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Interaction of Tritium-Labeled H_2DIDS (4,4′-Diisothiocyano-1,2,Diphenyl Ethane-2,2′Disulfonic Acid) with the Ehrlich Mouse Ascites Tumor Cell

Charles Levinson, Rebecca J. Corcoran, and Ellen H. Edwards

Department of Physiology, The University of Texas Health Science Center, San Antonio, Texas 78284

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Summary. The experiments reported in this paper were undertaken to explore the interaction of tritiated H_2DIDS (4,4′-diisothiocyano-1,2,diphenyl ethane-2,2′-disulfonic acid) with Ehrlich ascites tumor cells. Addition of (^{3}H)H_2DIDS to tumor cell suspension at 21 °C, pH 7.3, resulted in: (i) rapid reversible binding which increased with time and (ii) inhibition of sulfate transport. Tightly bound H_2DIDS, i.e., reagent not removed by cell washing, also increased with time. Binding of 0.02 nmol H_2DIDS/mg dry mass or less did not affect sulfate transport, but, at greater than 0.02 nmol and up to 0.15 nmol the relationship between tight binding and inhibition of transport is linear. The fact that H_2DIDS could bind to the cell and yet not affect anion transport suggests that binding sites exist unrelated to those concerned with the regulation of anion permeability. Support for this is the observation that H_2DIDS is spontaneously released from cells even after extensive washings by a temperature-sensitive process. The most important source of released H_2DIDS is the cell surface coat which labels rapidly (within 1 min) and is then spontaneously released into the medium. A second source is derived from H_2DIDS that slowly entered the cells. Consequently, at least four modes of interaction exist between H_2DIDS and ascites tumor cells. These include both reversible and irreversible binding to membrane components which regulate anion permeability, irreversible binding to cell surface proteins or glycocalyx, and finally incorporation of H_2DIDS into the intracellular phase.

Investigations concerning the regulation of anion permeability in the erythrocyte have lead to the hypothesis that a single class of transmembrane proteins is involved (Cabantchik & Rothstein, 1972, 1974, a,b; Zaki *et al.*, 1975; Lepke *et al.*, 1976; Passow, 1977). These proteins have an apparent molecular weight of 95,000 and are often referred to as Band 3 (Fairbanks, Steck & Wallach, 1971) from their position on sodium dodecylsulfate polyacrylamide gel electrophoretograms. Evidence supporting the involvement of Band 3 proteins has been obtained primarily from studies of the interaction of a group of substituted disulfonic acid stilbenes, potent and specific inhibitors of anion transport, with intact red blood

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cells or ghosts (Cabantchik & Rothstein, 1975; Rothstein, Cabantchik & Knauf, 1976). One of the substituted stilbenes, DIDS (4,4′-diisothiocyano-stilbene-2,2′-disulfonic acid) and its dihydro-derivative H_2DIDS (4,4′-diisothiocyano-1,2,diphenyl ethane-2,2′-disulfonic acid) have been particularly useful. These agents do not enter the red blood cell but have the capacity to bind both reversibly and irreversibly (covalently) to the membrane and in so doing inhibit both chloride and sulfate transport. The extent of transport inhibition is linearly related to the number of molecules bound to Band 3 proteins (Rothstein *et al.,* 1976; Lepke *et al.,* 1976; Ship *et al.,* 1977).

These stilbenes and the related compound SITS (4-acetamido-4′-isothiocyano-stilbene,2,2′-disulfonic acid) have been shown to be inhibitors of anion transport in turtle bladder (Ehrenspeck & Brodsky, 1976) snail neuron (Thomas, 1976), squid giant axon (Russell & Boron, 1976), and rabbit kidney slices (Hong *et al.,* 1978).

In Ehrlich ascites tumor cells, SITS when bound to the cell is a potent inhibitor of sulfate self-exchange but is without effect on either chloride or phosphate transport (Villereal & Levinson, 1976). In a more recent study (Levinson, 1978), we demonstrated that H_2DIDS when present in the medium inhibits both chloride and sulfate transport. However, if H_2DIDS is removed from the environment and the cells washed, only sulfate transport is inhibited by H_2DIDS remaining associated with the cells. This differential effect of H_2DIDS led to the postulation that a single transport system possessing two sites might be responsible for regulating anion permeability in this cell.

Only the nonradioactive analog of H_2DIDS was used in the previous experiments. Consequently, no information concerning the kinetics of interaction between H_2DIDS and the tumor cell, the number of binding sites, or the relationship between bound H_2DIDS and inhibition of anion transport was obtained. Accordingly, in the experiments to be presented we have used the tritiated derivative of H_2DIDS to explore in more detail the interaction of this agent with Ehrlich ascites tumor cells.

Materials and Methods

Preparation of Tritium-Labeled H_2DIDS

DADS (4,4′-diamino-stilbene-2,2′-disulfonic acid) obtained commercially (K & K Laboratories, Plainview, N.Y.) was purified by dissolving it in water with the addition of 1 N NaOH and stirring for 5 min with activated charcoal. The charcoal was removed by filtration. The clarified solution was heated to 40 °C, and after the addition of $MgCl_2$ (1 g/g DADS), cooled in an ice bath. The precipitate was collected on a Buchner funnel, dissolved in water, and the solution then cooled to about 10 °C. Upon the addition of a small volume of cold 6 N HCl, a white precipitate formed which was washed twice with cold absolute ethanol. The yield was 30–38% of the starting material. Analysis of the compound calculated for $C_{14}H_{14}N_2S_2O_6$ is: 45.4% C; 3.8% H; 7.6% N; 17.3% S; found: 44.2% C; 3.7% H; 7.2% N; 17.4% S (Chemalytics, Tempe, Ariz.).

The di-tritio derivative of DADS [(^{3}H)H_2DADS] was prepared by catalytic reduction of the inner ring double bond of purified DADS using tritium gas (Amersham, Arlington Heights, Ill.). The specific activity was 1.6 Ci/mmol. Dihydro-DADS (H_2DADS) was prepared from the catalytic reduction of DADS using H_2 gas. DADS, dissolved in water at pH 7.0, was stirred with Palladium-Carbon (3 mg/mg DADS; Englehard Industries, Newark, N.J.) and H_2 gas bubbled slowly through the mixture for 60 min. After filtration and cooling to 0 °C, a small volume of N HCl was added and the precipitate washed as described. The yield is 43–48% of the starting material. Analysis of H_2DADS calculated for $C_{14}H_{16}N_2S_2O_6$ is: 45.2% C, 4.3% H, 7.5% N, 17.2% S; found: 46.8% C, 4.6% H, 7.3% N, 17.2% S. The absorption spectra of DADS, H_2DADS and (^{3}H)H_2DADS are shown in Fig. 1.

