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Membrane Proteins Related to Anion Permeability of Human Red Blood Cells

I. Localization of Disulfonic Stilbene Binding Sites in Proteins Involved in Permeation

Z. I. Cabantchik* and A. Rothstein

Research Institute, The Hospital for Sick Children, Toronto, Ontario, Canada

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Summary. (^{3}H)DIDS (4,4′-diisothiocyano-2,2′-ditritiostilbene-disulfonate) was used as a covalent label for membrane sites involved in anion permeability. The label binds to a small, superficially located population of sites, about 300,000 per cell, resulting in almost complete inhibition of anion exchange. The relationship of binding to inhibition is linear suggesting that binding renders each site nonfunctional. In the inhibitory range less than 1% of the label is associated with lipids, but at higher concentrations of DIDS, the fraction may be as high as 4%. In ghosts, however, treatment with (^{3}H)DIDS results in extensive labeling of lipids. In cells, a protein fraction that behaves on SDS acrylamide gels as though its molecular weight is 95,000 daltons (95K) is predominantly labeled by (^{3}H)DIDS. The only other labeled protein is the major sialoglycoprotein which contains less than 5% of the total bound (^{3}H)DIDS. Because of the linear relationship of binding to inhibition and the unique architecture of the site, it is suggested that the (^{3}H)DIDS-binding site of the 95K protein is the substrate binding site of the anion transport system. The 95K protein is asymmetrically arranged in the membrane with the sites arranged on the outer face accessible to agent in the medium. In "leaky" ghosts, only a few additional binding sites can be reached from the inside of the membrane in the 95K protein, in contrast to the extensive labeling of other membrane proteins in ghosts as compared to cells.

The disulfonic stilbene derivative, SITS[1], was used by Maddy (1964) as a nonpenetrating fluorescent marker presumed to react with amino

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1 *Abbreviations:*

DADS	4,4′-Diamino-2,2′-dihydrostilbene disulfonic acid
DIDS	4,4′-Diisothiocyano-2,2′-stilbene disulfonic acid
(^{3}H)DADS	4,4′-Diamino-2,2′-ditritiostilbene disulfonic acid
(^{3}H)DIDS	4,4′-Diisothiocyano-2,2′-ditritiostilbene disulfonic acid

groups of the membrane surface. Its effects on permeability are particularly interesting (Knauf & Rothstein, 1971). Unlike other amino reactive reagents such as DNFB, MNT and TNBS, which inhibit anion permeability and increase cation permeability, SITS is specific for anions with no effect on cations. The different behavior of SITS led to the conclusion that two different populations of membrane amino groups are involved in permeability with a superficial population that controls the anions accessible to all of the agents, and a second, more internal population, that controls the cations accessible to many agents, but not to SITS.

SITS contains an isothiocyanate group, potentially capable of covalent bond formation with amino groups. On this basis, and because extensive washing caused little reversal of binding or inhibition, Maddy (1964) and Knauf and Rothstein (1971) assumed that the interaction of the compound with the surface of the membrane was covalent. Cabantchik and Rothstein (1972), however, in attempting to use radioactive forms of SITS as a label for the anion controlling sites provided evidence that only a small and variable fraction of the SITS was covalently bonded. They prepared a series of other disulfonic stilbene analogues, some with covalent binding groups and some without. All were inhibitory, demonstrating that the inhibition of anion permeability was related to electrostatic interactions of the charged core of the disulfonic stilbenes with positively charged groups of the membrane and not to covalent binding.

The inhibitory potency of the stilbenes could be manipulated by the addition of different substituents permitting conclusions to be drawn concerning the chemical nature and geography of the sites controlling anion permeability. The most potent of the compounds, DIDS, binds covalently and irreversibly to a small number of ligands on the outer surface of the cell membrane. In the form of (^{131}I) DIDS or (^{3}H)DIDS, it can be used to label the sites of interest. After the membrane is solubilized and the

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SITS	4-Acetamido-4′-isothiocyano-2,2′-stilbene disulfonic acid
DNFB	1-Fluoro-2,4-dinitrobenzene
MNT	2-Methoxy-5-nitropone
TNBS	2,4,6-Trinitrobenzene sulfonic acid
SDS	Sodium dodecyl sulfate
EDTA	Ethylene diamine tetra acetic acid
PBS	Phosphate buffer saline (NaH_2PO_4 50 mM, NaCl 75 mM)
TBS	Tris buffer saline (Tris-Cl 25 mM, NaCl 125 mM)
DTT	Dithiothreitol (Cleland's reagent)
K	1,000
CB	Coomassie Blue
PAS	Periodic Acid-Schiff

components separated by acrylamide gel electrophoresis, most of the labeled DIDS was found in a particular component of the membrane that behaves in the gels as though it had a molecular weight of 95,000 daltons (95K). In the present paper, (^{3}H)DIDS was used as a marker to explore, in detail, the inhibitory sites in terms of their location within the membrane, their location in membrane components, their number in relation to anion permeability, and the relationship of the sites to the inhibitory effect.

Materials and Methods

Source of Compounds

DADS (4,4′-diamino-2,2′-stilbene-disulfonic acid disodium salt), a gift from Sumitomo Chemical Co. Ltd. (Osaka) was crystallized several times from 1% NaCl solution. It was also purified on a silica gel column (0.05 to 0.2 mm, Merck A.G. –

