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THE INTERACTION OF AN ANIONIC PHOTOREACTIVE PROBE WITH THE ANION TRANSPORT SYSTEM OF THE HUMAN RED BLOOD CELL

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SUMMARY

N-(4-azido-2-nitrophenyl)-2-aminoethyl[^{35}S]sulfonate is employed as a photoreactive probe for the anion transport system in the human erythrocyte. In the dark and at 37 °C the probe penetrates the membrane via a pathway sensitive to specific inhibitors of anion permeability. It reversibly inhibits sulfate and chloride fluxes but the inhibition is reduced by higher concentrations of sulfate. Upon photolysis to produce a reactive nitrene (at 0 °C to minimize penetration), the probe reacts covalently with outer membrane components resulting in an irreversible inhibition of anion permeability. Under appropriate conditions the degree of inhibition after photoactivation (irreversible) is almost the same as that in the dark (reversible). The binding sites for the radioactive probe are largely found in proteins of 95 000 apparent molecular weight (band 3). After pronase treatment of the labelled cells, most of the probe is found in a 65 000 molecular weight segment derived from the 95 000 molecular weight protein. In this respect the photoreactive probe resembles another potent irreversible inhibitor of anion transport, 4, 4′-diisothiocyano-2, 2′ stilbene disulfonate. In fact, most of the binding sites for each probe are common to both. Thus, in the dark, the azido derivative protects the anion system from inhibition by DIDS and substantially reduces the binding of DIDS to band 3 protein. Conversely, pretreatment with DIDS substantially reduces the binding of the photoreactive probe to the same protein. The fact that an apparent substrate for the anion permeation system competes for binding sites with a specific non-penetrating inhibitor of anion permeability suggests that the inhibitory and transport sites may be closely related and implicates the 95 000 molecular weight protein as the element of the anion transport system which contains the substrate binding site.

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Abbreviations: DIDS, 4,4′-diisothiocyano-2,2′-stilbene disulfonate; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid.

INTRODUCTION

The anion permeation system of the red blood cell is responsible for the rapid exchange of Cl^- and HCO_3^-. Its properties include saturation kinetics, competition between pairs of substrates [1–3], and inhibition by low concentrations of specific reagents [4–7], all implying the existence of a carrier site with which the anions must bind in order to be transported. Because the normal substrates interact reversibly and with relatively low affinity (based on the K_m values of their transport [3, 8]), they cannot be directly used to identify either the binding site of the transport system or the molecular species which contains the site. For this reason, attempts to identify the membrane components involved in anion transport have depended on covalently reacting probes that specifically inhibit anion transport. These include the disulfonic stilbene, DIDS [7, 9, 10] and 1-isothiocyanate-4-benzene sulfonic acid [11]. DIDS in particular is largely localized in a protein component known as band 3 (based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis [12]), leading to the conclusion that this particular component may be involved in anion transport. Covalent probes, such as DIDS or isothiocyano-sulfanilic acid, are inhibitors, but are not substrates for the anion transport system. Their mechanism of inhibition has not been defined and does not necessarily involve a direct modification of the transport site. For example, the probes may interact with neighboring, or even distant sites, and affect transport indirectly through a conformational change or through some steric or charge effect. The fact that the probes are anions and that they would be expected to interact with anion binding sites, such as the transport site, is to some degree countered by evidence that neutral and even cationic substances can also inhibit anion transport [5].

Pyridoxal phosphate is an inhibitor of anion transport that slowly penetrates the membrane and that can be fixed in an irreversible bond at any particular time by reduction with $NaBH_4$ [13]. Its penetration is inhibited by DIDS at concentrations that also inhibit inorganic anion transport, suggesting that it may enter the cell by a common DIDS-sensitive mechanism. This conclusion must, however, be tempered by the knowledge that no evidence exists for a direct interaction of inorganic anions with the pyridoxal phosphate binding sites. Furthermore, pyridoxal phosphate is not highly specific in its binding to membrane components. After reduction with $NaBH_4$ it is localized not only in band 3, but also in three other bands identified as surface glycoproteins. Only band 3, however, is common to DIDS and to pyridoxal phosphate. The pyridoxal phosphate data are consistent with but do not prove that the probe-binding sites of band 3 are anion transport sites.