Catalytic reduction of DADS to form the dihydro- or ditritio-derivative results in significant changes in the absorption spectrum. DADS exhibits a single absorption maximum at 340 nm and at pH 7.0 the molar extinction is 3.1×10^4. The dihydro- and ditritio-derivatives under the same conditions exhibit two maxima, 245 and 295 nm. The molar extinction coefficient at 245 nm is 2.05×10^4. This change in absorbance, that is, the elimination of the 340-nm peak, was used to verify that the reduction of the inner double bond of DADS by tritium or H_2 was complete.

The diisothiocyanate derivative of both (^{3}H)H_2DADS and H_2DADS was prepared by reacting the precursors (65–100 μmol) at pH 6.8–7.0 with a 50-fold excess of thiophosgene (Aldrich Chemical Company, Milwaukee, Wisc.) with vigorous stirring for 30 min at room temperature (Lepke *et al.*, 1976). Excess thiophosgene was removed by repeated extraction with ether and the aqueous phase dried in a vacuum dessicator over P_2O_5 and KOH. The dry yellow-amber solid was stored in a dessicator at −20 °C. The specific activity of

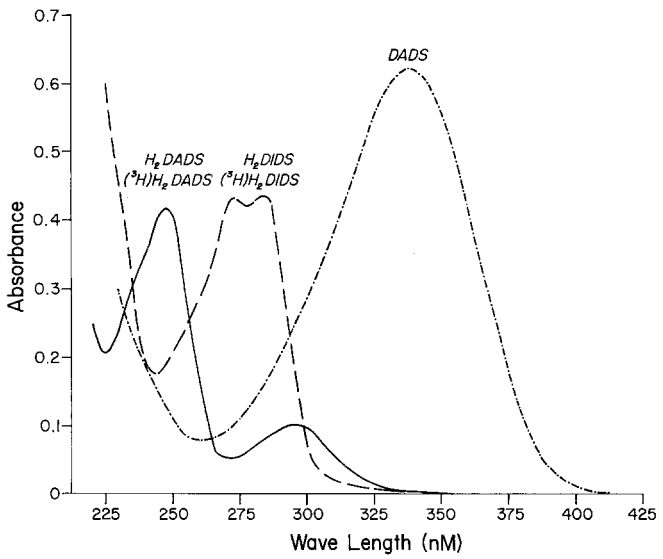

Fig. 1. Absorption spectra of DADS, H_2DADS, H_2DIDS and (^{3}H)H_2DIDS. Absorbance is plotted *vs.* wavelength (nm). All compounds were dissolved in 0.055 M phosphate buffer, pH 7.0

(^{3}H)H_2DIDS is assumed to be the same as that of its precursor (^{3}H)H_2DADS, since the introduction of isothiocyanate groups should not alter the amount of tritium contained within the molecule. The absorption spectra of (^{3}H)H_2DIDS and H_2DIDS shown in Fig. 1 are identical and exhibit maxima at 273 and 285 nm. The molar extinction coefficient is 3.45×10^4.

Experimental Procedures

Cell Suspension: Experiments were performed with Ehrlich-Lettré ascites tumor cells (hyperdiploid strain) which were maintained in Ha/ICR male mice by weekly transplantation. Tumor bearing animals with growth of between eight and eleven days were used. Cells were removed from unanesthetized animals by peritoneal aspiration and washed free of ascitic fluid by gentle centrifugation and resuspension. The wash and incubation medium had the following composition: 135 mM NaCl, 10 mM Na_2SO_4, 6 mM KCl and 10 mM Hepes-NaOH (pH 7.2–7.3; 296–301 mosm). Cell suspensions (20–40 ml; 14–17 mg dry mass/ml) were incubated in 1 liter Ehrlenmeyer flasks under an air atmosphere at 22–25 °C on a gyrorotary shaker set for 45 oscillations per min. The length of incubation depended on the nature of the experiment. After equilibration the cell suspension was centrifuged at $1,200 \times g$ and the packed cells resuspended to the same cell density with fresh medium. A small aliquot of cell suspension was mixed with medium containing 5 mg/100 mg Trypan Blue. Routinely 2 to 5% of the cells stained, which indicated that at least 95% were viable.

H_2DIDS Binding: Total H_2DIDS binding (reversible plus tight) to the tumor cell was assessed by adding (^{3}H)H_2DIDS to cell suspension. Periodically during the next 90 min samples of cell suspension were removed and centrifuged at $12,000 \times g$ for 1 min. An aliquot of the supernatant was diluted 1:100 with water and subsequently assayed for ^{3}H-activity by liquid scintillation counting.

H_2DIDS not readily removed from the cells by washing was determined by incubating cell suspension with (^{3}H)H_2DIDS for between 1 and 90 min. The interaction between H_2DIDS and the tumor cells was stopped by the addition of ice-cold medium containing 0.5% bovine serum albumin (Sigma Chemical Co.). Usually 40 ml of cold albumin wash solution was added per 10 ml cell suspension. The cells were separated from the medium by centrifugation at $1,500 \times g$ for 1 min. The packed cells were then washed once in ice-cold albumin wash solution, twice in ice-cold albumin-free medium, and finally resuspended in medium at 23 °C. One-ml aliquots were removed for the determination of wet and dry weight (Levinson & Villereal, 1975). Additional aliquots were removed, centrifuged, and after aspiration of the supernatant, Protosol (New England Nuclear, Boston, Mass.) was added. Usually 3 hr at 50–55 °C was necessary to completely digest the cell pellet. The Protosol digest was then assayed for ^{3}H-activity by liquid scintillation counting.

Anion Transport: To study the effect of H_2DIDS on anion transport, two types of experiments were performed. In the first, designed to study the effect of tightly bound H_2DIDS, i.e., H_2DIDS not removed by the standard washing procedure, cell suspension equilibrated for at least 90 min was divided into two portions. To one, (^{3}H)H_2DIDS was added, while the second served as the control. After incubation for from 1 to 90 min at 22 °C the cells were washed and resuspended in H_2DIDS-free medium as described. Aliquots were removed for wet and dry weight analysis and for the determination of H_2DIDS binding.

