Chemical Modification of Membranes

I. *Effects of sulfhydryl and amino reactive reagents on anion and cation permeability of the human red blood cell*

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ABSTRACT Four different amino-reactive reagents, 4-acetamido-4′-isothiocyano-stilbene-2,2′-disulfonic acid (SITS),[1] 1-fluoro-2,4-dinitrobenzene (FDNB), 2,4,6-trinitrobenzene sulfonic acid (TNBS), and 2-methoxy-5-nitrotropone (MNT) decrease the anion permeability of the human red blood cell, as measured by sulfate fluxes, whereas the sulfhydryl agent, parachloromercuriphenyl sulfonic acid (PCMBS), does not. In contrast, PCMBS increases the cation permeability as measured by K^+ leakage, whereas SITS does not. Of the other agents, FDNB increases the cation permeability to the same extent as PCMBS but MNT and TNBS produce smaller increases. PCMBS does not protect against FDNB as it does against other sulfhydryl agents (X-irradiation) and the FDNB effect on cations is attributed to amino groups. Studies of the binding of SITS indicate that it does not penetrate into the membrane and its failure to influence cation permeability is attributed to its inability to reach an internal population of amino groups. It is concluded that two ion permeability barriers, both involving proteins, are present in the red blood cell. The more superficial barrier contains amino groups and controls anion flow; the more internal barrier contains sulfhydryl and amino groups and controls cation flow. The amino groups contribute to the control of permeability by virtue of their positive charges, but the role of sulfhydryl groups is not clear. Only a small fraction of the membrane protein amino and sulfhydryl is involved in the barriers.

INTRODUCTION

Based primarily on studies of the pH and ionic strength dependence of anion (1, 2) and of cation (1–4) permeability, Passow has proposed that positively

[1] The following abbreviations are used in this paper: SITS, 4-acetamido-4′-isothiocyano-stilbene-2,2′-disulfonic acid; FDNB, 1-fluoro-2,4-dinitrobenzene; TNBS, 2,4,6-trinitrobenzene sulfonic acid; MNT, 2-methoxy-5-nitrotropone; PCMBS, parachloromercuriphenyl sulfonic acid; PCMB, parachloromercuribenzoate; PCV, packed cell volume.

charged amino groups are directly involved in the control of ion permeation through the red cell membrane. The tremendous selectivity of the red cell membrane for anions over cations (more than a millionfold) is, however, far in excess of that exhibited by artificial fixed charge membranes, and it must therefore be assumed that the effectiveness of the positive groups is enhanced in a unique way by the membrane structure of which they are a part (5). Because of such structural complexity, the usual kinetic analysis of ion flows has not led to suitable models with respect to the nature, location, arrangement, and the amount of the fixed charged groups of the membrane.

A more direct test of the potential role of positively charged amino groups in anion-cation selectivity has involved the use of chemical modifiers having a high affinity for amino groups. Berg et al. (6) observed that 1-fluoro-2,4-dinitrobenzene (FDNB) as well as its bifunctional analogue, 1,5-difluoro-2,4-dinitrobenzene (DFDNB), increased permeability of red cells to sodium and potassium, and Passow (7) found that FDNB caused a corresponding decrease in sulfate exchange permeability. Although these results are consistent with the hypothesis that amino groups regulate anion and cation permeability of the red cell, there are still several unanswered questions. First, Passow (3) found that the response of anion and cation permeability to increasing doses of FDNB was different, with anion permeability being affected at lower FDNB concentrations than cation permeability. This observation suggests that the FDNB effects on anions and cations might be due to interactions with different sites rather than the result of modification of a single population of amino groups. Second, the conclusion that FDNB produces its effects by reaction with amino groups is far from certain. FDNB is a relatively unspecific reagent, reacting with sulfhydryl, imidazole, and phenolic hydroxyl groups as well as with amino groups. In the course of a study of glucose transport, for example, Stein (8) measured the amount of dinitrophenylated (DNP) amino acid residues in hydrolysates of ghost protein from FDNB-treated red cells. After reaction with 10 mM FDNB for 1 min at pH 6.05 and 25°C, 18 nmoles of S-DNP-cysteine were formed per milliliter of packed cells, as well as 3 nmoles of ϵ-DNP-lysine, 2 nmoles of Im-DNP-histidine, and other uncharacterized products. Marfey (9) isolated cross-linked amino acids from the protein of cells which had been exposed to DFDNB, and found that more cysteine-lysine and tyrosine-lysine were formed than lysine-lysine. Because of the lack of specificity of FDNB, it is possible that its effects on anion and cation permeability might be due to reaction with ligands other than amino groups. Sulfhydryl groups in particular could be involved, since the binding of organic mercurial reagents to a small number of SH groups inside the membrane causes an increase in cation permeability very similar to that seen with FDNB (10, 11).

A further difficulty with FDNB is that, due to its appreciable lipid solubility, it penetrates the membrane rapidly (12), interacting with a large variety of proteins in the cell as well as in the membrane. It is therefore of little value in

determining the number or the location within the membrane of the sites which affect passive permeability.

