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Synthesis of Tritiated 4,4′-Diisothiocyano-2,2′-Stilbene Disulfonic Acid ([^{3}H]DIDS) and its Covalent Reaction with Sites Related to Anion Transport in Human Red Blood Cells

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Summary. The potent and specific inhibitor of anion permeability, 4,4′-diisothicyanostilbene-2,2′-disulfonic acid (DIDS) was synthesized in tritiated form ([^{3}H]DIDS) from tritiated 5-nitrotoluene-o-sulfonic acid. Its reactions with and effects on red blood cells were compared with those of a reduced form ([^{3}H]H_2DIDS), previously used as a tracer for DIDS. The rate of covalent reaction of [^{3}H]DIDS was substantially faster than that of [^{3}H]H_2DIDS at all temperatures tested. With both agents, the rate of reaction was increased in alkaline media, although the response occurred at a lower pH with [^{3}H]DIDS. On the other hand, the relationship of irreversible membrane binding to the degree of inhibition of sulfate fluxes was linear and virtually the same for both agents, with 100% inhibition associated with the binding of approximately 1.2×10^6 molecules per cell. About 90% of the binding for each probe was to a particular membrane protein, known as band 3, equivalent to about 1 mole of agent per mole of protein.

The disulfonic stilbenes were recently introduced as potent and specific inhibitors of anion transport in red blood cells (Knauf & Rothstein, 1971; Cabantchik & Rothstein, 1972). One of them, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS)[1], reacts irreversibly by formation of a covalent bond. It has been used to tentatively identify an abundant transmembrane protein known as band 3 (nomenclature of Fairbanks, Steck & Wallach, 1971), as the anion transport protein (Cabantchik & Rothstein, 1972, 1974*a, b*; Passow *et al.*, 1975/6; Zaki *et al.*, 1975; and Lepke *et al.*, 1976), and to help ascertain the molecular basis of the anion transfer mechanism (Rothstein, Cabantchik & Knauf, 1976).

1 *Abbreviations:* DIDS—4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; H_2DIDS—dihydro DIDS; SDS—Sodium dodecyl sulfate; DADS—4,4′-diaminostilbene-2,2′-disulfonic acid; HEPES—N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; Tris—Tris (hydroxymethyl) amino methane.

The disulfonic stilbenes seem to be specific anion inhibitors in a number of other cellular systems including ascites cells (Villereal & Levinson, 1976), pancreatic islet cells (Hellman *et al.*, 1973), turtle bladder (Ehrenspeck & Brodsky, 1976) and tubular cells of the kidney (Ullrich, 1976). In the case of the epithelial systems these agents appear to be of particular value, not only because they are potent and specific inhibitors of anion transport, but also because they are effective only when applied on the serosal side to the baso-lateral membranes.

Although DIDS is obviously a useful probe for anion transporting systems, its full effectiveness has been hampered by its unavailability in isotopically labelled form. Labelling studies have, therefore, been made with the tritiated reduced analog, dihydro DIDS ([^{3}H]H_2DIDS) (Cabantchik & Rothstein, 1974*a, b*; Lepke *et al.*, 1976). Although this derivative was thought to function in an identical fashion to DIDS and thus to act as a suitable radioactive marker, recently reported differences in reactivity of the two compounds (Lepke *et al.*, 1976) have shown this assumption to be inaccurate. Accordingly, we have synthesized tritiated DIDS and have used it to characterize the DIDS-membrane interaction and to re-evaluate the relationship between the number of sites per cell and the inhibitory effect.

Materials and Methods

The precursor for DIDS synthesis, 4,4′-diaminostilbene-2,2′-disulfonate (DADS) was obtained from the Sumitomo Chemical Co. (Osaka) or from the Eastman Kodak Co. (Rochester, N.Y.). It was used as received or was purified by a single recrystallization from 1% sodium chloride solution. 5-Nitrotoluene-o-sulfonic acid was obtained from Eastman Kodak Co. and was purified by recrystallization from water. Thiophosgene was obtained from Canadian Laboratory Supplies Ltd. (Toronto) and was distilled prior to use. Aquasol, Protosol and Liquifluor (Trade names) were obtained from New England Nuclear Co. (Boston). Albumin (bovine serum, fraction V) was obtained from Sigma Chemical Co. (St. Louis). All other chemicals were obtained from Fisher Scientific Co., Ltd. (Toronto).