The initial influx of $^{35}SO_4$ (New England Nuclear) was then measured as follows. One ml of cell suspension was added to each of a series of centrifuge tubes containing 3 ml of medium. At time zero and at 1-min intervals, 0.1 ml $^{35}SO_4$ solution (15 μCi/ml) was added. After 5 min, ice-cold isosmotic choline dihydrogen citrate solution (30 g CDHC in 900 ml H_2O; pH adjusted to pH 7.2 with conc. NH_4OH and made to 1 liter) was rapidly added and the cell samples centrifuged for 30 sec at 1,500 × *g*. The wash solution was decanted and the cells washed once. To correct for $^{35}SO_4$ trapped between the cells as well as that transported during centrifugation, a "zero time" sample was prepared along with the experimental samples. Seven milliliters of ice-cold CDHC was solution containing 0.1 ml $^{35}SO_4$ was rapidly added to a mixture of 1 ml cell suspension and 3 ml medium. This sample was centrifuged and the cells washed along with other cell samples. The $^{35}SO_4$ radioactivity found in this "zero time" sample was presumably present in all other cell pellets from the same experiment and consequently subtracted from each.

The packed washed cell pellets were mixed with 3.5 ml of 5% (vol/vol) ice-cold perchloric acid and maintained in an ice bath for at least 30 min. All samples were subsequently centrifuged at 1,500 × *g* for 5 min to remove the perchloric acid insoluble residue.

The second type of transport experiment, designed to study the effect of H_2DIDS when present in the medium, was performed in essentially the same manner except that cell suspension was added to centrifuge tubes containing (^{3}H)H_2DIDS or H_2DIDS and the uptake of $^{35}SO_4$ measured immediately thereafter. Radioactivity of the perchloric acid extract of the cells was measured by liquid scintillation counting. $^{35}SO_4$ was counted in the presence of ^{3}H by adjusting the discriminator settings so that ^{3}H activity was confined to only one channel.

The exchange flux of SO_4^{2-}, expressed as mmol per kg dry wt min^{-1}, was calculated as described previously (Villereal & Levinson, 1976).

Results

If (^{3}H)H_2DIDS is to serve as a tracer for H_2DIDS, then it is important to establish that both compounds have identical properties. As noted in the *Methods* section the absorption spectrum of (^{3}H)H_2DIDS is indistinguishable from that of H_2DIDS. These two compounds were also identical on thin layer chromatograms utilizing the solvent system *n*-propanol/NH_4OH/water (6:3:2; Cabantchik & Rothstein, 1972). Both compounds are effective inhibitors of steady-state SO_4^{2-} exchange. The data given in Fig. 2 which shows the relationship between SO_4^{2-} transport and the concentration of either (^{3}H)H_2DIDS or H_2DIDS in the medium, demonstrates that on a molar basis both compounds inhibited transport to the same extent. Furthermore, as shown in Fig. 3, the amount of (^{3}H)H_2DIDS bound to the tumor cell is reduced in a proportional manner by dilution with H_2DIDS, thereby demonstrating its use as a tracer for binding sites.

The inhibition of anion transport by DIDS or H_2DIDS in erythrocytes suggested that irreversible binding of these compounds to the membrane was preceded by reversible interaction. The occurrence of a similar pattern is demonstrated in the experiments presented in Fig. 4. In these experiments the total amount of H_2DIDS associated with the cell as a function of time was determined from the decrease in H_2DIDS concentration in the medium. Irreversible binding,

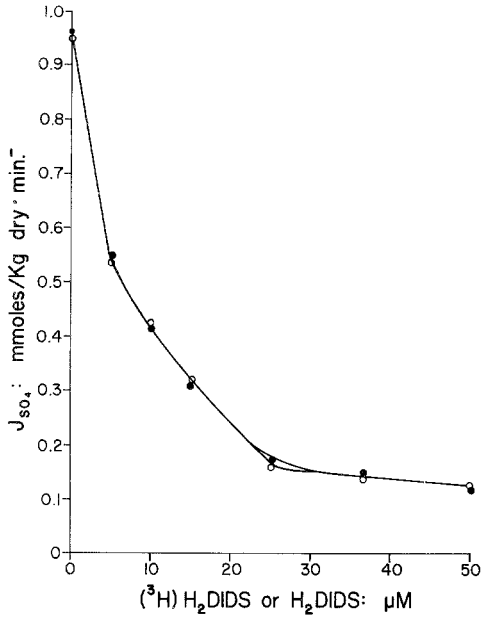

Fig. 2. Inhibition of SO_4^{2-} transport ($J_{SO_4}{}^2$: mmol/kg dry mass·min^{-}) by H_2DIDS or (^{3}H)H_2DIDS when present in the medium. Cell suspensions equilibrated for 90 min at 21–23 °C were added to medium containing either H_2DIDS (closed circles) or (^{3}H)H_2DIDS (open circles). Final concentration of reagent was from 5–50 μM. (^{35}S)SO_4-uptake was then measured each min for the next 5 min and the flux determined

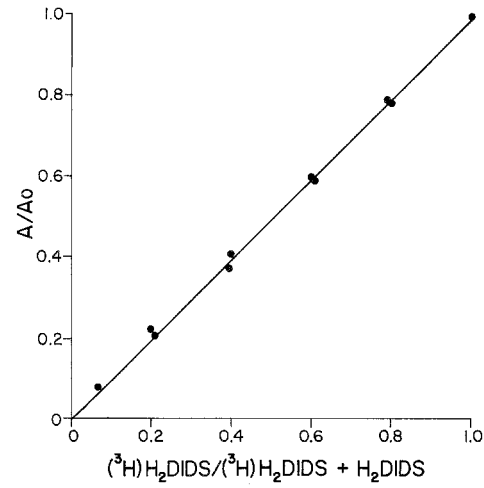

Fig. 3. Effect of diluting (^{3}H)H_2DIDS with H_2DIDS on the labeling of ascites cells. The ratio of ^{3}H activity determined with each diluted sample to that found with undiluted (^{3}H)H_2DIDS (*A/AO*) is plotted *vs.* the ratio of (^{3}H)H_2DIDS to the sum (^{3}H)H_2DIDS + H_2DIDS. The total concentration was 25 μM. Cells were incubated with the reagent for 30 min at 21 °C. Following the standard washing procedure, they were digested with Protosol and assayed for ^{3}H-radioactivity