In attempting to identify the anion transport sites in a more direct manner, it would be advantageous to utilize a probe (i) that is transported by the same system as Cl^- and other inorganic anions and (ii) that can be fixed at the site of its binding to the transport system by an irreversible bond. A compound that possibly meets such requirements is the organic anion, *N*-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate, an unreactive aryl azide derivative of taurine which is converted to a highly reactive nitrene on exposure to light [14, 15]. At low temperatures in the dark, its penetration into the cell is very slow [14, 15], but at 37 °C it penetrates quite rapidly [16, 17], so that it might be a substrate for the anion transport system. Unlike most conventional chemical modifiers the nitrene resulting from exposure of the azido derivative to

light does not require the presence of specific ligands (such as amino or sulfhydryl groups) for reaction. It can even react with carbon-hydrogen bonds [18]. Thus, if it is transported by the anion permeation system, it should, after photolysis, react with membrane components in that pathway, regardless of their specific chemical structures. The possibility that this photoreactive anion might be a useful probe for the anion transport system is reinforced by the observation that, despite the non-specificity of the nitrene reaction, it is highly localized in the intact red blood cell in the same band 3 protein [14, 15] as the disulfonic stilbenes [7, 9, 10, 19].

On the basis of the above considerations, the permeability properties of *N*-(4-azido-2-nitrophenyl)-2-aminoethyl[^{35}S]sulfonate were determined in the dark and in the presence of specific inhibitors of anion transport. Its effect on the transport of inorganic anions was determined both in the dark and after photoactivation. The inhibitory effects were then correlated with its irreversible binding to surface proteins of the membrane. The relationship of the binding sites for the photoreactive probe to binding sites for the disulfonic stilbenes was investigated by measuring the effect of one probe on the binding of the other, and by use of the proteolytic enzyme, pronase, to more precisely define the segment of the protein that binds these agents. The results support the view that the pronase-resistant 65 000 molecular weight segment derived from band 3 protein is directly involved in anion permeation.

METHODS

DIDS was prepared as previously described [7, 9]. *N*-(4-azido-2-nitrophenyl)-2-aminoethyl[^{35}S]sulfonate was synthesized from 4-fluoro-3-nitrophenylazide (Pierce) and [^{35}S]taurine (Amersham-Searle, 65–102 Ci/mol), as described [14]. The yields were usually 30–40 % of the original [^{35}S]taurine. The non-radioactive compound was either synthesized using unlabelled taurine (Sigma) or purchased (Pierce). The synthesis, as well as other light-sensitive steps, was carried out under a dim white light.

For measurements of the flux of the photoreactive probe, human erythrocytes were normally prepared in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid)-buffered saline (20 mM HEPES, 145 mM NaCl), pH 7.4. They were then washed in Tris/sulfate buffer (25 mM Tris · HCl, 5 mM Na_2SO_4, 20 mM NaCl, 200 mM sucrose or 25 mM Tris · HCl, 50 mM Na_2SO_4, 20 mM NaCl, 100 mM sucrose), pH 6.8, and incubated in the dark with various concentrations of ^{35}S labelled probe for a 10-h loading period at 37 °C. The cells were subsequently adjusted to pH 7.4 at 0 °C and washed to remove the remaining extracellular probe. In the cold, as reported [14], the permeation was very slow so that washing in the cold to remove external agent does not influence the intracellular concentration. At this stage a portion of the cells was reacted with 25 μM DIDS (20 min reaction at 0 °C) and washed. The efflux of labelled probe from normal and DIDS-modified cells (5 % hematocrit) into Tris/sulfate buffer, pH 7.4, at 37 °C was measured. The effect of dipyridamole (Persantin, obtained through the courtesy of Ciba-Geigy or Boehringer-Ingelheim) on the fluxes was measured with the drug present in the medium during the flux measurements.

[^{35}S]Sulfate efflux was measured in cells pre-equilibrated for two or more hours with Tris/sulfate buffer containing 5 mM Na_2SO_4, pH 7.1, 37 °C as previously

described [9]. Aliquots of the suspension were used to measure the effect of the azido derivative in the dark. Other aliquots were exposed to light at 0 or 27 °C to photoactivate the probe prior to the flux measurements. A volume of 0.5–1.0 ml of suspension (at specified hematocrit) was placed in an 8 ml pyrex test tube to which the probe was added. The tube was connected to a Buchler horizontal rota-evaporator and immersed in circulated water at the specified temperature for 5 min in the dark with continuous rotation. Unless otherwise specified, the test tube was then illuminated for 20 min with a General Electric D X 8 photospot lamp placed 10–12 cm from the target. The temperature in the tube did not vary by more than 1–2 °C during the exposure to light. After the irradiation, the cells were washed three times with buffer containing 0.5 % albumin at 0 °C prior to the [^{35}S]sulfate efflux measurements. The photolytic reaction was also carried out using ^{35}S-labelled probe in order to determine its distribution in membrane components. Proteolysis of cells (30 % hematocrit) was performed with 0.1 mg/ml pronase (Calbiochem, dialysed overnight at 5 °C against buffer) at 32 °C for 10 min. The cells were subsequently washed three times with 0.5 % albumin in buffer to stop the proteolysis [19] and then photoreacted. In one instance, the cells were first reacted with ^{35}S-labelled probe and subsequently proteolysed.

