Transmembrane Effects of Irreversible Inhibitors of Anion Transport in Red Blood Cells

Evidence for Mobile Transport Sites

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ABSTRACT Experiments were designed to determine whether band 3, the anion transport protein of the red cell membrane, contains a mobile element that acts as a carrier to move the anions across a permeability barrier. The transport site-specific, nonpenetrating irreversible inhibitor 4,4'-diisothiocyanato-2,2'-stilbene disulfonate (DIDS) was found to be effective only when applied extracellularly. It was used to sequester transport sites on the extracellular side of the membrane in intact cells. The membranes were then converted into inside-out vesicles. The number of anion transport sites available on the cytoplasmic side of the vesicle membranes was then estimated by measuring the binding of N-(4-azido-2-nitrophenyl)-2-aminoethyl-sulfonate (NAP-taurine), a photoreactive probe. Pretreatment with DIDS from the extracellular side substantially reduced the binding of NAP-taurine at the cytoplasmic side. Since NAP-taurine does not appear to penetrate into the intravesicular (normally extracellular) space, a transmembrane effect is apparently involved. About 70% of the DIDS-sensitive NAP-taurine binding sites are located in band 3, with the remainder largely in a lower molecular weight (band 4) region. A similar pattern of reduction in NAP-taurine binding is produced by high concentrations of Cl-, but this anion has little or no effect in vesicles from cells pretreated with DIDS. Thus the DIDS-modulated sites seem to be capable of binding either NAP-taurine or Cl. It is suggested that band 3 contains a mobile transport element that can be recruited to the extracellular surface by DIDS, thus becoming unavailable to NAP-taurine at the cytoplasmic face of the membrane. The results are consistent with a model of carrier-mediated transport in which the movement of the transport site is associated with a local conformational change in band 3 protein.

INTRODUCTION

A current model to explain anion transport across the red blood cell membrane is based on the concept of a mobile carrier in which an anion binding entity or transport site within the membrane is alternately exposed at the opposite faces of the membrane by movement of the anion-carrier complex (Wieth, 1972; Gunn et al., 1973; and Dalmark, 1976). This model has been recently modified...
to accommodate the finding that a particular transmembrane protein, band 3, has been implicated as the transporting element of the membrane (Rothstein et al., 1976; 1978; Cabantchik et al., 1978). It has been suggested that the protein undergoes a conformational change so that an anion-binding site (the transport site) alternates between inside- and outside-facing positions within a protein channel. This hypothesis involves predictable behavior that can be tested experimentally. If, for example, the reaction of a relatively high potency competitive inhibitor from one side of the membrane leads to a reduced mobility of the sites, they should tend to accumulate on that side and should consequently be unavailable at the opposite side. Following this rationale the transmembrane effects of slowly penetrating inhibitors have been investigated. Thus, pyridoxal phosphate present inside red cells decreases the binding of the nonpenetrating inhibitor, 4,4'-diisothiocyanato-2,2'-stilbene disulfonate (DIDS), to the outside (Rothstein et al., 1976). Similar protection against DIDS interaction from outside has also been observed with 2-(4'-aminophenyl)-6-methylbenzenethiazol-3',7-disulfonate (APMB) sealed within ghosts (Passow and Zaki, 1978). Furthermore, APMB present on either the inside or the outside protects against inhibition by the penetrating agent fluorodinitrobenzene (FDNB), suggesting that inhibitory sites are accessible at either side and that they may therefore be mobile.

Although the above observations are consistent with the concept of a mobile transport site, the interpretations are subject to certain reservations, including the fact that it is not precisely clear how the test probes inhibit the transport. They are organic anions, so they might be expected to interact with anion-binding sites of the transport system. On the other hand, kinetic studies suggest the existence of two anion-binding sites called the transport site and the modifier site (Dalmark, 1976). Interactions of anions with the transport site result in a competitive inhibition, but interactions with the modifier site result in a noncompetitive inhibition (Dalmark, 1976). The probes might inhibit by binding to either site or, perhaps, to other unspecified sites. Their behavior does not, therefore, provide unequivocal information concerning the mobility of the transport sites.

Recent information delineating the mechanisms of inhibition by two probes, DIDS and N-(4-amino-2-nitrophenyl)-5-aminoethylsulfonate (NAP-taurine), provides the opportunity for a more detailed analysis of transmembrane effects and their interpretation in terms of a mobile transport site. DIDS and its reduced analog, dihydro DIDS (H₂DIDS), bind covalently to sites in band 3, producing an irreversible inhibition of anion transport (Cabantchik and Rothstein, 1972, 1974; Lepke et al., 1976). Their covalent reaction is, however, preceded by a reversible binding to the same sites (Cabantchik and Rothstein, 1972; Lepke et al., 1976; Ship et al., 1977). The kinetics of the inhibitory effect of H₂DIDS on Cl⁻ transport can be determined at 0°C during a time period when its binding is almost completely reversible (Shami et al., 1978). Under these conditions, H₂DIDS appears to compete with Cl⁻ for binding to the transport sites. Similar observations, based on an analysis of transport kinetics, indicate that another DIDS analog, 4,4'-dinitro-2,2'-stilbene disulfonic acid (DNDS), is also a reversible inhibitor, competing with Cl⁻ for the transport site (Barzilay and Cabantchik, 1978). Although a direct kinetic analysis of inhibition
would be difficult with DIDS because of its rapid covalent reaction, experimental evidence indicates that it reacts with the same sites as $\text{H}_2\text{DIDS}$ (Lepke et al., 1976; Ship et al., 1977). Because DIDS is a nonpenetrating agent (Cabantchik and Rothstein, 1974) that reacts rapidly and irreversibly (Ship et al., 1977), it would seem to be especially well suited to immobilize anion transport sites at the outer face of the membrane.

