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The Nature of the Membrane Sites Controlling Anion Permeability of Human Red Blood Cells as Determined by Studies with Disulfonic Stilbene Derivatives*

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Summary. The disulfonic acid stilbene derivative SITS reported to be covalently bonded to the membrane of the red blood cell, was found to be largely reversibly bound. Reversal of its specific inhibitory effect on anion permeability was attained by washing the cells with buffer containing albumin. The small fraction of covalently bonded SITS could be increased by prolonging the time of exposure of the cells or by multiple exposures. A series of other disulfonic stilbene derivatives was synthesized. All of them specifically inhibited anion permeability whether or not they are capable of forming covalent bonds. Their inhibitory effectiveness, however, varied over a 5,000-fold range, allowing certain conclusions to be made concerning the chemical architecture of the binding site. Certain of the compounds were almost entirely covalently bonded. One of them was labeled with ^{125}I and used to determine to which membrane proteins the compound is bound. Over 90% was found in a protein band on acrylamide gels of 95,000 mol wt. The most effective compound against sulfate permeability was equally effective against chloride permeability, producing a maximum inhibition of over 95%. The residual anion fluxes respond differently to pH and temperature than do the fluxes of unmodified cells.

The permeability of the red blood cells to anions and to cations can be modified by a large variety of chemical agents (Passow, 1969; Deuticke, 1970; Gunn & Tosteson, 1971; Knauf & Rothstein, 1971). For many of

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these agents the interaction with the membrane would seem to involve reaction with positively charged groups, an assumption based on the fact that the agents are themselves anions or because they have the capacity to interact covalently with amino groups. From such observation and from earlier studies, the concept has been developed that fixed positive charges in the membrane, probably amino groups can account for the high degree of selectivity of the red blood cell membrane for anions over cations (Passow, 1969). The concept is supported, particularly, by the findings that the agents that would reduce the positive charge by interaction with amino groups, produce the expected reciprocal effects on anion and cation permeability, the former being reduced and the latter increased. For example, with increasing pH in a range that would be expected to result in conversion of $-NH_3^+$ to $-NH_2$, the anion permeability is reduced and the cation permeability is increased. Many of the agents that can interact covalently with anion groups, including DNFB[1], TNBS, MNT (Passow & Schnell, 1969; Knauf & Rothstein, 1971; Poensgen & Passow, 1971) and DASA (Berg, 1969 for cations and Takeshita and Rothstein, 1971 for anions) also reciprocally affect anion and cation permeability. Reciprocal effects are also produced by many simple anionic substances that would be expected to bind electrostatically with positive sites in the membrane. These include a lyotropic series of anions (Wieth, 1971) and TNC (Gunn & Tosteson, 1971). On the other hand, a series of unrelated amphiphilic compounds which should not react with amino groups, nevertheless, produce reciprocal effects on anion and cation permeability (Deuticke, 1970).

An important exception to the pattern of reciprocal anion-cation effects is found with the disulfonic stilbene derivative, SITS, developed by Maddy (1964) as a covalent bonding agent for amino groups of the cell surface. This agent is just as effective as others in reducing anion permeability, but has *no effect* on cation permeability. The unique specificity of SITS has

1 Abbreviations used throughout are:

BSA	Bovine serum albumin
DNFB	1-fluoro-2,4-dinitrobenzene
DOC	Na-deoxycholate
Hct	Hematocrit
HEPES	N-2-hydroxy-ethylpiperazine-N′-2-ethanesulfonic acid
MES	2-(N-morpholin)-ethanesulfonic acid
MNT	2-methoxy-5-nitropone
RBC	Red blood cells
SITS	4-acetamido-4′-isothiocyano-2,2′-disulfonic stilbene
SLS	Sodium lauryl sulfate
TNBS	1-sulfonic-2,4,6-trinitrobenzene
TNC	2,4,6-trinitro-*m*-cresol.

been attributed to its inability to penetrate into the membrane. It reacts with a small, finite population of sites, superficially located on the outer face of the membrane, some of which are concerned with anion permeability. It apparently cannot reach the sites that are concerned with cation permeability (Knauf & Rothstein, 1971). From the studies with SITS it is clear that the same population of sites in the membrane cannot control both anion and cation permeability.

The unique specificity of SITS, its limited accessibility to membrane amino groups, and its amphiphilic properties, offers the potential for a chemical exploration of the nature of the superficial "anion permeation sites". A series of structurally related compounds have been prepared. All have the stilbene core with two sulfonic acid groups. Some bind reversibly whereas others have one or two covalent binding groups (either isothiocyano, or diazonium). The various compounds have been characterized in terms of their effects on anion permeability (sulfate or chloride), and their binding to the membrane. A radioactive label was inserted into one of the covalently bonding compounds to determine the particular membrane proteins to which it would bind. From comparisons of the behavior of the different compounds conclusions can be made concerning the chemical architecture and nature of the "anion permeation sites" of the membrane.

Materials and Methods

Source of Compounds

The compounds used as chemical modifiers are, with two exceptions, disulfonic-stilbene derivatives. Their names are listed in Table 1; chemical structures and pathway of synthesis are listed in Fig. 1.

Compound I was a gift from Sumitomo Chemical Co. Ltd. (Osaka). It was purified by repeated crystallizations from 1% NaCl solutions, and by chromatography on a silica gel [0.05 to 0.2 mm Merck AG (Darmstadt)] column using pyridine/acetic acid/water (10:1:40) [Solvent A] or *n*-propanol/NH_4OH/water (6:3:2) [Solvent B] as eluents. The final product was concentrated by flash evaporation, de-ionized with ion exchange resin (Dowex 50 W-2, H^+-form), boiled with charcoal, precipitated as the free acid by addition of HCl and by cooling, and finally crystallized from hot water. Its purity was confirmed by thin-layer chromatography (Silica Gel F 254 Merck) using Solvents A, B and others. All steps in this and the following procedures were performed with minimal exposure to light.

Compound II was obtained by mild benzoylation of Compound I with benzoylchloride followed by chromatographic separation using Solvent A (Kotaki, Naoi & Yagi, 1971) and Solvent B. Further purification was accomplished by crystallization steps similar to those used for Compound I.