Nonradioactive DIDS was prepared from DADS as described by Cabantchik and Rothstein (1972). [^{3}H]H_2DIDS, with a specific activity of 1.7 Ci/mmole, was the kind gift of Professor H. Passow (Max Planck Institute, Frankfurt, Germany). [^{3}H]DIDS was synthesized as described below.

Preparation of [^{3}H]DIDS

Four unsuccessful attempts were made to label the rings in DADS by catalytic tritiation without reduction of the inter-ring double bond. A synthesis was, therefore, developed using a tritiated single ring compound as a precursor. 5-Nitrotoluene-o-sulfonic acid was reacted (by New England Nuclear Co.) with tritiated water in the presence of trifluoroacetic acid and rhodium on charcoal. A solution of this compound (0.20 g) in distilled water

(2.5 ml) was stirred at 50°, and a 35% (by weight) sodium hydroxide solution (0.13 ml) was added over 30 min, followed by gradual addition of a mixture of 4.85% sodium hypochlorite solution (2.52 ml) and 35% sodium hydroxide solution (0.27 ml) over 1.5 hr (Fierz-David & Blangey, 1949). The reaction was stirred at 50–55° for a further 2.5 hr and then cooled. After addition of sodium chloride (0.5 g) and storage at 5° overnight, filtration gave bright orange cystals of 4,4′-dinitrostilbene-2,2′-disulfonic acid (90 mg).

To this product was added fine grain tin metal (0.18 g) and then, gradually, concentrated hydrochloric acid (2.0 ml). The mixture was stirred at 100–105° for 30 min. After cooling, water (2 ml) was added, followed by sufficient sodium bicarbonate to bring the mixture to neutral pH, and the resulting suspension was filtered to remove the tin hydroxide. The filtrate was brought to pH 1 with concentrated hydrochloric acid and cooled, to give [^{3}H]DADS as a fine yellow precipitate (40 mg). The specific activity of the well washed and dried product was determined to be 1.40 mCi/mmole by measuring the radioactivity of an aliquot of an aqueous solution of known concentration. This product exhibited an absorption maximum at 340 nm and was indistinguishable from samples of commercial DADS by thin layer chromatography with pyridine/acetic acid/water (10:1:40, by volume), or with ammonium hydroxide/*n*-propanol/water (3:6:2). Furthermore, autoradiography of both chromatograms showed coincidence of the radioactivity with the fluorescent spots.

Thiophosgene (0.4 ml) was added to a solution of the [^{3}H]DADS product in 1% sodium chloride (1 ml) containing sodium hydroxide (0.20 ml of 1 N solution) and the mixture was stirred vigorously for 1 hr. After repeated extraction with ether, the mixture was cooled to yield [^{3}H]DIDS as a white crystalline precipitate (10 mg); λ_{max} 340 nm (ε16,000).

General Procedures

Fresh or recently outdated blood, obtained from the Blood Bank, was washed at 0–5°, twice with 170 mM sodium chloride solution and twice with 10 mM HEPES buffer pH 7.4 containing 75 mM potassium chloride, 50 mM sodium sulfate and 40 mM sucrose. For measurements of sulfate fluxes, the suspension was equilibrated for 3 hr with labelled $SO_4^{=}$. Routinely, the erythrocytes were resuspended in the same buffer at 25% hematocrit and reacted with DIDS for a specified time. The cells were then washed three times with the HEPES buffer containing 0.5% albumin at 0°, in order to remove noncovalently bonded probe, and then once with HEPES buffer. (Further washes did not remove any additional reagent, suggesting that the remainder was irreversibly associated with the membrane.)

In experiments designed to measure the time course of the labelling reaction, the unreacted isothiocyanate was destroyed by addition of 2-mercaptoethanol in isosmolar HEPES buffer, to give a concentration of 40 mM of the thiol. The suspension was quickly cooled to 0° and, after several minutes, was washed as described above. Stopping of the reaction by this technique did not significantly alter the pH of the red cell suspension and gave amounts of DIDS labelling that differed from those obtained by the standard procedure (albumin washes alone) only at short times.