Isolated membranes (hemoglobin-free) were prepared as previously described [20]. Membrane protein was measured by a modified microbiuret method [21]. Radioactivity was measured with a toluene scintillation mixture containing 4.2 % Liquifluor and 10 % Protosol (New England Nuclear). Sodium dodecyl sulfate gel electrophoresis, staining of gels for proteins (Coomassie blue) or for carbohydrates (periodic acid-Schiff), and analysis of radioactivity profiles of gels were performed as previously described [9, 19] using primarily the procedures of Fairbanks et al. [12]. Specific activities for ^{35}S-labelled probe were based on the reported specific activity of the precursor ([^{35}S]taurine, Amersham) as well as on direct measurements of radioactivity and absorbance.

RESULTS

In the dark, the release of *N*-(4-azido-2-nitrophenyl)-2-aminoethyl[^{35}S]-sulfonate from preloaded cells containing 175 nmol per ml of packed cell volume is illustrated in Fig. 1. The rate of efflux was 80 nmol per ml of cells per h for the first 20 min, but fell to lower levels thereafter. The release was somewhat slower (about 25 %) from cells equilibrated with 50 mM SO_4^{2-} than from cells equilibrated with 5 mM SO_4^{2-}, and was inhibited in either case to the extent of about 90 % by pretreatment of the cells with DIDS or by addition of dipyridamole, another inhibitor of anion permeability [5].

The azido derivative inhibits sulfate fluxes in the dark (Table I). The degree of inhibition is, however, dependent on the concentration and nature of other anions that may be present. Citrate, a non-penetrating anion, has no effect on the inhibition, whereas Cl^-, NO_3^- and SO_4^{2-}, which permeate, reduce the inhibition.

The inhibition in the dark is almost completely reversible by washing three times with buffer containing albumin to sequester the agent. Upon photolysis of the cell suspension containing the photoreactive probe, however, the inhibitory effect becomes irreversible inasmuch as it is no longer affected by extensive washing with

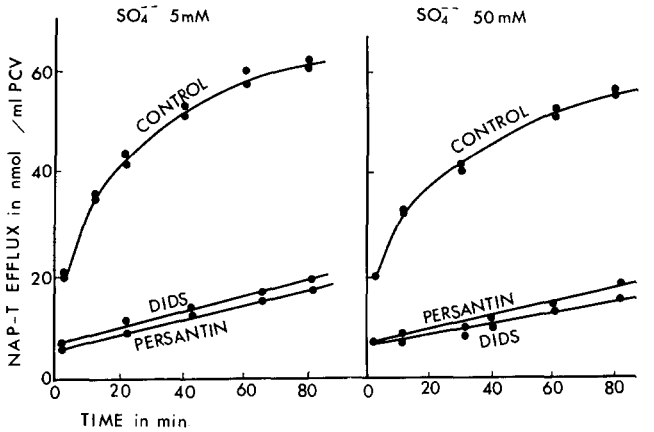

Fig. 1. The effect of DIDS and of dipyridamole on *N*-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate fluxes from red blood cells. The efflux of ^{35}S-labelled probe was measured in cells preloaded by exposure to 5 mM probe for 10 h at 37 °C as described in Methods. The probe content of the loaded cells was 175 nmol per ml of packed cell volume. Experiments were carried out at 37 °C in the dark. Inhibitions were determined in cells pretreated with 25 μM DIDS followed by washing, or in the presence of 25 μM dipyridamole (Persantin). Buffer included either 5 or 50 mM Na_2SO_4 as indicated.

TABLE I

INHIBITION OF $^{35}SO_4$ EFFLUX BY *N*-(4-AZIDO-2-NITROPHENYL)-2-AMINOETHYLSULFONATE IN THE DARK IN THE PRESENCE OF VARIOUS ANIONS

Values represent percent inhibition. The low sulfate buffer contained 5 mM Na_2SO_4 and 25 mM Tris base titrated to pH 7.4 at room temperature with H_2SO_4. Sucrose was added to make the solution isotonic. The various anions were added by isosmotic replacement for sucrose. Cells were equilibrated in the various media and loaded with [^{35}S]sulfate prior to exposure to the probe. In Expts. A and B, the temperature was 37 °C, in Expt. C it was 28 °C. Inhibition is expressed as the percent decrease in ^{35}S efflux produced by 70 μM probe relative to a control in the same medium without the probe.

