

# Transmembrane Effects of Intracellular Chloride on the Inhibitory Potency of Extracellular H<sub>2</sub>DIDS

## *Evidence for Two Conformations of the Transport Site of the Human Erythrocyte Anion Exchange Protein*

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**ABSTRACT** The ping-pong model for the red cell anion exchange system postulates that the transport protein band 3 can exist in two different conformations, one in which the transport site faces the cytoplasm ( $E_i$ ) and another in which it faces the outside medium ( $E_o$ ). This model predicts that an increase in intracellular chloride should increase the fraction of sites in the outward-facing, unloaded form ( $E_o$ ). Since external H<sub>2</sub>DIDS is a competitive inhibitor of chloride exchange that does not cross the membrane, it must bind only to the  $E_o$  form. Thus, an increase in  $E_o$  should cause an increase in H<sub>2</sub>DIDS inhibition. When intracellular chloride was increased at constant extracellular chloride, the inhibitory potency of H<sub>2</sub>DIDS rose, as predicted by the ping-pong model. This increase was not due to the concomitant changes in intracellular pH or membrane potential. When the chloride gradient was reversed, the inhibitory potency of H<sub>2</sub>DIDS decreased, again in qualitative agreement with the ping-pong model. These data provide support for the ping-pong model and also demonstrate that chloride gradients can be used to change the orientation of the transport protein.

### INTRODUCTION

The disulfonic stilbenes, such as SITS (4-acetamido-4'-isothiocyano-stilbene-2,2'-disulfonic acid) (Knauf and Rothstein, 1971) and DIDS (4,4'-diisothiocyano-stilbene-2,2'-disulfonic acid) (Cabantchik and Rothstein, 1974) have proven to be very useful in probing the mechanism of the anion exchange system in human

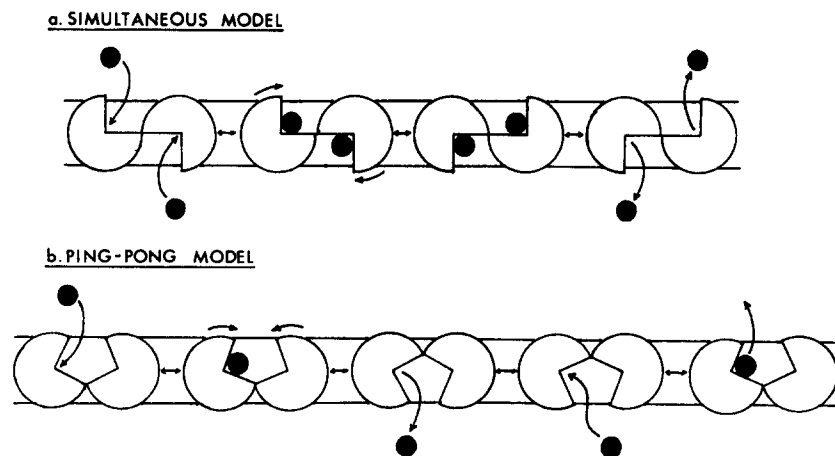
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erythrocytes, and have led to the discovery that anion transport is mediated by the 95,000-dalton, asymmetric, integral protein known as band 3 (Cabantchik and Rothstein, 1974; Passow et al., 1975). The versatile bimodal inhibitor  $H_2DIDS$  (4,4'-diisothiocyano-1,2-diphenylethane-2,2'-disulfonic acid), a reduced analogue of DIDS, was found to act as a reversible inhibitor at  $0^\circ C$  (Shami et al., 1978), thus being useful in kinetic studies, and as an irreversible inhibitor at  $37^\circ C$  (Cabantchik and Rothstein, 1974) for use in labeling studies. Chloride competition data (Shami et al., 1978) have shown that  $H_2DIDS$  most likely acts at the substrate (transport) site of the anion exchange system.



**FIGURE 1.** Models to explain the one-for-one exchange of anions. (a) Simultaneous model: two chloride ions (black circles) must bind to transport sites at opposite sides of the membrane before the two ions are simultaneously translocated across the membrane. The number of transport sites at each side of the membrane is constant. (b) Ping-pong model: there is only one transport site, which can face either the cytoplasm ( $E_i$  conformation) or the external medium ( $E_o$ ). The transition from  $E_i$  to  $E_o$  or vice versa can only occur if a chloride ion (or other anion) is bound to the transport site. Note that these models are only schematic: nothing is implied about the detailed molecular mechanism of the transport-related change in conformation. In particular, the diagrams should not be taken to imply that anion transport involves a dimer of band 3. Since all evidence to date indicates that each band 3 monomer functions as an independent transport site, the conformational changes involved in anion transport probably involve a reorientation of domains of a single band 3 monomer.

The one-to-one exchange of anions across the red cell membrane may be explained (Fig. 1) by one of two basic models (Knauf, 1979; Gunn and Fröhlich, 1979). The simultaneous model (terminology of Sachs, 1977) postulates the existence of two anion binding sites, one on each side of the membrane. When anions are bound to both sites, the two bound anions are translocated at the same time to opposite sides of the membrane, where they are then released into the adjacent solutions.

The ping-pong model (Cleland, 1963; Sachs, 1977) postulates that there is

only a single transport site,<sup>1</sup> which can exist in either an outward-facing  $E_o$  form or an inward-facing  $E_i$  form. In this model, the tightly coupled one-for-one exchange of anions is explained by postulating that the interconversion from  $E_o$  to  $E_i$  or vice versa can only occur after binding of a chloride to form the corresponding  $ECl_o$  or  $ECl_i$  form. Since the unloaded transport sites cannot cross the membrane, the band 3 protein "remembers" the direction of the last transport event by means of the conformation (inward- or outward-facing) of the transport site. The next transport event must therefore be in the opposite direction, giving rise to a one-for-one exchange of anions.

Several studies have been performed to try to detect changes in the conformation of the carrier from  $E_o$  to  $E_i$ , giving support for the ping-pong model. Studies by Rothstein et al. (1976), Passow and Zaki (1978), Grinstein et al. (1979), and Passow et al. (1980*a, b*) all indicated a transmembrane effect of binding of an inhibitor at one side of the membrane on the binding of another inhibitor at the opposite side of the membrane.

A possible complication in these studies is that chemical probes were used to induce a change in conformation of the transport site. Such probes may well cause other perturbations of membrane structure and function, besides those related to the change in transport site conformation (Grinstein et al., 1979). To avoid such problems in this study, one of the natural substrates of the anion exchange system, chloride, was used to induce an asymmetry in the distribution of the protein conformations.

Preliminary reports of parts of this study have previously appeared elsewhere (Furuya and Knauf, 1979; Knauf et al., 1980*a, b*; Knauf, 1982), and in thesis form (Furuya, 1980).

#### THEORY

As first shown by Dalmark (1975*a*), if the transport system is assumed to be symmetrical, with equal amounts of  $E_i$  and  $E_o$  when the chloride concentrations inside the cell and in the medium are equal, then the ping-pong model predicts that a change in the chloride ratio will lead to an asymmetric distribution of  $E_i$  and  $E_o$ , according to the equation:

$$\frac{E_o}{E_i} = \frac{Cl_i}{Cl_o} \quad (1)$$

Such an alteration of the chloride ratio would be expected to have no such effect according to the simultaneous model, since in this model there are always equal numbers of inward- and outward-facing sites, regardless of the chloride ratio (see Fig. 1).

Since external H<sub>2</sub>DIDS is a competitive inhibitor of the anion exchange system, and since it does not cross the membrane, it must bind exclusively to the outward-facing, unloaded form ( $E_o$ ). Under conditions where the covalent reaction of

<sup>1</sup> Models in which a chain of sites participates in a conformational change such that at any time only one site is accessible either to cytoplasmic or medium anions, but not to both, also exhibit ping-pong kinetics (see, e.g., Wieth et al., 1983).