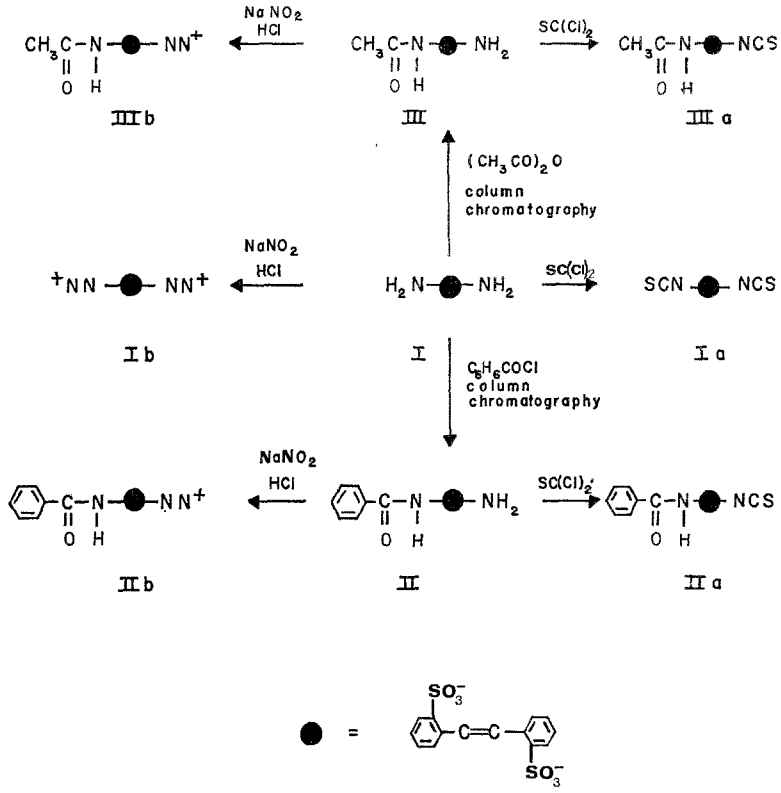

Fig. 1. Chemical structures and scheme of synthesis of probes. (For chemical nomenclature *see* Table 1)

Table 1. List of compounds

Symbol	Name*
0	2-2′-disulfonic-stilbene
I	4-4′-diamino-2-2′-disulfonic-stilbene
Ia	4-4′-diisothiocyano-2-2′-disulfonic-stilbene
Ib	4-4′-didiazonium-2-2′-disulfonic-stilbene
II	4-benzoamido-4′-amino-2-2′-disulfonic-stilbene
IIa	4-benzoamido-4′-isothiocyano-2-2′-disulfonic-stilbene
IIb	4-benzoamido-4′-diazonium-2-2′-disulfonic-stilbene
III	4-acetamido-4′-amino-2-2′-disulfonic-stilbene
IIIa	4-acetamido-4′-isothiocyano-2-2′-disulfonic-stilbene (SITS)
IIIb	4-acetamido-4′-diazonium-2-2′-disulfonic-stilbene
IV	Fluorescein-isothiocyanate (Isomer I + II)
V	4-4′-dihydroxy-2-2′-disulfonic-stilbene + 4′-4′-dichloro-2-2′-disulfonic-stilbene + 4-hydroxy-4′-chloro-2-2′-disulfonic-stilbene
TI	Iodinated derivative of I
TIb	Iodinated derivative of Ib

* For structural formulas *see* scheme of syntheses of probes (Fig. 1).

Compound III was obtained by mild acetylation of I in pyridine DMSO with acetic anhydride. The mixture of I, II and diacetylated I was precipitated by the addition of acetone/ether (1:3), washed with ether and dissolved in Solvent A. Further purification steps were similar to those used for Compounds I and II.

The *isothiocyano* derivatives were obtained by addition of excess thiophosgene to a vigorously stirred solution of the starting material in 1% NaCl. After 1 hr, the excess thiophosgene was removed by washing with ether and centrifugation. The final precipitate was washed with 0.01 N HCl and ether, and kept in the dark after dehydration over P_2O_5 (Maddy, 1964).

Compound IIIa (SITS) was also purchased from the British Drug House. The *diazonium* salts of I, II and III were obtained by the direct method (Saunders, 1949; Berg, 1969) using about a 15% normal excess of $NaNO_2$. The precipitates were washed twice with a minimal amount of cold 0.01 N HCl, cold water, acetone/ether (1:3) and ether, and dried in vacuum over P_2O_5 at 4 °C.

Compound TI was obtained from I by the ICl-iodination method (Helmkamp & Sears, 1970) using a 0.05 N HCl, 1.0 M NaCl solution instead of 1.0 N HCl, and a 5:1 molar ratio of ICl to I. TI was collected from repetitive acid precipitations, dehydrated with acetone/ether (1:3), followed by ether and kept in the dark in vacuum.

Compound TIb was obtained by diazotization of I as described above.

Compound V is a mixture of chlorinated and hydroxylated derivatives of I, obtained from Ib by the $NaNO_2$-HCl-H_2SO_4 method (Saunders, 1949) or by boiling Ib in 0.1 N NaOH. No attempt has been made to separate or to quantitate the relative yields of the individual species.

Compound IV was purchased from Sigma Chemical Co.

^{125}I-TIb was obtained by exchanging Na ^{125}I, carrier free (New England Nuclear) with TI according to the method of Helmkamp and Sears (1970), then diazotizing with a 1:1.2 normal excess of $NaNO_2$ followed by 5 equivalents of HCl. The efficiency of isotopic exchange was higher than 90%.

^{14}C-II was prepared by reacting I with ^{14}C-Benzoylchloride (6.1 mC/mmole) (Amersham/Searle) and purifying by the same procedure as cold II.

^{14}C-III was prepared essentially by the same procedure as for cold III, using ^{14}C-acetic anhydride (20 mC/mmole) in benzene/pyridine (1:4) (New England Nuclear). The final products were analyzed for radiopurity by using Whatman 3 MM paper chromatography (strips) with Solvent A and B and scanned for radioactivity with a Baird Atomic Radio-chromatogram Scanner. The mono- and disubstituted derivatives were clearly separated and obtained in a ratio of 4:1. The isotopic yield for II was 60% and for III was 25%.