After reaction with DIDS the red blood cells were washed with 300 mOsm phosphate buffer pH 7.4 and ghosted by incubation with 60 mOsm phosphate at 0°, centrifugation, and resuspension in 20 mOsm phosphate buffer. The ghosts were subsequently washed three times with the latter buffer, each time centrifuging at 12,000 rpm for 20 min. Incorporated radioactivity was determined by dissolving an aliquot of the ghost suspension in Aquasol (10 ml) for counting on a Packard Liquid Scintillation Counter. Protein content was determined by a modification of the method of Lowry *et al.* (1951).

The labelled membranes were solubilized and applied to 3.3% or 5.6% SDS polyacrylamide gels as described by Cabantchik and Rothstein (1974*a*). Gels were fractionated by

hand slicing or by means of a Maizel Autogel Divider (Savant Instruments, Inc). The fractions were digested with Protosol (45%) and water (5%) in toluene at 60° for 24 hr and then solubilized with Protosol (10%) and Liquifluor (4.2%) in toluene by leaving at 40° for at least 24 hr. The total recovery of radioactivity in the gel fractions was close to 100%.

The rates of sulfate (^{35}S) efflux from erythrocytes were measured as described by Cabantchik and Rothstein (1972).

Results

The [^{3}H]DIDS synthesized by the procedure described in Materials and Methods was indistinguishable from well characterized nonradioactive DIDS on thin layer chromatography or high voltage paper electrophoresis. With both techniques, radioautography confirmed the coincidence of the major radioactivity spots of [^{3}H]DIDS with the fluorescent spots of the DIDS. No differences were found in ultraviolet spectrum or extent of reaction with hemoglobin. A colorimetric test for amino groups (Bratton & Marshall, 1939) showed that both compounds contained less than 2 mole % of free amino groups.

Furthermore, the quantity of [^{3}H]DIDS covalently attached to the erythrocyte surface was reduced by dilution with unlabelled DIDS in a proportional manner (Fig. 1), thus demonstrating the validity of its use as a radioactive label for binding studies[2].

The rate of reaction of both [^{3}H]DIDS and [^{3}H]H_2DIDS with red cells is markedly increased at higher temperatures (Fig. 2). For both agents, the reaction as a function of time approximately follows first order kinetics and for both the maximal binding is about the same. On the other hand [^{3}H]DIDS reacts appreciably more rapidly than the reduced form, especially at lower temperatures (up to five times as fast).

The rate of the irreversible reaction increases with rising pH for both compounds, but not according to the same pattern (Fig. 3). Whereas the reaction of [^{3}H]DIDS at 5° is more or less complete in 10 min

2 Some lots of DIDS prepared from commercial precursor (DADS), characterized by a single colorless fluorescent spot on thin layer chromatography and by a high absorption coefficient at 340 nm, appear to be essentially the pure trans-isomer (DeTar & Carpino, 1956). The DIDS synthesized from 5-nitrotoluene-o-sulfonic acid, on the other hand, is probably a mixture of trans- and cis-isomers because a second fluorescent spot is visible on thin layer chromatograms and the absorption coefficient at 340 nm is reduced (about 50%). The degree of inhibition of sulfate fluxes in red cells is also slightly reduced at a particular concentration of probe (10% at 10 μM DIDS). Thus it appears that the potency of the cis-isomer is slightly less than that of the trans-isomer. Attempts at preparative separation of the isomers on thin layer chromatography proved unsuccessful due to continuous isomerization.