H<sub>2</sub>DIDS with band 3 is slow, H<sub>2</sub>DIDS is in reversible equilibrium with the  $E_o$  form:

$$ED = \frac{(E_o)(D_o)}{K_d}, \quad (2)$$

where  $K_d$  is the dissociation constant,  $D_o$  is the external H<sub>2</sub>DIDS concentration, and  $ED$  is the concentration of the band 3-H<sub>2</sub>DIDS complex. The amount of inhibition of anion exchange is directly related to the fraction of the total number of band 3 molecules that are complexed with H<sub>2</sub>DIDS (i.e., in the  $ED$  form). As can be seen from Eq. 2, for a given concentration of H<sub>2</sub>DIDS, the amount of  $ED$  and hence the inhibition of anion exchange will be directly related to the amount of  $E_o$  present. Thus, the H<sub>2</sub>DIDS inhibitory potency can be used to monitor the amount of  $E_o$  present and to determine whether or not the ratio of  $E_o$  to  $E_i$  is

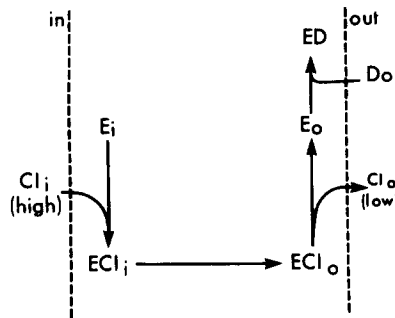


FIGURE 2. Effect of a chloride gradient on H<sub>2</sub>DIDS inhibition. If a chloride gradient is set up with  $Cl_i > Cl_o$ , the reversible interactions of the transport system ( $E$ ) with chloride and with external H<sub>2</sub>DIDS ( $D_o$ ) are displaced in the directions shown by the arrows. This results in a greater fraction of transport sites trapped in the inhibited  $ED$  complex, and therefore in a greater apparent inhibitory potency of H<sub>2</sub>DIDS.

altered by changes in the chloride ratio, as predicted by the ping-pong but not by the simultaneous mechanism.

An example of the expected effect of an outwardly directed chloride gradient is shown in Fig. 2. Because of the differences in internal and external chloride concentrations, the reversible equilibria of the various forms of the transport system are displaced in the direction shown by the arrows. Under these conditions,  $E_o$  will increase and so the amount of the  $ED$  complex at a given H<sub>2</sub>DIDS concentration will increase. This in turn means that the inhibitory potency of H<sub>2</sub>DIDS will increase.

This effect can be expressed mathematically in terms of the concentration of H<sub>2</sub>DIDS required to inhibit chloride exchange by 50% with a chloride gradient present ( $ID_{50}$ ), as compared with that required to cause 50% inhibition with no chloride gradient ( $ID_{50}^*$ ). For the situation where the external chloride concentration is held constant, one obtains the following equation for a symmetrical transport system (see Appendix, Eq. A14):

$$\frac{ID_{50}}{ID_{50}^*} = \frac{(Cl_o/Cl_i) + 1 + 2(Cl_o/K_c)}{2 + 2(Cl_o/K_c)}, \quad (3)$$

where  $K_c$  is the concentration of chloride required to half-saturate the transport system when  $Cl_o = Cl_i$ . It is apparent from this equation that the largest effects of chloride gradients will be seen when the terms  $Cl_o/K_c$  in the numerator and denominator of Eq. 3 are small, that is, at low external chloride concentrations. For this reason, the external chloride concentration was kept as low as practicable in these experiments. For the simultaneous mechanism, the corresponding equation predicts that  $ID_{50} = ID_{50}^*$  (see Appendix, Eq. A22). Thus, such experiments can provide a critical test to distinguish between these two possible models.

#### METHODS

##### *Preparation of Cells with $Cl_i > Cl_o$*

Red cells were obtained from freshly drawn blood from apparently healthy adults and were washed three or four times with 160 mM NaCl, 5 mM HEPES, pH 7.4 (H-N). For some experiments, they were then incubated at 25% hematocrit in 2.5–6  $\mu$ M DIDS in H-N buffer for 30 min at 37°C. The cells were then washed twice with H-N containing 0.5% bovine serum albumin and then twice in H-N. This pretreatment did not appear to have any systematic effect on the inhibitory potency of H<sub>2</sub>DIDS, but it did inactivate some of the transport sites, thereby reducing the chloride flux and permitting more precise flux measurements. The lack of effect of H<sub>2</sub>DIDS reaction with some sites on binding of H<sub>2</sub>DIDS to other band 3 molecules is compatible with the finding of Kampmann et al. (1982) that binding of H<sub>2</sub>DIDS to one band 3 monomer does not affect the cross-linking reaction of H<sub>2</sub>DIDS to an adjacent band 3 monomer. With disulfonic stilbene derivatives having bulkier substituents at the 4 and 4' positions, however, binding to one band 3 monomer does seem to affect interactions at the adjacent monomer (Macara and Cantley, 1981; Verkman et al., 1983).

Chloride gradients were set up as follows: washed cells were divided into two groups, H and L (high and low internal chloride). The H cells were washed with 150 mM KCl, 20 mM HEPES, 27 mM sucrose, pH 7.2, at 0°C (150K), and the L cells with 10 mM KCl, 20 mM HEPES, 245 mM sucrose, pH 7.2 (10K-Hi). The L cells were then incubated at 10% hematocrit for 10 min at 0°C in 10K-Hi containing 75  $\mu$ g/ml nystatin (Mycostatin; E. R. Squibb and Sons, Inc., Princeton, NJ). After centrifugation, the cells were resuspended in 10 mM KCl, 20 mM HEPES, 27 mM sucrose, pH 7.2 (10K-Lo), with 75  $\mu$ g/ml nystatin and incubated at 0°C for a further 10 min. The cells were then washed at least five times at room temperature in 10K-Lo. H cells were treated with nystatin in a similar fashion, but all incubations and washes were in 150K medium, except for the last wash and the isotope loading, which were done in 10K-Hi buffer. For some experiments, as indicated, the HEPES concentration was 5 mM and the sucrose concentration was 33 mM (Freedman and Hoffman, 1981). Cells were loaded with <sup>36</sup>Cl (Amersham Corp., Arlington Heights, IL; or ICN Chemical and Radioisotope Division, Irvine, CA) and fluxes were determined at 0°C as described by Knauf et al. (1978), using 10K-Hi as the flux medium for the H cells and 10K-Lo for the L cells. H<sub>2</sub>DIDS, kindly provided by Dr. M. Ramjeesingh, was used to inhibit chloride exchange. Chloride ratios were measured (Knauf et al., 1978) using [<sup>3</sup>H]methoxyinulin (New England Nuclear, Boston, MA) as a marker for extracellular space. Chloride ratios ( $Cl_i/Cl_o$ ) for the L cells ranged from 0.82 to 1.07; for the H cells they are indicated in the figures and tables. Because of the increase

in intracellular pH, and possibly because of an increased cation permeability caused by nystatin treatment, the H cells suspended in 10K-Hi medium were slightly shrunken. When H cells were suspended in 150K buffer, however, addition of 245 mM sucrose caused a small increase in the  $ID_{50}$  for  $H_2DIDS$ , in contrast to the decrease in  $ID_{50}$  seen when  $Cl_i > Cl_o$  (Table I). Thus, the effects of  $Cl^-$  gradients cannot be attributed to cell shrinkage.

#### *Preparation of Ghosts with $Cl_i < Cl_o$*

Resealed ghosts were prepared by a modification of the methods of Schwach and Passow (1973) and Funder and Wieth (1976). Fresh red cells were washed three times in 165 mM NaCl, the white cells were removed, and the red cells were made up to 50% hematocrit in 165 mM NaCl. A 35-ml volume of lysing solution (3.5 mM acetic acid, 4 mM  $MgSO_4$ , 1 mM EGTA) was added to 3.5 ml of 50% cells. All of the lysing and resealing solutions were kept at 0°C in ice with added salt to ensure that the ghosts would be tightly resealed. To obtain ghosts with 10 mM internal chloride, 3.37 ml of 25 mM Tris base, 124 mM KCl, 1.16 M sodium citrate was added to the lysed cells, and after 10 min at 0°C, the ghosts were resealed by incubation for 45 min at 37°C. For ghosts with 60 mM chloride, 3.37 ml of 25 mM Tris base, 745 mM KCl, and 745 mM sodium citrate was added to the lysing solution. Both groups of resealed ghosts were washed twice in medium containing 60 mM sodium chloride, 60 mM sodium citrate, 5 mM HEPES, 5 mM glucose, and 4  $\mu M$   $MgSO_4$ , pH 7.2, at 0°C. Chloride fluxes and chloride ratios were measured in this medium as described above.

#### *Preparation of Cells with $Cl_i < Cl_o$ by Changing pH*

Red cells from the blood bank were washed in 165 mM NaCl and brought to 50% hematocrit. They were then washed in 60 mM KCl, 10 mM HEPES, 99 mM sucrose, pH 7.2, at 0°C (60K-Hi) and then treated with 2.5 or 5  $\mu M$   $H_2DIDS$  for the low and high pH cells, respectively, at 10% hematocrit for 45 min at 37°C. They were then washed with 60K-Hi medium containing 0.5% albumin and incubated in 60K-Hi medium with 75  $\mu g/ml$  nystatin for 10 min at 0°C. After centrifugation, they were then incubated in medium with 60 mM KCl, 10 mM HEPES, 54 mM sucrose, pH 7.2 (60K-Lo), with nystatin for a further 10 min at 0°C. They were then washed several times with 60K-Lo to remove the nystatin. The low pH cells were then washed twice in 60K-Lo, while the high pH cells were washed twice in 60K-Lo that had been titrated to pH 9.0. Chloride fluxes and chloride ratios were measured in the pH 7.2 and pH 9.0 media as described above.