Medium	Expt. A	Expt. B	Expt. C
Low sulfate buffer	62.1	66.3	
+20 mM NaCl		61.9	56.6
+60 mM NaCl	38.8	33.6	
+134 mM NaCl	30.6	36.0	35.8
+134 mM $NaNO_3$	25.4		
+85 mM Na_2SO_4		44.3	
+87 mM trisodium citrate	65.8		61.0

buffer containing albumin. Some comparisons of reversible and irreversible inhibition are shown in the experiment of Fig. 2. At first inspection, the relationship of the two forms of inhibition seems complex. At a low hematocrit (5 %) and a low probe concentration (0.17 mM) as in panel A, the reversible and irreversible inhibitions are almost the same (67 and 70 %). On the other hand, at high concentrations of probe (1.7 mM), or at high hematocrits (40 %), as in panel B, the irreversible inhibition after photolysis and washing was considerably lower than the reversible inhibition with the agent

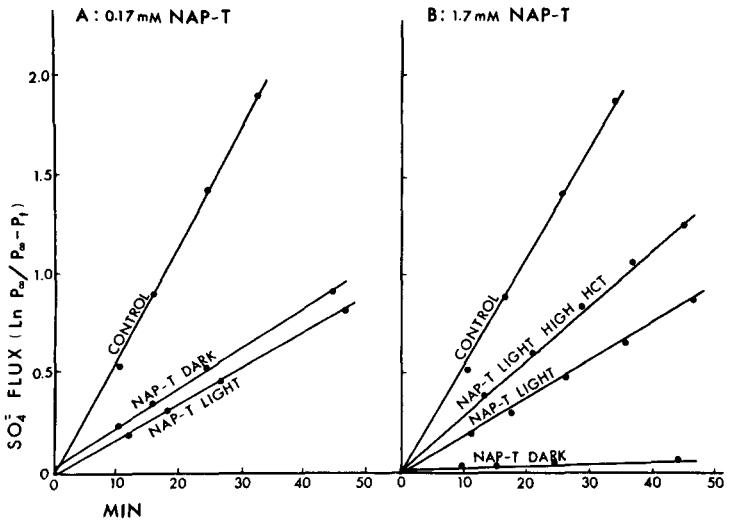

Fig. 2. The inhibition of sulfate permeability by *N*-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate in the dark and after exposure to light. Cells preloaded with [^{35}S]sulfate (5 mM) were incubated in the presence of either 0.17 or 1.7 mM probe. In one aliquot, the fluxes of sulfate were measured in the dark. In another, the suspension was exposed to light for 20 min at 0 °C followed by extensive washing before the flux measurements (as described in Methods). The hematocrit was 5 % except in the line marked HIGH HCT where it was 40 %. The efflux of [^{35}S]sulfate was measured at 37 °C and computed as previously described [9]. The ordinate is ln $[P(\infty)/(P(\infty)-P(t))]$, where $P(\infty)$ is the total [^{35}S] in the cell suspension and $P(t)$ is the [^{35}S] in the medium at time t.

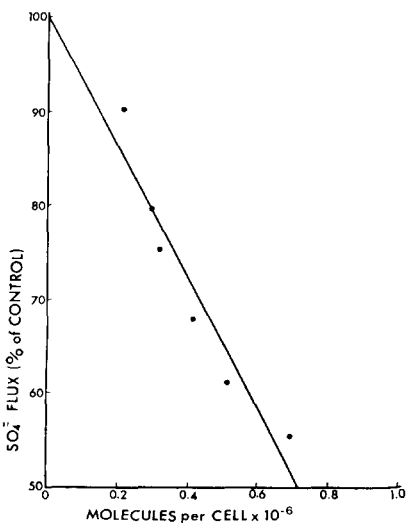

Fig. 3. The relationship of inhibition of sulfate fluxes to the binding of *N*-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate after exposure to light. Cells at 5 % hematocrit loaded with [^{35}S]sulfate (5 mM) were exposed to concentrations of ^{35}S labelled probe ranging from 3 to 30 μM and exposed to light as described in Methods at 0 °C for 20 min. They were then washed extensively in the cold and the sulfate fluxes were measured as described in the legend of Fig. 2. At the end of the flux measurements, well washed ghosts were prepared and their radioactivity was measured by the procedures outlined in Methods. The washed ghosts retain little [^{35}S]sulfate so the counts can be attributed entirely to the bound ^{35}S-labelled probe. It was assumed that each ghost contains $6 \cdot 10^{-10}$ mg of protein. Protein was determined in this case by the method of Lowry et al. [46].