Sucrose, SLS, MES, HEPES, TRIS and BSA (fraction V, crystalline) were obtained from Sigma Chemical Co.; DOC was from Mann Chemicals.

Treatment of Cells with Isotopically Labeled Modifier

Labeling of cells was accomplished by reacting 1 mM of ^{125}I-TI_b (1.5×10^9 cpm/mmole) with prewashed RBC (40% final hematocrit) in an isotonic phosphate-NaCl buffer (pH 7.4) for 20 min at 4 °C. After treatment, the cells were washed four times with 10 volumes of isotonic Tris-NaCl buffer (pH 7.4), followed by two washes with albumin 0.5% in isotonic Tris-NaCl buffer (pH 7.4). The cells were finally washed with the phosphate buffer and ghosts prepared by the Dodge, Mitchell and Hanahan (1963) method. The protein content of ghosts was estimated by a modified micro biuret method

(Itzhaki & Gill, 1964) using DOC as the membrane solubilizer or by fluorescence spectroscopy (Resch, Imm, Ferber, Wallach & Fischer, 1971) using albumin and tryptophane, respectively, as standards. Polyacrylamide-SLS-gel-electrophoresis and protein staining were performed according to Fairbanks, Steck and Wallach (1971) using 0.8×13.0 cm gels. The gels were stained with Coomassie Brilliant Blue and sliced with a home-made slicer. Membranes and gel slices were counted for radioactivity in a Packard γ-Scintillation Counter.

SO_4^{--} *and* Cl^- *Fluxes*

SO_4^{--} and Cl^- fluxes were measured by the isotopic back exchange method (Gunn & Tosteson, 1971; Lepke & Passow, 1971) using $Na_2\ ^{35}SO_4$ (New England Nuclear) and Na ^{36}Cl (Amersham-Searle). Pre-washed cells at 30% hematocrit were equilibrated at 37 °C with sulphate-chloride medium (Na_2SO_4 50 mM, KCl 75 mM, HEPES 10 mM, and sucrose to 350 mosm, pH 7.4, unless specified otherwise). After 1 hr they were washed and resuspended in the same medium, a trace of $Na_2\ ^{35}SO_4$ added, followed by incubation for another 2 hr (sufficient for complete isotope equilibration). The cells were then brought to 5 °C, centrifuged, washed twice with cold medium (to remove any traces of Hb), and reacted with the chemical modifiers at 5 °C (20% final Hct). The unreacted chemical was removed by successive washes with 3 volumes of cold medium or with one wash of cold medium, two with cold medium + 0.5% albumin, and two with cold medium, unless specified otherwise. The cells were finally resuspended at a 4 to 5% Hct in a pre-warmed medium. The $^{35}SO_4$ content was measured in deproteinized (5% TCA) cell supernatants ($3{,}000 \times g$ for 3 min at 5 °C) taken at different times ($P(t)$). The supernatant counts at the time of isotopic equilibration (P_∞) was estimated from an aliquot of a deproteinized (5% TCA) cell suspension. Effluxes were measured at room temperature, unless specified otherwise.

^{36}Cl exchange was performed in a similar way, but the cells were loaded with Na ^{36}Cl at 37 °C for 30 min after being chemically modified, washed and resuspended at a 50% final Hct. The cells were then brought to a 0.5% final Hct with cold medium (5 °C) and quickly transferred to a syringe. Supernatant samples were obtained by filtering the suspension through a Millipore Swinnex filter unit that contained an AP 250 pre-filter and a 0.45 filter. All the operations were done at 5 °C.

When pH was used as a variable the media contained 5 mM MES (pK 6.15) and 5 mM HEPES (pK 7.55) (Good *et al.*, 1966). Cells were loaded and reacted at pH 7.4 then extensively washed and resuspended in the medium of appropriate pH for another 20 min. During the flux measurements the pH remained constant within ± 0.06 pH units.

The supernatants and cells were analyzed for hemoglobin (Dacie & Lewis, 1968). During the flux measurements the per cent hemolysis did not exceed 0.80 per cent. At extreme pH's (6.0 to 6.4, 7.8 to 8.0) and high temperatures (39 °C) the per cent hemolysis reached a value of about 2% after 100 min. The results were corrected accordingly.

Radioactivity was measured with a Packard Liquid Scintillation spectrometer.

Sulfate and chloride fluxes of normal and chemically modified cells were calculated according to Knauf and Rothstein (1971) using a linear least-square program with an IBM 360 computer. The program included a correction for hemolysis, and data are reported as per cent inhibition based on the appropriate control.

Fluorescence

Fluorescence measurements were done at room temperature in 1-cm quartz cuvettes at right angles to the excitation beam, using an Aminco-Bowman Spectrophotofluorimeter. No correction was included for lamp output or photomultiplier response.

Results

When Maddy (1964) studied the interaction of SITS with ox red blood cells, he assumed that the binding was covalent because SITS contains an isothiocyanate moiety capable of covalent reaction with amino groups and because after washing with buffer SITS-fluorescence was still present in the ghosts. The same assumption was made by Knauf and Rothstein (1971) for interaction of SITS with human red blood cells (RBC). Extensive washing with buffer did not reduce the inhibitory effect on anion permeability. In both studies, binding of SITS to the cells, and its presence in ghosts, could be qualitatively determined by SITS-fluorescence, but quantitative estimates of binding could only be measured indirectly by determining how much SITS disappeared from the medium using a spectrophotometric method. In human RBC, the amount bound was about 4×10^6 molecules per cell (or 60 nmols per ml PCV).

To provide an accurate and suitable method of determining the membrane-bound SITS, ^{14}C-labeled compound (^{14}C-IIIa) was prepared. In preliminary studies the binding to the cell was found to be about the same as reported by Knauf and Rothstein (1971), but recovery in ghosts was surprisingly small, 5×10^5 molecules per cell, or only about 10% of the amount originally bound to the cells. This finding indicated that much of the compound was reversibly rather than covalently bound. It seemed likely that the reversible component of SITS was removed during exposure to hemoglobin during the ghosting process. SITS would be expected to react readily with protein.