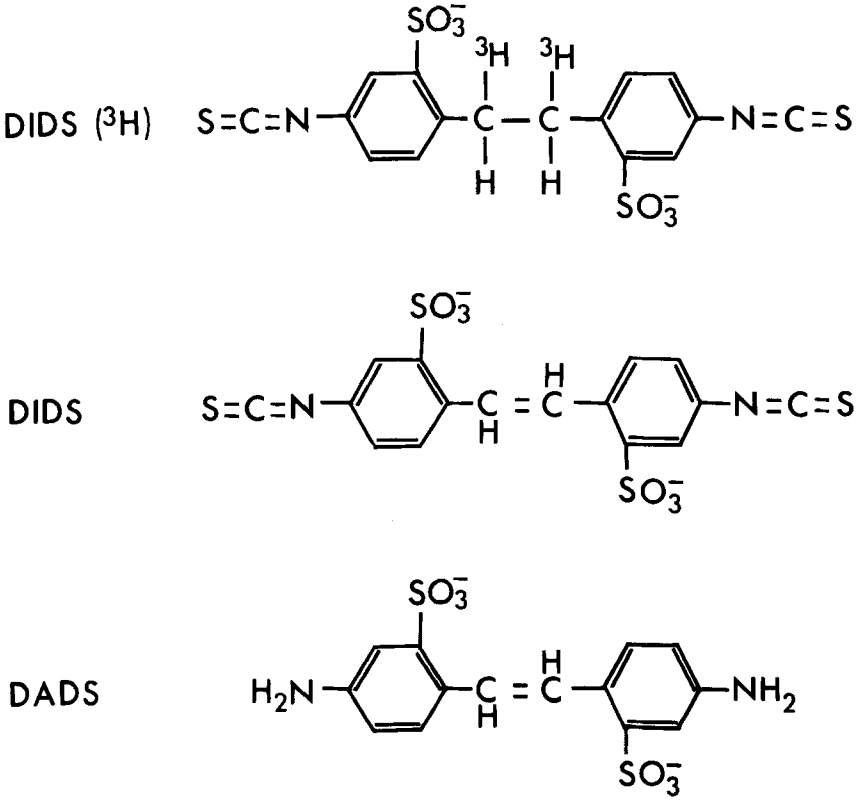

Fig. 1. Chemical structures of surface labels. (^{3}H)DIDS: 4,4′-diisothiocyano-2,2′-ditritiostilbene disulfonate; DIDS: 4,4′-diisothiocyano-2,2′-stilbene disulfonate; DADS: 4,4′-diamino-2,2′-stilbene disulfonate

Darmstadt) using pyridine/acetic acid/water (10:1:40) and *n*-propanol/ammonium hydroxide/water (6:3:2) as elution solvents. The final product was concentrated by flash evaporation, deionized on a Dowex 50W-(H^+ form) column, boiled in charcoal and precipitated as the free acid by the addition of HCl and cooling. The purity of the final product was confirmed by thin-layer chromatography using different solvents. All steps in this and the following procedures were conducted with minimal exposure to light.

(^{3}H)DADS (4,4′-diamino-2,2′-dihydrostilbene-disulfonic acid disodium salt) was obtained by catalytic hydrogenation (Palladium on Carbon) of DADS with 5 curies of tritium gas (New England Nuclear, Tritium Labelling Services) and obtained with a specific activity of 1.47 mC/μmole. 100 μmoles of (^{3}H)DADS dissolved in 2.0 ml of 0.1% NaCl were reacted with 0.5 ml of thiophosgene (Baker Chemical Company) under vigorous stirring for 30 min at room temperature. As the solution acidifies an orange precipitate of (^{3}H)DIDS (4,4′-disothiocyano-2,2′-dihydrostilbene-disulfonate) is readily formed. The excess thiophosgene is removed by repeated extraction with 1.0 ml of ether and centrifugation. The final precipitate is washed with 0.5 ml of cold (~5 °C) 0.01 N HCL and subsequently with 0.5 ml of cold water. The final precipitate is quickly placed in vacuum and dehydrated over P_2O_5, and finally obtained as a red-brown fine powder. The radioactive yield was not less than 30 per cent. The specific activity of (^{3}H)DIDS should be the same as that of (^{3}H)DADS (1.47 mC/μmole), based on the assumption that reaction of the latter with thiophosgene (introduction of isothiocyano groups at the terminal amino groups) does not change the amount of tritium in the compound. All the materials were of either analytical or electrophoresis grade, unless specified otherwise. The structural formulas of the DADS, DIDS and (^{3}H)DIDS are given in Fig. 1.

Labeling of Red Blood Cells

(^{3}H)DIDS is stable under anhydrous conditions, low temperatures (−10 °C) and minimal exposure to light for at least 8 months, but decomposes at room temperature in aqueous solutions to the diamino derivative (positive diazonium test), H_2S and CO_2. Its use for chemical modification must, therefore, be conducted at low temperatures and for short periods of time (15 to 30 min). As a polyaromatic fluorophore, DIDS absorbs light efficiently (ξ_{340} 3.4×10^4 M^{-1} cm^{-1} at pH 8.0) but undergoes trans-cis isomerization as do other substituted stilbenes (Schulte-Frohlinde, Blume & Gusten, 1962; Maddy, 1964). Both isomers are equally reactive with red blood cells and both produce the same inhibitory effect.

Fresh or recently outdated blood was obtained from the Toronto General Hospital Blood Bank. The red cells were extensively washed and isolated in PBS (phosphate buffer-NaCl, 300 milliosmolar, pH 7.4) at 5 °C. A 5 to 30% cell suspension in PBS was reacted with different concentrations of (^{3}H)DIDS at 5 °C for 15 to 30 min in the dark. The cells were subsequently washed 3 times with 20 volumes of TBS (Tris buffer saline 300 milliosmolar, pH 7.4) for 10 min at room temperature and centrifuged to remove the free reagent and to neutralize the nonreacted groups of monofunctionally bound DIDS. The procedure was followed by similar washes with TBS containing 0.5% albumin (Fraction V, essentially fatty acid free, Sigma Chemical Company) and finally with PBS. All the steps following the labeling were done at room temperature. As reported earlier (Cabantchik & Rothstein, 1972) the presence of albumin was found necessary in order to remove the noncovalently bonded probes from the membrane of the intact cell. The modified cells are stable (no hemolysis) for at least 36 hr when kept at 5 °C and in the presence of 0.5% albumin.

Since DIDS is a bifunctional agent, it was necessary to destroy free isothiocyanate groups of monofunctionally bound probes prior to the albumin washes. This was accomplished by extensively washing the labeled cells with TBS at 5 °C or at room temperature. When a highly radioactive ^{125}I albumin was present in the washing solution no incorporation of ^{125}I label was found in the membrane preparation.

In one set of experiments the cells were first reacted with 1×10^{-6} M DIDS, washed and subsequently reacted with 1×10^{-6} M (^{3}H)DIDS and were finally subjected to the same washing steps as mentioned above.

"In situ" cross-linking of labeled ghosts was performed by reacting ghosts (3 mg/ml protein) in 20 milliosmolar Na-phosphate buffer, pH 8.0, with O-phenanthrolene-100 μM, $CuSO_4$-20 μM (Steck, 1972) for 30 to 60 min at room temperature, and centrifuged at $17{,}000 \times g$ for 5 min after addition of 3 volumes of the same buffer containing 1 mM EDTA.