present in the dark. The lack of correspondence in these cases seems to be due to the screening of light by either the high concentrations of hemoglobin or of probe so that photolysis is incomplete. This interpretation is supported by similar experiments in which the exposure to light was prolonged (40 rather than 20 min). At low hematocrit and probe concentrations, where a correspondence was found between the light and dark inhibitions, a longer exposure had little effect. At high hematocrits and probe concentrations, however, in which the irreversible inhibition was substantially lower than that in the dark, a longer exposure considerably increased the degree of irreversible inhibition.

Chloride exchange is also inhibited by similar concentrations of the probe, with the effect also being irreversible after exposure to light (data not shown).

The total irreversible binding of probe associated with varying degrees of inhibition of sulfate fluxes is illustrated in Fig. 3. The extrapolated value for the number of bound probe molecules associated with 50 % inhibition was 720 000 per cell. If the relationship is linear, 100 % inhibition would require the binding of about 1.4 million molecules per cell*.

The distribution of irreversibly bound probe in membrane proteins separated by sodium dodecyl sulfate-acrylamide gel electrophoresis is shown in Fig. 4. Under conditions similar to those reported previously [14, 15] (relatively high hematocrit, high probe concentrations and with exposure at 0 °C), the observed labelling pattern was confirmed (Fig. 4A), with approx. 70 % of the probe associated with a protein of 95 000 apparent molecular weight (band 3 according to the nomenclature of Fairbanks et al. [12]). The labelling pattern was further characterized by treating cells with pronase. This enzyme cleaves the proteins of band 3, so that new bands appear at 65 000 and 35 000 molecular weights [19]. The pronase treatment results in a loss of about 30 % of previously bound probe. The 70 % that remains in the membranes is largely located in band 3′ (65 000 daltons) with most of the minor peaks having disappeared (Fig. 4B). Approx. 80 % of the label of band 3 (or about 55 % of the total membrane labelling) seems to have been conserved in band 3′ (based on the areas under the peaks of Figs. 4A and 4B). If the photolysis is carried out after the pronase treatment, the binding of the probe to the cells is increased by about 30 % but the gel patterns are the same as in Fig. 4B. As reported previously [14], the labelling of lipid components at the detergent front (labelled "Sodium dodecyl sulfate front" in Fig. 4) was small compared to the labelling of protein components.

* Other values have been reported for the number of binding sites in band 3. The original value of 300 000 sites per cell [9] was based on the use of reduced (tritiated) DIDS. Records from the firm that carried out the tritiation now reveal that reduction was not complete so that the material used was a mixture of DIDS and reduced DIDS. A reevaluation of the binding of reduced DIDS gives a value of 1 700 000 sites per cell. [22] A new preparation of labelled DIDS (not reduced) gives a value of 1 200 000 sites per cell (Ship, S., Shami, Y. and Rothstein, A., unpublished observations). This value agrees with another recent calculation based on the total amount of DIDS necessary to achieve complete inhibition of anion transport[30]. The estimate of $1.4 \cdot 10^6$ per cell based on Fig. 3 is a maximal value, for it may include non-functional as well as transport sites. For example, preliminary data based on the observation that Cl^- partially reverses NAP-taurine inhibition and also prevents some of the NAP-taurine binding [23] suggest that approximately half of the NAP-taurine sites may be related to transport (assuming that only the Cl^--dependent component of binding is associated with transport).