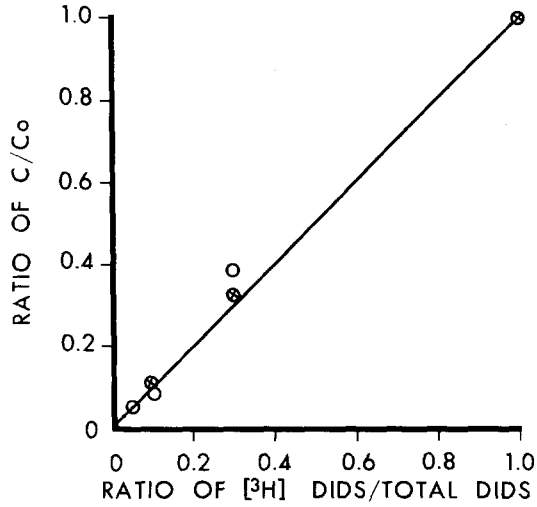

Fig. 1. The effect of dilution of [^{3}H]DIDS with nonradioactive DIDS on the extent of radioactive labelling of membranes. The ordinate represents the ratio of the counts determined with each diluted sample to that obtained with undiluted [^{3}H]DIDS. The total concentration of reagent (DIDS+[^{3}H]DIDS) was 10 μM in each case. Red blood cells (25% hematocrit) in HEPES buffer (pH 7.4) were reacted with the reagent for 10 min at 37 °C. Ghosts were prepared and counted (for tritium content) as described in Materials and Methods. Two separate preparations of [^{3}H]DIDS were utilized, each marked by a different symbol

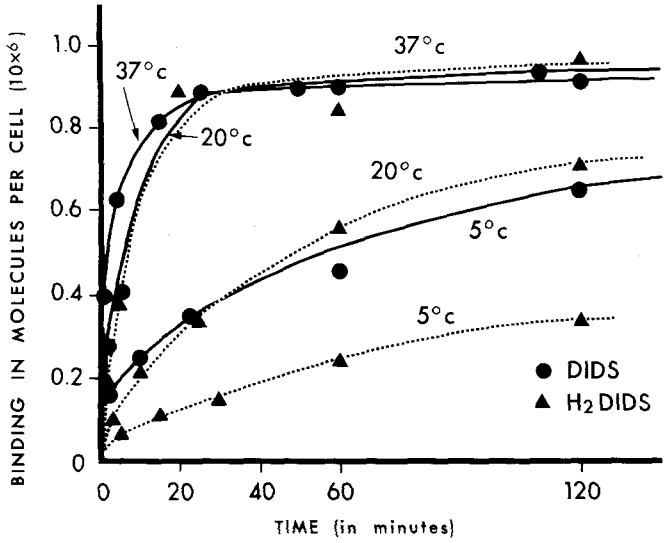

Fig. 2. A comparison of the time course of the reaction of [^{3}H]DIDS and of [^{3}H]H_2DIDS at different temperatures. Red blood cells (25% hematocrit) in HEPES buffer (pH 7.4) were reacted with 10 μM reagent at the indicated temperatures. The reactions were terminated at the indicated times by rapid addition of 2-mercaptoethanol (40 mM final concentration) and by rapid cooling. Ghosts were prepared and counted as described in Materials and Methods. The number of molecules incorporated per cell was calculated from the counts per minute, the specific activity of the [^{3}H]DIDS and the protein content of the white ghosts using a value of 6.0×10^{-10} mg protein per ghost (Dodge *et al.*, 1963)

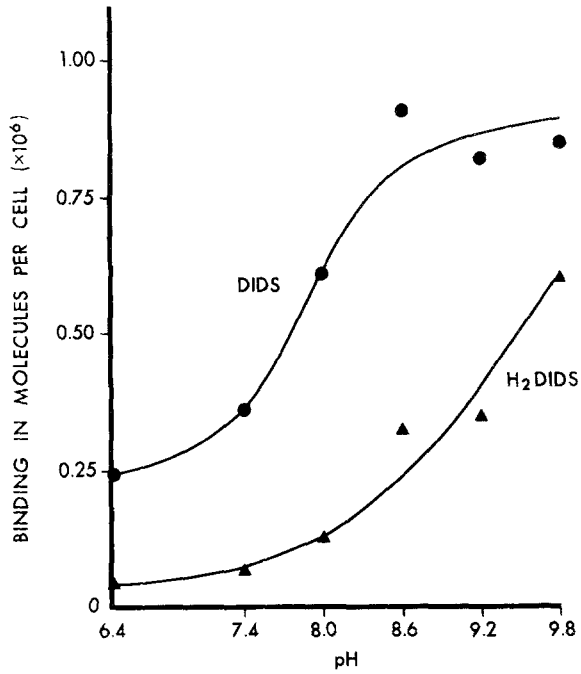

Fig. 3. The effect of pH on the labelling of erythrocytes with [^{3}H]DIDS or [^{3}H]H_2DIDS. Red blood cells (25% hematocrit) were suspended in buffer at the indicated pH and reacted with 10 μM of reagent at 5 °C for 10 min followed by two rapid washes with buffer containing 0.5% albumin. Buffers contained 150 mM sodium chloride plus: pH 6.4, 20 mM 2-(N-morpholino)-ethane sulfonic acid; pH 7.2–8.6, 20 mM Tris; pH 9.2–9.8, 20 mM boric acid. Ghosts were prepared and counted as described in Materials and Methods

at pH 8.6, the reaction with the same concentration of [^{3}H]H_2DIDS is still incomplete at this time even at pH 9.8.

