiocyanate of a benzene sulfonic acid. They showed that there exists a linear relationship between binding to the protein in band 3 and inhibition. However, no more than about 50% of the label was associated with sites on that protein. The rest was bound to the lipids and to other membrane proteins including spectrin, which is located at the inner membrane surface. The binding to the membrane constituents, other than the protein in band 3, was not linearly related to inhibition. Ho and Guidotti found that the maximal number of binding sites on the protein in band 3 for the monoisothiocyanate is 300,000 per cell. This value agreed with that published by Cabantchik and Rothstein [4] but is considerably different from the revised number reported in this paper.

4 The turnover number for 25 °C was calculated using the V_{max} for chloride transfer obtained by Dalmark [5] at 0 °C (900 mequiv/10^{13} cells/min) and an E_A value of 30 kcal/mol [24].

18] it is unlikely that much of the H_2DIDS inhibitable anion exchange proceeds via gramicidin-like channels. Hence one would expect that the number of transport sites is close to the upper limit indicated above. Our new data on H_2DIDS binding would agree much better with this expectation than the earlier ones of Cabantchik and Rothstein, and suggest that the binding sites on the protein in band 3 are involved in anion transport. However, it would be premature to conclude that anion transport takes place by a valinomycin-like carrier system, the turnover of which is largely determined by diffusion across the highly viscous lipid double layer. Membrane proteins could possibly accomplish anion transport by means of a rotating side chain or a bimolecular exchange mechanism [16] much faster than a valinomycin-like carrier which diffuses through the whole of the thickness of the lipid bilayer. We conclude, therefore, that the current ignorance about the molecular mechanism of anion transport does not allow the determination of the expected minimal number of transport sites with sufficient accuracy to decide whether the protein in band 3 or other membrane constituents are involved.

Time Course and Stoichiometry of H_2DIDS Binding

In addition to the clarification of some of the problems associated with the earlier work, the experiments described above lead to the discovery of two striking features of H_2DIDS and DIDS binding. The first is the observed biphasicity of the time course of binding to both the whole cell and the protein in band 3. Despite this biphasicity, inhibition correlates closely with binding in either phase, and therefore, even if the site of inhibition should not be in band 3, it must show similar binding kinetics. The second observation is that the extent of irreversible binding at the time of cessation of the reaction varies with the reagent concentration up to the highest levels tested.

It would be tempting to attribute the kinetic biphasicity to reactions with sites in the membrane which differ in chemical nature or accessibility. Although isothiocyanates are mostly used for the modification of amino groups, they are also capable of reacting with SH groups, serine or tyrosine OH groups,[5] and, less easily, with guanidino groups (for litera-

5 The reaction with OH groups on the proteins would lead to the formation of hemithiocarbaminates which are labile in aqueous media and thus would not result in a permanent fixation to the membrane. In a control experiment we assured ourselves that even at 37 °C the hydrolysis of the isothiocyanate in aqueous media in the presence or absence of red cells is too slow to influence our binding studies.

ture *see* [14]). The widely different relative proportions of the two phases for the chemically and geometrically closely similar DIDS and H_2DIDS (Fig. 13) would argue against the involvement of sites of differing chemical natures or accessibilities.

Another explanation for the biphasicity of the time course of binding could be provided by the presence of various isomers of DIDS or H_2DIDS in the media. DIDS like other stilbenes exhibits olefinic cis-/trans isomerism [14, 19] while H_2DIDS, an ethane derivative, does not. Since both DIDS and H_2DIDS show biphasicity of binding, it cannot be attributed to different rates of reaction with olefinic cis- or trans isomers. However, H_2DIDS as well as DIDS may exist in other isomeric forms which differ with respect to the orientation of the isothiocyanate groups relative to each other and to the sulfonate groups (Fig. 15). Under moderate conditions these forms are unlikely to be interconvertible since rotation about the aromatic carbon-isothiocyanate nitrogen bond will be strongly disfavored by the loss of resonance stabilization energy on passage to the transition state. Hence their relative proportions will be dependent on the course of the synthetic reaction. We would not expect strikingly different reactivities between these isomers, since the distance between the isothiocyanate carbon and the center of the sulfate group is approximately 5.6 Å for the β-trans form and 6.4 Å for the α-trans form – not a highly significant difference. The cis-form contains both separations, one on each ring. Also, the proportions of the isomers formed from DIDS and H_2DIDS could be expected to be nearly identical, since the synthetic pathways are the same. Yet the proportion of total binding that takes place during the fast phase is quite different for the two compounds, amounting to 20% for H_2DIDS and more than 60% for DIDS.