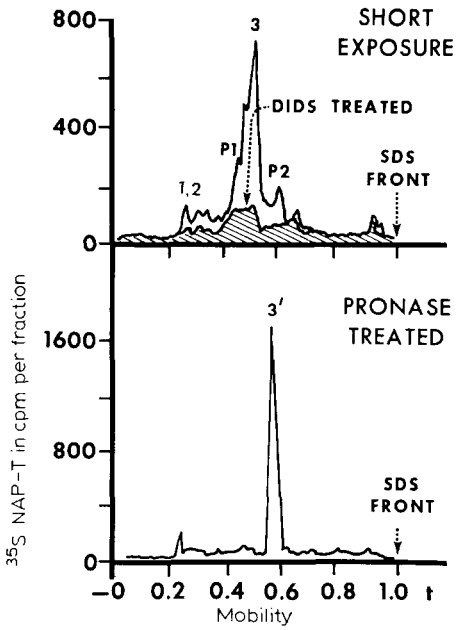

Fig. 4. Labelling of erythrocytes by [^{35}S]NAP-taurine photolysis as analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. (A) Control cells and cells modified with 25 μM DIDS (hatched area) at 30 % hematocrit were photo-reacted with 1 mM [^{35}S]NAP-taurine for 20 min at pH 8, 0 °C, solubilized and electrophoresed on SDS-polyacrylamide gels (3.3 % acrylamide). P_1 and P_2 designate glycoproteins stainable with periodic acid-Schiff, and 3 designates the 95 000 molecular weight protein stainable with Coomassie blue. Gels were sliced after fixation and counted for ^{35}S activity. (B) The same cells as "control" in A (30 % hematocrit) were digested with pronase (0.1 mg/ml for 10 min at 32 °C) after photolysis of the [^{35}S]NAP-taurine and then were analyzed as in A. Essentially the same profile was found if the cells are first exposed to pronase and then labelled.

The labelling pattern in cells exposed under more optimal conditions for completion of the light-sensitive reaction (low hematocrit (5 %) and NAP-taurine concentrations ($< 30\ \mu$M)) was not substantially different from that in Fig. 4. The specificity of labelling of band 3 relative to other peaks was, however, somewhat greater.

The potent and specific inhibitor of anion permeability, DIDS, also binds largely to band 3 and in pronase-treated cells to band 3' [7, 9, 19, 22]. Therefore, attempts were made to determine whether the same membrane sites were involved in interactions with DIDS and the photoreactive probe. In cells pretreated with DIDS (25 μM at 10 % hematocrit) the total binding of the azido derivative was reduced by 70 %. A comparable reduction in its specific binding to band 3 was also observed (Fig. 4A). Conversely, the binding of reduced tritiated DIDS (4 μM at 0 °C for 5 min at 10 % hematocrit) was substantially reduced by the azido derivative (100 μM). In the dark, its presence resulted in a reduction of stilbene binding of 60–70 % (two experiments). Exposure to light prior to addition of stilbene, resulted in a greater reduction, 80–85 %. Thus a substantial fraction of membrane binding sites are common to the photoreactive probe and to reduced DIDS. Among the common

TABLE II

THE PROTECTIVE EFFECT OF *N*-(4-AZIDO-2-NITROPHENYL)-2-AMINOETHYLSULFONATE ON THE INHIBITION OF SULFATE EFFLUX BY DIDS

Cells (10 % hematocrit) preloaded with [^{35}S]sulfate (5 mM as described in Methods) were exposed to DIDS for 5 min at 10 °C in the presence or absence of the probe. They were then washed twice with albumin (0.5 %) containing buffer and twice with buffer. In some aliquots of cells, 50 or 100 μM probe was present in the dark during DIDS treatment; in others it was added and then washed out in the dark or after exposure to light (two Mazda long-wave ultraviolet lamps at 6 cm) before DIDS treatment. In one sample, treated with the probe in the light, no DIDS was used. Control samples of blood cells were subjected to the same washing procedures. Fluxes were measured at 37 °C at 5 % hematocrit after the washing procedure.

	Inhibition in percent of control	
	8 μM DIDS, 50 μM probe	4 μM DIDS, 100 μM probe
DIDS only	88,92	72,83
Probe (dark) + DIDS, then washed	63,58	22,25
Probe (dark) then washed + DIDS	95,	66,78
Probe (light) then washed + DIDS		88,92
Probe (light) then washed	39,44	72,

sites is a sub-population that is related to the inhibition of anion fluxes. This conclusion is based not only on the finding that both agents are effective inhibitors, but also on the observation that the azido derivative in the dark, reversibly associated with the sites, can protect against the irreversible inhibition by DIDS (Table II). Thus, cells exposed to DIDS in the presence of the azido compound in the dark and then washed were afforded considerable protection. In contrast, cells exposed to the azido compound in the dark, washed and then exposed to DIDS were not protected.