The irreversible interaction of DIDS with the cells results in an irreversible inhibition of anion fluxes (Cabantchik & Rothstein, 1972). The degree of inhibition of sulfate flux as a function of DIDS concentration is presented in Fig. 4. Over a range of concentrations the relationship is linear and the extrapolation value for 100% inhibition is 9 μM reagent. From the known number of cells, determined by means of a Celloscope Cell Counter, it can be calculated that this concentration represents 1.5×10^6 molecules per cell. This value represents the maximal number of binding sites for DIDS that might be associated with its inhibitory effect, but it is an overestimate, because not all of the DIDS in the suspension is covalently bound. To determine the relationship between the degree of inhibition of the anion efflux and the extent of irreversible binding, an erythrocyte suspension which had been loaded with [^{35}S]-

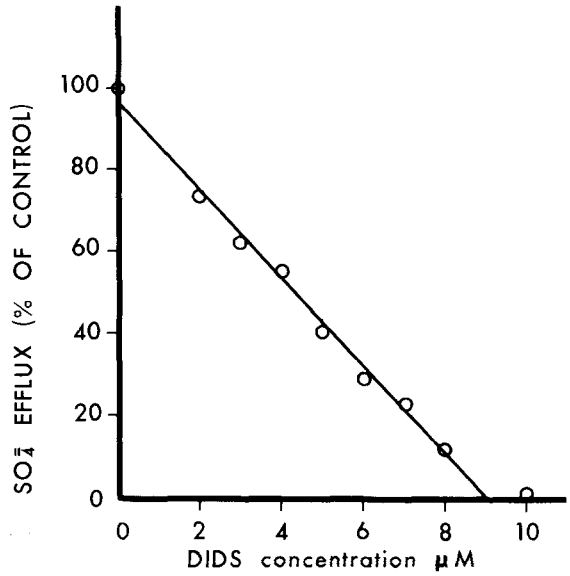

Fig. 4. The relative rates of sulfate efflux as a function of the initial DIDS concentration. Red blood cells (25% hematocrit) pre-equilibrated with labelled $SO_4^{=}$ were exposed to the designated concentrations of DIDS for 30 min at 37 °C. After washing to remove reversibly bound DIDS, the sulfate efflux was measured

sulfate was reacted with a series of concentrations of [^{3}H]DIDS. Aliquots were utilized to measure sulfate fluxes and to prepare ghosts for tritium counting. The results plotted in Fig. 5*a* show that the relative rate of efflux and the number of [^{3}H]DIDS molecules bound per cell are linearly related. By extrapolation, 1.16×10^6 molecules of [^{3}H]DIDS per cell are associated with 100% inhibition. Analogous modification of red cells with [^{3}H]H_2DIDS gave a similar amount of binding, 1.15×10^6 molecules per cell associated with complete inhibition (Fig. 5*b*).

The SDS polyacrylamide gel patterns of the radioactively labelled membrane proteins (Fig. 6) show that the great majority of the inhibitor molecules have become attached to band 3, the major penetrating membrane protein (*see also* Cabantchik & Rothstein (1974*a*)). For both [^{3}H]DIDS and [^{3}H]H_2DIDS approximately 90% of the total label is routinely found in this band (amounting to about 1×10^6 sites per cell), with less than 5% in an adjacent shoulder, and with the remaining 5% distributed along the gel. The shoulder presumably contains DIDS-labelled glycophorin (Cabantchik & Rothstein, 1974*b*) and a "satellite" band (Lepke *et al.*, 1976).