Ghost Preparation

Ghosts were prepared from normal and chemically modified cells by the method of Dodge, Mitchell and Hanahan (1963). A portion of these ghosts were dissolved immediately after preparation in an equal volume of 20% sucrose, 2% SDS, 20 mM Tris, 2 mM EDTA, 80 mM Dithiotreitol, pH 8.0, boiled for 4 min and kept frozen. The protein content of ghosts was determined by measuring the fluorescence of the membrane fluorophores in phosphate buffer pH 7.4 (20 milliosmolar) with an Aminco Bowman Spectrofluorimeter with Ratio Photometer (excitation wavelength: 285 nm, emission wavelength 340 nm). The instrument was calibrated with tryptophane standards and by adjusting the high voltage on the photomultiplier to an adopted arbitrary value equivalent to 100 μg/ml ghost protein. The "real" content of protein in the ghosts was first established by comparing the value obtained from the fluorescence technique with the value obtained by a modified microbiuret method (Itzhaki & Gill, 1964) using 3 × crystallized bovine serum albumin (Sigma Chemical Company) as standard and in the presence of 0.1% Na deoxycholate. Since the latter method seems to be less dependent on the protein composition, it was found to be a reliable reference for standardization of the fluorimetric method for determinations of membrane proteins. This technique is basically a modification of a previously published study (Resch, Imm, Ferber, Wallach & Fischer, 1971).

Fractionation of Ghosts

The isolated ghosts were extracted with chloroform/methanol (2:1) (Hamaguchi & Cleve, 1972) into an aqueous phase that contained the glycoproteins, a protein interphase and an organic phase that contained part of the lipids. The protein interphase was extensively extracted with ethanol/ether (3:1) (Rosenberg & Guidotti, 1968), and the organic phases combined and concentrated under nitrogen. The protein precipitate was dissolved subsequently by incubation at 40 °C for 40 hr in SDS 4%, Tris 2.0 mM, EDTA 2 mM and Dithiothreitol 80 mM, pH 8.0.

Polyacrylamide-SDS Gel Electrophoresis

Acrylamide gels, 3.3, 7.5 and 10.5%, were prepared according to a modified Fairbanks, Steck and Wallach (1971) method. The N-N′-methylenebisacrylamide (Bio-Rad) was maintained at 3.75% of the acrylamide (Bio-Rad) monomer concentration. The gels were cast in columns (0.6 × 20 cm) on a 0.5-cm height underlay of 40% sucrose, 1% SDS, 10 mM Tris acetate buffer (pH 7.8), 1 mM EDTA, 0.15% ammonium persulfate.

After polymerization they were equilibrated with electrophoresis buffer for 4 hr. Pyronin Y and Bromophenol Blue were used as tracking dyes. Phosphorylase A and β-galactosidase (*Escherichia coli*) were purchased from Worthington, bovine serum albumin 2 × crystallized, ovalbumin 2 × crystallized and cytochrome *c* were purchased from Sigma Chemical Company, and used as molecular weight markers.

The gels were stained for proteins with Coomassie Blue (Sigma Chemical Co.) as described by Fairbanks *et al.* (1971). The glycoproteins were stained by the periodic acid-Schiff technique (Fairbanks *et al.*, 1971) with the following modification: before the addition of the Schiff reagent, the gels were soaked twice with 0.1% sodium metabisulfate, 0.01 N HCl for 5 min each time. This eliminates any extraneous background of nonspecific staining, as well as enabling the re-use of the Schiff reagent for at least four more times. The appearance of the rose-pink bands is evident only after soaking the gels that were reacted with the Schiff reagent in 0.1% sodium metabisulfate, 0.01 N HCl for at least 30 min. The stain intensifies with time and upon soaking the gels with fresh metabisulfate, and is stable for at least 36 hr. The intensity and sharpness of the bands are more pronounced than in the original procedure. Stained gels were photographed and also scanned for protein and carbohydrate patterns using a Gilford 240 spectrophotometer with a 2410 linear transport accessory. Proteins were scanned at 530 nm and carbohydrate at 560 nm.

Fractionation of Gels and Counting of Radioactivity

Gels with radioactive labeled proteins (stained or unstained) were fractionated on a Maizel Autogel Divider (Savant Instruments Inc.) into 40 to 90 fractions that were collected directly in scintillation vials (Savant Unifrac Collector). Some gels were first stained for glycoproteins, counterstained for proteins and subsequently crushed. This enables marking the bands of main concern by pricking them on the gels with drafting ink for easy identification of their location in the vials after the fractionation.

Samples with ^{3}H were incubated first in 15% hydrogen peroxide for 8 hr at 80 °C (digestion × bleaching of stain) in closed vials, then dried at 60 °C under vacuum (about 1 hr), and finally redissolved in 0.1 to 0.2 ml water. A sample of 10 ml of toluene/PPO containing 10% Protosol (New England Nuclear) was added. Alternatively, but with the same results, gel fractions were first dried under vacuum at 50 °C, wet with 0.1 to 0.2 ml H_2O and digested with 10% Protosol (in PPO-toluene) mixture for 18 hr at 60 °C. Under these conditions all the gel and biological materials are dissolved, the recovery of label is not less than 95% and variations due to quenching are minimized. The recovery of counts after polyacrylamide-electrophoresis and fractionation was not less than 90% of the sample initially loaded on the gels.

The polyacrylamide gels were calibrated for molecular weights by using ^{125}I-iodinated samples of phosphorylase A, bovine serum albumin and ovalbumin. The iodination was accomplished by the iodine-monochloride method of Bale *et al.* (1966).

Permeability of Anions and Labeling of Cells

Sulfate (^{35}S) exchanges of normal and chemically modified cells were determined as described previously (Cabantchik & Rothstein, 1972). The cells were equilibrated with a medium of 50 mM Na_2SO_4 containing ($^{35}SO_4^{2}$) at 37 °C for 3 hr. The loaded cells were subsequently brought to 5 °C, centrifuged, washed 4 times (× 10 volumes) with cold medium (no $^{35}SO_4^{2}$) and finally resuspended in a prewarmed incubation medium for flux measurements.

Sulfate-loaded cells were also labeled with (^{3}H)DIDS and examined for sulfate fluxes (Cabantchik & Rothstein, 1972) as well as for the bound probe recovered in the ghost. The distribution of label in the proteins of the membrane was studied as previously described.