#### *Statistical Analysis*

In each experiment, the  $ID_{50}$  was determined from the  $x$ -intercept of the line that best fit the Dixon plot data by the method of least squares (see, e.g., Fig. 3). The ratio of the  $ID_{50}$  for cells with a chloride gradient to the  $ID_{50}$  in cells with  $Cl_i \cong Cl_o$  ( $ID_{50}^*$ ) was determined for each experiment. The mean for the ratio was calculated by taking the mean of the  $ID_{50}$  values and dividing by the mean of the  $ID_{50}^*$  values. The 95% confidence interval (CI) for this ratio was determined by the method of Goldstein (1964, pp. 184–187), using Fieller's Theorem as applied to correlated data.

#### *Net KCl Flux with Valinomycin*

Fresh red cells were washed twice in 150K buffer, then once in 10K-Hi. Net  $K^+$  efflux was measured at 0°C at 0.1% hematocrit in 10K-Hi buffer with 1.33% (vol/vol) total ethanol and various concentrations of valinomycin, as described previously (Knauf et al., 1983).

*<sup>86</sup>Rb Exchange with Valinomycin*

Fresh red cells were washed three times in 150 mM NaCl, 2 mM KCl, 20 mM HEPES, pH 7.4, and then once in loading solution (5 mM adenosine, 150 mM NaCl, 2 mM KCl, 20 mM HEPES, 1  $\mu$ g/ml chloramphenicol, pH 7.4) at room temperature. The cells were incubated 17 h at 37°C at 50% hematocrit in the loading buffer with  $\sim 5$   $\mu$ Ci/ml <sup>86</sup>Rb (Amersham Corp.). The cells were then washed five times in ice-cold flux buffer consisting of 48 mM NaCl, 112 mM KCl, 5 mM HEPES, pH 7.2, at 0°C. <sup>86</sup>Rb efflux was measured at 0°C at 0.1% hematocrit in flux buffer containing 1.33% ethanol, with various valinomycin concentrations (valinomycin was added last). Samples of 1 ml were taken at various times and were centrifuged in an Eppendorf model 3200 Microcentrifuge (Eppendorf Division, Brinkmann Instruments, Westbury, NY). The supernatants were then counted by liquid scintillation, using Aquasol (New England Nuclear). Duplicate samples of the suspension were also counted for infinity values, and the rate constant was determined as described earlier for chloride fluxes (Knauf et al., 1978). The chloride ratio (from <sup>36</sup>Cl distribution) and the intracellular potassium concentration (by flame photometry) were measured as previously described (Knauf et al., 1978, 1983).

*pH Equilibration*

High chloride (H) cells as prepared above were suspended at 1% hematocrit in 10 mM KCl, 280 mM sucrose, 0.2 mM HEPES, pH 7.2, at 0°C. The pH of the suspension was measured as a function of time for up to 10 min, after which the suspension was centrifuged and a measured aliquot of supernatant was removed and titrated with measured amounts of NaOH back to the original pH. This titration curve was used to calculate the buffer capacity, that is, the millimoles of OH<sup>-</sup> required per milliliter of solution to cause a given change in pH. From this and the rate of change of pH in the original cell suspension, together with the cell concentration, the rate of OH<sup>-</sup> flow per liter of packed cell volume was determined. Assuming a hemoglobin content of 34 g/100 ml packed cell volume and assuming that 10 eq of base is required to titrate 1 mol of hemoglobin 1 pH unit in the physiological range (Dalmark, 1975b), an intracellular buffer capacity of 53 meq/liter packed cell volume·pH unit can be calculated. The OH<sup>-</sup> flux divided by the intracellular buffer capacity gives the rate of change of intracellular pH, which for these experiments was <0.04 pH unit/min.

**RESULTS***Cells with Cl<sub>i</sub> > Cl<sub>o</sub>*

To determine whether or not a chloride gradient across the membrane has any effect on the inhibitory potency of H<sub>2</sub>DIDS, two groups of cells were prepared. One group (H) was loaded with 150 mM Cl<sup>-</sup> by the nystatin technique (see Methods), while the other group (L) was loaded with 10 mM Cl<sup>-</sup>. Both groups were then washed and loaded with <sup>36</sup>Cl in flux media containing 10 mM Cl<sup>-</sup>, the only difference between the two groups of cells being that sucrose was added to the medium for the H cells to maintain osmotic balance. The rate of chloride exchange at 0°C was then measured in both groups of cells in the presence of various concentrations of H<sub>2</sub>DIDS, and the results were plotted on a modified Dixon plot.

The Dixon plot for a typical experiment is shown in Fig. 3. In this plot the slope is equal to 1/ID<sub>50</sub> and is therefore directly related to the potency of the

inhibitor. The  $x$ -intercept is the negative of the  $ID_{50}$  value. For the H cells (broken line) with  $Cl_i > Cl_o$ , the slope of the Dixon plot was larger and the  $x$ -intercept ( $-ID_{50}$ ) was closer to zero than for the L cells (solid line) with  $Cl_i \cong Cl_o$ . Thus,  $H_2DIDS$  was a more effective inhibitor in the cells with an outwardly directed chloride gradient.

Since the only difference between the external media for the two groups of cells was the presence of sucrose, which has no known effect on the anion exchange system, and since the chloride concentration at the external (*cis*) side of the membrane was constant, the increase in  $H_2DIDS$  inhibitory potency for

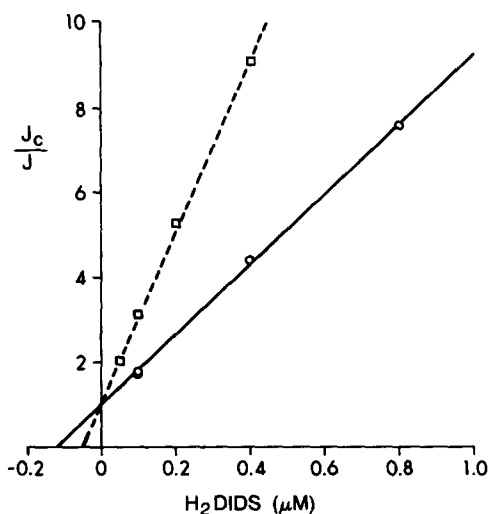


FIGURE 3. Modified Dixon plot of the effects of  $H_2DIDS$  on chloride exchange in cells with high and low intracellular chloride concentrations. On the ordinate is plotted the control chloride exchange flux,  $J_c$ , divided by the flux in the presence of  $H_2DIDS$ , against the concentration of  $H_2DIDS$  on the abscissa. For the high chloride (H) cells (squares, broken line), the chloride ratio ( $Cl_i/Cl_o$ ) was 7.16 and for the low chloride (L) cells (circles, solid line) it was 0.90. For clarity, the points at 0  $H_2DIDS$  are not plotted. For the L cells, with nearly equal chloride concentrations inside and outside, the  $ID_{50}$ , determined from the  $x$ -intercept, was  $0.119 \mu M$ , with a 95% CI of 0.002. For the H cells, with a large outwardly directed chloride gradient, the  $ID_{50}$  was  $0.052 \pm 0.003$ . The external chloride concentration was 10 mM and the temperature was  $0^\circ C$ .

the H cells could not have been due to any change in the interactions of  $Cl^-$  and  $H_2DIDS$  at the *cis* side of the membrane. The data therefore suggest that there is a transmembrane effect of the internal chloride concentration on the inhibitory potency of external  $H_2DIDS$ .

Table I summarizes the data from nine such experiments. There was considerable scatter in the absolute values of the  $ID_{50}$  for  $H_2DIDS$  in different experiments, which did not seem to be related to the source of either the blood or the  $H_2DIDS$ , and whose cause is still obscure. Also, in a few experiments, the Dixon plots exhibited some apparent curvature. For example, of the 14 Dixon



plots represented in Table I, two exhibited some upward concavity and one some downward concavity. As this was not a consistent finding and was generally minimal and of dubious statistical significance, all of the data were fitted to straight lines to determine the ID<sub>50</sub> values. Despite these experimental difficulties, in every case the ID<sub>50</sub> for cells with a chloride gradient was less than the value (ID<sub>50</sub><sup>\*</sup>) for cells with a chloride ratio near 1, and for the entire set of data the difference was statistically significant (paired *t* test) at the *P* < 0.01 level. The mean ID<sub>50</sub> value with an average chloride gradient (Cl<sub>o</sub>/Cl<sub>i</sub>) of 0.167, relative to that with no chloride gradient, was 0.48, a somewhat larger decrease than would be predicted from the ping-pong model for a symmetrical transport system, in which case a ratio of 0.64 would be expected. As will be shown in the following paper (Knauf et al., 1984), this discrepancy can be explained if there is significant asymmetry in the transport system.