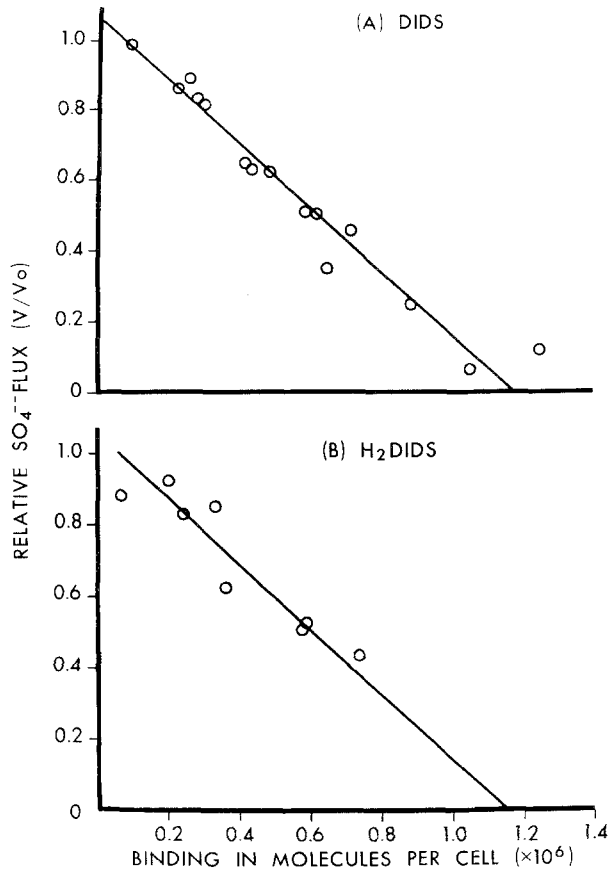

Fig. 5. The relationship between the number of molecules of [^{3}H]DIDS (*A*) or [^{3}H]H_2DIDS (*B*) bound per cell and the associated inhibition of [^{35}S]-sulfate efflux. Measurements of the two parameters were made on aliquots of the same sample of red blood cells (25% hematocrit) in HEPES buffer (pH 7.4) which had been reacted with different concentrations of probe ([^{3}H]DIDS, 1–15 μM; [^{3}H]H_2DIDS, 1–25 μM) for 10 min at 37 °C. Calculation of the number of molecules bound per cell was as described in the legend of Fig. 2

Discussion

The experiments described in this article were conducted not only to provide an appropriate radioactive form of DIDS, but also to clarify certain discrepancies which have arisen out of use of a reduced form of DIDS ([^{3}H]H_2DIDS) to determine the number of binding sites in the red blood cell that were associated with inhibition of anion transport. Using the reduced form, Cabantchik and Rothstein (1974*a*) reported 300,000 sites per cell associated with complete inhibition. More recently,