Results

In most experiments (^{3}H)DIDS was used to measure the binding and DIDS was used to demonstrate the inhibition of sulfate permeability. The hydrogenation to produce (^{3}H)DIDS results in the reduction of the double bond between the two rings (Fig. 1). It was, therefore, necessary to establish that the two compounds bind to the same sites and that they produce the same effects. The identity of the binding sites was established by the demonstration that pretreatment with DIDS completely prevented the binding of (^{3}H)DIDS. The substantially similar effects on sulfate permeability are illustrated in Fig. 2. The inhibition curve represents the effect of DIDS. At

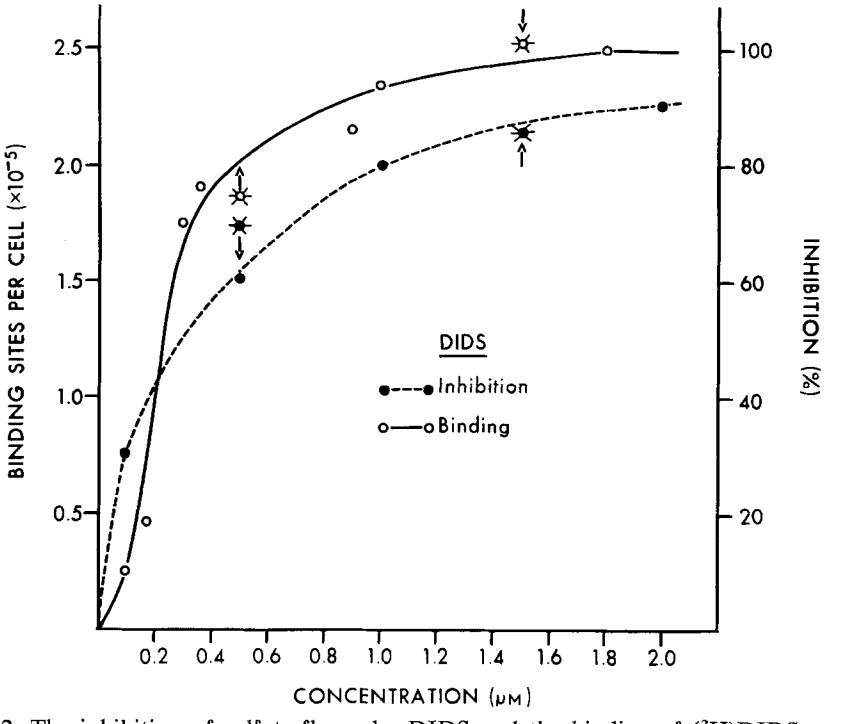

Fig. 2. The inhibition of sulfate fluxes by DIDS and the binding of (^{3}H)DIDS to red blood cells as a function of the concentration in the medium. At two concentrations the inhibition by (^{3}H)DIDS (starred closed points) was compared with the inhibition curve for DIDS. Because binding was measured in PBS medium, and fluxes in sulfate medium, the binding in the sulfate media was also compared at two concentrations (starred open points). The number of binding sites per cell was obtained from the amount of probe associated with the isolated ghosts, assuming a value of 9×10^{-10} mg protein per ghost (Hoogeveen *et al.*, 1970)

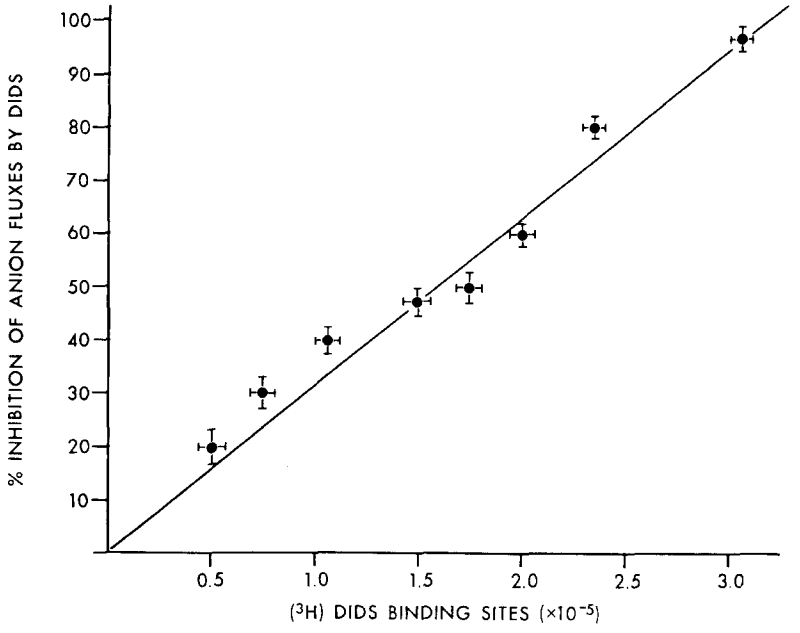

Fig. 3. The relationship between (^{3}H)DIDS binding to the red blood cell, and its effects on sulfate permeability. Data include that in Fig. 2 and two other similar experiments

two concentrations tested, the effects of (^{3}H)DIDS (starred points), were found to be comparable. Thus the reduction of the double bond in (^{3}H)DIDS (Fig. 1) does not cause a substantial difference in the inhibitory potency. The cis and trans isomers of DIDS were also found to be equally effective.

The reaction between (^{3}H)DIDS or DIDS and red blood cells is fast and efficient. At temperatures as low as 5 °C the binding of (^{3}H)DIDS showed no further increase beyond 5 min of exposure at pH values higher than 7.0. The inhibitory effect on anion permeability caused by DIDS was also completed in the same period of time. The effect is virtually irreversible, in that extensive washing with medium (in the presence or absence of albumin) did not reduce the inhibitory effect (Cabantchik & Rothstein, 1972).

The binding of (^{3}H)DIDS to red blood cells and the parallel effect of DIDS on anion permeability as a function of the concentration of the probe in the external medium, are displayed in Fig. 2. Both curves tend toward a maximum value. The relationship is more clearly expressed in Fig. 3 by plotting binding versus inhibition for three experiments (including that of Fig. 2). The points can be fitted by a straight line. By extrapolation

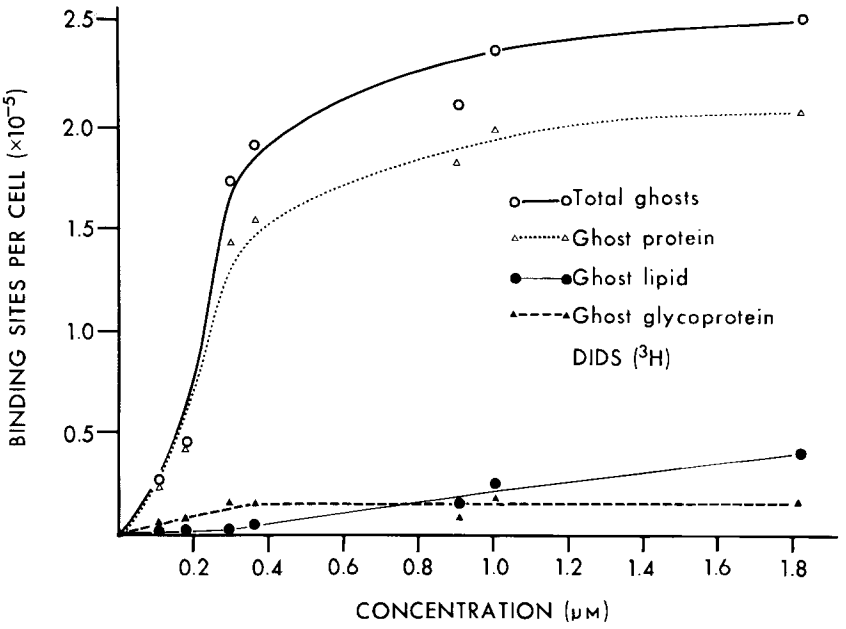

Fig. 4. The distribution of (^{3}H)DIDS in the components of ghosts isolated from labeled red blood cells as a function of (^{3}H)DIDS concentration. Ghosts were partitioned into glycoprotein, proteins and lipids as described in Materials and Methods. The number of sites was calculated as described in Fig. 2