TABLE I  
*Effect of Chloride Gradient on H<sub>2</sub>DIDS Inhibition in Cells with Cl<sub>i</sub> > Cl<sub>o</sub>*

Experiment	ID <sub>50</sub> <sup>*</sup> μM	ID <sub>50</sub> μM	ID <sub>50</sub> /ID <sub>50</sub> <sup>*</sup>	Cl <sub>o</sub> /Cl <sub>i</sub>
1	0.12	0.05	0.44	0.210
2	0.11	0.09	0.77	0.175
3	0.16	0.04	0.26	0.142
4	0.42	0.28	0.66	0.157
5	0.22	0.11	0.48	0.176
6	0.12	0.05	0.44	0.140
7	0.32	0.09	0.27	—
Mean	0.210	0.101	0.48	0.167
SEM	0.045	0.031	—	0.011
95% CI	{ 0.099 0.320	{ 0.025 0.178	{ 0.20 0.69	{ 0.139 0.194

The extracellular chloride concentration was 10 mM and the temperature was 0°C. For measurements of ID<sub>50</sub><sup>\*</sup>, the intracellular Cl<sup>-</sup> concentration ranged from 8.2 to 10.7 mM; for measurements of ID<sub>50</sub>, the intracellular Cl<sup>-</sup> averaged 61.2 mM.

The data are completely in contradiction to the simultaneous model (see Theory and Appendix, Eq. A22), which would predict no effect of the chloride gradient on the inhibitory potency of H<sub>2</sub>DIDS. If, however, one were to modify the simultaneous model by assuming that the binding of chloride to the site at the cytoplasmic side of the membrane causes an increase in the affinity of the external site for H<sub>2</sub>DIDS, it would be possible to rationalize these data if chloride binding at the inside surface of the membrane were to increase the H<sub>2</sub>DIDS affinity at the outside by a factor of 12.5 (see Appendix). While this model would fit the results of the chloride gradient experiments shown in Fig. 3 and Table I just as well as the ping-pong model, it would imply that internal Cl<sup>-</sup> increases H<sub>2</sub>DIDS inhibitory potency, even though Shami et al. (1978) have shown that an increase of chloride concentrations at both sides of the membrane causes a

decrease in the effectiveness of H<sub>2</sub>DIDS (i.e., an increase in the ID<sub>50</sub>). In Fig. 4, the calculated Hunter-Downs plot for such a modified simultaneous model with interacting sites is compared with the data of Shami et al. (1978). If the ID<sub>50</sub> values are scaled to the value of ID<sub>50</sub> at zero chloride (lower solid line), the conflicting actions of chloride as a *cis* competitive inhibitor and *trans* facilitator of H<sub>2</sub>DIDS binding result in a much less pronounced dependence of ID<sub>50</sub> on chloride concentration than was actually observed. Since no data are available in the range from 0 to 19 mM chloride, however, it would also seem permissible to scale the ID<sub>50</sub> values to the observations at 19 mM (broken line), in which case the modified form of the simultaneous model does fit the data reasonably well.

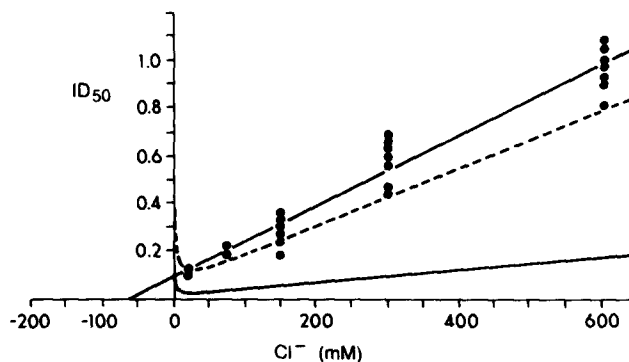


FIGURE 4. Comparison of Hunter-Downs plot predictions of the modified simultaneous model with experimental data. The ID<sub>50</sub> for H<sub>2</sub>DIDS inhibition of chloride exchange at 0°C is plotted on the ordinate against the chloride concentration on the abscissa, under conditions where Cl<sub>i</sub> ≅ Cl<sub>o</sub>. The points and least-squares best fit through them are taken from Shami et al. (1978). The lower solid line is calculated from the simultaneous model, assuming that the dissociation constant for chloride at the transport site is 26.9 mM at either side of the membrane (which would give half-saturation at the value of 65 mM observed by Brazy and Gunn, 1976) and assuming that Cl<sup>-</sup> binding to the inside site increases the affinity for H<sub>2</sub>DIDS binding to the outside by a factor of 12.5. The ID<sub>50</sub> values are scaled by matching the predicted to actual values at 0 chloride. The broken line is calculated according to the same model, but the predicted values are scaled to the observed data at 19 mM chloride. Predictions calculated on the basis of a chloride dissociation constant of 65 mM provided a slightly poorer fit to the data.

It does so, however, only at the cost of the ad hoc and rather improbable assumption that chloride binding to the internal site increases the binding affinity of the external site for H<sub>2</sub>DIDS by over 10-fold. In theory, this model could be tested by measuring the effects of H<sub>2</sub>DIDS at very low chloride concentrations where the ID<sub>50</sub> should increase dramatically, but in practice, clumping of the cells and difficulties in maintaining a chloride ratio of 1 under these conditions made such experiments impossible. Even in the absence of these data, it seems clear that the ping-pong model provides a much simpler explanation of the effects of chloride gradients than does this modified simultaneous model.

*Possible Effects of Changes in Internal pH*

Although the experiments in the preceding section indicate that changes in internal concentration have a strong effect on the inhibitory potency of external H<sub>2</sub>DIDS, these experiments do not tell us whether the chloride gradient directly affects the anion exchange system, as predicted by the ping-pong model, or whether some other mechanism is involved. When the internal chloride concentration is higher than the external concentration, chloride-hydroxyl and chloride-bicarbonate exchange can occur via the anion exchange system, and this may cause pH shifts which in turn could affect the inhibitory potency of H<sub>2</sub>DIDS. In the experiments described above, the external medium is heavily buffered, so equilibration of hydroxyl ions with the chloride gradient would primarily result in an alkalization of the cell interior. That such a rise in intracellular pH does occur is indicated by the fact that the measured chloride ratios (Table I) are less than those that would be predicted from the initial ratio of intracellular to extracellular chloride concentration. This is probably due at least in part to the rise in intracellular pH, which increases the negative charge on hemoglobin and other intracellular proteins, thereby causing a decrease in the intracellular chloride concentration.

The simplest way to test for possible effects of increasing intracellular pH would be to perform experiments under conditions where the intracellular pH does not change. This can be done if the rate of chloride-hydroxyl exchange is so slow that chloride exchange measurements can be done before any appreciable change in intracellular pH has taken place. To determine the rate of change of intracellular pH, high chloride (H) cells were added to flux medium with 10 mM chloride, in which the buffer concentration was reduced to 1/25 of the normal value, and the change in external pH was measured. After several minutes, the cell suspension was centrifuged and an aliquot of the supernatant was titrated with NaOH to determine the buffer capacity of the supernatant so that the number of moles of OH<sup>-</sup> entering the cell could be calculated. Even for media in which no special precautions were taken to minimize the concentration of bicarbonate, which catalyzes the chloride-hydroxyl exchange, the pH change was very gradual, corresponding to a calculated change in internal pH of ~0.04 pH unit/min. Since chloride fluxes can be easily measured within 2.5 min, it should therefore be possible to determine the effects of H<sub>2</sub>DIDS under conditions where the internal pH changes by <0.1 unit.

Fig. 5 shows the inhibition of chloride exchange caused by various concentrations of H<sub>2</sub>DIDS when high chloride cells are suspended in 10 mM chloride media. The circles are for cells that were washed and loaded with <sup>36</sup>Cl in 10 mM chloride medium containing 280 mM sucrose, the same treatment that was used for the experiments described in the preceding section. The squares depict results for high chloride cells that were loaded with <sup>36</sup>Cl in high chloride medium and then plunged into low chloride medium at the beginning of the flux measurement. In this group of cells, there was not sufficient time for the internal pH to change by more than 0.1 pH unit, but the inhibitory effect of H<sub>2</sub>DIDS is just as great as in the cells that had been pre-equilibrated to permit Cl<sup>-</sup>/OH<sup>-</sup> exchange. Pre-incubation of high chloride cells in high chloride medium with a

pH 0.3 unit lower than the usual flux medium, prior to plunging the cells into low chloride medium, also had no significant effect on the inhibition caused by H<sub>2</sub>DIDS (data not shown). Since the internal pH in these cells is lower than usual, even after 2.5 min in the 10 mM chloride medium, the internal pH would not have risen to the value at which it began under the usual flux conditions. Thus, the internal pH in these cells never rose above the usual control value, and yet the inhibition of chloride exchange by H<sub>2</sub>DIDS was just as large as under the usual conditions. This rules out the possibility that the system might exhibit such an extraordinary pH sensitivity that the 0.1-unit rise in internal pH could cause the increase in H<sub>2</sub>DIDS inhibitory potency seen when high chloride cells are plunged into low chloride medium.