The question of the reversibility of SITS inhibition of anion permeability was re-examined by comparing cells that were washed with buffer alone or with buffer plus bovine serum albumin (BSA), the latter being used to scavenge reversibly bound SITS. The inclusion of albumin in the washing buffer had no effect on the control, but drastically reversed the inhibition of the modified cells (Fig. 2). On the other hand, washing with buffer alone did not change the inhibition (not shown in Fig. 2, but *see* Section *B*, Fig. 3). The reversal by albumin was not complete. Even after several washes, a residual inhibition of 10 to 15% of the total remained, about the same proportion as the percentage recovery of ^{14}C-SITS in ghosts.

These observations suggest that about 85 to 90% of the SITS bound to the membrane under the conditions of exposure is not covalently reacted, but is reversibly (but firmly) bound. It is removable by washing with buffer containing extracellular protein but not by buffer alone. A maximum of 10 to 15% of the membrane-associated SITS might be covalently reacted.

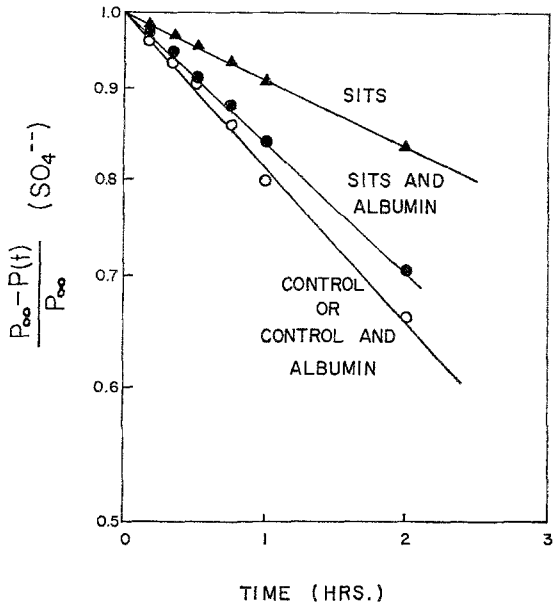

Fig. 2. The reversal of SITS-inhibition by washing with buffered albumin. Cells were loaded with ^{35}S-sulfate, treated with SITS (1×10^{-4} M for 15 min at 5 °C) and washed with buffer as described in Materials and Methods. Aliquots (labeled "control" or "SITS") were washed twice more with cold buffer, whereas aliquots (labeled "SITS + albumin" or "control plus albumin") were washed once with buffered albumin and once with buffer. The cells were finally resuspended in prewarmed buffer to a 5% Hct and effluxes measured at room temperature

The fraction of the SITS inhibition that was not reversed by BSA could be increased either by a more prolonged exposure to SITS or by a sequential series of exposures (Fig. 3). For example, the nonreversible component of inhibition increased from about 10% of the total after 10 min of SITS exposure to about 50% after 90 min. In contrast, the inhibition in buffer-washed preparation did not change with time of exposure. Similarly, if cells washed with albumin were retreated with SITS, washed again with albumin and retreated a third time, the irreversible component was again increased from 10% to 50% of the inhibition.

The data of Figs. 2 and 3 indicate that SITS can be bound in two ways to the membrane sites. Immediately after exposure most of the binding is reversible, but over a prolonged period of time, an increasing proportion of SITS becomes irreversibly bound. The degree of inhibition does not seem to depend on the nature of the binding, but rather on the total amount bound, whether reversible or irreversible.

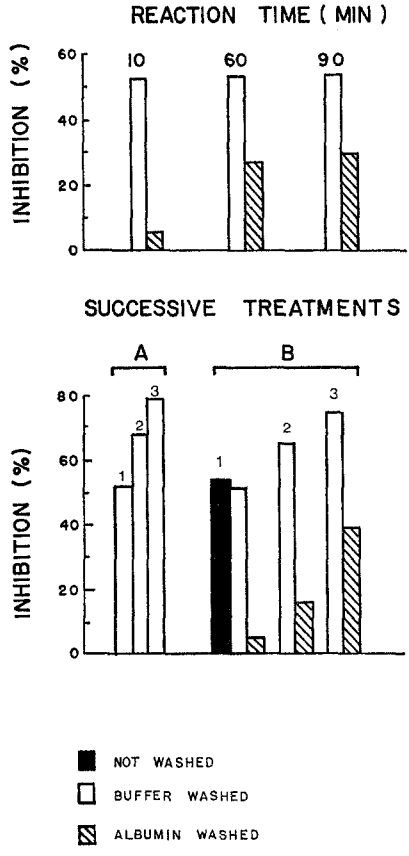

Fig. 3. The effect of reaction-time and successive SITS treatments on the reversibility of SITS-inhibition. Values are given as inhibition in per cent of sulfate fluxes of modified cells relative to nonmodified cells exposed to equivalent treatments. *Upper diagram:* Cells (20% Hct) were exposed for 10 min to 10^{-4} M SITS, washed with buffer, resuspended (20% Hct) and left for stated times before they were washed again with buffer or buffered albumin. "Reaction-time" refers to the time elapsed between the first exposure to the modifier and the wash with albumin. Fluxes were measured as described in Materials and Methods. *Lower diagram:* (*A*) Cells (20% Hct) were exposed to 1, 2 or 3 successive treatments with 10^{-4} M SITS for 10 min each time. After each reaction they were washed with buffer. Fluxes were measured as described in Materials and Methods. (*B*) Cells (20% Hct) were first reacted with 10^{-4} M SITS for 10 min and either immediately resuspended to a 5% Hct (not washed), washed 3 times with buffer, or washed once with albumin and twice with buffer (1). The albumin-washed cells of (1) were exposed to one or two more similar cycles of exposure to SITS and washing, labeled (2) and (3), respectively. Fluxes were measured as described in Materials and Methods

The relationship of the inhibition to the nature of the binding was explored by comparing the inhibitory effects of two series of disulfonic stilbene derivatives, one with no covalent bonding groups, (Fig. 4) and the other with groups capable of covalent interaction (Fig. 5). All of the derivatives