the number of binding sites associated with virtually complete inhibition is approximately 300,000 per cell. The number is based on the estimation of the amount of (^{3}H)DIDS per mg of ghost protein and a value of 9×10^{-10} mg of protein per ghost based on several estimates that agree with the published value of Hoogeveen, Juliano, Coleman and Rothstein (1970). Several other estimates based on treatments with sufficient (^{3}H)DIDS to achieve 98 to 99% inhibition of anion permeability rather than by extrapolation as in Fig. 3, ranged from 250,000 to 300,000 sites per cell.

The distribution of bound (^{3}H)DIDS in membrane components was determined by extracting the lipids and the sialoglycoproteins from the remaining protein as outlined in Materials and Methods. Cells were treated with a series of concentrations of the probe. At all concentrations most of the probe was found in the protein fraction (Fig. 4). In the concentration range associated with inhibition of anion permeability (up to 0.5 μM), the amount in protein was over 93%, with less than 5% in sialoglycoprotein and less than 1% in lipid. At very high concentrations, the amount in lipid could be increased to about 4%.

Table 1. Comparison of (^{3}H)DIDS labeling of ghosts *vs.* labeling of intact cells. The distribution of label in the components of the erythrocyte ghost

Treatment[a]	Total ghosts[b]	Protein fraction[b]	Lipid fraction[b]
Labeling of cells	2.2	2.0	0.12
Labeling of ghosts	22.5	10.2	9.4

[a] Labeling performed with 0.28 μM (^{3}H)DIDS as described in Materials and Methods. Results are the average of two experiments.
[b] nmoles $\times 10^{-10}$ per ghost.

The limited binding of DIDS by the intact cell demonstrated in Figs. 2 and 4 is probably due to the inability of the compound to penetrate and to reach other potential binding sites. If "leaky" ghosts rather than cells were exposed to the probe, the binding was increased by an order of magnitude (Table 1), with a disproportionate increase in the lipids (100 ×) compared to protein (5 ×).

The data on distribution of (^{3}H)DIDS based on extraction of components are subject to some reservations. The solubilization of sialoglycoproteins by the method of Hamaguchi and Cleve (1972) is incomplete (70 to 80%) and the solubilization of DIDS-modified phospholipid may be changed due to introduction of two sulfonic acid groups. However, the distributions were also determined by dissolving the membrane components in detergent and separating by SDS-gel electrophoresis, with essentially the same results as reported above. Little (^{3}H)DIDS was found at the SDS front (lipids), or associated with the sialoglycoprotein bands.

In Fig. 5A gels stained for protein with Coomassie Blue (CB) and for glycoprotein by periodic acid Schiff reaction (PAS) are compared with the distribution of (^{3}H)DIDS. In Fig. 5B another similar experiment is presented with densitometric tracings of the CB- and PAS-stained gels. The gel concentration was 7.5% rather than the usual 5% in order to achieve better separation of the major sialoglycoprotein band (I) and the 95,000 dalton protein band (95K). Most of the label seems to be related to the 95K band with only a fraction on the right-hand shoulder possibly associated with the sialoglycoprotein.

An even more convincing separation of the 95K protein and the sialoglycoprotein cross-linking of the former was achieved by the method of Steck (1972). The 95K protein was largely shifted to higher molecular weight polymers, mostly a dimer that appeared as a new band at about