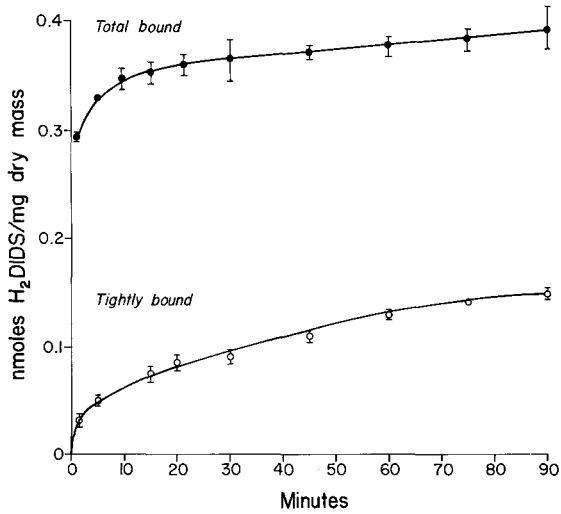

Fig. 4. Total H_2DIDS binding as a function of time at 21–23 °C. The upper curve (closed circles) represents the sum of reversible (readily washed away) plus tightly bound. The lower curve (open circles) represents tightly bound H_2DIDS, i.e., not removed after the standard washing procedure. The H_2DIDS concentration was 25 μM. Error bars = ± 1 SEM

i.e., H_2DIDS not removed by the standard washing procedure, was determined by measuring the (^{3}H)H_2DIDS associated with the same cells. The reaction between H_2DIDS and the cells was stopped by the addition of ice-cold medium containing 0.5% serum albumin and subjecting the cells to the standard washing procedure. The cells were digested with Protosol prior to the measurement of (^{3}H)H_2DIDS.

Note that the addition of H_2DIDS to the cell suspension resulted in a rapid association of the probe with the cells. For example, within one minute after the addition of H_2DIDS the total binding amounted to approximately 0.30 nmol/mg dry mass. Binding, however, continued to increase and by 90 min had reached 0.40 nmol/mg dry mass. H_2DIDS molecules not removed by cell washing and therefore tightly bound, also increased during the entire period of experimental observation. Within 1 min after H_2DIDS addition this binding was 0.024 ± 0.007 nmol/mg dry mass and increased to 0.151 ± 0.02 by 90 min.

The fact that both total H_2DIDS binding as well as that not removed by cell washing increased with time suggests that H_2DIDS enters the tumor cell or that this cell possesses more than a single population of reactive sites, or both.

To assess the relationship between H_2DIDS binding and the effect on SO_4^{2-} transport, tumor cells were exposed to 25 μM H_2DIDS at 22 °C. After incubation for from 1 to 90 min the interaction between H_2DIDS and the cells was stopped by the standard washing procedure. The cells were then resuspended at 22–24 °C and aliquots removed for the measurement of bound H_2DIDS and SO_4^{2-} transport. The data presented in Fig. 5 shows that the binding of 0.02 nmol H_2DIDS/mg dry mass or less was without detectable effect on transport. However, at

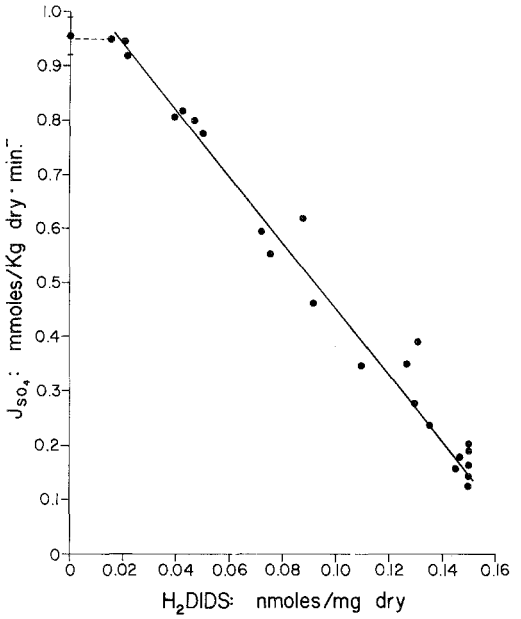

Fig. 5. The relationship between SO_4^{2-} transport ($J_{SO_4}^{2-}$: mmol/kg dry mass·min) and H_2DIDS tightly bound (nmol/mg dry mass). Cells were incubated with H_2DIDS (25 μM) for from 1 to 90 min at 21 °C and subjected to the standard washing procedure. The SO_4^{2-} flux and bound H_2DIDS were then measured

greater than 0.02 nmol the relationship between binding and inhibition of transport is linear. Under these experimental conditions the binding of 0.15 nmol was associated with 85–88% inhibition of transport. The observation that a fraction of bound H_2DIDS was without detectable effect on transport raised the suspicion that these cells possessed a population of binding sites distinctly different from those concerned with regulating SO_4^{2-} permeability.

To this point in the investigation we had assumed that exposure of cells to H_2DIDS followed by the standard washing procedure removed all of the reversibly bound probe. Consequently, that remaining would be irreversibly fixed to membrane binding sites. However, it has recently been shown in these cells that surface associated glycoproteins are slowly released when cells are incubated in physiological salt solution (Rittenhouse, Rittenhouse & Takemoto, 1978). This raised the possibility that a fraction of the H_2DIDS bound to the cells might be associated with these surface components and that these constituents represent nontransport related binding sites. To test this possibility cells were exposed to 25 μM H_2DIDS for 90 min at 21 °C. After standard washing the cells were resuspended and incubated in H_2DIDS-free medium at 1, 23, and 37 °C. The amount of H_2DIDS associated with the cells after washing ranged from 0.135–0.152 nmol/mg dry mass. Periodically during the next 2.5 hr samples of cell suspension were removed, centrifuged at 15,000 × g for 1 min, and the supernatant was assayed for ^3H-radioactivity. The viability of the cells as judged by Trypan Blue exclusion remained at between 94–96%. The results of these experiments, shown in

Fig. 6, demonstrate that H_2DIDS is released from washed cells and that the rate of release is temperature sensitive. For example, after 2 hr incubation H_2DIDS release expressed as nmol/mg dry mass, was 0.005 at 1 °C and 0.030 at 23 °C. Experiments conducted at 37 °C were limited to 60 min because of the deleterious effects of prolonged incubation at this temperature on cell viability. However, by 60 min at 37 °C approximately the same amount of H_2DIDS was released to the medium as that observed after incubation for 2 hr at 23 °C. The amount of H_2DIDS lost from the cells expressed as the fraction of H_2DIDS associated with the cell after washing was 21.7% (37 °C; 1 hr), 20.3% (23 °C, 2 hr) and 3.5% (1 °C, 2 hr). If we assume that washing removes reversibly bound H_2DIDS then these results are consistent with the contention that H_2DIDS released into the medium is derived from superficial binding sites or glycocalyx. They, however, do not rule out the possibility of loss from an intracellular pool.