The failure to account for the slow and fast phases of binding by the assumption of either different binding sites in the membrane or of isomers of DIDS or H_2DIDS in the medium directs the attention to the fact that both modifiers are agents capable of interacting either covalently or Coulombically. The Coulombic interactions could be reinforced by interactions between other parts of the modifier and hydrophobic groups in the membrane. Reversible as well as irreversible combination of H_2DIDS with the red blood cell membrane was in fact observed and shown to cause inhibition of anion movement (Figs. 2, 6; Table 1). It is possible, therefore, that reversible binding could affect the rate of irreversible binding.

This possibility can be visualized in the simple case of a single site by two similar but kinetically distinct hypotheses. The fast phase of

irreversible binding would be represented by immediate covalent binding to such single sites. This would be accompanied by simultaneous reversible binding through Coulombic forces (Fig. 16). According to hypothesis A, after establishment of the equilibrium of the reversible binding of H_2DIDS to the membrane, the number of uncombined membrane sites available for irreversible reaction is considerably diminished and hence the rate of reaction is reduced. Under hypothesis *B*, the establishment of the equilibrium for reversible binding is instantaneous and the slow phase of the irreversible reaction is governed by the rate of dissociation of the reversibly bound molecules. Under either hypothesis, the extent of irreversible binding during the initial, fast phase would be determined by the relative rates of Coulombic binding and covalent linkage. These in turn could depend either on relative reactivities or on the probabilities that the H_2DIDS molecule strikes the membrane site with one or the other orientation. The latter explanation seems more likely in view of the low temperature dependence of the extent of fast irreversible binding. Although these two hypotheses explain the biphasicity of the binding kinetics it is obvious that they fail to account for the concentration dependence of the stoichiometry of the irreversible reaction.

To illustrate the problem of concentration dependence more specifically, one may note in Fig. 2 that the extent of binding of H_2DIDS at 90 min and 30 °C is a function of the H_2DIDS concentration. At this temperature the rate constant of the slow phase corresponding to 19.2 μM, obtained by interpolation of the Arrhenius plot of Fig. 4, is 0.0436 min^{-1}. (An ambient concentration of 19.2 μM corresponds to the initial concentration of 25.0 μM used in the temperature studies, corrected for the effect of reversible binding). At 90 min a reaction with this rate constant will be more than 98% complete, yet when the reaction took place at higher H_2DIDS concentrations, significantly higher numbers of binding sites were occupied.

The change of stoichiometry of the reaction with increasing H_2DIDS concentration can be accounted for on the assumption that H_2DIDS reacts with adjacent pairs of identical ligands rather than with single ligands in the membrane. Under these conditions, for the same reasons as discussed for the reaction with a single site, one would observe biphasicity of binding. However, in contrast to the single site model, the reaction may reach completion not only when both sites of the pair are occupied by two H_2DIDS molecules covalently bound (denoted $S{=}{=}D_2$) but also if, for example, each pair reacted with one H_2DIDS molecule only, one site being attached via a thiourea bond, the other via Coulombic interactions (denoted $S{-}{-}D$).

Fig. 17 shows the full set of possible compounds between H_2DIDS and two contiguous sites including the compounds $S\!=\!-\!-\!-D$ and $S\!=\!=\!=D_2$ at which the reaction could come to a standstill. It is obvious that the sum $S\!=\!-\!-\!-D+2\ S\!=\!=\!=D_2$ which represents total H_2DIDS binding will vary with the concentration in the medium. If the reaction takes place at low concentrations of H_2DIDS, the predominant species formed will be $S\!=\!-\!-\!-D$ and the stoichiometrical ratio will be close to one H_2DIDS molecule bound per pair of binding sites in the membrane. If the reaction takes place at high concentrations of H_2DIDS, the predominant species formed would be $S\!=\!=\!=D_2$ and the stoichiometrical ratio would tend to approach 2.0. Assuming that anion transport depends on the availability of both sites in a pair S, it is feasible, therefore, that equal numbers of bound H_2DIDS molecules may produce different degrees of inhibition or different numbers of bound molecules may produce the same degree of inhibition. We have formulated the reactions depicted in Fig. 17 in mathematical terms and made a quantitative estimate of the possible number of pairs of sites S available for H_2DIDS binding. Unfortunately, one of the reviewers of this paper wanted us to remove the originally included abbreviated version of this mathematical treatment. Thus, it must suffice to state that the quantitative analysis of the data presented in Fig. 2 (curve labeled 90 min) would suggest the presence of $1.3\times10^6-1.8\times10^6$ pairs of H_2DIDS binding sites per cell.