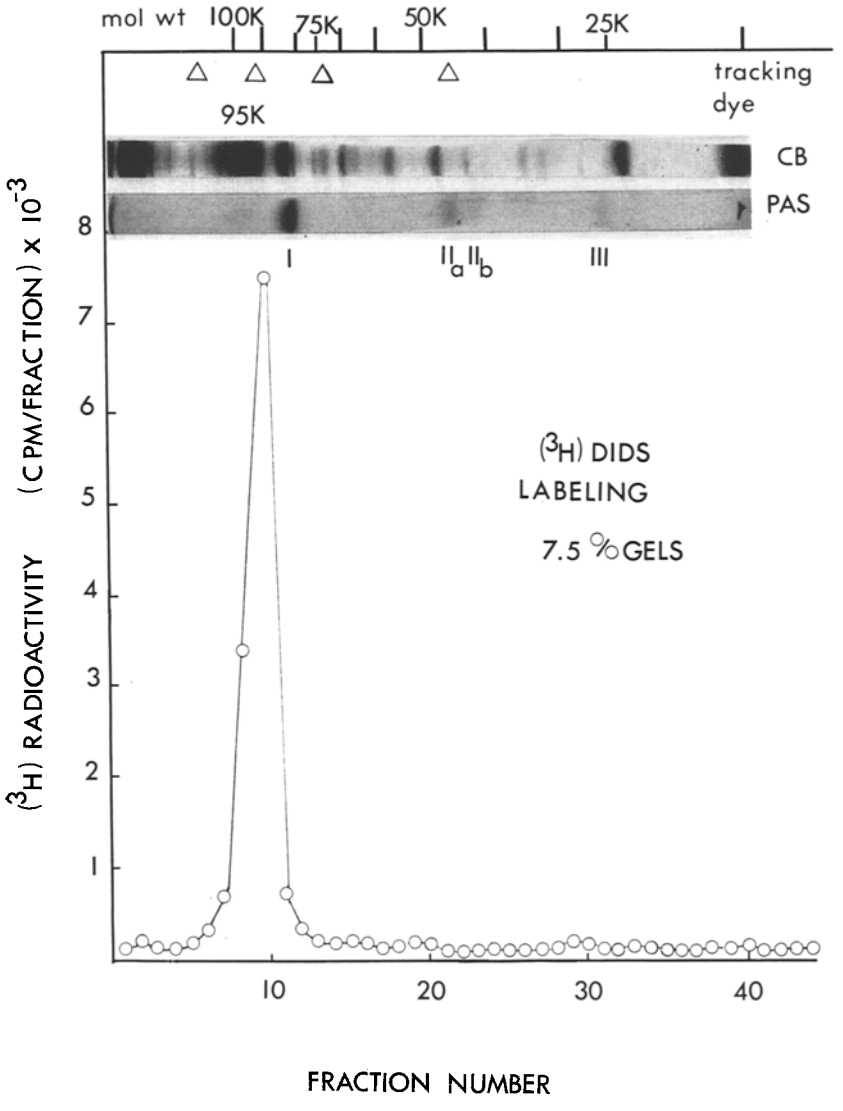

Fig. 5*A*

Fig. 5. (*A*) The labeling and staining profiles of ghost proteins isolated from (^{3}H)DIDS-labeled cells. Ghosts isolated from red blood cells labeled with 0.1 μM (^{3}H)DIDS (30 min, 5 °C) were analyzed for protein and label distribution by polyacrylamide SDS electrophoresis. Proteins were stained with Coomassie Blue (CB) and glycoproteins with periodic acid-Schiff (PAS). Gels with radioactive material were fractionated, digested and analyzed for distribution of (^{3}H)DIDS. The molecular weight (mol wt) scale of the gels was obtained with the following protein markers (open triangles): β-galactoside, phosphorylase A, bovine serum albumin and ovalbumin. (*B*) The labeling and staining profiles of ghost proteins isolated from (^{3}H)DIDS-labeled cells. Densitometry of gels shown on Fig. 3. *Upper figure:* Densitometry tracings of gels stained for proteins with Coomassie Blue (CB) (full line) and distribution of ^{3}H label (broken line). *Lower figure:* Densitometry tracings of the same gel as in the upper figure, but stained for carbohydrates (PAS: Periodic Acid-Schiff method)

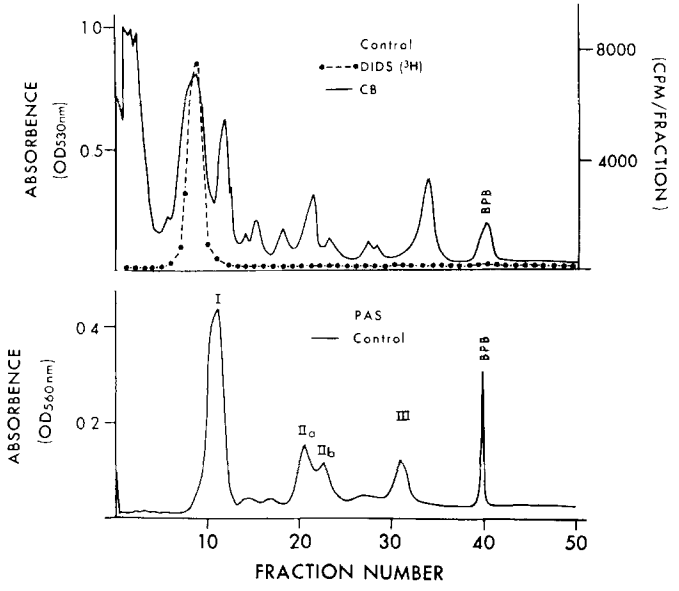

Fig. 5*B*

190K, with some high molecular weight material that did not enter the gel (Fig. 6). No other protein bands were shifted appreciably and the PAS bands remained in their normal position indicating no cross-linking of the sialoglycoproteins. The (^{3}H)DIDS label was, however, shifted in parallel to the 95K protein, most of it appearing at 190K, with some at the top of the gel. The separation of the bulk of the label from the sialoglycoprotein bands is unequivocal in this case, supporting the conclusion that only a small fraction of DIDS-binding can be attributed to sialoglycoprotein based on separation by selective solubilization (Fig. 4). If the cross-linked ghosts were reduced by treatment with DTT, the stained bands and the (^{3}H)DIDS returned to their normal position at 95K.

The pattern of Fig. 5 was essentially the same with concentrations of DIDS between 0.1 and 0.5 μM, the latter quantity being sufficient to produce a maximal effect on anion permeability. Above 0.5 μM, however, a small amount of label was seen at the SDS front. The observation confirms the data obtained by lipid extraction presented in Fig. 4 indicating that in the inhibitory range of DIDS concentration, binding to lipids is minimal, but with a small but finite interaction at higher concentrations.

When ghosts, rather than cells, were treated with (^{3}H)DIDS, the pattern of labeling was entirely different. The total amount of label was increased by a factor of ten as demonstrated in Table 1, with more than half of the

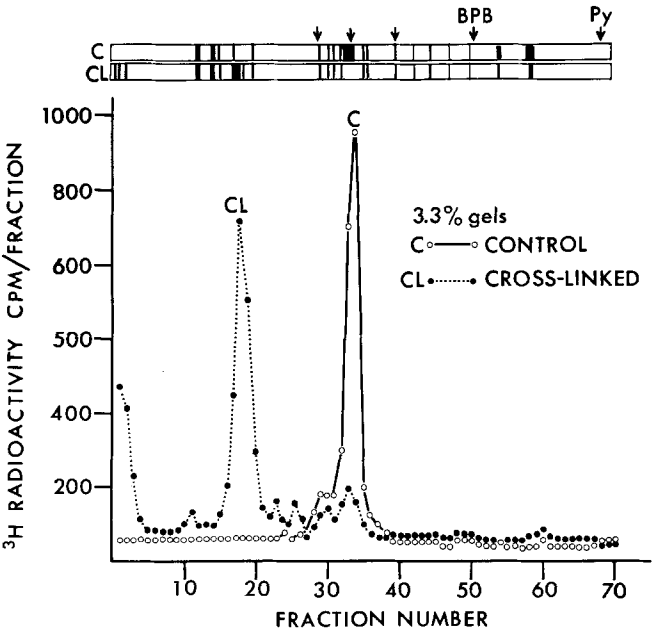

Fig. 6. The "in situ" cross-linking of the major polypeptide of the red blood cell membrane. Polyacrylamide gel electrophoresis of cross-linked ghosts. Ghosts from labeled cells (3 mg/ml protein) in PBS-20 mosm, pH 7.8, were reacted with 20 μM $CuSO_4$, 100 μM O-phenantroline for 45 min at room temperature. The reaction was stopped by addition of an equal volume of solubilizing solution (80 °C) (no DTT), boiled for 3 min, cooled and applied to 3.3% gels. The three arrows represent the position of three molecular weight markers (from left to right): β-Galactosidase: 130,000, Phosphorylase A: 98,000, and Bovine Serum Albumin: 67,000

increase representing labeling of lipids. This finding was confirmed by the acrylamide gel technique with the appearance of a large peak at the SDS front (Fig. 6) that could be eliminated if the ghosts were delipidated before they were solubilized for electrophoresis. In the case of the proteins, the increased labeling in ghosts was associated with binding to components that were not accessible in the intact cell. In fact, (^{3}H)DIDS was found in almost every part of the gel, with many peaks. Every visible protein band (seen with Coomassie Blue) seems to have acquired some label. It is of some significance, however, that in the 95K band (the only one substantially labeled in the intact cell), only a small increase, about 15% to 20%, was found.