If H_2DIDS-labeled surface proteins were in fact slowly lost from washed cells, then this material should be recoverable from the medium. To test this, cell suspensions were exposed to 25 μM H_2DIDS for from 1 to 90 min. After subjecting the cells to the standard washing procedure, they were resuspended in medium and incubated for 60 min at 22 °C. The cells were then separated from the medium by low speed centrifugation, and the supernatant was centrifuged twice at 50,000 × g for 60 min at 0 °C. The supernatant was dialyzed for 40–48 hr against cold water, lyophilized, and the residue dissolved in 2% sodium dodecyl sulfate solution. Aliquots were removed for protein analysis using bovine serum albumin as the standard (Hartree, 1972) and for the assay of ^{3}H-activity. Table 1 summarizes the results of these experiments. Note that the protein recovered in the supernatant represented only about 0.75% of the total starting cell protein. The specific radioactivity of the whole cell protein increased with increasing time of exposure to H_2DIDS. This reflects the time-dependent nature of H_2DIDS binding. By contrast, the specific radioactivity of the protein recovered in the supernatant remained virtually constant after 5 min. A comparison of the specific activities of the whole cell protein to that of the supernatant protein is revealing. For example, after 1 min exposure to H_2DIDS followed by washing, the protein released from the cell into the medium had a specific activity 21 times that of the whole cell. However, with increasing times of exposure this ratio declined and after 90 min was 8.3.

The fact that the specific activity of the released protein is almost constant after 5 min exposure to H_2DIDS while that of the whole cell increased with time indicates that H_2DIDS reacts first with loosely adherent surface proteins and then with other cellular constituents.

Although the data shown in Table 1 demonstrates that H_2DIDS released from washed cells (Fig. 6) is at least in part associated with surface protein, we could not discount an intracellular source. Because of this uncertainty it was necessary to determine whether H_2DIDS entered ascites tumor cells under the conditions employed in our labeling studies.

To assess this possibility, cells were incubated for 60 min in medium containing 1 mM ^{14}C-labeled α-amino-isobutyric acid (α-AIB), washed twice, and resuspended in α-AIB-free medium. This amino acid, although actively transported by the Na^+-dependent pathway, is not metabolized by the tumor cell and therefore

Table 1. Release of H_2DIDS-labeled surface proteins

Time in (^{3}H)$_2$DIDS (min)	Protein recovered in supernatant (mg)	% Total cell protein	SA:total cell protein	SA:protein recovered in supernatant
			(cpm/ng protein × 10^{-5})	
1	1.49	0.74	0.91	19.8
5	1.38	0.75	1.42	24.1
30	1.18	0.74	2.12	26.1
60	1.30	0.77	2.68	27.5
90	1.63	0.84	3.09	25.5

Cell suspension (25 mg protein/ml) was incubated at 21–23°C with 25 μM (^{3}H)H_2DIDS for from 1–90 min. Following the standard washing procedure, the cells were resuspended in fresh medium and an aliquot was removed for the analysis of protein and ^{3}H-radioactivity. After incubation for 60 min the cells were pelleted at 3,000 × *g* for 5 min and the supernatant was centrifuged twice at 50,000 × *g* for 60 min. After dialysis and lyophilization, the protein and ^{3}H-radioactivity were measured.

accumulates in the cell water (Jacquez, Sherman & Terris, 1970; Jacques, 1973). (^{3}H)H_2DIDS was then added to a final concentration of 25μM, and the cells were incubated for 90 min at 22 °C. After subjecting the cells to the standard washing procedure, they were resuspended and incubated in medium at 23 °C. More than 96% of the cells excluded Trypan Blue, which indicated a high percentage of viable cells. Periodically during the next 29 min samples of cell suspension were removed, centrifuged at 15,000 × *g* for 1 min, and the supernatant was assayed for both ^{14}C and ^{3}H-radioactivity. At 30 min a small volume of medium containing the lytic agent, saponin, was added to one-half of the cell suspension, and sampling continued for an additional 30 min. Results from a representative experiment are shown in Fig. 7.

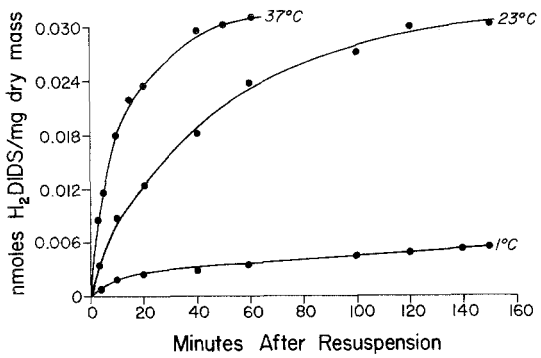

Fig. 6. Release of H_2DIDS (nmol/mg dry mass) as a function of time and temperature. Cells were incubated with 25 μM $^3H_2$DIDS for 90 min at 23 °C, washed, and resuspended in medium maintained at 1, 23, or 37 °C. The appearance of H_2DIDS in the medium was then measured as a function of time

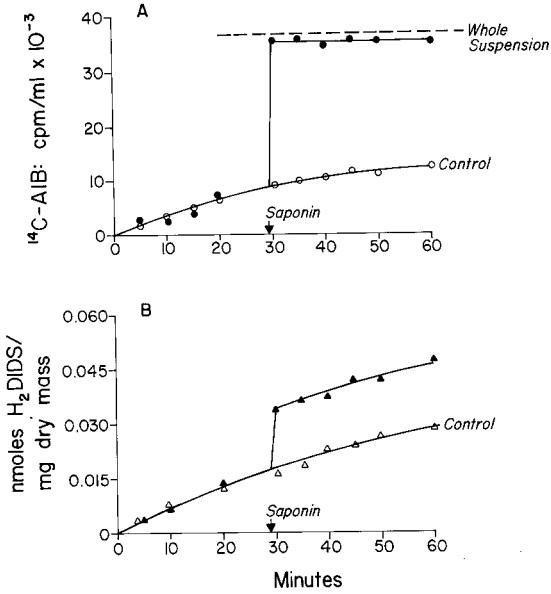

Fig. 7. Simultaneous release of (^{14}C)–AIB and H_2DIDS by saponin. Cells were incubated in medium containing 1 mM ^{14}C-AIB for 60 min at 22 °C, washed twice with cold medium, and incubated an additional 90 min with 25 μM H_2DIDS at 22 °C. Following the standard washing procedure, the cells were resuspended in medium and the appearance of H_2DIDS and AIB measured as a function of time. At 30 min saponin (100 μg/ml cell suspension) was added. (*A*): Time course of AIB release; (*B*): time course of H_2DIDS release

Incubation of cells labeled with both H_2DIDS and α-AIB resulted in the slow release of both agents into the environment. In the case of α-AIB the intracellular phase was the source of this loss since there is no evidence that this amino acid binds to the cell surface. However, with H_2DIDS both intracellular as well as that associated with the cell surface are potential sources. the addition of saponin caused within 1 min the release of 98.6% of the total cellular pool of α-AIB and greatly stimulated the release of H_2DIDS. Furthermore, all cells exposed to saponin immediately stained with Trypan Blue. These data are consistent with the suggestion that saponin very rapidly and in a nonspecific manner increases the permeability of the plasma membrane. Consequently α-AIB which is confined to the intracellular phase diffuses from the cell to the medium. Similarly H_2DIDS, which entered the cells during this labeling period, diffuses out. The continuous loss of H_2DIDS to the medium is presumably related to the release of labeled surface components.