The photosensitive agent NAP-taurine is a reversible inhibitor of anion transport in the dark (Cabantchik et al., 1976; Rothstein et al., 1977; Knauf et al., 1978b). When present inside cells or resealed ghosts it appears to compete with $\text{Cl}^-$ for the transport site (Knauf et al., 1978b). On exposure to light, the azido group of NAP-taurine is converted to a nitrene that reacts irreversibly at the binding site with high efficiency (Rothstein et al., 1977; Knauf et al., 1978b). Because irreversible binding to cells after exposure to light closely reflects the reversible binding in the dark, NAP-taurine should be well suited to assess the number of transport sites exposed at the cytoplasmic surface.

In the present study, DIDS and NAP-taurine were used to test the hypothesis that a transport site in band 3 is alternately exposed to opposite sides of the membrane. It was first established that DIDS can inhibit only from the extracellular side of the membrane. Intact cells were then reacted with DIDS from the outside to determine whether such treatment resulted in a diminution in the number of NAP-taurine binding sites accessible from the cytoplasmic side of the membrane. Direct access to the cytoplasmic face, for the titrations with NAP-taurine, was achieved by allowing vesiculation of red cell ghosts under conditions where inside-out vesicles are formed (Steck and Kant, 1974). It was then determined which membrane proteins were involved in the observed effects. The hypothesis was further tested by evaluating the effects of a transported anion, $\text{Cl}^-$, on NAP-taurine binding to the cytoplasmic side of membranes of normal and DIDS-treated cells. The results are discussed in terms of the mobile site hypothesis.

**MATERIALS AND METHODS**

Recently outdated human red cells were used for all the experiments. The cells were washed three times with 5 mM Na phosphate-buffered saline, pH 8 (PBS) before each experiment.

The general protocol used to determine the effects of external DIDS on the NAP-taurine binding to the cytoplasmic surface is illustrated in the diagram of Fig. 1. Washed cells (15 ml) were resuspended in PBS at 25% hematocrit and reacted for 30 min at 37°C with 0.08 $\mu$M $^3\text{H}_2\text{DIDS}$ (step I, Fig. 1). Under these conditions, <5% of the maximum binding occurred but it was sufficient for subsequent quantification of the amount of ghost and vesicle material based on the labelling of band 3 by the probe. When $^3\text{H}$NAP-taurine was used to titrate binding sites (see below), the $^3\text{H}_2\text{DIDS}$-labelling step was omitted and the amount of vesicle material was estimated on the basis of phospholipid content by phosphate analysis. After washing, the cells were split into two batches and one of them was reacted with 5 $\mu$M nonradioactive DIDS (step II) using the same conditions as in step I. This treatment blocks virtually all of the DIDS-sensitive sites resulting in over 99% inhibition (Cabantchik and Rothstein, 1974; Ship et al., 1977). After removal of the excess DIDS, both the control and DIDS-treated cells were transformed into inside-out (IO) vesicles and the sealed vesicles were separated from leaky membrane...
fragments by flotation on a dextran density step, using basically the method of Steck and Kant (1974) with only minor modifications (Grinstein et al., 1978) (Step III). The sealed vesicles were washed once with 5 mM Na phosphate, pH 8 (5P8), and then extracted for 5 min at 0°C with 10 mM NaOH and 0.1 mM EDTA to remove extrinsic proteins (Yu and Steck, 1975), and washed once more with 5P8. This treatment removes extrinsic proteins unrelated to anion transport, that might bind NAP-taurine and so obscure the specific interactions of NAP-taurine at the cytoplasmic face. In agreement with previous findings (Steck and Yu, 1973), the extraction procedure removed bands 1, 2, 4.2, 5, and 6 almost completely (as examined by staining with Coomassie Blue on acrylamide gels) and left bands 3, 4.1, 4.5, and 7 as the major polypeptides in the vesicles. Glycoproteins such as

![Diagram representing the protocol used for the irradiation of the vesicles in the presence of NAP-taurine. IO and RO stand for inside-out and right-side-out vesicles, respectively. See Methods for specific details.](image-url)
glycophorin are also retained. The extraction does not interfere with the anion transport system for such “stripped” vesicles remain sealed, with sulfate fluxes essentially unchanged (Grinstein et al., 1978). In a few cases, as indicated in the text, the vesicles were washed and equilibrated overnight in media other than 5P8. Step IV in the diagram was carried out only in some experiments, as specified in the results. It consists of the additional of 20 μM DIDS to the extracted IO vesicle preparation which had been resuspended in 5P8 at a final concentration of 1 to 3 mg protein/ml. This step largely eliminates NAP-taurine binding to the outside facing sites (Cabantchik et al., 1976) in the small fraction of right-side-out (RO) vesicles that are present in the IO preparations. 1-ml aliquots of the vesicle suspension containing 0.23 mM of either [35S]- or [3H]NAP-taurine were irradiated (step V) to fix the probe to its binding sites (Staros and Richards, 1974). Irradiation for various times (as indicated in Results) was performed using the experimental setup described by Knauf et al. (1978 a). The temperature during this part of the procedure was 0°C to minimize the penetration of NAP-taurine (Staros et al., 1975; Cabantchik et al., 1976). Immediately after the exposure to the light, the vesicles were diluted with 9 ml of ice cold PBS containing 0.5% wt/vol albumin and centrifuged for 10 min at 48,000 g to remove unreacted and photolysed probe. Washing the PBS-albumin was repeated three more times. Finally, before counting of 35S and 3H, phosphate analysis, or electrophoresis, the vesicles were washed twice with saline to remove inorganic phosphate that would interfere with the phospholipid P1 determinations, and albumin which would contaminate the protein electrophorograms.