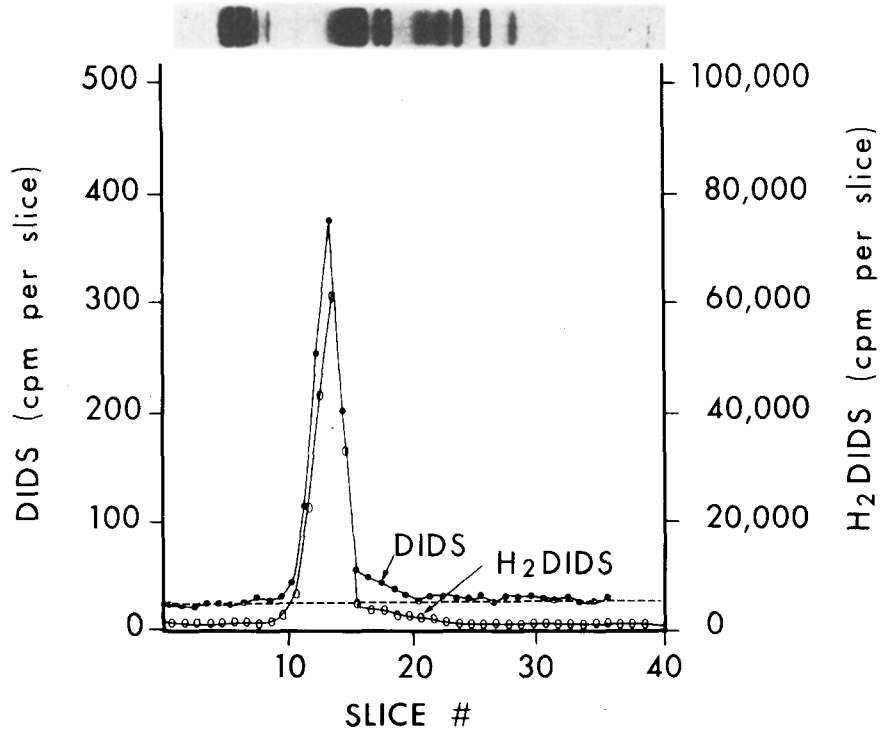

Fig. 6. The radioactive profiles of ghost proteins isolated from red blood cells labelled with [^{3}H]DIDS or [^{3}H]H_2DIDS, separated by SDS-acrylamide gel (5.6%) electrophoresis. Red blood cells (25% hematocrit) in HEPES buffer (pH 7.4) were reacted with 10 μM reagent for 15 min at 37 °C. Parallel gels stained with Coomassie Brilliant Blue showed that the principal labelled band corresponds to band 3. The counting background was not subtracted from the experimental values. The average background for [^{3}H]DIDS is shown as a dotted line. On the scale used for [^{3}H]H_2DIDS, the background values are insignificant

Lepke *et al.* (1976), also using [^{3}H]H_2DIDS as radioactive probes, have suggested that the correct number is 1.9×10^6 molecules per cell, of which 1.2×10^6 molecules are bound to band 3. The reason for the lower value of Cabantchik and Rothstein (1974*a*) became clear as a result of a discussion between the two groups of investigators. A check of the records of the company that tritiated the DIDS precursor (DADS) indicated that reduction was only about 20% complete so that the end product was a mixture of 80% DIDS and 20% [^{3}H]H_2DIDS. Such a mixture would account for the results originally reported. Because DIDS reacts more rapidly than H_2DIDS (Lepke *et al.*, 1976, and Fig. 2 of this paper), the inhibitory potency would be only slightly less for the mixture than for DIDS alone, but the apparent binding as measured

by [^{3}H]H_2DIDS would be low thus giving a substantial underestimate in the number of binding sites per cell.

Our values for the binding of DIDS and H_2DIDS were 1.1 to 1.2×10^6 sites per cell (Fig. 5) for total binding, and about 1×10^6 sites per cell for binding to band 3. The results for binding to band 3 are in rather good agreement with those reported by Lepke *et al.* (1976), which were 1.23×10^6 molecules per cell for H_2DIDS by direct measurements and 1.09×10^6 molecules per cell for DIDS by an indirect estimate. The relatively small differences in values from the two laboratories could arise from errors in the determinations of specific activities of the probes, counting efficiencies, protein determinations on ghost suspensions, and the value for membrane protein per cell used as a factor in the calculations[3]. On the other hand, Lepke *et al.* (1976) report a significantly higher quantity of total binding of H_2DIDS to the cell (1.9×10^6 molecules per cell) and a much higher fraction of binding to components other than band 3 (over 30%, compared to our finding of 10%). This greater extent of nonspecific binding may be due to longer exposures to the probe at lower hematocrit employed by those authors.

The maximal total number of DIDS sites that can possibly be involved can be determined from inhibition curves such as that presented in Fig. 4. Such estimates are free of potential sources of error encountered in the determination of radioactivity incorporated into red cell ghosts but depend only on knowledge of the amount of DIDS added to a cell suspension, the number of cells in the suspension, and the determination

3 For the quantity of membrane protein per cell, we have made use of the number of 6.0×10^{-10} mg protein per ghost determined by Dodge, Mitchell and Hanahan (1963), whose ghosting procedure we have followed. This figure, determined by cell counting and protein determination on ghosts carefully prepared so as to give a near quantitative yield, is probably as reliable as any, considering that all procedures for determining the factor are subject to some uncertainty. Dr. P. Knauf of this Institute, using cells from a single donor, has determined the factor to be 7.2×10^{-10} mg protein per ghost by comparison of the quantity of [^{3}H]H_2DIDS per washed erythrocyte cell with the quantity of probe per mg of protein recovered in the ghosts prepared by the procedure of Dodge *et al.* (1963). Lepke *et al.* (1976), who have utilized a different ghosting procedure, make use of a factor of 5.1×10^{-10} mg protein per ghost, determined by them.