The present study is an attempt to clarify the nature of the FDNB effects, particularly with regard to the involvement of amino and sulfhydryl groups in the control of anion and cation permeability, and with regard to the location of the controlling ligands in the membrane. The effects of FDNB are directly compared with those of other sulfhydryl (PCMBS) and amino (MNT, TNBS and SITS) reactive reagents. In addition, agents are used in pairs to determine whether they interact with the same membrane sites in producing permeability changes. The data suggest that two distinct rate-limiting barriers exist, one predominantly for anions and the other for cations. It is concluded that the anion barrier is controlled by amino groups located superficially, whereas the cation barrier is controlled by amino and sulfhydryl groups located deeper within the membrane.

METHODS

Cell Preparation Human blood was obtained by venipuncture from hematologically normal adults and was defibrinated by stirring. The blood was centrifuged and the plasma and white cells were removed by aspiration. The red cells were then washed three times in isotonic (165 mM) sodium chloride. In some experiments, recently outdated blood bank blood was used. The effects of inhibitors did not differ significantly from those observed with fresh blood.

Sulfate Exchange Sulfate exchange was chosen as a convenient measure of anion permeability since it can be measured without using the complicated techniques required to study the more rapidly penetrating ions (e.g., chloride). Since chloride, phosphate, and other ions compete with sulfate for entry, changes in sulfate permeability may be used as an indicator of anion permeability in general (1, 2).

Washed packed red cells were incubated in a medium containing ^{35}S-labeled sodium sulfate for at least 3 hr at 37°C with constant stirring. This time is sufficient to allow sulfate to reach equilibrium distribution. The washed cells were resuspended in 10 ml of nonradioactive medium which had been prewarmed to 37°C (hematocrit = 10%). The cells were stirred constantly and the pH was adjusted intermittently with a pH stat (Radiometer). At various times 1 ml samples were removed and centrifuged in a modified hematocrit centrifuge. A 0.3 ml aliquot of the supernatant was then added to 0.3 ml of 10% (w/v) trichloroacetic acid (TCA), centrifuged, and 0.4 ml of the supernatant was counted in a liquid scintillation counter (Packard).

In the experiments with chemical modifiers, the agents that reacted slowly (requiring exposure of over 15 min) were added to the cells suspended in radioactive sulfate prior to washing, in order to prevent large losses of labeled sulfate before the start of the experiments. In cases in which exposure to the chemical modifiers could be short (less than 15 min), the reaction was carried out in washed labeled cells.

The dependence of sulfate permeability on the composition of the medium is quite complex (1, 2). For convenience, incubation conditions were chosen so that the half-time of the control would be 15–45 min. If potassium efflux was simultaneously meas-

ured, the cells were suspended in a solution of 10 mM sucrose, 50 mM Na_2SO_4, and 85 mM NaCl. When it was desirable to minimize net cation movements, however, the incubation solution consisted of 30 mM sucrose, 50 mM K_2SO_4, 16.5 mM NaCl, and 57 mM KCl. Other media were also used as indicated.

Since it has been demonstrated that incubation of red cells at 37°C in the absence of substrate causes changes in the physical properties of the cells, especially after 10 hr (13), it was important to determine whether the incubation periods used in this study, although they were of shorter duration, caused any alteration in the anion transport properties of the cells. Cells preincubated in media containing 30 mM glucose in place of the sucrose normally present, exhibited no significant differences in anion permeability. LaCelle (14) has also reported that there is no significant change in anion permeability after 5 hr of incubation at 37°C. Length of preincubation also had little influence on the effects of modifiers. For example, the effect of FDNB was similar after 5 hr to that observed with short preincubation periods.

The permeability of anions, including sulfate, is strongly pH dependent (1–3). With the statting techniques used in these experiments, the pH of the individual cell suspensions at the end of an experiment varied by less than 0.1 pH unit. When discrepancies existed, the cells exposed to chemical modifiers had a lower pH than controls. Since any lowering of the pH should increase the sulfate permeability, pH variations could not have accounted for the decrease in sulfate permeability observed after treatment with these reagents.

In order to insure that the isotope was measuring true unidirectional sulfate flux and not sulfate-sulfate exchange diffusion, results with and without external sulfate were compared. This was done at a sulfate concentration of 10 mM where effects due to volume changes resulting from sulfate-chloride exchange (3) would be minimized. The rates of efflux with and without sulfate in the external medium were identical. Doubling the specific activity of the sulfate also had no effect on the rate of efflux. In agreement with Passow's results (1–3) the efflux of sulfate from the cells was a first-order process. All the results were consistent with the assumption that the internal sulfate is well stirred and that more than 90% of the sulfate exchanges with a single rate constant.