The sidedness of the vesicles was determined by measuring their acetylcholinesterase activity in the presence and absence of 0.1% Triton X-100 (Rohm & Haas Co., Philadelphia) as described by Steck and Kant (1974). Inasmuch as the yield and protein content of the vesicles vary somewhat in the different preparations, parameters other than protein analysis must be used to estimate the amount of membrane material used in each experiment. Based on the observation that neither lipids nor intrinsic proteins are lost during the preparation of the vesicles (Grinstein et al., 1978), the phospholipid content (based on phosphate analysis) and the band 3 protein content (based on amount of 3H2-DIDS labelling, step I, Fig. 1) were determined for the vesicles used in each experiment. The ratio of 3H2-DIDS to phosphate does not change during the procedure as outlined in Fig. 1, so that experimental data could be normalized to the amount of membrane or band 3, or calculated on a per cell basis.

In some experiments, IO vesicles loaded with either albumin or hemoglobin were prepared. This was achieved by forming the vesicles in a medium containing 0.5 mM Na phosphate, 0.1 mM EDTA, and 1-2% wt/vol of the desired protein. The pH of this solution was 9 at 0°C, the temperature used for vesiculation and homogenization. The hemoglobin used was obtained by hemolyzing red cells with 1 vol of distilled water, followed by centrifugation of the membranes. The supernate was then dialyzed at 4°C against a solution containing 0.5 mM Na phosphate and 0.1 mM EDTA, pH 9.0. The dialyzate was finally diluted with dialysis buffer to obtain the desired concentration of protein in the vesiculation medium. Only small changes were noted in the yield and purity of the vesicles due to the addition of the proteins.

For 35SO4 efflux measurements, IO vesicles were prepared as described above, whereas RO vesicles were obtained by the addition of 0.2 mM MgSO4 to the vesiculation medium before centrifugation, as recommended by Steck and Kant (1974). The vesicles were resuspended to a final concentration of 2-5 mg protein/ml in a medium containing 10 mM Na2SO4, 0.1 mM MgSO4, and 1 mM Tris-H2SO4, pH 7.4 (SO4 medium). This suspension was equilibrated overnight at 4°C with 4-8 μCi/ml 35SO4. After equilibration, removal of extravascular isotope was accomplished by filtering 1 ml of the suspension through a 3-ml column of Dowex AG 1X4, that had been washed and equilibrated with
SO₄ medium. The vesicles were eluted from the column with 3 ml of SO₄ medium, and the eluate diluted with the same solution to the desired final concentration. Efflux determinations were started immediately after this dilution by filtering 0.5-ml aliquots through smaller (1.2 ml) anion exchange columns as described above, and washing with 1.6 ml of ice-cold SO₄ medium. The filtrate, which contained the vesicle suspension free of extravesicular isotope was collected directly into scintillation vials and counted with 11 ml Aquasol (New England Nuclear, Boston, Mass.).

Electrophoresis of membrane proteins in 7.5% polyacrylamide gels (or where noted in 3.5% polyacrylamide) with 0.1% SDS was performed according to Weber and Osborn (1969). Pyronin Y was used as the tracking dye. The gels were stained with Coomassie Blue and scanned in a Beckman Acta II Spectrophotometer adapted with a Beckman R II C scanning device (Beckman Instruments Inc., Fullerton, Calif.). For counting, the unfixed gels were sliced manually and the slices (1.5 mm wide) were eluted overnight at 60°C in 1 ml of 1% SDS. In some experiments, slicing was performed after fixing the gels overnight in a solution containing 450 ml methanol, 51.6 g sulfosalicylic acid and 172.5 g trichloroacetic acid per liter water. The slices were in this case digested for 48 h with protocol: toluene: water (9:10:1) at 60°C before counting. Similar results were obtained with both methods.

Phospholipid phosphorus (Rouser et al., 1966) and protein determinations (Lowry et al., 1951) were performed as described before (Grinstein et al., 1978). Radioactivity was counted in a Packard Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.), using Aquasol (New England Nuclear) as the scintillation fluid.

[³H]NAP-taurine, [³⁵S]NAP-taurine, ³H₂-DIDS and DIDS were synthesized in our laboratory by Doctors S. Ship and M. Ramjeesingh by methods described previously (Staros and Richards, 1974; Cabantchik and Rothstein, 1972, 1974). ³⁵SO₄ was purchased from New England Nuclear. Bovine serum albumin was obtained from Sigma Chemical Co., St. Louis, Mo. Dowex AG1X4, 20-50 mesh, chloride form, was purchased from Bio-Rad Laboratories, Richmond, Calif.

RESULTS

The Sidedness of the Inhibitory Action of DIDS

The use of DIDS to demonstrate a transmembrane effect requires that it react only on the external face of the membrane under the particular conditions of the experiments. In intact cells, DIDS is reported to produce its inhibitory effect on the external surface by interacting with sites in band 3 with minimal penetration through the membrane (Cabantchik and Rothstein, 1974). Furthermore, the cytoplasmic face may be insensitive to the disulfonic stilbenes. A reversibly binding analog, 4,4’-diacetamido-stilbene-2,2’-disulfonic acid (DAS), which inhibits anion transport in cells, has no inhibitory effect when sealed inside red cell ghosts (Zaki et al., 1975).

The relative inhibitory effectiveness of DIDS when applied to the extracellular or cytoplasmic surface of the membrane was assessed by comparing RO and IO vesicles. The efflux of ³⁵SO₄ from RO vesicles (which were > 95% pure, as evidenced by their acetylcholinesterase activity; see Methods) is illustrated in Fig. 2. The rate of ³⁵SO₄ efflux under equilibrium conditions was very fast at the beginning of the experiment but fell to lower values thereafter, presumably due to heterogeneity in vesicular size. Addition of DIDS to the vesicles at the onset
of the efflux measurements produced a marked reduction in the efflux. The inhibition increased as the concentration of DIDS was raised with > 95% inhibition observed with 5 μM DIDS. There was no apparent delay in the onset of inhibition after the addition of the stilbene, suggesting that the inhibitor-binding sites are externally located, as has also been reported in the intact cell (Shami et al., 1978).