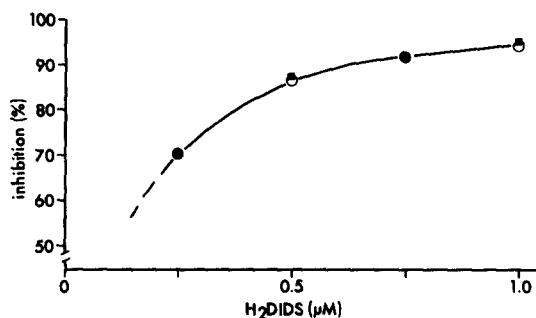


FIGURE 5. Inhibition of Cl<sup>-</sup> exchange by H<sub>2</sub>DIDS in low chloride medium. Cells in this experiment were not treated with nystatin, but were divided into two groups. One group (preincubated, circles) was washed with 150 mM KCl, 33 mM sucrose, 5 mM HEPES, pH 7.2, at 0°C, and then incubated for 10 min at 0°C, washed, and loaded with <sup>36</sup>Cl in the flux buffer containing 10 mM KCl, 280 mM sucrose, 5 mM HEPES, pH 7.2, at 0°C. Chloride efflux was then measured at 0°C in the flux buffer. A second group of cells (plunged, squares) was washed and loaded with <sup>36</sup>Cl in the 150 mM KCl buffer, and then plunged directly into the flux buffer for measurement of <sup>36</sup>Cl exchange in the presence of various H<sub>2</sub>DIDS concentrations. The intracellular Cl<sup>-</sup> concentration for the preincubated cells was 78.9 mM; for the plunged cells it was 133.7 mM. The intracellular pH for the plunged cells at time zero was 7.15; for the preincubated cells it was 8.1 (assuming complete Cl<sup>-</sup>/OH<sup>-</sup> equilibration). A second experiment gave similar results.

The experiments in which high chloride cells are plunged into low chloride media also argue against the possibility that changes in cell volume, caused by the cation permeability increase that occurs in low ionic strength media (La Celle and Rothstein, 1966), affect the H<sub>2</sub>DIDS inhibitory potency. The KCl efflux at 14°C in 10 mM chloride media is <10 mmol/liter packed cells·h and decreases with temperature (La Celle and Rothstein, 1966). At 0°C during the <3-min flux measurements, the KCl efflux would be <0.5 mmol/liter packed cells, which should cause a <0.5% decrease in cell volume. Changes in cell volume are therefore unlikely to be responsible for the increase in H<sub>2</sub>DIDS inhibition.

*Possible Effects of Membrane Potential Changes*

Although most of the chloride transport across the red cell membrane involves an electroneutral exchange of chloride for another anion, there is a conductive (net) chloride permeability which, while much smaller than the chloride exchange permeability, is still far larger than the permeabilities for cations such as sodium and potassium (Hunter, 1971, 1977; Knauf et al., 1977). Because of this net chloride flow, when red cells with high internal chloride are suspended in a medium with low chloride, a chloride diffusion potential results that depolarizes the membrane, that is, makes the membrane potential positive inside with respect to outside. Insofar as the anion exchange system permits very little net flow of anions, such changes in membrane potential would not be expected to affect directly the distribution of the various forms of the band 3 protein ( $E_i$ ,  $E_o$ , etc.). Indirect effects of potential on the band 3 protein structure are possible, however, and these might alter the affinity for external H<sub>2</sub>DIDS.

Under the conditions of the preceding experiments, chloride ions were always in equilibrium with the membrane potential, which makes it difficult to distinguish effects that depend on the chloride gradient from those related to membrane potential changes. We therefore made use of the ionophore valinomycin, together with appropriate potassium gradients, to establish a potassium potential across the membrane that would differ from the potential expected from the chloride gradient.

Such experiments are only possible if the potassium permeability can be increased to a much larger value than the net chloride permeability. This can easily be done at 37°C (Hunter, 1971, 1977; Knauf et al., 1977), but since the net chloride permeability (Hunter, 1977; Knauf et al., 1983) and the valinomycin-induced K<sup>+</sup> permeability (Hunter, 1974; Kaplan and Passow, 1974; Kaplan et al., 1976) are both dependent on temperature, the situation is less clear at 0°C. From calculations based on extrapolations of literature data, Gunn and Fröhlich (1979) assumed that at 0°C it would be necessary to use 10 μM valinomycin to achieve the necessary increase in K<sup>+</sup> permeability, but the  $P_K/P_{Cl}$  ratio was not experimentally verified.

To determine whether or not valinomycin can make  $P_K$  much greater than  $P_{Cl}$  under the conditions of these experiments, we took nystatin-treated high chloride (H) cells, washed them once in 10K-Hi buffer, and measured the net KCl efflux from these cells into 10K-Hi buffer in the presence of various concentrations of valinomycin (Fig. 6). At low valinomycin concentrations (<2 μM), the flux rose sharply with increasing valinomycin concentration, but it reached a plateau between 5 and 20 μM valinomycin. These data are similar to those obtained by us earlier at 37°C, except that at 0°C a much larger valinomycin concentration was required to achieve the plateau. As previously discussed (Knauf et al., 1977), this plateau suggests that under these conditions the membrane potential nearly reaches the potassium potential,  $E_K$ , and the rate of net KCl efflux is limited by the net chloride permeability. In the presence of 10 μM DIDS, the plateau flux was greatly reduced (Fig. 6). As DIDS is an inhibitor of net Cl<sup>-</sup> flux, but does not inhibit valinomycin-induced K<sup>+</sup> flux (Knauf et al., 1977; Kaplan et al., 1980),

these data are consistent with the hypothesis that the net chloride permeability limits the rate of net KCl efflux at high valinomycin concentrations, and therefore that under these conditions the membrane potential is close to  $E_K$ .

To further ensure that the plateau in net KCl efflux is not due to a leveling off of the valinomycin-induced  $K^+$  conductance, we measured the valinomycin-induced  $Rb^+$  exchange flux (as an indication of the  $K^+$  flux) at  $0^\circ C$  as a function

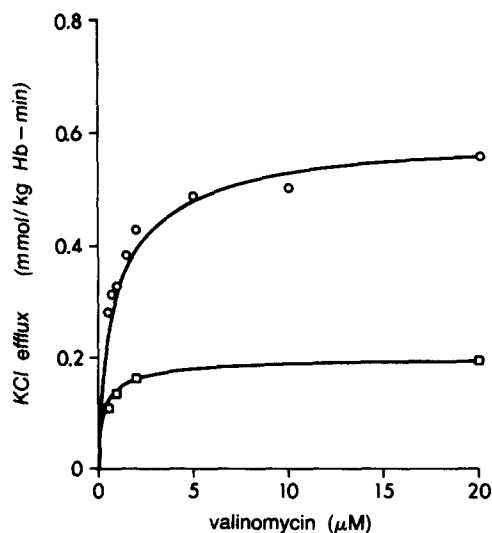


FIGURE 6. Net chloride efflux into low chloride media. High chloride (H) cells were prepared (with nystatin) and net KCl efflux was determined as described in Methods. The net  $K^+$  efflux was determined by flame photometry and was assumed to reflect net KCl efflux, since fluxes of other ions were negligible when measured under similar circumstances (Knauf et al., 1977). Flux measurements were started by addition of the valinomycin concentrations shown on the abscissa. The circles are data for control cells, while the squares represent fluxes measured in the presence of  $10 \mu M$  DIDS. The solid lines were calculated from the Goldman equation according to the method of Knauf et al. (1977, 1983). For the control cells,  $P_K$  was assumed to vary linearly with valinomycin concentration, and to be  $0.0913 \text{ mmol/kg Hb} \cdot \text{min} \cdot \text{mM}$  at  $20 \mu M$  valinomycin.  $P_{Cl}$  was calculated from the point at  $20 \mu M$  and was found to be  $0.00137 \text{ mmol/kg Hb} \cdot \text{min} \cdot \text{mM}$ . The corresponding  $P_K$  and  $P_{Cl}$  values with  $10 \mu M$  DIDS present were  $0.09216$  and  $0.00045$ .  $P_{Na}$  was assumed to be negligible. The  $P_K$  values were based on the data at  $10 \mu M$  valinomycin in Fig. 8, assuming that  $P_K = 0.5 P_{Rb}$ .

of valinomycin concentration (Fig. 7), under conditions where  $E_K$  was approximately equal to  $E_{Cl}$ . Although there was some nonlinearity at low concentrations, from  $1$  to  $10 \mu M$  valinomycin the  $Rb^+$  exchange rate constant was nearly linear with valinomycin concentration. DIDS ( $10 \mu M$ ) had no significant effect on  $Rb^+$  exchange. From the data at  $10 \mu M$  valinomycin, the value of  $P_{Rb}$ , calculated by assuming that  $P_K \gg P_{Cl}$ , was  $0.091 \text{ mmol/kg Hb} \cdot \text{min} \cdot \text{mM}$ . In the presence of

DIDS, the value was 0.092. Both calculated values were affected by <2% if  $P_{\text{Cl}}$  was assumed to be much greater than  $P_{\text{K}}$ .