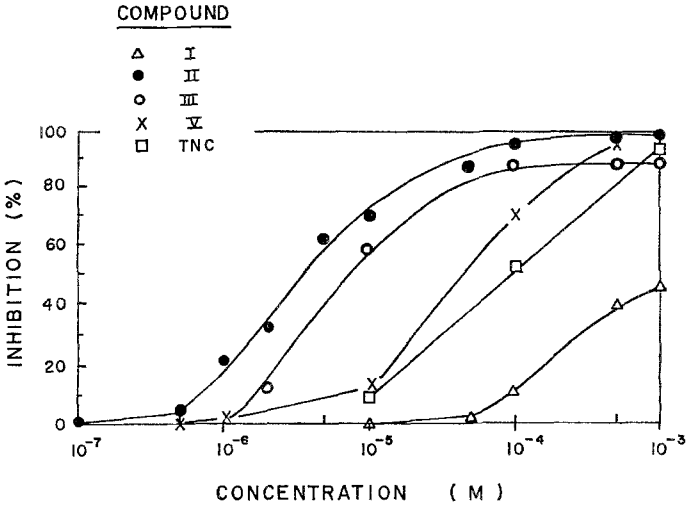

Fig. 4. The effect on sulfate permeability of probes that bind reversibly. Fluxes were measured in the presence of the probes as described in Materials and Methods. ^{35}S-loaded and washed cells were resuspended in a prewarmed medium containing different concentrations of probes and preincubated for 5 min before fluxes were measured. Concentrations are given in moles per liter of extracellular volume. TNC: Data from Gunn & Tosteson, 1971, done on Cl^- fluxes in the presence of the inhibitor

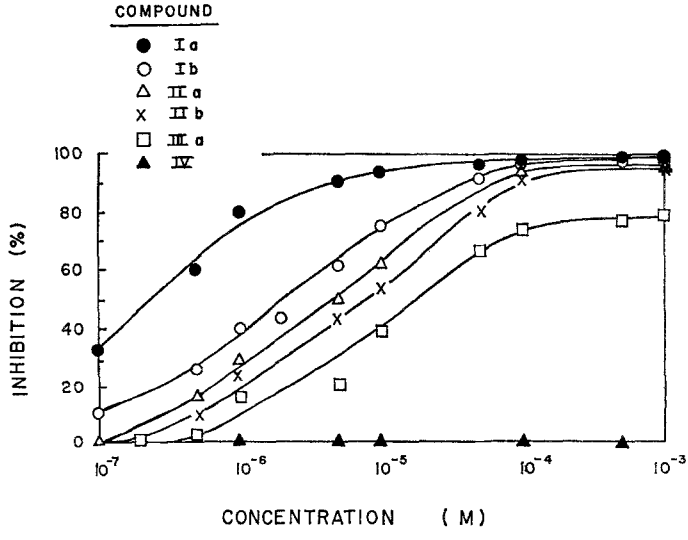

Fig. 5. The effect on sulfate permeability of probes that have the capacity for covalent bonding. Fluxes were measured after exposing the cells to different concentrations of probes for 30 min at 5 °C, and washed 3 times with 5 volumes of buffer. (*See* Materials and Methods)

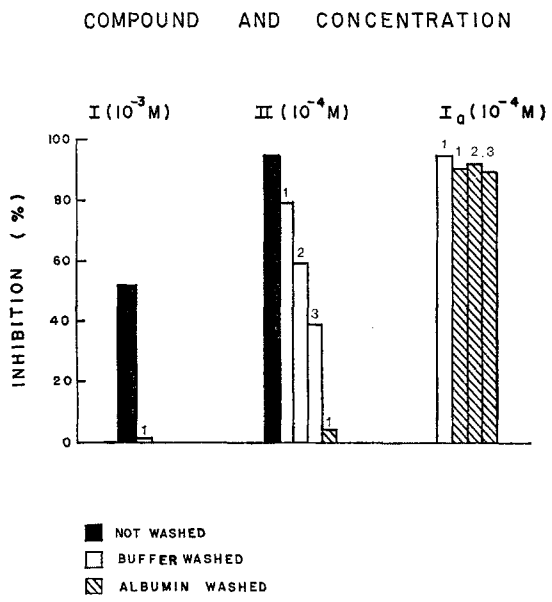

Fig. 6. Reversibility of inhibition of anion permeability produced by several probes. ^{35}S-loaded, washed cells were resuspended in a prewarmed medium containing the probe (I and II) (5% Hct). Fluxes were measured after a 10-min preincubation period. Others were exposed for 30 min to the probe (20% Hct) at 5 °C and washed 1, 2 or 3 times with 5 volumes buffer or buffered albumin, then they were finally resuspended in a prewarmed medium. Fluxes were measured as described in Materials and Methods

were capable of producing inhibition, so it is clear that covalent binding is not essential. Binding studies with a compound from each group, one that binds reversibly (II) and one that binds covalently (Ib) indicate that the same number of sites are involved in each case, about 4×10^6 per cell (the same as found for SITS). On the other hand, the effectiveness of the compounds on a concentration-inhibition basis varies considerably. The most effective compound (Ia) produces 50% inhibition at 2×10^{-7} M and the least effective (I) at 1×10^{-3} M, a difference of 5,000-fold. Large differences in reversibility were also evident. The inhibition by the least effective compound (I), was reversed by a single wash with buffer (Fig. 6). The most effective noncovalent bonding compound (II) was only partially reversed by several buffer washes, but was virtually completely reversed by an albumin wash. With the most effective compound of all (Ia), the inhibitory effect was not reversible even with three successive washes with albumin. Presumably, it is virtually all covalently bonded.