As in the case of RO vesicles, the efflux of 35SO₄ from IO vesicles was nonlinear (Fig. 2B) presumably for the same reason, i.e., heterogeneity in size.
distribution of vesicles. In contrast to the RO vesicles, however, $^{35}$SO$_4$ efflux from IO vesicles was only marginally affected even by the highest concentration of DIDS used (5μM). Furthermore, in that the vesicle preparations used for these experiments were only 88-92% in the IO form, the small effect of DIDS can be accounted for, if it is assumed that only the 8-12% contaminant RO vesicles are reacting with the inhibitor. Indeed, consistent with this assumption, the less successful preparations in which the purity of the IO preparation was below 80% showed significantly greater degrees of inhibition by the stilbene (not illustrated). It can be concluded that the inhibitory sites are accessible to DIDS from the outside but not from the inside, so that this agent is ideal for the studies of the anion transport system reported here.

**Figure 3.** Time-course of NAP-taurine binding to IO vesicles obtained from normal (●) and DIDS-treated (○) cells. Each point is the average of three determinations. Vesicles were suspended in a solution containing 230μM NAP-taurine and irradiated for the times indicated in the abscissa, as described in Methods. The amount of NAP-taurine bound after repeated washing is given for the amount of membrane equivalent to one cell. This was calculated on the basis of the phospholipid phosphorus content of the vesicle suspension and using a reported value of phospholipid phosphorus content per red cell (Reed et al., 1960).

**The Effect of DIDS on the Binding of NAP-Taurine Applied to the Cytoplasmic Face**

The binding of NAP-taurine to vesicles from normal and DIDS-treated membranes after different irradiation intervals is shown in Fig. 3. The time dependence presumably reflects the total amount of light necessary to activate all of the NAP-taurine, with complete activation requiring something > 5 min for the particular light source used (about the same time is required for low concentrations of cells [Knauf et al.; 1978 a]). Thus, values after 5 min or more of irradiation can be taken to estimate the maximal binding for 230μM NAP-taurine. The average binding was $1.79 \pm 0.29$ (SE) $\times 10^6$ sites per cell ($n = 8$, including the values in Fig. 3). Of the total, $0.70 \pm 0.10$ (SE) $\times 10^6$ sites per cell
or about 40%, were made unavailable by DIDS pretreatment. The effect of DIDS was highly significant with a P value of <0.001.

Measurements of the sidedness of the IO vesicle population indicated that ~10% of the membranes retain their original RO configuration. In view of the fact that DIDS reduces the binding of NAP-taurine when both agents are present on the same (extracellular) side (Cabanthchik et al., 1976), it was conceivable that the decrease in NAP-taurine binding described in Fig. 3 originated in the contaminating RO vesicles. The following considerations, however, argue against this possibility. (a) The maximum number of external NAP-taurine binding sites which are associated with complete inhibition of anion transport is around $1.4 \times 10^6$ (Cabanthchik et al., 1976). Inasmuch as only ~10% of the vesicles are RO, it follows that a maximum of $0.14 \times 10^6$ sites per cell could be accounted for by contaminating RO vesicles. This number is small compared to the experimentally determined values. (b) Because DIDS will prevent most of the binding of NAP-taurine to the outside of cells (Cabanthchik et al., 1976), selective elimination of NAP-taurine binding sites of the RO vesicles was achieved by treating ressealed and alkali-extracted vesicles with DIDS (20 μM), before addition of the photoreactive label (step IV, Fig. 1). It was assumed that the added DIDS would not influence NAP-taurine binding sites of IO vesicles, based on the lack of effect of the stilbene on their anion transport (Fig. 2). The concentration of DIDS used for blocking the RO vesicles was higher than that used in the inhibition experiments (Fig. 2) because the vesicle concentration was about 10 times as high. It is important to maintain an appropriate ratio of DIDS to protein, rather than a constant concentration of the stilbene, because its reaction is irreversible. The DIDS treatment of the control and experimental vesicles reduced NAP-taurine binding in both cases by a similar and small amount (~15%). However, the difference in the number of NAP-taurine binding sites induced by pretreating the cells with DIDS was virtually unchanged.