If one assumes that  $P_{\text{K}}$  is one-half the value of  $P_{\text{Rb}}$ , as in artificial membranes (Szabo et al., 1973), one can calculate the expected results for net KCl efflux experiments such as that shown in Fig. 6. To do this, the value of  $P_{\text{Cl}}$  was determined from the measured flux at the highest valinomycin concentration,

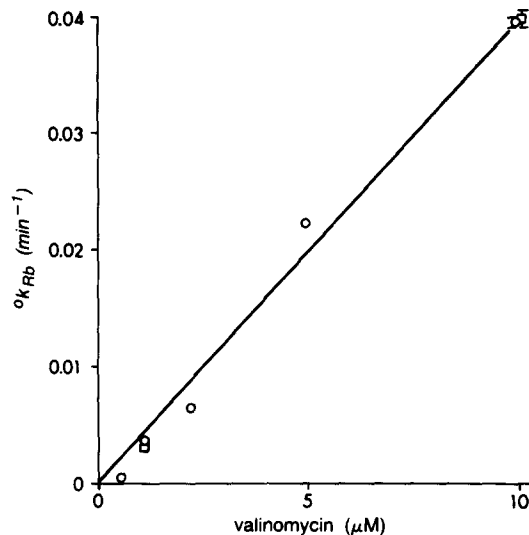


FIGURE 7. Rubidium efflux rate constant as a function of valinomycin concentration. Cells were loaded with  $^{86}\text{Rb}$  and efflux was determined as described in Methods.  $\%k_{\text{Rb}}$  is the rate constant for  $\text{Rb}^+$  efflux. From the intracellular and extracellular  $\text{K}^+$  and  $\text{Cl}^-$  concentrations,  $E_{\text{K}}$  was calculated to be  $-2.94$  mV, while  $E_{\text{Cl}}$  was  $-3.79$  mV. For this reason, different assumptions about the relative values of  $P_{\text{K}}$  and  $P_{\text{Cl}}$  had little effect upon the membrane potential and hence upon the value of  $P_{\text{Rb}}$  calculated as described by Knauf et al. (1977). The circles represent data for control cells, while the squares are for fluxes measured with  $10 \mu\text{M}$  DIDS present. The bars at  $10 \mu\text{M}$  valinomycin show SEM for three observations. The data for DIDS at  $10 \mu\text{M}$  are displaced slightly to the right for clarity. The line is drawn between the origin and the point for control cells at  $10 \mu\text{M}$  valinomycin. From the  $10 \mu\text{M}$  data,  $P_{\text{Rb}}$  for control cells was  $0.09134$  mmol/kg Hb  $\cdot$  min  $\cdot$  mM, and with DIDS it was  $0.09216$ .

and it was assumed that  $P_{\text{K}}$  varies linearly with valinomycin. The fit of the theoretical curves to the data in Fig. 6 is very good and is consistent with a  $P_{\text{K}}/P_{\text{Cl}}$  ratio at  $10 \mu\text{M}$  valinomycin of 33. In other experiments in which the medium contained  $150$  mM NaCl and  $10$  mM KCl, the fit was not quite as good, but the  $P_{\text{K}}/P_{\text{Cl}}$  ratio was always  $>20$ . This means that if cells with  $150$  mM internal KCl are suspended in medium with  $10$  mM KCl, addition of  $10 \mu\text{M}$  valinomycin should change the membrane potential from  $+63.7$  mV ( $E_{\text{Cl}}$ ) to  $-50.6$  mV, quite near to the value of  $E_{\text{K}}$  ( $-63.7$  mV).

Therefore, to determine whether or not membrane potential changes were involved in causing the increase in H<sub>2</sub>DIDS inhibitory potency when high chloride cells were suspended in low chloride medium, we measured the H<sub>2</sub>DIDS inhibition of chloride exchange under these conditions in the presence or absence of 10  $\mu$ M valinomycin. If the change in membrane potential from its resting value near 0 mV to about +64 mV in the low chloride medium were the cause of the increased inhibitory effect of H<sub>2</sub>DIDS, then membrane hyperpolarization by valinomycin to  $-50$  mV would be expected to decrease H<sub>2</sub>DIDS inhibition. As can be seen from the data in Fig. 8, however, valinomycin caused no significant change in H<sub>2</sub>DIDS inhibition of chloride exchange at any H<sub>2</sub>DIDS concentration tested. These data therefore demonstrate that the change in inhibitory potency caused by a chloride gradient cannot be attributed to concomitant changes in the membrane potential.

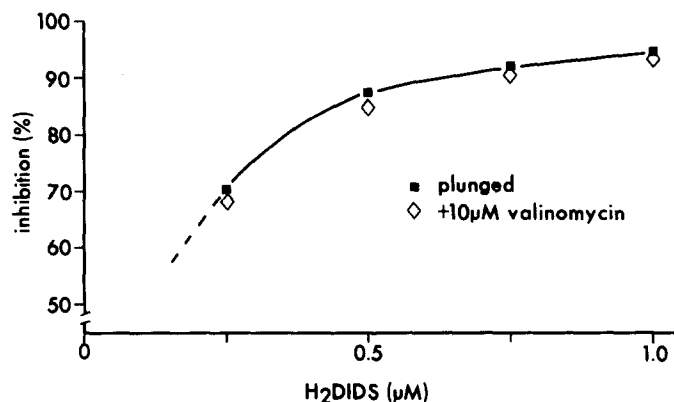


FIGURE 8. Inhibition of chloride exchange by H<sub>2</sub>DIDS with and without valinomycin. Fresh cells were washed and chloride exchange was measured as described for the "plunged" cells in Fig. 5 (solid squares). The data for the open diamonds were obtained in the presence of 10  $\mu$ M valinomycin, with 1.33% ethanol in the medium. From the data in Figs. 6 and 7, it can be calculated that valinomycin should change the membrane potential from 60.9 to  $-57.5$  mV under these conditions.

#### *Experiments with Reversed Gradients ( $Cl_o > Cl_i$ )*

Eq. 3 (see Theory) predicts that if the chloride gradient is reversed, that is, if  $Cl_o$  is greater than  $Cl_i$ , the  $ID_{50}$  for H<sub>2</sub>DIDS will increase, so H<sub>2</sub>DIDS will become a less potent inhibitor. According to the ping-pong model, this will happen because under these circumstances the relative amount of the band 3 protein in the  $E_o$  form, the form that interacts with H<sub>2</sub>DIDS, will decrease. To test this prediction, we have made use of two systems for altering the chloride gradient, one involving the use of resealed ghosts and the other involving changes in the pH of the medium.

Establishment of conditions with low chloride inside the cell requires that some other anion be substituted for chloride. The anion chosen must not interact strongly with the transport system and must be introduced into the cell interior.



To do this, we have resealed ghosts in the presence of citrate, an anion that has a very low affinity for the transport system (Schnell et al., 1978). Such experiments with ghosts should be comparable to experiments with cells, since anion fluxes in ghosts are quantitatively almost identical to those in intact cells (Funder and Wieth, 1976). Unfortunately, the ghost experiments were not very reproducible, perhaps because of the fact that by microscopic observation the ghosts seemed shrunken, probably because of an osmotic effect of citrate addition to the lysed ghosts. Also, according to the theory, the external Cl<sup>-</sup> concentration had to be kept low and the internal Cl<sup>-</sup> concentration even lower. It was difficult to establish a large stable chloride gradient under these conditions, since the ghosts rapidly took up chloride, causing the gradient to dissipate within 5 min. Despite these problems, the majority of experiments (Table II) showed a definite

TABLE II  
*Effect of Chloride Gradient on H<sub>2</sub>DIDS Inhibition in Ghosts*

Experiment	ID <sub>50</sub> * μM	ID <sub>50</sub> μM	ID <sub>50</sub> /ID <sub>50</sub> *	Cl <sub>o</sub> /Cl <sub>i</sub>
1	0.19	0.18	0.94	1.77
2	0.17	0.20	1.17	1.87
3	0.13	0.10	0.75	2.83
4	0.13	0.24	1.84	1.83
5	0.14	0.15	1.05	1.61
6	0.16	0.15	0.94	2.90
7	0.16	0.26	1.62	1.67
8	0.12	0.26	2.17	3.30
9	0.13	0.33	1.70	3.37
Mean	0.15	0.21	1.41	2.35
SEM	0.01	0.02	—	0.25
95% CI	0.13	0.15	1.05	1.78
	0.17	0.26	1.79	2.92

The extracellular Cl<sup>-</sup> concentration was 60 mM and the temperature was 0°C. For measurements of ID<sub>50</sub>\*, the intracellular Cl<sup>-</sup> was ~60 mM; for measurements of ID<sub>50</sub>, the intracellular Cl<sup>-</sup> averaged 27.8 mM.

increase in ID<sub>50</sub> with Cl<sub>o</sub> > Cl<sub>i</sub>. A paired *t* test showed that the increase was marginally significant (*P* = 0.055). The mean value of the ID<sub>50</sub> ratio (with gradient/without) was 1.41, with a 95% CI from 1.05 to 1.79. These results, while not very precise, are in qualitative agreement with the predictions of the ping-pong model.