In comparing the effects of the different stilbene compounds, one additional point should be made. In previous studies (Knauf & Rothstein,

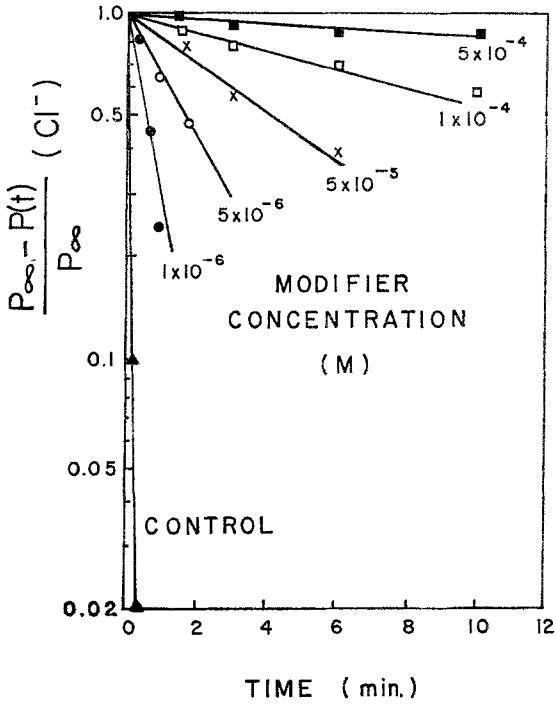

Fig. 7. Effects of different concentrations of compound Ia on chloride fluxes. Cell modification and measurements of chloride fluxes are described in Materials and Methods. Temperature for flux measurements was 5 °C

1971) and in the present study, the maximum inhibition with SITS was 80 to 85%. Most of the other compounds, whether reversibly or covalently bonded, produced a higher maximal inhibition, of the order of 95 to 98% (Figs. 4 and 5).

Compound Ia, the most effective tested against sulfate permeability (Fig. 5), is about equally effective as an inhibitor of chloride permeability (Fig. 7), with 50% inhibition falling between 10^{-6} and 10^{-7} M, about the same range as in the case of sulfate (Fig. 5). This identity of effect is subject to one reservation. The treatment with reagent was the same for cells used for sulfate or for chloride measurements, but for technical reasons (to slow the fluxes) the measurements with chloride were carried out at 5 °C, whereas those with sulfate were carried out at 22 °C.

None of the stilbene compounds studied had any measurable effect on cation permeability measured by K^+ leakage, confirming the findings of Knauf and Rothstein (1971) with SITS.

It was of some interest to determine whether the residual anion permeability in fully modified cells was altered appreciably in its properties.

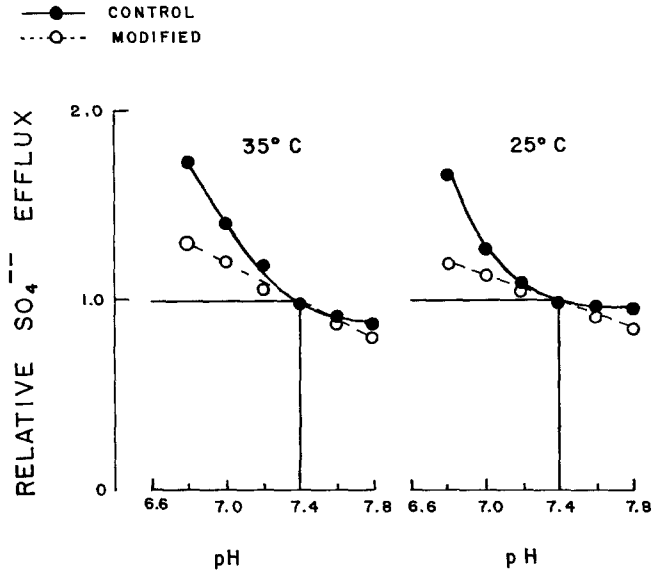

Fig. 8. pH profiles of sulfate fluxes in normal and modified cells. Cells were modified with compound Ia (10^{-4} M) at pH 7.4, re-equilibrated to the desired pH and finally resuspended for flux measurements as described in Materials and Methods. The values of fluxes at different pH's are given as fluxes relative to values at pH 7.4

Cells were treated with the most effective compound (Ia) which is also a covalently binding compound (Fig. 6) resulting in an inhibition of sulfate permeability of over 95%. The SO_4^{--} efflux in the inhibited cells was considerably less dependent on pH (Fig. 8). The temperature dependence of modified cells was also shifted (Fig. 9). In control cells, the Arrhenius plot has at least two components. In the modified cells the slopes are steeper than in the control indicating that the degree of the inhibition increases as the temperature is decreased. The calculated energy of activations for the left-hand component is about 8 kcal per mol larger in the modified cells than in the control.

The covalently binding compounds can be used to label the binding sites of the membrane. An iodine (^{125}I) labeled derivative of compound Ib was prepared. After reaction with the cells, ghosts were isolated and the proteins dissociated as described in Materials and Methods. In Fig. 10, the pattern of distribution of proteins on acrylamide gel electrophoresis is displayed together with concomitant counts of ^{125}I in slices of the gels prepared in parallel. More than 90% of the counts in the gel was coincident with a major protein peak of 95,000 mol wt. Less than 15% of the count was extractable from the labeled ghosts by repeated lipid extraction with ethanol/ether (3:1).

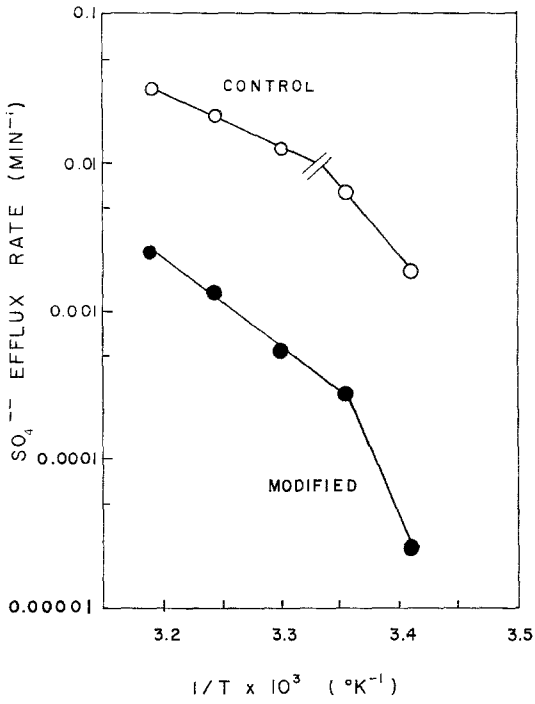

Fig. 9. Arrhenius plots of sulfate fluxes of normal and modified cells. ^{35}S-cells (20% Hct) were modified with probe Ia (10^{-4} M) for 30 min at 5 °C, washed 3 times with buffer and finally resuspended in prewarmed buffer (pH 7.4) for flux determinations