The distribution of covalently bound NAP-taurine among the proteins of IO vesicles separated by SDS polyacrylamide gel electrophoresis is shown in Fig. 4. The top graph (Fig. 4 A) shows a density scan of the Coomassie Blue-stained proteins. The pattern is similar to that already reported for alkali “stripped” vesicles (Steck and Yu, 1973). Small variable amounts of bands 1 and 2 are retained (the doublet of low mobility on the far left of the scan); band 3 is the highest peak, mobility 0.20; band 4.1 is just to the right of band 3, mobility 0.23 (it is a separate peak, but in the scan it shows up as a shoulder on band 3); band 4.5, a broad zone between mobilities 0.26 and 0.33; and band 7, mobility 0.43. Glycophorin can be visualized by staining with periodic acid-Schiff reagent (PAS). It is located just to the right of band 3 in about the same location as band 4.1. Most of the NAP-taurine was localized in band 3 (Fig. 4 B). Smaller amounts also appeared to the left and right of band 3 in the higher molecular weight region and in a lower molecular weight region (band 4 region). The counts were near background in the region of the gel corresponding to a mobility of 0.6 or greater (the right-hand half), and no discrete stained bands were evident. In the experiment illustrated in Fig. 4, that particular portion of the gel was not sliced.
FIGURE 4. (A) Gel electrophoretic patterns of “stripped” IO vesicles. The gel was stained with Coomassie Blue and scanned at 575 nm. The arrow indicates the position of the tracking dye pyronin Y. The deflection in the region immediately behind the tracking dye corresponds to scattering due to lipids. (B) Labelling of control IO vesicles by [3H]NAP-taurine photolysis. Vesicles were irradiated with 0.23 mM of the probe for 5 min at 0°C followed by extensive washing as described in Methods. Data from four individual gels are presented. The solid line connects the average of the four determinations for each slice. The patterns were aligned by matching the peaks of band 3. In some gels, this required lateral displacement of one or at most two slices. The dotted lines separate the regions of the gel used for the computations in Table I. The region of the gel corresponding to relative mobilities > 0.6 contained a low number of counts and was therefore not illustrated. Abscissa: relative mobility. Ordinate: counts per minute per slice. (C) Labelling of IO vesicles obtained from DIDS-treated cells by [3H]NAP-taurine photolysis. The same amount of vesicles as in (B) were irradiated and subjected to electrophoresis under identical conditions. For details see (B) above.
and counted. To obtain a more precise quantitation of the distribution of radioactivity in the different regions, the gel-to-gel variability was minimized by applying four aliquots of labelled proteins from control (Fig. 4 B) and from DIDS-treated membranes (Fig. 4 C) to separate gels which were simultaneously electrophoresed and carefully sliced. The counts for each individual slice are plotted in Fig. 4. In the preparation from DIDS-pretreated cells, a large decrease in the NAP-taurine labelling of band 3 was evident. Some diminution in the band 4 (mobility about 0.3) and in the higher molecular weight regions was also observed. To quantitate the effects, regions of the gels were designated as indicated by the dotted lines of Fig. 4, with an integration of the total count in each region calculated for each gel. The results, presented in Table I, indicate that ~70% of the DIDS-induced reduction in NAP-taurine binding is located in band 3, with the remainder largely accounted for by the band 4 region. The smaller effect found in the higher molecular weight region was not statistically significant, but in another experiment a distinct DIDS effect was noted in both the band 4 and higher molecular weight region (as well as the large effect in band 3). Thus, a small effect in the high molecular weight region cannot be excluded.

The small high molecular weight peak of NAP-taurine binding has a mobility of 0.085 (average of four estimates). This is not far from the location of residual band 2 (mobility 0.08, average of two estimates), although the uncertainty in measuring the mobility of the high molecular weight peaks is such that the correspondence cannot be considered as strong evidence that the radioactivity is in fact in band 2. The small peak of NAP-taurine binding to the right of band 3 has a mobility of 0.28 (average of four estimates) falling close to the middle of band 4.5 a broad band (mobility range 0.26-0.33 with a midpoint of 0.3). The radioactive peak has a distinctly higher mobility than either band 4.1 or glycophorin (PAS 1). In the case of glycophorin, the relative mobility could be altered by changing the amount of acrylamide in the electrophoresis system. In 3.5% acrylamide gels, in which the glycophorin has a slightly lower mobility than band 3, the small peak of NAP-taurine binding is still found in the band 4.5 region with a mobility higher than that of band 3.

Effect of Chloride on NAP-Taurine Binding

When present on the cytoplasmic side of red blood cells (in the dark), NAP-taurine is reported to compete with Cl\textsuperscript{−} for binding to the anion transport site (Knauf et al., 1978 b). Because NAP-taurine behaves as a photoaffinity probe (Knauf et al., 1978 a), on exposure to light it should therefore bind irreversibly to the transport sites, and the binding to those sites should depend on the Cl\textsuperscript{−} concentration in a predictable manner.\textsuperscript{1} Furthermore, because DIDS added to

\textsuperscript{1} It might appear that because the photoactivated reaction of NAP-taurine is irreversible, whereas the Cl\textsuperscript{−} interaction is reversible, that the Cl\textsuperscript{−} would modulate the rate but not the final amount of the NAP-taurine binding in the light. Knauf et al. (1978 a), however, demonstrated that the amount of the irreversible reaction in the light is approximately equal to the amount of reversibly bound NAP-taurine in the dark under the same circumstances. This equivalence obtained because as the covalent interaction of the reversibly bound NAP-taurine proceeds, a parallel destruction of the NAP-taurine in solution occurs largely via hydrolysis. A kinetic analysis explaining the equivalence is given in the appendix of the cited paper. Experimental verification is given by demonstrating that
the external face of the membrane also seems to react with the transport sites (Shami et al., 1978), DIDS and Cl\(^-\) would be expected to modulate NAP-taurine binding in a similar manner, and in DIDS-treated cells the Cl\(^-\)-modulation should be suppressed. To test these predictions light-induced NAP-taurine binding to "stripped" IO vesicles was determined in the presence and absence of 150 mM Cl\(^-\) (Fig. 5 A and B). In the Cl\(^-\)-free medium ionic strength was maintained with sodium citrate and osmolarity by sucrose (composition given in legend of Fig. 5). Citrate seems to have little interaction with the anion transport system and little effect on inhibition by external NAP-taurine (Rothstein et al., 1977; Schnell et al., 1978). The vesicles were preequilibrated with the two media. Other experimental procedures were identical to those used in the DIDS experiment illustrated in Fig. 4.

The binding of NAP-taurine to the vesicles was substantially reduced by 150 mM Cl\(^-\) (33%). The protein pattern was similar to that induced by DIDS, with most of the reduction in the band 3 region (66%), a significant portion in the band 4 region (25.7%). The reversible inhibition of Cl\(^-\) fluxes by NAP-taurine in the dark is approximately equal to the irreversible inhibition after exposure of the same cells to light and, furthermore, that the light-induced covalent binding of NAP-taurine to band 3 is decreased by high Cl\(^-\) concentrations in proportion to the effect of the Cl\(^-\) on the degree of inhibition (reversible or irreversible).