DISCUSSION

The main premise of this paper is that the organic photoreactive anion, *N*-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate can permeate the red cell membrane by the same transport system as the inorganic anions, and that it may, therefore, be a useful probe for identifying the transport components. Evidence relating to the permeation of the probe via the inorganic anion transport system can be briefly summarized: (1) Its flux in the dark is blocked to the extent of about 90 % by two specific and effective inhibitors of inorganic anion permeability, DIDS [7, 9] and dipyridamole [5]. Because these two agents are chemically dissimilar and presumably inhibit anion transport by different mechanisms, their common action on the flux of the probe suggests that it permeates to a large degree via the normal anion pathway; (2) The flux of the probe is somewhat reduced (about 25 %) in the presence of high SO_4^{2-} concentrations (Fig. 1), perhaps due to competition for a common transport system; (3) In the dark, the probe inhibits sulfate (Fig. 2) and chloride fluxes (data not shown). The extent of the inhibition is reduced by high concentrations of Cl^-, NO_3^-, or SO_4^{2-}, but not by the non-penetrating anion, citrate (Table I); (4) Other

organic anions also permeate across the red cell membrane [24–27] and their fluxes are largely inhibitable by agents that also inhibit inorganic anion transport [27–30].

Because of the non-specificity of the reaction of the nitrene that is formed [18], the photoactivated probe should react with the transport and inhibitory sites to which it is reversibly bound in the dark. This expectation is supported by the close correspondence (at low hematocrits and probe concentrations where photolysis is relatively complete) between the reversible inhibition of sulfate fluxes by the probe in the dark and the irreversible inhibition after exposure to light (Fig. 2A). The data support the premise that the irreversible binding reflects the specificity of its interactions with the membrane as an anion, rather than the completely non-specific interactions of the nitrene: (1) Under conditions in which the agent is restricted to the extracellular compartment, it is largely localized in band 3 (Fig. 4) which contains most of the accessible positively charged sites that bind other slowly penetrating or non-penetrating anions such as derivatives of sulfanilic acid [11, 32], pyridoxal phosphate [13, 33], trinitrobenzene sulfonic acid [34] and disulfonic stilbenes [7, 9, 10]. In contrast, another surface probe, the lactoperoxidase-catalysed iodination [31], labels the sialoglycoproteins as well; (2) Pretreatment with the highly specific disulfonic stilbene, DIDS, depresses the binding of the photoreactive probe to band 3 by about 80 % (Fig. 4). Conversely, pretreatment of cells with the photoreactive probe in the dark or after exposure to light reduces the binding of DIDS by up to 85 %. Furthermore, the binding site for the photoreactive probe is largely located in a pronase-resistant 65 000 molecular weight segment derived from band 3, as is the DIDS-binding site [19]; (3) The binding of the photoreactive probe to band 3 is reduced when the Cl^- concentration is raised [23].

The anion transport mechanism is not fully understood in molecular terms, but kinetic studies suggest that anion binding to a membrane site is a prerequisite for transfer across the membrane [35], with one proposed model involving fixed positive charged groups [36] and a second, a mobile carrier [8]. The irreversible inhibitor, DIDS, is not itself transported, so the only substantive conclusion that can be reached on the basis of its use is that the 65 000 molecular weight segment contains an "inhibitory site". The photoreactive probe, on the other hand, seems to be a substrate for transport as well as an inhibitor. Since it can also protect the inhibitory sites from interaction with DIDS (Table II), the latter may inhibit by direct interaction with the transport mechanism.

The data presented in this paper are consistent with and support the view that some fraction of the 65 000 molecular weight segment of band 3 protein is directly involved in anion transport. Other evidence favoring this view has been reported recently [37]. Extracts from normal or pronase-treated cells enriched in band 3 or in the 65 000 molecular weight pronase-resistant segment enhance anion transport in lecithin vesicles, whereas the same proteins from DIDS-treated cells do not. The properties of band 3 are consistent with its involvement in transport. It is hydrophobically associated with the membrane lipids [38] and is reported to "span" the membrane so that it is accessible from inside, as well as outside [39–45]. The findings in this paper, therefore, support the view that anion transport may involve a continuous protein channel through the membrane, a concept that is presented in more detail elsewhere [23].