To avoid some of the problems encountered with ghosts, the internal Cl<sup>-</sup> concentration in cells was lowered by raising the pH. This increases the negative charge on hemoglobin, thereby decreasing intracellular chloride. To test for direct effects of the increased pH on the binding of H<sub>2</sub>DIDS, ID<sub>50</sub> measurements were made at pH 7.2 and pH 9.0 in ghosts, where the chloride ratio is nearly invariant with pH (Funder and Wieth, 1976). High pH caused only a 30% increase in ID<sub>50</sub>, which suggests that pH itself has only a small effect on the

interactions of H<sub>2</sub>DIDS with the anion exchange system, possibly because amino groups near the H<sub>2</sub>DIDS binding site are titrated.

The results of experiments with intact cells are presented in Table III. The gradients obtained in different experiments differed somewhat because of the very sharp variation in chloride ratio with pH at high pH. In this case, all experiments showed an increase in ID<sub>50</sub> with a reversed chloride gradient, and the paired *t* test indicated significance at the *P* < 0.01 level. The mean increase in ID<sub>50</sub> was twofold, with a 95% CI from 1.34 to 2.84. Again, these results are in qualitative agreement with the predictions of the ping-pong model.

TABLE III  
*Effect of a Chloride Gradient Induced by Increasing pH on H<sub>2</sub>DIDS Inhibition of Chloride Exchange in Red Blood Cells*

Experiment	ID <sub>50</sub> * μM	ID <sub>50</sub> μM	ID <sub>50</sub> /ID <sub>50</sub> *	Cl <sub>o</sub> /Cl <sub>i</sub>
1	0.26	0.40	1.51	3.70
2	0.28	0.34	1.21	3.20
3	0.13	0.40	3.76	4.70
4	0.26	0.72	2.79	4.28
5	0.19	0.30	1.53	5.55
6	0.19	0.83	4.28	3.27
7	0.38	0.55	1.44	2.88
8	0.24	0.32	1.36	5.10
9	0.21	0.41	2.88	9.30
Mean	0.24	0.47	2.00	4.66
SEM	0.02	0.06	—	0.65
95% CI	{ 0.18 0.29	{ 0.33 0.62	{ 1.34 2.84	{ 3.16 6.17

The extracellular Cl<sup>-</sup> concentration was 60 mM and the temperature was 0°C. For measurements of ID<sub>50</sub>\*, the intracellular Cl<sup>-</sup> was ~60 mM; for measurements of ID<sub>50</sub>, the intracellular Cl<sup>-</sup> averaged 14.4 mM.

#### DISCUSSION

The data presented here provide evidence that changes in the chloride concentration inside the red cell strongly affect the ability of external H<sub>2</sub>DIDS to inhibit chloride exchange (Fig. 3 and Tables I–III). This transmembrane effect of internal chloride on external H<sub>2</sub>DIDS potency cannot be attributed to the concomitant changes in transmembrane pH or potential gradients that usually occur when a chloride gradient is imposed across the membrane (Figs. 5 and 8). In this respect the nonconductive chloride exchange system differs sharply from transport systems engaged in net ion flow, such as the Na-glucose carrier from the intestinal brush border, whose orientation is strongly affected by the membrane potential (Toggenburger et al., 1978). The data fit well to the predictions of the ping-pong model for anion exchange, thereby adding evidence from chemical probe experiments to the evidence from chloride flux kinetics (Gunn and Fröhlich, 1979) favoring this model. The data are not sufficient to exclude an ad hoc variation of the simultaneous model, in which internal Cl<sup>-</sup> increases

the binding affinity of external H<sub>2</sub>DIDS (Fig. 4). This model, however, is much more complex and would not fit with the chloride exchange data of Gunn and Fröhlich (1979). As with Gunn and Fröhlich's data, however, it is not possible to exclude certain degenerate forms of the simultaneous model, such as a model in which one site is always occupied with chloride. Such a model, however, is only semantically different from the ping-pong model, since only one site is actually involved in transport.

The data are consistent with the idea that H<sub>2</sub>DIDS binds to the transport site and can only do so when the transport site is in the outward-facing conformation. There is good evidence that H<sub>2</sub>DIDS is a competitive inhibitor (Shami et al., 1978), but it is by no means certain that it binds to the transport site itself. For example, H<sub>2</sub>DIDS might bind to a different site, but might prevent Cl<sup>-</sup> binding to the transport site (and vice versa). This kind of "allosteric competition" would fit the available data on the inhibition of chloride exchange by H<sub>2</sub>DIDS as well as the model in which H<sub>2</sub>DIDS binds to the transport site (Passow et al., 1980a). The conclusion that the anion exchange system is a ping-pong mechanism, with a single transport site that can alternate between an inward-facing (*E<sub>i</sub>*) and outward-facing (*E<sub>o</sub>*) form, however, is not dependent on the nature of the H<sub>2</sub>DIDS binding site. Even if H<sub>2</sub>DIDS binds to a site distant from the transport site, whose binding affinity is affected by the conformation of the transport site, the evidence still shows that a chloride gradient across the membrane can affect the orientation of the transport system, as predicted by the ping-pong model.

Similarly, these experiments do not provide further evidence that H<sub>2</sub>DIDS binds to the transport site. That it interacts with some site that is affected by changes in conformation of the transport site is all that can be deduced. As we show elsewhere (Knauf et al., 1984; Knauf and Mann, 1984), the effects of a chloride gradient would be exactly the same for such a model as for the case where H<sub>2</sub>DIDS binds directly to the transport site.

Although the data can be most simply interpreted in terms of a transport site that is alternately accessible to the cytoplasmic and external sides of the membrane, the evidence is actually compatible with any model in which the transport protein can exist in two conformations, which differ in that one permits binding of anions from the cytoplasm, while the other permits binding of external anions. It is not necessary that a single "site," defined in the restricted sense of a single amino acid residue of the band 3 protein, should have access to either side of the membrane. Models in which a chain of sites participates in a conformational change that affects the ability of anions to bind from the outside or inside (e.g., see Wieth et al., 1982), but in which no site actually "sees" both the cytoplasm and the external medium, are not excluded by these data.

One of the most important results of this study is the demonstration that chloride gradients can be used to change the orientation of the unloaded forms of the chloride exchange system. These results thus extend and confirm the findings of Jennings (1980, 1982) that the orientation of the anion exchange system, as well as the inhibitory potency of an analogue of H<sub>2</sub>DIDS, DNDS (4,4'-dinitro-2,2'-stilbene disulfonate), can be altered by putting chloride on one side of the membrane and sulfate on the other. Such gradients can be used to see

whether or not the binding of other probes is affected by the conformation of the transport site and also should provide a means for investigating structural differences between the two conformations of the transport protein band 3. Some of these topics are treated at greater length in the accompanying papers (Knauf et al., 1984; Knauf and Mann, 1984).

#### APPENDIX

#### Derivations of Equations for Predicting the Effect of a Chloride Gradient on H<sub>2</sub>DIDS Inhibitory Potency

##### *The Effect of a Chloride Gradient on H<sub>2</sub>DIDS Inhibition Assuming a Ping-Pong Mechanism*

The effect of a chloride gradient on the inhibition by H<sub>2</sub>DIDS is schematically shown in Fig. 2. The fluxes in either direction can be described as follows:

$$\text{outward flux: } J_o = kECl_i \quad (\text{A1})$$

$$\text{inward flux: } J_i = k'ECl_o \quad (\text{A2})$$

where  $k$  and  $k'$  are the rate constants for translocation of ions in the outward and inward direction, respectively.