Furthermore, by subtraction of the quantity of unbound [^{3}H]DIDS (determined by scintillation counting of the supernatants in the washing procedure) from the total quantity of [^{3}H]DIDS added to a cell suspension, it has been determined that approximately 1.0×10^6 molecules of irreversibly bound [^{3}H]DIDS per red cell are associated with 100% inhibition of sulfate flux (S. Ship & A. Gaarn, *unpublished data*). This value corresponds quite well with that of 1.16×10^6 molecules per cell (Fig. 5) calculated on the basis of the Dodge *et al.* (1963) value for protein per ghost (6.0×10^{-10} mg), suggesting that the latter number is essentially correct.

of the inhibition, all of which can be estimated with reasonable accuracy. The data of Fig. 4 indicate that addition of 1.5×10^6 molecules of DIDS per cell is associated with 100% inhibition. Other estimates calculated from similar experiments ranged from 1.2 to 1.5×10^6 molecules per cell. Halestrap (1976) reported a value of 1.2×10^6 molecules per cell for DIDS inhibition of pyruvate flux and the same value has been observed by P.A. Knauf and P. Marchant (*personal communication*) for DIDS inhibition of iodide fluxes. The reported values provide an upper limit because all of the added DIDS does not necessarily react covalently with the cells. They are entirely consistent with the value of 1.2×10^6 sites per cell estimated in Fig. 5 from the total irreversibly bound DIDS required for 100% inhibition.

The calculated number of DIDS molecules bound to band 3 required to achieve 100% inhibition, about 1×10^6 per cell, corresponds to the number of monomers of band 3 per cell, as estimated by Steck (1974). The tempting conclusion that all band 3 monomer units are, therefore, involved in anion transport must, however, be tempered by other information. The value of 1×10^6 monomers is a rather crude estimate; band 3 probably exists as a dimer (Yu & Steck, 1975); band 3 is heterogeneous with a major and several minor components (Steck, 1974; Rothstein *et al.*, 1976; Anselstetter & Horstmann, 1975; Conrad & Penniston, 1976); and there may be more than one DIDS-binding site per monomer (Passow *et al.*, 1974/75).

One other difference between the results reported here and those of Lepke *et al.* (1976) is in the shape of the reaction profiles at different temperatures. Lepke *et al.* reported a biphasic curve with a substantial rapid component, whereas the curves of Fig. 2 can, within limits, be fitted to a first order curve. The difference may be due to the method used for stopping the DIDS-binding reaction. We used a thiol, 2-mercaptoethanol which does not change the pH of the medium, whereas they used ethylenediamine. In our hands, the latter compound, at concentrations sufficient to react quickly and completely with DIDS, gave a higher amount of labelling than either the albumin washing procedure or mercaptoethanol (especially with DIDS), because it increased the pH of the medium (*see* Fig. 3). The reaction profiles of Fig. 2 are consistent with the reaction sequence proposed by Cabantchik and Rothstein (1972), the rapid formation of an agent-protein complex followed by a slower irreversible reaction.

The nature of the amino acid residue modified by reaction with the isothiocyano group is still in question. Since DIDS shares sites with

pyridoxal phosphate, a compound known to form specific Schiff bases with free amino groups, a lysine residue was suspected (Rothstein *et al.*, 1976). This possibility is supported by the pH profiles in Fig. 3, which corresponds to the dissociation of groups with apparent pKa's of approximately 8 to 9, consistent with the values for amino groups of proteins (Edsall & Wyman, 1958).

Under defined conditions, DIDS and H_2DIDS have equal specificity for the band 3 proteins. The higher reactivity of the former, particularly at low temperatures, is due to the change in chemical structure associated with hydrogenation of the central double band. Thus, the H_2DIDS molecule has a slightly increased overall length and a greatly modified electronic structure. In addition, the increased conformational mobility would be expected to result in a higher entropy of activation for the chemical reaction. Both agents are useful as irreversible inhibitors of anion transport and to designate potential inhibitory sites. DIDS has the advantage that under appropriate conditions it reacts more rapidly and completely. On the other hand [^{3}H]DIDS is more difficult to prepare than is [^{3}H]H_2DIDS and the efficiency of tritiation is lower so that its specific activity is lower by a factor of 10^3.

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