At the concentrations used to obtain the bulk of the data on the relationship between H_2DIDS binding and inhibition (Fig. 9), the model predicts a variation of the stoichiometry of the reaction between H_2DIDS and one pair of membrane sites between 1.1 and 1.4. Hence under our specific conditions no great deviations from linearity are to be expected in individual experiments, and the overall effect on all of the data should be of some additional scatter in the points. When the binding data of Fig. 9 are corrected to number of sites occupied, by division by the stoichiometry predicted at the concentration used, and replotted, a slight reduction in scatter is achieved. The straight lines extrapolate to 2.1×10^6 binding sites per cell, of which 1.4×10^6 are on the protein in band 3, at complete inhibition.

Despite the ability of the model described above to account for our observations on the relationship between H_2DIDS or DIDS binding and inhibition of anion exchange, we do not believe that it represents a final explanation, and further work in this laboratory is directed towards testing alternative hypotheses. We have chosen, however, to discuss the model in Fig. 17 in some detail, since it serves to demonstrate three important points. Firstly, it shows that there is no need to assume the

existence of chemically inhomogeneous populations of binding sites to account for the observed phenomena, despite superficial evidence to the contrary. Secondly, it illustrates the complex behavior, including biphasic and nonstoichiometric reactions, that can be expected when reagents with mixed functions react with a homogeneous population of multi-ligand sites, such as those postulated for H_2DIDS binding by Cabantchik and Rothstein [4] or by Gunn [7] for the anion carrier. The final point is the most instructive: a linear relationship between inhibition and binding to one among several sets of sites does not prove that this set of sites mediates the process inhibited. Instead, sites at which a nonlinear relationship or no exact relationship is observed may be involved (*cf.* footnote 2).

Regardless of the exact stoichiometry of the reaction and its dependence on concentration, our results strongly suggest that at least one H_2DIDS molecule is bound per molecule of protein in band 3; according to Steck [22] the number of peptide molecules of molecular weight of about 100,000 Daltons in band 3 is about 900,000 per cell. Our figure of about 1.7×10^6 H_2DIDS bound/cell as measured at 25 μMH_2DIDS is about twice as large. If we take into account that about 16% of the H_2DIDS binding which we measure at the location of band 3 is due to binding to glycophorin, this would yield 1.5 H_2DIDS/molecule of protein. Whatever the final result will be, it is clear that only very few of the 57 lysine residues reported to be present in the protein in band 3 [23] are available for reaction with H_2DIDS.

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Note Added in Proof

In the present paper, binding had been measured as μM/mg of membrane protein. The data were converted to numbers of molecules bound per cell, using a constant factor which relates estimates of membrane protein to corresponding numbers of cells. The factor was taken from the literature (Dodge *et al.*, 1963). The conversion factors used for similar calculations in different laboratories vary from 1.1×10^9 (Cabantchik & Rothstein, 1974), to 2.0×10^9 (Bodemann & Hoffman, 1976. *J. Gen. Physiol.* **67**:497). We recently redetermined the conversion factor and found it different from that of Dodge *et al.* It amounted to 1.95×10^9 cells/mg instead of 1.41×10^9 cells/mg as used in the text. Multiplication of the binding data in figures, figure legends and text (but not in the summary where the numbers were changed in the proofs) by 0.725 leads to the absolute values corresponding to the new conversion factor. For H_2DIDS binding to the membrane as a whole and to the protein in band 3, this yields, respectively, 1.88×10^6 and 1.23×10^6 molecules/cell. The figures for DIDS are 1.38×10^6 and 1.09×10^6 molecules/cell. The stoichiometrical ratio for binding to the protein in band 3 assumes a value of about 1.1–1.2.