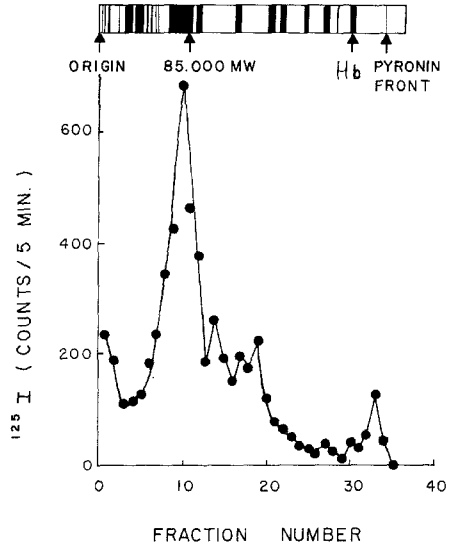

Fig. 10. Distribution of compound ^{125}I-TIb in the membrane proteins of modified RBC. Ghosts were prepared (as described in Materials and Methods) from cells modified with ^{125}I-TIb (10^{-3} M). The membrane proteins were separated on polyacrylamide gels (5.4%), fixed and stained with Coomassie Blue. In parallel, gels were fixed, sliced and counted as described in Materials and Methods. The given values of radioactivity per fraction are the average of counts on three gels, each containing 120 µg of membrane protein

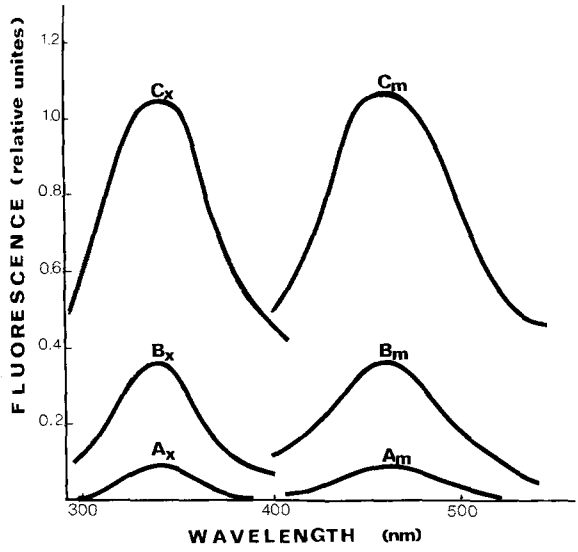

Fig. 11. The interaction of compound II and ghosts as observed by fluorescence spectroscopy. *X* refers to excitation (460 nm emission); *m* refers to emission (340 nm excitation). Probe concentration 10^{-5} M in 0.02 M phosphate buffer, pH 7.2 *A*: no ghosts; *B*: 65 μg/ml of ghost protein; *C*: 200 μg/ml of ghost protein

One of the noncovalent binding compounds (II) has proved to be particularly useful as a fluorescent probe for evaluating protein conformation (Kotaki *et al.*, 1971). When added to RBC ghosts the intensity of its fluorescence is increased by a factor of 10 or more, depending on the ghost concentration (Fig. 11). The large enhancement as well as the additional increase in fluorescence obtained after addition of $CaCl_2$ (100 μM), indicates an especially strong interaction between the probe and the RBC membrane. In parallel, the high potency of the same compound as an inhibitor of anion permeability (Fig. 4) is also a manifestation of its high affinity for membrane sites.

Discussion

Although the initial studies with SITS (Maddy, 1964; Knauf & Rothstein, 1971) led to the assumption that its binding to the membrane, and its specific effect on anion permeability were associated with covalent bond formation, the data presented in this paper indicate otherwise. Most of the binding and most of the inhibitory effect can be readily reversed by the addition of protein (serum albumin). The previous failure to demonstrate reversal can be attributed to the relatively high affinity of SITS for the

membrane sites. Even extensive washing with buffer failed to appreciably reverse the inhibitory effect. Furthermore, no adequate method was available for determining the amount of SITS that remained after preparation of ghosts. The observed fluorescence in the ghosts was a strictly qualitative indication that some SITS was present.

A single wash with albumin in buffer seems to differentiate between reversibly associated agents and covalently bonded agents. Thus, with compound II with no covalently bonding group, virtually all of the inhibitory effect can be washed out; with SITS (compound IIa), 85 to 90% can be washed out and with compound Ia, virtually none can be washed out (Figs. 3 and 6). In parallel, if ghosts are prepared, recoveries of the compounds show a similar pattern of binding properties. In the case of compound Ib, the ghosts were dissolved in SDS and run on acrylamide gels and yet 85% of the compound was recovered in the protein bands (the rest was associated with lipid). It seems evident, from these findings, that the albumin irreversible component is covalently bonded.

Although their effectiveness varies considerably, all of the disulfonic stilbene derivatives produce the same specific effect, an inhibition of anion permeability with no effect on cation permeability. Because some of the compounds have no capacity to form covalent bonds (and their inhibitory effect is completely reversible), it must be concluded that covalent interaction is not a prerequisite to binding to the membrane or to inhibition. On the other hand, certain of the compounds such as Ia and Ib are covalently bonded. Thus, if the same sites are involved for both classes of compounds, they must be sites capable of either reversible binding or covalent binding. The evidence favors the concept of a common site. First, the basic core of all of the molecules is the same (disulfonic stilbene), so that they all might be expected to react electrostatically. Second, the specific effect produced is the same for all of the derivatives. Third, the number of binding sites are the same (about 4×10^6 per cell) for a noncovalently bonded compound (II), a covalently bonded compound (Ib) and for SITS (IIIa) which is partly covalently bonded. Fourth, in the case of SITS, the amount of irreversible component increases markedly with time but the total amount of inhibition is not changed. It would, therefore, appear as though it is the amount bound that determines the inhibition independent of whether the binding is reversible or covalent.