<table>
<thead>
<tr>
<th></th>
<th>High molecular weight region</th>
<th>Band 3 region</th>
<th>Band 4 region</th>
<th>Total in all regions</th>
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<tr>
<td>Control</td>
<td>1,840 ± 39</td>
<td>5,761 ± 126</td>
<td>2,079 ± 131</td>
<td>10,802</td>
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<tr>
<td>DIDS</td>
<td>1,750 ± 126</td>
<td>2,608 ± 129</td>
<td>1,122</td>
<td>4,370</td>
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<tr>
<td>% of total difference</td>
<td>2.1</td>
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<td>25.7</td>
<td>100</td>
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The values are the mean ± 1 SE of four determinations from a single batch of vesicles. The numbers indicate the integrated counts in the regions of the gel demarcated by the dotted lines in Figs. 3 and 4. "High molecular weight" refers to the area between the y axis and the first dotted line. The first slice was not included because it contains aggregated material. "Band 3 region" is the area between the first and second dotted lines, and "Band 4 region" is encompassed by the second and third lines. The last line refers to the percent of the total difference contributed by the individual regions. The region of the gel corresponding to mobilities higher than 0.6 contained very few counts and no Coomassie Blue-stained bands and is therefore not included. Thus the total (last column) is the sum of the counts in the three marked regions. Student's t test was used to calculate the significance of the differences.
band 4 region, and a smaller fraction in the high molecular weight region (Fig. 5, Table I). Furthermore, Cl\textsuperscript{−} had no measurable effect on NAP-taurine binding to IO vesicles prepared from DIDS-pretreated cells (data not shown). These findings indicate that the DIDS-sensitive sites can bind Cl\textsuperscript{−} whereas the DIDS-insensitive sites cannot, implying specificity for the transported anion. They suggest that the DIDS- and Cl-sensitive NAP-taurine binding sites are largely the same.

**Is NAP-Taurine Permeating across the IO Vesicle Membrane?**

Although NAP-taurine was added to the cytoplasmic (external) side of the IO membranes, it was necessary to determine the degree of permeation of the probe across the membrane, because penetration into the intravesicular space and reaction on the extracellular membrane surface of the cell could explain the
observed results without invoking a transmembrane effect of DIDS. Nonspecific leaks are not evident in the IO vesicles. Albumin or hemoglobin sealed within the vesicles (see Methods) do not escape even after eight washings. Nor is the membrane permeable to the small molecule acetylthiocholine (mol wt 167) used to determine the sidedness of the vesicles based on the accessibility of cholinesterase (see Methods). The vesicles are also sealed to small cations such as Rb⁺, since the half time of its equilibration is several hours at 22°C and longer at 0°C (Grinstein and Rothstein, 1978).

NAP-taurine can permeate across the membrane of intact red cells at 37°C (Staros et al., 1975; Cabantchik et al., 1976; Knauf et al., 1978 b), but at 0°C it is

reported to be virtually impermeant. To decide, however, whether sufficient penetration was occurring during the course of the irradiation of the IO vesicles to account for the DIDS effect (Fig. 3, Table I), several additional types of experiments were undertaken. In the experiment of Fig. 6, vesicles were incubated at 0°C in the dark for periods of time ranging from 0 to 6 min and then irradiated for 2 min at 0°C. After washing, the vesicles were counted for both ³H₂-DIDS and [³⁵S]NAP-taurine. (□) Control vesicles. (■) Vesicles obtained from cells pretreated with 5 μM DIDS. The lines were calculated by the least squares method. The points are the mean of duplicate determinations. Abscissa: total incubation time in minutes (including the irradiation period). Ordinate: counts per minute of [³⁵S]NAP-taurine per ³H₂-DIDS.

![Graph showing NAP-taurine binding as a function of incubation time with the probe. Vesicles obtained from the same batch of ³H₂-DIDS-labelled cells were incubated in the dark at 0°C for periods of time ranging from 0 to 6 min and then irradiated for 2 min at 0°C. After washing, the vesicles were counted for both ³H₂-DIDS and [³⁵S]NAP-taurine.](image)

From the slopes, it can be seen that the amount of NAP-taurine that might permeate the membrane after 8 min is an insignificant fraction of the DIDS-sensitive component of binding during the 2-min irradiation period.
Even if the upper limit of the 95% confidence interval for the slope of the lines in Fig. 6 is taken to represent permeation through the membrane, the uptake of NAP-taurine at different times would still be considerably smaller than the DIDS-sensitive component of NAP-taurine binding after equivalent periods of time. It is evident, therefore, that a slow penetration of NAP-taurine could not account for the observed reduction in binding caused by the stilbene.

The data of Fig. 6 are consistent with a rapid binding to outside sites and an almost zero rate of influx. It is also possible, however, that NAP-taurine can move so rapidly across the membrane that it has already fully equilibrated into the intravesicular space before the first point, at 2 min (the irradiation period). The lines of Fig. 6 would then represent the complete equilibration of NAP-taurine with binding sites on both sides of the membrane and also with the intravesicular space. This possibility was tested by incubating vesicles with NAP-taurine for 10 min at 0°C, after which the temperature was quickly raised to 37°C. An additional increment of uptake (measured by filtration of the vesicles on Millipore filters, Millipore Corp., Bedford, Mass.) was observed at the higher temperature during the next 8 min amounting to 31.2 ± 2.2% (SE) (n = 11). This statistically significant temperature-dependent increase (P < 0.001) was not observed in vesicles from DIDS-treated cells, suggesting that it represents penetration into intravesicular space (NAP-taurine fluxes in cells are inhibited by DIDS [Cabantchik et al., 1976]).

A further test was carried out by preparing IO vesicles with relatively large amounts of hemoglobin (2% wt/vol) trapped in the intravesicular space (see Methods). Hemoglobin is labelled by NAP-taurine in intact cells (Staros et al., 1975). Thus, any hemoglobin-probe interaction in the vesicles would identify NAP-taurine that had reached the intravesicular space.