One other difference in the response to DIDS of ghosts as compared to cells was evident. DIDS contains two isothiocyanate groups and is therefore potentially a cross-linking reagent. Nevertheless no evidence of

cross-linking was found in DIDS-treated cells. No bands were shifted and no protein or label was found on the top of the gel (indicating the presence of high molecular weight polymers) even when a high concentration (10^{-4} M) of DIDS was used. In DIDS-treated ghosts, however, some protein and label does not enter the gels indicating cross-linking into high molecular weight aggregates (compare Figs. 5 and 6).

Discussion

Maddy (1964) for ox red cells and Knauf and Rothstein (1971) for human red cells proposed that SITS did not penetrate the membrane but reacted only with superficial sites. The conclusion was based on the finding that uptake of the agent was virtually complete in a short period of time, that the maximal binding involved a small fraction of the potential binding sites of the membrane and that little SITS was found in hemoglobin. The methods based on the spectrophotometric estimation of the disappearance of SITS from the medium or on qualitative estimates of fluorescence were relatively insensitive or inaccurate requiring concentrations of SITS considerably greater than necessary for maximal inhibition of anion permeability. By use of isotope procedures with (^{3}H)DIDS, the binding curves were accurately determined in the inhibitory range as well as at higher concentrations (Fig. 2). Only a small number of sites react with the agent, less than 300,000 per cell. If, however, ghosts rather than cells are treated with the same concentration of (^{3}H)DIDS, the binding is increased more than 10-fold (Table 1) and many additional components of the membrane become accessible (compare Figs. 5 and 6). These observations support the conclusion that the intact cell membrane is impermeable to the disulfonic stilbenes under conditions studied and that their interaction is therefore limited to a small population of superficial sites.

Because DIDS is covalently bonded (Cabantchik & Rothstein, 1972) (^{3}H)DIDS can be used to locate the binding sites among the various membrane components. In the range of concentrations of agent that influences permeability, the maximal amount of (^{3}H)DIDS in the membrane lipids is exceedingly small (less than 1 % of the total) and is not dose-related (Fig. 4). At very high concentrations, considerably above those required for virtually complete inhibition, the binding to lipids increases somewhat (as high as 4% of the total). It seems unlikely, therefore, that binding to lipids is a factor in the inhibitory effect. The failure of (^{3}H)DIDS to bind to lipids in the intact cells is also due to accessibility factors, because in ghosts the

binding is equal to that of protein (Table 1). Similar findings have recently been reported by Bretscher (1972) using another surface label.

In the intact cell, (^{3}H)DIDS binds substantially to only two components, the major sialoglycoprotein and the 95K protein (Figs. 4 and 5). Although a maximum of 7% of the binding is found to be associated with the sialoglycoprotein it is unlikely that these sites are associated with the inhibition. Low concentrations of pronase and other proteolytic enzymes that do not affect permeability, or change the inhibition of permeability produced by DIDS, can digest the sialoglycoprotein of the intact cell, removing most of the sugar moieties (PAS staining on acrylamide gels is eliminated), much of the sialic acid, and substantially all of the DIDS bound to this component (Cabantchik & Rothstein, 1974).

The only component of the membrane that might account for the DIDS-inhibition is in the 95K protein band. It binds about 95% of the DIDS at inhibitory concentrations, and the relationship of binding to inhibition is linear (Fig. 2). The latter finding suggests that the binding sites behave in a one-to-one fashion with respect to anion permeability, with each molecule of DIDS completely blocking one site. The only other possibility is that another component that also binds (^{3}H)DIDS in a linear relationship with inhibition is not observed by the reported procedures because the number of sites is below the limits of sensitivity, that is, less than 1 to 2% of the number of binding sites in the 95K protein.

The sites not only display a linear relationship to inhibitors but they also possess a relatively unique molecular architecture that has been explored in some detail by determining the inhibitory potencies of a series of analogues of the stilbenes (Cabantchik & Rothstein, 1972). It was concluded that the inhibition is associated with the electrostatic binding of the two sulfonic acid groups of the compounds to two positively charged groups on the membrane. Because addition of a hydrophobic group to one of the rings on the stilbene core, markedly increased the inhibitory potency, the membrane was presumed to contain a corresponding hydrophobic center near the positively charged groups. The presence of a covalent bonding group such as isothiocyanate is not required for inhibition. In fact, since the covalent bond formation occurs subsequent to the electrostatic binding, it is suggested that an affinity-labeling type of reaction takes place between the covalent probes and anion binding sites on the membrane. The covalent bonding could involve an amino group. The arylisothiocyano group forms stable covalent bonds (at pH values above neutrality) with amino or sulfhydryl groups but the latter is unlikely because Knauf and Rothstein (1971) found that anionic sulfhydryl reagents had no effect on anion permeability

and also because DIDS-binding (and the parallel inhibitory effect) is increased with pH, a property of the amino reaction. Thus, the inhibitory site involves a hydrophobic center, at least two positively charged groups (probably amino), and an adjacent group capable of covalent bonding, either amino or imidazole.

The DIDS-binding site has also been probed with a slowly penetrating anion pyridoxal phosphate, capable of Schiff base formation with amino groups (Cabantchik, Balshin & Rothstein, *submitted for publication*). The fixation of this anion to the outer surface of the cell membrane by short exposure to sodium borohydride results in a drastic inhibition of anion permeability. Furthermore, labeling studies with sodium borotritide show that the pyridoxal-phosphate is in fact bound to the 95K protein and that DIDS prevents this labeling. These recent results reinforce the concept that the inhibitory sites are the anion-binding sites of the transport mechanism.

The nature of anion transfer in the red cell is not precisely known, but it has been characterized as a specific saturable, anion exchange system (Gunn, Dalmark, Tosteson & Wieth, 1973), presumably involving an anion-binding site. The DIDS-site with its cluster of positive groups would be a good candidate for such an anion-binding site. The one-to-one relationship of (^{3}H)DIDS binding and inhibition would also be compatible with the hypothesis that the DIDS-site is the substrate binding site of the anion transferring system.