It is suggested that the primary inhibitory effect is caused by reversible binding and that the covalent binding, if it occurs, is incidental, a secondary reaction. Certainly in the case of SITS, the sequence of an initial reversible binding followed by covalent reaction seems clear. The binding and the

inhibitory effect are apparent in a few minutes or less. Despite the fact that the surplus SITS is washed away with buffer, an increasing amount of the inhibition becomes irreversible over the next 1½ hours. It seems likely that SITS first binds reversibly and that the bound SITS slowly interacts covalently with adjacent ligands, a process commonly known as affinity labeling (Singer, 1967).

The binding of the compounds under study probably involves an electrostatic interaction of the sulfonic acid groups with positive groups on the surface of the membrane. This conclusion is based on a number of lines of evidence:

(1) The exceptionally high permeability to anions can be explained by assuming a role for fixed positive charges (Passow, 1969).

(2) In the higher pH range, the anion permeability is decreased in a manner compatible with dissociation of $-NH_3^+$ to $-NH_2$ (Lepke & Passow, 1971).

(3) A variety of amino-reactive reagents (most of them anionic) of differing structures, including DNFB, MNT, TNBS, DASA as well as the disulfonic stilbene derivatives will inhibit anion permeability. None of the agents is absolutely specific, but amino groups are the most likely candidates to account for the common effect, especially since anionic sulfhydryl agents such as PCMBS have no effect on anion permeability. As an exception, the neutral compound fluorescein-isothiocyanate, a protein amino reactive reagent, has shown no effect on anion permeability (Fig. 5). This failure may suggest an additional requirement of anionic characteristics for the effectiveness of an amino reagent as an inhibitor of anion permeability.

(4) Other anions such as ANS, TNC, salycilate (Fortes & Hoffman, 1971; Gunn & Tosteson, 1971; Wieth, 1971) also inhibit anion permeability.

(5) In the case of compound Ia, the covalent reaction (irreversible effect on inhibition) is increased if the pH is increased from 6 to 8. This would be the expected behavior for reaction with amino groups, because the reactant form, $-NH_2$, as well as the specificity of the $-NCS$ group for amino groups would increase at higher pH.

In the above discussion, the positively charged groups have been identified as amino groups, but they might also be guanido groups.

Many of the noncovalent reacting disulfonic stilbene derivatives are exceptionally potent inhibitors of anion permeability compared to monovalent compounds that have been studied. It can be suggested that the high potency relates to the presence of two negative groups (sulfonic acid) which could chelate with two fixed positive charges. One or more additional amino

groups would have to be present in the micro environment to account for the covalent reaction of the isothiocyano or diazonium derivatives. A micro environment with a number of positive groups in a cluster would seem to be indicated. Such a clustering of positive charge has been proposed as an essential part of a "fixed charge" theory of anion permeability (Passow, 1969). It might explain also why the diamino derivative (I) is relatively ineffective.

In addition to the positively charged center, the "site" must have an adjacent neutral or lipophilic center to account for the high potency of reversibly bound compounds that have an additional aromatic ring (II) or acetyl group (III). Such a lipophilic center would also account for the high relative potency of TNC, salycilate and ANS as compared to inorganic ions (Fortes & Hoffman, 1971; Gunn & Tosteson, 1971; Wieth, 1971). It would also account for the large enhancement of fluorescence found with compound II (benzoyl derivative), demonstrated in Fig. 11.

Maddy (1964) observed that the diacetylated derivative of I did not bind to the membrane. In our hands it showed a negligible inhibitory effect on anions. The dibenzoylated derivative is also a relatively ineffective inhibitor of anion permeability. These findings can be accounted for by possible steric factors or might be a reflection of a large, highly charged (positive) site which will exclude a large molecule with two terminal hydrophobic moieties.

The mechanism of the inhibitory effect is not clear because the mechanism of permeation of anions is not clear. In particular, recent evidence that a large proportion of the Cl-flux is electrically silent involving a one-for-one anion exchange (Hoffman & Lassen, 1971) and that the process is saturable (Deuticke, 1970; Wieth, 1971) raises many questions about the mechanism. In the present paper, it has been demonstrated that the fluxes of both sulfate and chloride are inhibited to the same extent by the diisothiocyano-disulfonic stilbene derivative. In the present study, the measurements of anion fluxes were made at equilibrium by isotope techniques so they probably represent largely the anion exchange component (electrically silent). On the other hand, Fuhrman, Knauf and Rothstein (1972, *unpublished observations*), have demonstrated that the net movements of sulfate and of chloride out of the cell (accompanied by cation) is also effectively inhibited by SITS and especially by compound Ia (same relative inhibition as compared with the isotopic exchange method).

There is no reason to believe that the inhibitory effect of the stilbene derivatives on anion permeability is different in mechanism than that of other amino reactive reagents or anionic substances. It would be difficult

at this time to distinguish between effects associated with the reduction in fixed positive charge, steric blocking, charge screening caused by the presence of sulfonic groups, and/or inhibition of a "carrier" type of mechanism.

The disulfonic stilbenes offer distinct advantages over other compounds inasmuch as they do not penetrate into the cell. They reach a small and finite population of sites among which are those concerned with anion permeability. They have a very high affinity for these sites. They do not reach the cation-controlling sites or react with them. In addition, certain of them can form covalent bonds so they can be used as radioactive markers for sites on the external surface of the membrane. For example, compound Ib is found predominantly in a particular protein fraction (Fig. 10) that is known to be partially exposed on the outer surface (Bretscher, 1971; Phillips & Morrison, 1971). Potentially, the covalently bound derivatives can be used to chemically identify the fixed positive charges that control anion permeability. In addition, because Ia and Ib are bifunctional agents they can be used to internally cross-link those sites in the membrane that possess a high density of amino groups (within a 12-Å range), or to cross-link neighboring proteins in the membrane matrix. In addition, one of the noncovalent derivatives (II) is a highly site specific hydrophobic, fluorescent probe as demonstrated in Fig. 11.

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