The distribution of NAP-taurine in various proteins in these experiments is illustrated in Fig. 7. At the top (Fig. 7 A) the absorbance curve for the membrane proteins in the acrylamide gel shows the band 3 peak at the left. The intravesicular hemoglobin shows as a large peak toward the right, that is absent in control vesicles (Fig. 4 A). In hemoglobin-loaded vesicles exposed to the probe at 0°C, the amount and pattern of distribution of [³⁵S]NAP-taurine is similar to that of the control curve (Fig. 4 B) except that an additional small peak is seen in the hemoglobin region (Fig. 7 B). Preexposure at 37°C before irradiation on the other hand, results in an increase in all of the labelled peaks, but especially in the hemoglobin peak, indicating that much of the probe that reached the intravesicular space at the higher temperature is associated with the hemoglobin.

These experiments support the view that the temperature-dependent increase in NAP-taurine uptake represents, at least in part, a penetration of the probe into intravesicular space (defined as binding to trapped protein). Thus the NAP-taurine could not yet be at equilibrium distribution after 5 min of exposure at 0°C. Therefore the horizontal lines in Fig. 6 must represent rapid binding to the outside (cytoplasmic) surface rather than a rapid equilibration with the intravesicular space. Although no significant influx is detectable at 0°C by direct measurement (Fig. 6), a small amount of influx is detectable as hemoglobin-
Figure 7. (A) Gel electrophoretic pattern of IO vesicles loaded with 2% hemoglobin. The gel was stained with Coomassie Blue and scanned at 575 nm. The positions of band 3 and hemoglobin are indicated. The arrow indicates the position of the tracking dye, pyronin Y. (B) Labelling of hemoglobin-loaded IO vesicles with [35S]NAP-taurine. Vesicles like those in (7A) were irradiated with 0.23 mM NAP-taurine for 5 min at 0°C. A fraction of the vesicles was preincubated with the photoreactive probe for 10 min at 37°C in the dark, before irradiation (upper curve). The rest of the vesicles were irradiated immediately after the addition of NAP-taurine (hatched area). The pH of the medium used was 6.0, to increase the affinity of hemoglobin for NAP-taurine. Abscissa and ordinate as in Fig. 4B.

bound NAP-taurine in protein-loaded vesicles (Fig. 7). The exact amount is hard to quantify from the gel distributions, but it is relatively small compared to the DIDS-sensitive increment of binding (Fig. 4).²

It can be concluded that the reduction in NAP-taurine binding resulting from

² The amount of NAP-taurine that might pass into the IO vesicles at 0°C can be calculated from data on the efflux of the probe from cells at 0°C (Staros et al., 1975). The calculated rate constant is 0.03 h⁻¹. On this basis the penetration into the vesicles would be 6.4 × 10³ molecules per cell equivalent.
the interaction of DIDS with the extracellular face is the result of a transmembrane effect.

**DISCUSSION**

The experiments reported in this paper were performed to test certain aspects of a particular model for the anion transporting system. The model described in detail elsewhere (Rothstein et al., 1976; Cabantchik et al., 1978) takes into account that the kinetics of anion transport are consistent with a mobile carrier model (Wieth, 1972; Gunn et al., 1973; and Dalmark, 1976), and that the transport seems to be associated with a particular membrane protein, band 3 (Cabantchik and Rothstein, 1974; Rothstein et al., 1976; Lepke et al., 1976; Cabantchik et al., 1978). Briefly, it is proposed that a 17,000 dalton transmembrane segment of band 3 provides a protein channel through the lipid bilayer, through which the anion transfer occurs. Because the anion flux is largely a nonconductive one-for-one exchange, it is also proposed that the channel is blocked by a diffusion barrier and that the transport site, when occupied by an anion, can, by a local conformational change in the protein, traverse the short distance across this diffusion barrier. Thus the transport site becomes alternately accessible from the inside and outside. To explain the fact that DIDS can only inhibit from the outside, it is proposed that the transport site is close to the external surface so that it can be reached by this large probe present in the medium, but it cannot be reached from the cytoplasmic side. The smaller anion, NAP-taurine, on the other hand, can diffuse from the cytoplasmic side through the channel within the protein matrix to reach the site. The model predicts that, at a given time, each transport site is accessible only from one side of the membrane. It follows that immobilizing the sites at one side should reduce the number accessible from the opposite side. This prediction was tested by the use of two probes applied to opposite sides of the membrane. The probes used, the disulfonic stilbene, DIDS, and NAP-taurine, are appropriate because they both appear to react with the Cl⁻-transport site based on studies of inhibition kinetics (Shami et al., 1978; Knauf et al., 1978 b); they both can react covalently; and under certain conditions they act predominantly on the side of the membrane to which they are applied.

The main finding in this paper is that reaction of the extracellular surface of red cells with DIDS significantly reduces the number of NAP-taurine binding sites at the cytoplasmic surface, most of the change being localized in band 3 (Fig. 3, Table I), the purported anion transport protein (Cabantchik et al., 1978). Further evidence supporting the view that the DIDS-modulated NAP-taurine binding to band 3 is related to the transport sites is provided by the observation that the NAP-taurine binding is also reduced by Cl⁻ concentrations (Table I, Fig. 5) in the range where competition for the transport site would be per minute, or ~1% of the amount bound in the same time. Thus, equilibration of NAP-taurine into intravesicular space would be insubstantial in the short term experiments reported in the paper. It should be pointed out that the concentration of NAP-taurine used by Staros et al. (1975) was 0.7 mM, whereas that used in the vesicle experiments was less, 0.23 mM. Because both values are below the $K_s$ for NAP-taurine efflux (Knauf et al., 1978 b), the rate of transport is approximately linear with substrate concentration, and the same rate constant, 0.08 h⁻¹, is applicable.
expected. Furthermore, in membranes from DIDS-treated cells no Cl\textsuperscript{-}-modulation of NAP-taurine binding is observed, suggesting that Cl\textsuperscript{-} and DIDS modulate the same binding sites. Although other explanations cannot be ruled out, these observations are consistent with the idea that the transport site is accessible from either side of the membrane, and that it is mobile in the sense that it can be "recruited" to the outside by reaction with DIDS with its consequent "disappearance" from the opposite side.