$E$  denotes the carrier (by analogy to enzyme-catalyzed reactions).  $K_i$  and  $K_o$  are the dissociation constants for chloride at the substrate (transport) site internally and externally, and are defined as follows:

$$K_i = \frac{(E_i)(Cl_i)}{ECl_i}; \quad (\text{A3})$$

$$K_o = \frac{(E_o)(Cl_o)}{ECl_o}. \quad (\text{A4})$$

The dissociation constant for external H<sub>2</sub>DIDS is  $K_d$ :

$$K_d = \frac{(E_o)(D_o)}{ED_o}. \quad (\text{A5})$$

Since for this system the net chloride flux is very small in comparison to the unidirectional fluxes,

$$J_i = J_o = kECl_i = k'ECl_o. \quad (\text{A6})$$

At very high chloride concentrations, if interactions at the modifier site are ignored:

$$E_t = ECl_o + ECl_i, \quad (\text{A7})$$

where  $E_t$  is the total "carrier" (band 3) present. Under these conditions, the flux reaches the maximum flux,  $J_m$ :

$$J_m = k(ECl_i)_m. \quad (\text{A8})$$

Substituting A7 and A6 into A8:

$$J_m = \frac{kE_t}{(1 + k/k')}. \quad (\text{A9})$$

With external H<sub>2</sub>DIDS present,  $E_t$  is given by:

$$E_i = E_i + E_o + ECl_i + ECl_o + ED_o. \quad (A10)$$

Substituting Eqs. A1, A3–A6, and A9 into Eq. A10, we obtain:

$$\frac{1}{J_o} = \frac{1}{J_m(1 + k/k')} \left[ \frac{K_i}{Cl_i} + \frac{K_o k}{Cl_o k'} + 1 + \frac{k}{k'} + \frac{D_o K_o k}{K_d Cl_o k'} \right]. \quad (A11)$$

If we make the simplest assumption, namely that the transport system is symmetric, then  $k = k'$  and  $K_i = K_o$ . Eq. A11 then becomes:

$$\frac{1}{J_o} = \frac{1}{2J_m} \left[ \frac{K_i}{Cl_i} + \frac{K_i}{Cl_o} + 2 + \frac{D_o K_i}{K_d Cl_o} \right]. \quad (A12)$$

If  $Cl_i = Cl_o$ , and if there is no H<sub>2</sub>DIDS present and modifier site effects are ignored, then the flux,  $J_o$ , reaches half of the maximal flux,  $J_m$ , when  $Cl_o = K_i$ . Thus,  $K_i = K_o = K_c$ , the chloride concentration at which flux is half-maximal.

When the external H<sub>2</sub>DIDS concentration,  $D_o$ , is equal to the ID<sub>50</sub>, the concentration required for 50% inhibition,  $1/J_o$  is doubled compared with its value without H<sub>2</sub>DIDS. Thus, the term in Eq. A12 dependent on H<sub>2</sub>DIDS concentration is equal to the terms without H<sub>2</sub>DIDS:

$$\frac{ID_{50} K_c}{K_d Cl_o} = \frac{K_c}{Cl_i} + \frac{K_c}{Cl_o} + 2, \quad (A13)$$

with  $K_c$  substituted for  $K_i$ . Solving for ID<sub>50</sub>:

$$ID_{50} = K_d \left[ \frac{Cl_o}{Cl_i} + 1 + 2 \left( \frac{Cl_o}{K_c} \right) \right]. \quad (A14)$$

This equation is unaffected by including interactions of chloride with the modifier site (Dalmark, 1976), so long as it is assumed that chloride can bind to the modifier site regardless of the orientation ( $E_i$ ,  $E_o$ , etc.) of the transport site.

Thus, under the conditions of these experiments, where  $Cl_o$  is constant and  $Cl_i$  is varied, the ID<sub>50</sub> for H<sub>2</sub>DIDS should show a dependence on the reciprocal of  $Cl_i$ . As  $Cl_i$  increases, the ID<sub>50</sub> should decrease until it reaches a limiting value, determined by the concentration of chloride in the external medium ( $Cl_o$ ).

#### *The Effect of a Chloride Gradient on H<sub>2</sub>DIDS Inhibition Assuming a Simultaneous Mechanism*

Assuming the validity of the simultaneous mechanism with  $K_i = K_o$ , and assuming that binding of chloride at the inside-facing site does not affect the affinity of the outward-facing site for H<sub>2</sub>DIDS, then,

$$J = k(Cl_i E Cl_o) \quad (A15)$$

and 
$$J_m = k(Cl_i E Cl_o)_m = kE_i. \quad (A16)$$

(An ion or probe to the left of  $E$  denotes binding at the inside site; to the right indicates binding to the outside.)

The dissociation constants are described as follows:

$$K_i = \frac{(E)(Cl_o)}{ECl_o} = \frac{(E)(Cl_i)}{Cl_i E} = \frac{(Cl_i)(ECl_o)}{Cl_i E Cl_o} = \frac{(Cl_o)(Cl_i E)}{Cl_i E Cl_o} = \frac{(Cl_i)(ED_o)}{Cl_i ED_o} \quad (A17)$$

$$K_d = \frac{(E)(D_o)}{ED_o} = \frac{(Cl_i E)(D_o)}{Cl_i ED_o}. \quad (A18)$$

The total carrier,  $E_t$ , is given by:

$$E_t = E + ECl_o + Cl_iE + Cl_iECl_o + ED_o + Cl_iED_o. \quad (A19)$$

Substituting Eqs. A15–A18 into Eq. A19 and solving for  $1/J$ ,

$$\frac{1}{J} = \frac{1}{J_m} \left[ \frac{K_i^2}{Cl_iCl_o} + \frac{K_i}{Cl_i} + \frac{K_i}{Cl_o} + 1 + \frac{D_o}{K_d} \left( \frac{K_i^2}{Cl_iCl_o} + \frac{K_i}{Cl_o} \right) \right]. \quad (A20)$$

When  $D_o = ID_{50}$ , then

$$\frac{ID_{50}}{K_d} \left[ \frac{K_i^2}{Cl_iCl_o} + \frac{K_i}{Cl_o} \right] = \frac{K_i^2}{Cl_iCl_o} + \frac{K_i}{Cl_i} + \frac{K_i}{Cl_o} + 1. \quad (A21)$$

Solving for  $ID_{50}$ ,

$$ID_{50} = 1 + \frac{Cl_o}{K_i}. \quad (A22)$$

Thus, if  $Cl_o$  is constant and  $Cl_i$  varies, there is no change in the  $ID_{50}$  for  $H_2DIDS$ .

*Modified Simultaneous Mechanism in Which  $Cl_i$  Affects the Binding Affinity of External  $H_2DIDS$*

It is assumed that binding of  $Cl_i$  to  $E$  changes the affinity for  $H_2DIDS$ , such that the dissociation constant for  $H_2DIDS$  becomes  $K_d'$  instead of  $K_d$ . Thus:

$$K_d = \frac{(E)(D_o)}{ED_o} \quad \text{but} \quad K_d' = \frac{(Cl_iE)(D_o)}{Cl_iED_o}. \quad (A23)$$

Inserting Eq. A23, together with Eqs. A15–A17 into Eq. A19, and solving for the  $ID_{50}$  of  $H_2DIDS$ :

$$ID_{50} = \frac{Cl_o}{K_i} \left( \frac{K_dK_d'Cl_i}{K_iK_d' + K_dCl_i} \right) \left( 1 + \frac{K_i}{Cl_i} \right) \left( 1 + \frac{K_i}{Cl_o} \right). \quad (A24)$$

If  $Cl_o$  is constant and  $Cl_i$  is varied, the ratio of the  $ID_{50}$  to the value of the  $ID_{50}$  with  $Cl_i = Cl_o$  ( $ID_{50}^*$ ) is given by:

$$\frac{ID_{50}}{ID_{50}^*} = \frac{(K_i + Cl_i)(K_i + Cl_oK_d/K_d')}{(K_i + Cl_o)(K_i + Cl_iK_d/K_d')}. \quad (A25)$$

Thus, if  $K_d \neq K_d'$ , the  $ID_{50}$  will change as  $Cl_i$  is varied.

The variation in  $ID_{50}$  with  $Cl^-$  concentration under conditions where  $Cl_i = Cl_o$  (as in Fig. 4) can be calculated by substituting  $Cl_o$  for  $Cl_i$  in Eq. A24 to yield:

$$ID_{50} = K_d \left( 1 + \frac{Cl_o}{K_i} \right) \left( 1 + \frac{K_i}{Cl_o} \right) \left( \frac{Cl_o}{K_i + Cl_oK_d/K_d'} \right). \quad (A26)$$

To generate the theoretical lines in Fig. 4, Eq. A25 was used to determine the value of  $K_d/K_d'$  corresponding to the observed mean  $ID_{50}/ID_{50}^*$  value from Table I, and the  $ID_{50}$  as a function of  $Cl_o$  was then calculated from Eq. A26.

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