Additional data supporting the view that H_2DIDS enters these cells is illustrated in Fig. 8. In these studies cells were incubated with 25 μM H_2DIDS for from 1 to 90 min, washed, and resuspended. The release of H_2DIDS from the cells to the medium was measured for 30 min prior to and 30 min after the addition of saponin. Note that the amount of H_2DIDS released upon the addition of saponin increased with increasing length of time of exposure to H_2DIDS. For example, after 1 min exposure, saponin addition resulted in only an insignificant

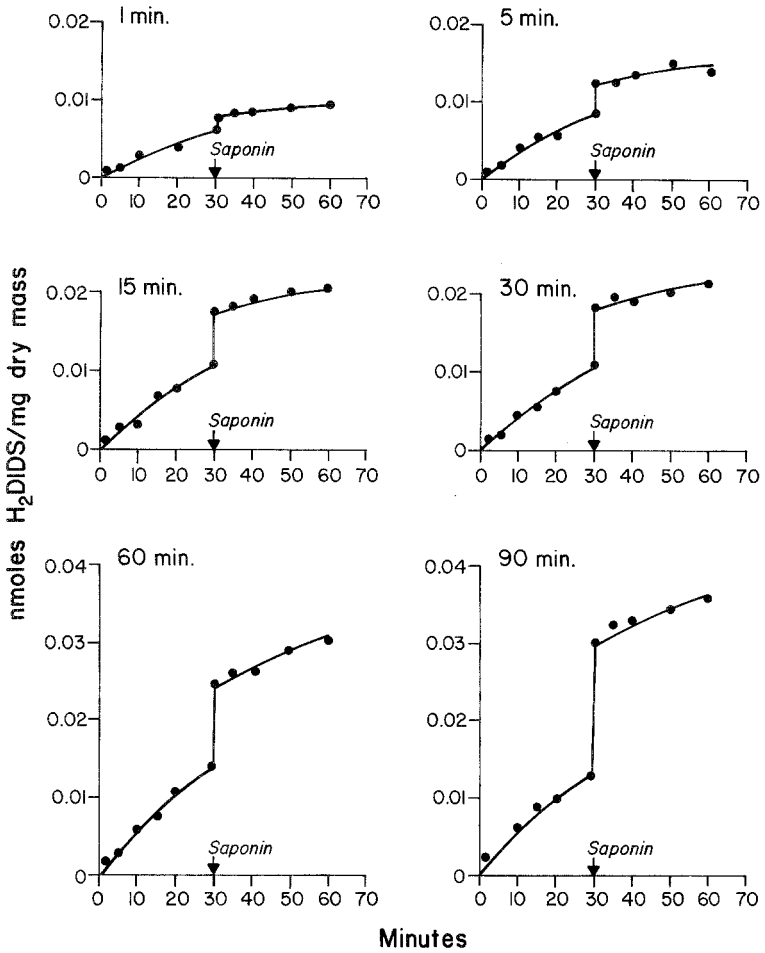

Fig. 8. Release of H_2DIDS (nmol/mg dry mass) induced by saponin as a function of time of exposure to H_2DIDS at 21 °C. Cells were incubated with 25 μM H_2DIDS for 1 to 90 min, washed, and resuspended in fresh medium. At 30 min after resuspension, saponin (100 μg/ml cell suspension) was added

amount to be released, while after 90 min more than 10% of the total H_2DIDS associated with cells after washing was released (0.017 nmol/mg dry mass out of a total of 0.152 nmol/mg).

While the addition of saponin to H_2DIDS-labeled ascites tumor cells resulted in the enhanced release of H_2DIDS, it was conceivable that this represented nothing more than an accelerated rate of release of a labile cell surface component. However, the data from a representative experiment shown in Fig. 9 shows this not to be the case. In these experiments, cell suspension was incubated at between 1–5 °C with 25 μM H_2DIDS for 30 min. Following the standard washing procedure, the cells were resuspended at 23 °C and the release of H_2DIDS was measured both before and after the addition of saponin. Note that the addition of saponin did not result in a significant increase in H_2DIDS release. This suggests

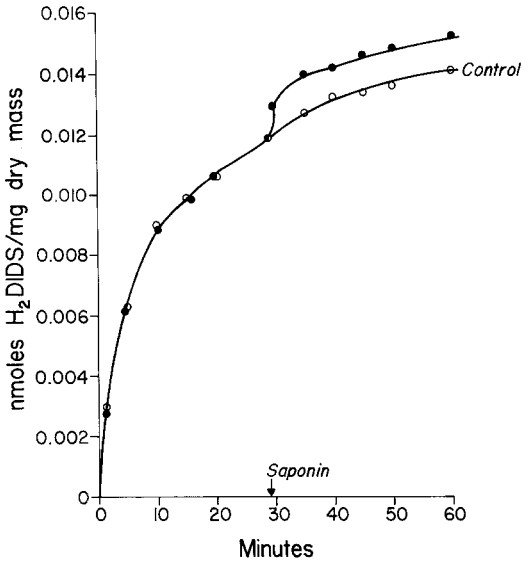

Fig. 9. Release of H_2DIDS (nmol/mg dry mass) as a function of time. Cells were incubated with 25 μM H_2DIDS for 30 min at from 1–5 °C. After the standard washing procedure they were resuspended at 23 °C and the release of H_2DIDS was measured. Saponin (100 μg/ml cell suspension) was added at 29 min

that incubation at a reduced temperature greatly depressed the incorporation of H_2DIDS into the intracellular phase but did not prevent H_2DIDS from interacting with surface binding sites.