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REFERENCES

1 Lepke, S. and Passow, H. (1971) J. Membrane Biol. 6, 158–182
2 Wieth, J. O. (1970) J. Physiol. Lond. 207, 581–609
3 Dalmark, M. (1976) J. Gen. Physiol. 67, 223–234
4 Passow, H. and Schnell, K. F. (1969) Experientia 25, 460–468
5 Deuticke, B. (1970) Naturwissenschaften 57, 172–179
6 Knauf, P. A. and Rothstein, A. (1971) J. Gen. Physiol. 58, 190–210
7 Cabantchik, Z. I. and Rothstein, A. (1972) J. Membrane Biol. 10, 311–330
8 Gunn, R. B. M., Dalmark, M., Tosteson, D. and Wieth, J. O. (1973) J. Gen. Physiol. 61, 185–206
9 Cabantchik, Z. I. and Rothstein, A. (1974) J. Membrane Biol. 15, 207–226
10 Zaki, L., Fasold, H., Schuhmann, B. and Passow, H. (1976) J. Cell Physiol., in the press
11 Ho, M. K. and Guidotti, G. (1975) J. Biol. Chem. 250, 675–683
12 Fairbanks, G., Steck, T. L. and Wallach, D. F. H. (1971) Biochemistry 10, 2606–2617
13 Cabantchik, Z. I., Balshin, M., Breuer, W. and Rothstein, A. (1975) J. Biol. Chem. 250, 5130–5136
14 Staros, J. V. and Richards, F. M. (1974) Biochemistry 13, 2720–2726
15 Staros, J. V., Haley, B. E. and Richards, F. M. (1974) J. Biol. Chem. 249, 5004–5007
16 Staros, J. V., Richards, F. M. and Haley, B. E. (1975) J. Biol. Chem. 250, 8174–8178
17 Cabantchik, Z. I., Ostwald, T., Knauf, P. A. and Rothstein, A. (1975) Biophys. J. 15, 209a
18 Knowles, J. K. (1972) Acc. Chem. Res. 55, 155–160
19 Cabantchik, Z. I. and Rothstein, A. (1974) J. Membrane Biol. 15, 227–248
20 Dodge, J. T., Mitchell, C. and Hanahan, D. (1963) Arch. Biochem. Biophys. 110, 119–130
21 Itzhaki, R. F. and Gill, D. M. (1964) Anal. Biochem. 9, 401–410
22 Lepke, S., Fasold, H., Pring. M. and Passow, H. (1976) J. Membrane Biol., in the press
23 Rothstein, A., Cabantchik, Z. I. and Knauf, P. A. (1976) Fed. Proc. 35, 3–10
24 Giebel, O. and Passow, H. (1960) Pflüg. Arch. Ges. Physiol. 271, 378–388
25 Schanker, L. S., Johnson, J. M. and Jeffrey, J. J. (1964) Am. J. Physiol. 207, 503–508
26 Deuticke, B. (1973) in Erythrocytes, Thrombocytes, Leucocytes (Gerlach, E., Moser, K., Deuticke, B. and Wilmanns, W., eds.), pp. 81–87, Georg Thieme, Stuttgart
27 Aubert, L. and Motais, R. (1975) J. Physiol. Lond. 246, 159–179
28 Knauf, P. A. and Rothstein, A. (1971) J. Gen. Physiol. 58, 211–223
29 Ross, A. H. and McConnell, H. M. (1975) Biochemistry 14, 2793–2797
30 Halestrap, A. P. (1976) Biochem. J. 156, 193–207
31 Phillips, D. R. and Morrison, M. (1971) Biochemistry 10, 1766–1771
32 Juliano, R. L. and Rothstein, A. (1971) Biochim. Biophys. Acta 249, 227–235
33 Cabantchik, Z. I., Balshin, M., Breuer, W., Markus, H. and Rothstein, A. (1975) Biochim. Biophys. Acta 382, 621–633
34 Arrotti, J. J. and Garvin, J. E. (1972) Biochem. Biophys. Res. Commun. 49, 205–211
35 Sachs, J. R., Knauf, P. A. and Dunham, P. B. (1975) in The Red Blood Cell (Surgenor, D. M., ed.), Vol. 2, 2nd edn. p. 1, Academic Press, New York
36 Passow, H. (1969) in Progress in Biophysics and Molecular Biology (Butler, J. A. V. and Noble, D., eds.), Vol. 19, pp. 425–467, Pergamon Press, New York
37 Rothstein, A., Cabantchik, Z. I., Balshin, M. and Juliano, R. L. (1975) Biochem. Biophys. Res. Commun. 64, 144–149
38 Steck, T. L. (1974) J. Cell. Biol. 62, 1–19

39 Bretscher, M. S. (1973) Science 181, 622–629
40 Reichstein, E. and Blostein, R. (1973) Biochem. Biophys. Res. Commun. 54, 494–500
41 Boxer, D. H., Jenkins, R. E. and Tanner, M. J. A. (1974) Biochem. J. 137, 531–534
42 Morrison, M., Mueller, J. J. and Huber, C. T. (1974) J. Biol. Chem. 249, 2658–2660
43 Shin, B. C. and Carraway, K. L. (1974) Biochim. Biophys. Acta 345, 141–153
44 Whiteley, N. M. and Berg, H. C. (1974) J. Mol. Biol. 87, 541–561
45 Jenkins, R. E. and Tanner, M. J. A. (1975) Biochem. J. 147, 393–399
46 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275