The data presented in this paper are derived from experiments with 230 \mu M NAP-taurine. For technical reasons no attempt was made to determine the binding at other concentrations. Below 230 \mu M NAP-taurine, the amount of bound radioactivity would become a limiting factor, whereas at higher concentrations the exposure to light would have to be considerably prolonged (Knauf et al., 1978 a). Thus the exact relationship between the number of DIDS binding sites and of DIDS-modulated NAP-taurine binding sites in band 3 is not known. Nevertheless, some rough estimates can be made. The number of DIDS binding sites in band 3 has been estimated at 1.1-1.3 \times 10^8 per cell (Lepke et al., 1976; and Ship et al., 1977). The DIDS-sensitive NAP-taurine binding sites per cell in the present experiments number \sim 0.7 \times 10^6 (Fig. 3) or \sim 0.5 \times 10^6 for band 3 (based on the finding that 70% of the DIDS-sensitive sites are located in that protein, Table I). It must be born in mind, however, that not all of the potential sites would be labelled by NAP-taurine. This situation arises because the concentration of the probe used (230 \mu M) is below the reported apparent \textit{K}_i at zero Cl\textsuperscript{-} (730 \mu M) for its binding to the cytoplasmic side (Knauf et al., 1978 b). From these values it can be calculated that only 24% of the inhibitory sites would be occupied. In reasonable agreement with this consideration, the SO\textsubscript{4} efflux for IO vesicles irradiated for 5 or more min with 230 \mu M NAP-taurine was inhibited by 34\% (n = 5). On this basis the total number of DIDS-sensitive sites might be underestimated by a factor of about 3. Those in band 3 might therefore be as high as 1.5 \times 10^6. Even though these calculations involve certain assumptions, and they are based on approximate data, they are not inconsistent with the proposition that binding of DIDS to the outside face might result in an equivalent reduction of binding of NAP-taurine at the cytoplasmic face.

Although the predictions of the mobile-site model are fulfilled with respect to the transmembrane effects of DIDS and Cl\textsuperscript{-} on NAP-taurine binding to band 3, additional effects were observed which were not predicted. Only 70% of the DIDS-sensitive NAP-taurine binding sites are localized in band 3 (Table I) with most of the remainder in the band 4 region and perhaps a small component in a high molecular weight region (bands 1-2 region). Furthermore, some of the NAP-taurine binding to proteins in those regions is Cl\textsuperscript{-}-sensitive. The basis for these unexpected results is not readily apparent, for DIDS binding to the outside of the cell is considerably more specific than the NAP-taurine binding to the cytoplasmic face. The stilbene is highly localized in band 3 (Cabantchik and Rothstein, 1974; Lepke et al., 1976; Ship et al., 1977) with virtually none in the bands 1-2 region and little in the band 4 region (a finding confirmed in the present study). The identity of the high and low mobility components of NAP-taurine binding can be tentatively made. The high molecular weight component
corresponds in mobility with band 2. Most of this component (and of band 1) is removed by the alkali stripping of the vesicles (Steck and Yu, 1973) but small, variable amounts remain with the membranes. From limited observations, it is our impression that the amount of NAP-taurine in this component is related to the amount of residual bands 1-2. The NAP-taurine peak in the band 4 region corresponds closely in mobility to band 4.5 but does not correspond with the mobility of band 4.1 or glycophorin (PAS-1), other components present in this region in alkali stripped vesicles (Steck and Yu, 1973).

It is not clear how DIDS binding localized largely to band 3 can modulate NAP-taurine binding to other protein components, nor what role, if any, those components play in anion transport. Fractions enriched with band 3 purified by various procedures are sufficient to support anion transport in phospholipid vesicles (Rothstein et al., 1975; Wolosin et al., 1977, and Ross and McConnell, 1977). Although these preparations are not “pure” in a chemical sense, they are free of substantial amounts of other proteins by the criterion of staining in acrylamide gels. Taken at face value, these results indicate that substantial amounts of other components are not essential for anion transport, though they may nevertheless play an ancillary role through associations with band 3. It has already been demonstrated that such associations do occur in the case of several proteins (Steck, 1974; Rothstein, 1978). It is probably significant that the interaction of DIDS or other disulfonic stilbenes with band 3 results in changes in its association with certain proteins and lipids. Thus, the cytoplasmic portion of band 3 is the site of interaction of the enzyme glyceraldehyde-3-phosphate dehydrogenase (band 6) (Steck, 1974, Yu and Steck, 1975) and under certain conditions, of hemoglobin (Salhany and Shaklit, 1978). The covalent binding of DIDS to band 3 modifies these protein-protein interactions. The interaction of DIDS with band 3 is also reflected in alterations of the transition temperature of associated lipids (Snow et al., 1978). Both effects are probably related to a DIDS-induced change in the conformation of band 3 that can be measured in terms of an altered electron paramagnetic resonance of probe molecules covalently bound to the protein. It is therefore conceivable that the DIDS-band 3 interaction might also result in changes in the anion binding properties of other associated proteins.

In summary, the specific prediction derived from the proposed model is fulfilled: DIDS bound to band 3 from the external face of the membrane reduces the binding of NAP-taurine to the same protein from the inner face. Furthermore, the DIDS-modulated sites also interact with the substrate, Cl−, whereas DIDS-insensitive sites do not. The conclusion that a mobile anion transport site is involved is therefore given support. Some caution should, however, be expressed because 30% of the DIDS-modulated effect, which is also Cl−-sensitive, is unexpectedly found in proteins other than band 3, a finding that can only be explained in a tentative fashion. If the mobile-site model is accepted as correct, the unexpected effect would imply the existence of protein interactions that we do not yet fully understand.

3 Eaton, J. Personal communication.
4 Ginzburg, H., and Z. I. Cabantchik. Personal communication.
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