Inhibition of SO_4^{2-} transport by H_2DIDS presumably occurs because of its irreversible fixation to surface membrane sites involved with the regulation of this process. However, since the data shown in Figs. 7 and 8 raises the possibility that H_2DIDS enters the ascites tumor cell, it is conceivable that inhibition of transport is a consequence of intracellular binding. To explore this possibility the effect of intracellular H_2DIDS on SO_4^{2-} transport was investigated. Cells were incubated in the presence and absence of H_2DIDS for 90 min at 21 °C. They were then washed, resuspended, and periodically during the next two hours aliquots were removed for the measurement of SO_4^{2-} transport and intracellular H_2DIDS. Intracellular H_2DIDS were estimated by measuring that release from the cells on the addition of saponin (*see* Figs. 7 and 8). the results of these experiments are summarized in Fig. 10. Even though the intracellular H_2DIDS content declined 70%, from 0.023 nmol/mg dry mass at 3 min after resuspension to 0.007 nmol at 120 min, SO_4^{2-} transport remained inhibited by 83%. In other words, a marked reduction in H_2DIDS content did not relieve the inhibition of SO_4^{2-} transport.

Discussion

Previous studies from our laboratory established that H_2DIDS when reversibly associated with the ascites tumor cell inhibited the transmembrane movement of

both Cl and SO_4^{2-}. However, prolonged exposure to H_2DIDS followed by extensive washing resulted in the inhibition of SO_4^{2-} but not Cl^- transport (Levinson, 1978). The observation that inhibition increased with increasing time of exposure to H_2DIDS suggested that this agent irreversibly interacted with and thereby blocked membrane sites responsible for the regulation of anion permeability. The experiments reported in this paper were undertaken to further explore the nature of the interaction of H_2DIDS with ascites cells.

Specifically we were interested in establishing the time course of H_2DIDS labeling, the number of binding sites, and the relationship between binding and inhibition of SO_4^{2-} transport.

Time Course of H_2DIDS Labeling

The addition of H_2DIDS to a suspension of ascites tumor cells results in a rapid uptake that can be partitioned into at least two phases. The first represents an almost instantaneous association that is complete by the time the first experimental sample was obtained, i.e., 1 min (Fig. 4). The second phase is considerably slower and, in fact, continued to increase throughout the 90-min period of experimental observation. The initial rapid association of H_2DIDS with the ascites cell accounts for approximately 80% of that measured at 90 min. If only a single population of superficial binding sites reacted with H_2DIDS, then one would expect to observe only a single rapid phase of association. Rather, these results suggested that either more than one population of binding sites were present or that this probe entered the cells or both. Much of the H_2DIDS could be removed from the cells by washing with albumin-containing media which indicates a reversible type of interaction. However, the amount removed was dependent on the length of time the cells were exposed to H_2DIDS. For example, when washing was initiated after 1 min exposure, 92% could be removed. If, on the other hand, cells were washed after 90 min, only 60% was removed by washing.

The fact that H_2DIDS can be removed from cells by washing indicates that covalent interaction is not a prerequisite for binding.

Both reversibly as well as tightly bound H_2DIDS inhibit SO_4^{2-} transport. In the case of reversibly bound H_2DIDS, inhibition occurs without delay and thereby reflects reversible interaction of the probe with membrane sites governing SO_4^{2-} permeability (Fig. 2). This, of course, does not discount the possibility of interaction with other populations of binding sites. As noted above, incubation of cells with H_2DIDS for increasing periods of time leads to increasing amounts of H_2DIDS tightly bound. This suggests a time-dependent transition from reversible to tight binding. Tightly bound H_2DIDS is also a potent inhibitor of SO_4^{2-} transport. However, the observation that some H_2DIDS molecules can bind to the cell and yet not affect transport also argues for the presence of more than a single class of receptive sites (Fig. 5).

Binding of H_2DIDS to Nontransport Related Sites

Numerous studies have appeared in the past few years that suggest that cellular components, primarily glycoproteins, associated with the cell surface coat or gly-

cocalyx are spontaneously released into the cellular environment (Kim *et al.*, 1975). Although the cell surface coast does not act as a permeability barrier to small molecules, this layer undoubtedly plays a major role in cell adhesion, cell recognition, and the establishment of the antigenic properties of cells (Stuhlmiller & Siegler, 1977). The glycocalyx is a labile structure that can be removed from a variety of cells, including tumor cells, by such mild treatment as repetitive cell washing (Kilarski, 1975). For example, in Ehrlich ascites tumor cells it has recently been shown that more than 50% of the surface-iodinated proteins from lactoperoxidase-labeled cells are spontaneously released into physiological salt solutions after incubation for 1 hr at 4 °C (Rittenhouse *et al.*, 1978). This surface layer was characterized by the presence of a highly active aminopeptidase, a variety of glycoproteins, and glycoaminoglycans. When compared to isolated, purified plasma membranes, the glycocalyx fraction possessed virtually no Na^+/K^+-ATPase activity and little or no cholesterol or sialic acid. Clearly, this material is biochemically different from plasma membrane. The demonstration that elements of the cell surface, including proteins, can be spontaneously "shed" by Ehrlich ascites tumor cells supports the contention that H_2DIDS spontaneously released from washed cells (Fig. 6) and bound to protein (Table 1) represents desorption of cell surface materials. This view is strengthened by the fact that the specific radioactivity of the protein released into the medium from H_2DIDS-labeled cells was practically independent of the initial time of exposure of cells to H_2DIDS. This suggests that H_2DIDS labels superficial surface binding sites much more rapidly than the membrane sites involved in the regulation of SO_4^{2-} transport (Table 1, Fig. 5).

Intracellular H_2DIDS

The results of a number of studies have led to the proposition that certain substituted stilbenes (SITS, DIDS and H_2DIDS) because of their electronic charge and size interact with a small population of surface binding sites of the red blood cell but do not cross the cell membrane. This is based primarily on the observations that (i) very little reagent is found associated with hemoglobin, (ii) uptake is complete within short periods of time, and (iii) in intact cells maximal binding involved only a small number of surface receptive sites while red cell ghosts exposed to the same concentration of probe bound ten times as much (Maddy, 1964; Knauf & Rothstein, 1971; Cabantchik & Rothstein, 1975; Lepke *et al.*, 1976; Ship *et al.*, 1977).

In our previous studies we assumed that both SITS and H_2DIDS were unable to cross the ascites tumor cell membrane and that the inhibitory effects of these agents on anion transport were due solely to interaction with membrane sites (Villereal & Levinson, 1976; Levinson, 1978). However, in the present studies two observations suggest that the assumption of impermeability may not be valid. The first was the finding that the kinetics of interaction between cells and H_2DIDS were not consistent with that of a saturating process. Rather, there was a continuous uptake of H_2DIDS during the entire periods of experimental observation (Fig. 4). This suggested that H_2DIDS crossed the membrane and thereby entered the cell at a slow rate. The second was the finding that a significant amount

of H_2DIDS bound to the cells did not lead to inhibition of SO_4^{2-} transport. Although this fraction could be associated entirely with the glycocalyx proteins, it was difficult to discount the possibility of contribution from an intracellular source.