Although there seem to be many more 95K monomers (Fairbanks *et al.*, 1971) than DIDS-sites (1×10^6 *vs.* 3×10^5), it is quite possible that the unusually broad 95K band is a collection of different proteins (Cabantchik & Rothstein, 1974). Alternatively, or in addition, one cannot exclude the possibility that a single DIDS molecule may inhibit several anion binding sites either by the presence of its bulk or by creating a localized negatively charged field which will exclude small anions as chloride or sulfate or other DIDS molecules.

Certain properties of the 95K protein are of interest in terms of their potential role in anion permeability. Perhaps the most interesting is the probability that it is hydrophobically bonded. This conclusion is based on the finding that it is one of the most difficult proteins to solubilize and separate from the membrane (Tanner & Boxer, 1972). It is not extractable with high or low ionic strength, EDTA and high pH, and after extraction in 8 M urea, it and the sialoglycoprotein are virtually the only proteins that still remain insoluble and associated with lipid vesicles (Juliano & Rothstein, 1971). At the same time, it must, at least in part, be exposed on the outer surface of the cell, for it reacts with nonpenetrating agents such as DIDS

(Cabantchik & Rothstein, 1972) and others (Bender, Garan & Berg, 1971; Bretscher, 1971), is iodinated by peroxidase (Phillips & Morrison, 1971) and is digested by pronase (Bender *et al.*, 1971). In fact, no other protein component except the sialoglycoprotein is known to be so exposed.

Although the sialoglycoprotein is highly susceptible to proteolytic digestion in the intact cell (Triplett & Carraway, 1972) and is highly reactive to surface labels (Bretscher, 1971; Phillips & Morrison, 1971), its reaction with (^{3}H)DIDS is relatively small. With the nonspecific surface labels, the presence of phenolic-hydroxyls, carbohydrate hydroxyls, histidyl or lisyl residues exposed at the outer surface of the sialoglycoprotein (Segrest, Kahane, Jackson & Marchesi, 1973) will lead to substantial iodination or alkylation. The failure of (^{3}H)DIDS to react extensively with the glycoprotein may be attributed either to an electrostatic repulsion exerted by the negatively charged sialyl residues on the disulfonic stilbene core of (^{3}H)DIDS or to protection of the targets by the bulky and nonreactive carbohydrate chains.

The asymmetric arrangement of the 95K protein with the anion site toward the outside and with a major portion of the protein associated with lipids is suggested by two other sets of information. Firstly, if DIDS binding to cells is compared to binding to ghosts (compare Figs. 5 and 7), in the former case only the 95K protein and to a small extent the sialoglycoprotein are labeled, whereas in the latter virtually all membrane proteins are labeled (as well as lipids). In the ghosts, however, the labeling of the 95K protein is increased by 15 to 20% over that in the intact cell treated with the same amount of (^{3}H)DIDS (1 μM). Thus, the (^{3}H)DIDS-binding sites of the 95K protein appear to be more accessible or reactive from the outside of the cell. A similar asymmetry with DNFB has recently been found by Passow (*personal communication*). Secondly, careful treatment of the intact cell with proteolytic enzymes indicates not only that the 95K band is digested to 65K as demonstrated by Bender *et al.* (1971) and by Triplett and Carraway (1972), but that a 35K segment can also be recovered. The behavior of the 35K segment toward proteolysis, towards DIDS labeling, and towards urea extraction, leads to the conclusion that the 95K protein is associated with the membrane with much of the 65K segment hydrophobically associated with the lipid and with the 35K segment, electrostatically associated on the outside surface (Cabantchik & Rothstein, 1974). The hydrophobic portion seems to be associated with intralipid particles seen by freeze-fracture electron-microscopy based on experiments in which ferritin particles are covalently linked to membrane-bound DIDS and on parallel extraction of the 95K protein and of particles by nonionic detergents (Rothstein &

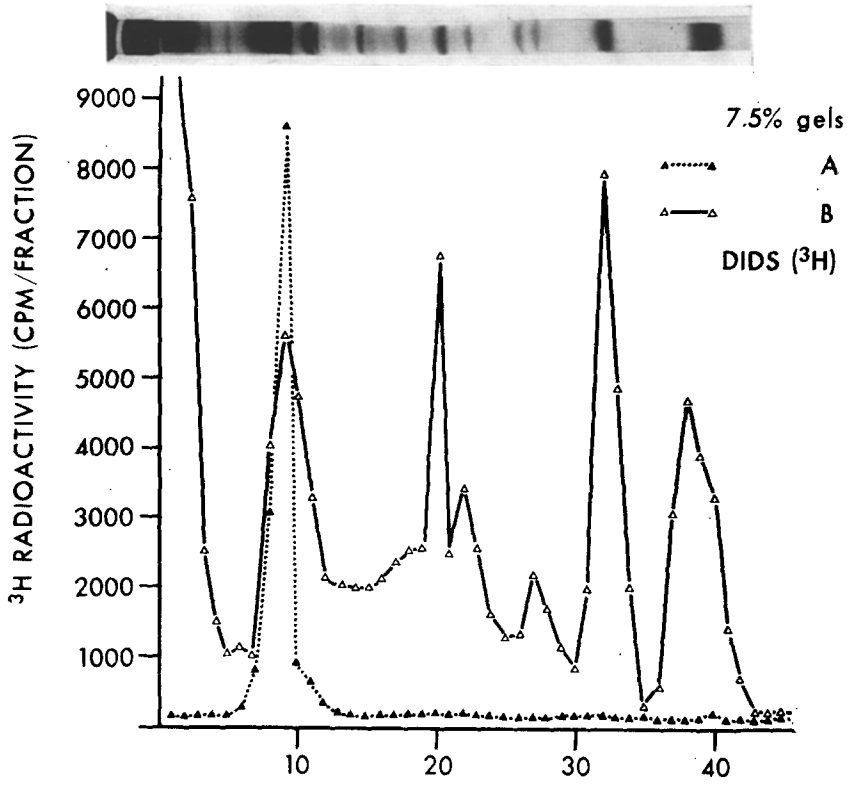

Fig. 7. The labeling of cells *vs.* labeling of ghosts. The labeling profile of red blood cells and isolated ghosts as seen by polyacrylamide-gel electrophoresis of membrane proteins and staining with Coomassie Blue. Intact cells and red blood cell ghosts were reacted with 1.0 μM (^{3}H)DIDS washed and analyzed as described in Materials and Methods. (*A*) Labeling of intact red blood cells (broken line). (*B*) Labeling of isolated ghosts (full line). The picture of the stained gel is from *A*

Cabantchik, 1973). These proteineous particles might provide the anion pathway through the lipid phase of the membrane. Bretscher (1971) has suggested on the basis of labeling studies in cells and ghosts, that the 95K protein spans the membrane.

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