Support for the view that H_2DIDS does indeed enter the intracellular phase of ascites cells is derived from the observation that saponin caused the simultaneous release of both cellular α-AIB and H_2DIDS (Fig. 7). This nonmetabolizeable amino acid served in these experiments as a marker for intracellular contents. Consequently, any agent or condition that increased the permeability of the membrane in a nonspecific manner would cause accumulated α-AIB to leave the cell. Since the addition of saponin resulted in the release of both α-AIB and H_2DIDS simultaneously into the medium, we conclude that the intracellular phase may serve as the source for H_2DIDS. This conclusion is further supported by two related observations. First, the amount of H_2DIDS released from cells by the addition of saponin is directly related to the time of exposure to H_2DIDS (Fig. 8). This indicates then that the uptake of H_2DIDS into the intracellular phase is minimal after 1 min exposure to H_2DIDS but increases thereafter and could account for the slow association of H_2DIDS shown in Fig. 4. Second, the data shown in Figs. 7 and 8 are based on experiments performed at room temperature (21-23 °C). However, when cells were labeled with H_2DIDS at 15 °C, washed, and incubated at 23 °C the addition of saponin had practically no effect on the release of H_2DIDS. This result is consistent with the idea that reduced temperature depressed the entry of H_2DIDS into the intracellular phase. Furthermore, although H_2DIDS binds to cells at low temperature, we have shown previously that the binding is virtually without effect on SO_4^{2-} transport (Levinson, 1978).

Although our finding that H_2DIDS may enter the tumor cell was unexpected, others have shown that SITS can label internal structures of a variety of cell types. For example, Benjaminson and Katz (1970) showed that SITS when added to the environment of a variety of cells in culture at 37 °C was bound to vesicles within the cytoplasm around the nucleus. A major criticism of this work, however, is the fact that the SITS concentrations used were very high and ranged from 0.5 to 50 mM. The binding of SITS by intact liver cells and cell fractions has also been studied (Marinetti & Gray, 1967). When intact liver cells were labeled with SITS at room temperature and then homogenized to obtain the cell fractions, nearly all of the SITS was found in the mitochondrial fraction and none detected in the cell cytoplasm or nuclei. In a more recent study Juliano and Behar-Bannelier (1975) studied the labeling of surface proteins of tissue-cultured cells at 0 °C by tritium-labeled H_2DIDS. Autoradiograms and cell fractionation studies indicated that the plasma membrane is preferentially labeled in most cells in the population. However, a small number of nonviable cells labeled internally as well as on the surface. These results are consistent with our finding that when cells are maintained at 0 °C labeling by H_2DIDS is confined almost exclusively to the cell surface.

The possibility that H_2DIDS enters the ascites tumor cells complicates the interpretation of the data relating binding to inhibition of SO_4^{2-} transport (Fig. 5). Since the time course of tight binding is slow, cells must be incubated for pro-

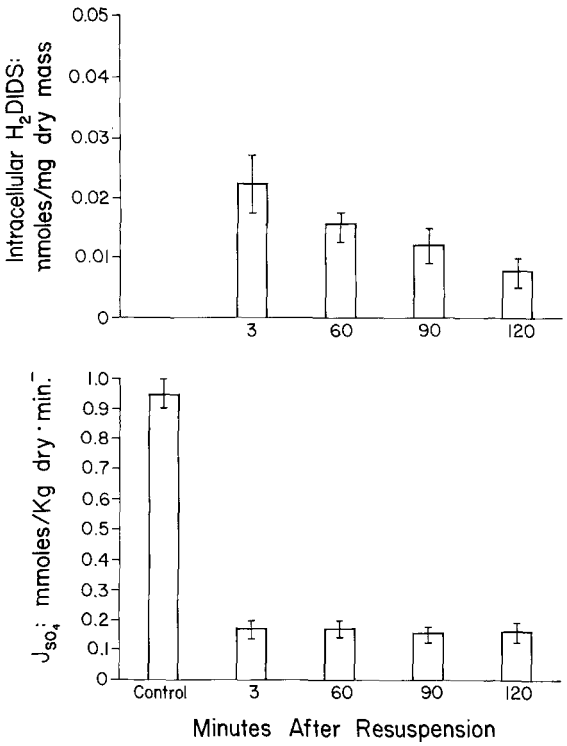

Fig. 10. The relationship between inhibition of SO_4^{2-} transport ($J_{SO_4}^{2-}$: mmol/kg dry mass·min) and intracellular H_2DIDS (nmol/mg dry mass). Cells were incubated with 25 μM H_2DIDS for 90 min at 21 °C, washed, and subsequently resuspended. Fluxes were measured at 3, 60, 90 and 120 min after resuspension. Intracellular H_2DIDS at each of these times was estimated by the addition of 100 μg saponin per ml of cell suspension and measuring the H_2DIDS released after 10 min. Error bars = ± 1 SEM

longed periods at room temperature which may favor accumulation of intracellular H_2DIDS. Consequently, inhibition of SO_4^{2-} transport could have resulted from the interaction of intracellular H_2DIDS with sites on the inner membrane surface. If this were the case then incubation of H_2DIDS-labeled cells in H_2DIDS-free medium would be expected to lead to the loss of incorporated H_2DIDS and thereby reverse the inhibition of SO_4^{2-} transport. However this was not observed. Rather, when cells were incubated for 90 min with H_2DIDS at 21 °C and subsequently washed, SO_4^{2-} transport was inhibited by 83%. Subsequent incubation in H_2DIDS-free medium for up to 2 hr resulted in the loss of 70% of the intracellular H_2DIDS, yet SO_4^{2-} transport remained inhibited by 83% (Fig. 10).

Taken together, the results of these experiments indicate that there are at least four modes of interaction between H_2DIDS and the Ehrlich ascites tumor cell. Two of these, a reversible interaction and an as yet unidentified irreversible binding to the membrane, are involved in the regulation of anion transport. An additional irreversible binding occurs with a component of the cell surface which we

have tentatively identified as the glycocalyx. Finally, H_2DIDS may enter the ascites tumor cell at room temperature and may interact with nontransport related binding sites.

Consequently, future investigations directed toward identification of the membrane component(s) concerned with the regulation of anion permeability must take into account the possibility of multiple interactions between H_2DIDS and most likely other substituted stilbenes and the ascites tumor cell.

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