Since sulfate exchange at Donnan equilibrium is a first-order process, $\ln\{[P(\infty) - P(t)]/P(\infty)\} = -kt$, where $P(\infty)$ represents the total counts per minute (cpm) of ^{35}sulfate in the medium at infinite time, $P(t)$ is the total counts per minute in the medium at time t, and k is the rate constant which characterizes the process. In the experiments with inhibitors, $P(\infty)$ was estimated from the number of counts per minute in a sample of the suspension which had been hemolyzed by exposure to TCA. After correction for the Donnan ratio, the resulting values agree closely with $P(\infty)$ as estimated by curve fitting.

The unidirectional sulfate flux may be calculated from the following equation (15)

$$m = \frac{kS_iS_o}{A(S_i + S_o)} = \frac{0.693\ S_iS_o}{t_{1/2}A(S_i + S_o)} \simeq \frac{0.693\ S_i}{t_{1/2}A}$$

where S_i is the total amount of sulfate (radioactive and nonradioactive) in the cells, S_o is the amount of sulfate in the medium, A is the total surface area of the cells, m is the

flux rate, and $t_{1/2}$ is the half-time of the efflux process. Equal numbers of cells which had been loaded with sulfate in the same preincubation solution were always used. Thus, S_i and A were constant for all the cell suspensions of a given experiment. This means that k and $t_{1/2}$ provide as good a measure of the degree of inhibition as does m. The sulfate concentration inside the cell and the membrane potential (as determined from the Donnan ratio) were nearly identical for the different samples of cells in an experiment, and so the driving force for sulfate efflux was also the same. Since flux = permeability × driving force, the rate constant provides a measure of the relative permeability as well as of the flux.

Potassium Efflux Net potassium efflux was determined as described in detail in the following paper (16). Cells were resuspended in either isotonic sodium chloride or choline chloride containing 5 mg % ouabain and 5% (v/v) isotonic Tris or phosphate buffer at pH 7.4. At various times potassium efflux was measured by flame photometry carried out on samples of the medium from which cells were removed by centrifugation.

SITS Binding Washed red cells were rewashed in phosphate-buffered saline (saline:isotonic phosphate buffer 9:1) at pH 7.4. 2 ml of cell suspension (hematocrit = 50%) were then added to 1 ml of freshly prepared SITS solution in buffer (usually 60 μM) and mixed by inversion. Cells were allowed to react for specified times (6 min to 5 hr). The suspensions were then centrifuged and 1 ml of supernatant was removed and added to 0.25 ml of 40% TCA. The optical density was read at 340 nm and compared with blanks without SITS and with standards containing various quantities of SITS in 8% TCA. All these procedures were carried out under the illumination of a 75 w bulb about 10 ft away, in order to prevent the conversion of the SITS from the *trans* form to the *cis* form which has a much lower extinction coefficient (17). Standards were kept in the same test tube rack as the experimental samples to ensure that they would be subjected to the same illumination. That the SITS remained in the *trans* form was indicated by the close agreement of the observed extinction coefficients with those already published (17). The amount of SITS bound by the cells was calculated from the decrease of its concentration in the medium.

SITS Binding by the Fluorometric Method When the concentrations of added SITS were too low to saturate the binding sites, the spectrophotometric method was not sufficiently sensitive to measure the unbound agent. In such cases the SITS was measured fluorometrically.

Since the fluorescence of the *trans* form of SITS is highly sensitive to light (17, 18) and is therefore unstable with time, samples were converted to an equilibrium mixture of *cis* and *trans* form by exposure to room light before measurement. Potassium hydroxide (14% final concentration) was added to enhance the fluorescence (17). The excitation wavelength was 350 nm and emission was measured at 483 nm. Under these conditions, standards showed a linear relationship between fluorescent intensity and concentration, but there was a slow decrease in intensity, due perhaps to irreversible breakdown of SITS (19). In order to correct for this effect, the time of measurement of each sample was recorded and the results were appropriately corrected. The results of duplicate samples were averaged.

This technique permitted measurements of SITS concentration in the lowest range, but it is far less reliable than the less sensitive spectrophotometric method. In the experiments in which this method was used, however, most of the SITS was bound so that the fluorometric determination provided only a small correction term in the binding calculation. Even if the total amount of SITS added to the cells were bound, the results would be qualitatively similar.

Sialic Acid Unbound sialic acid was determined by the thiobarbituric acid method (20). When peptide-bound sialic acid was to be measured, samples were first hydrolyzed in 0.1 N H_2SO_4 at 80°C for 1 hr, a procedure which releases bound sialic acids without degradation (21).

Lipid Chromatography Phospholipids and their reaction products were separated by chromatography on Whatman silica gel paper with a solvent system consisting of propionic acid: diisobutyl ketone: water (8:2:1) (22). Phospholipids were stained with a solution containing 0.01 N HCl, 0.2% uranyl acetate, and 0.001% acid fuchsin (23). Before staining, chromatograms were viewed under ultraviolet light and fluorescent spots were marked.

Reaction of Cysteine with FDNB The reaction mixtures containing cysteine, phosphate- or borate-buffered saline, and 1 mM EDTA were brought to 35–37°C. The reaction was started by adding 2.7 ml of this solution to 0.3 ml of a 1% solution of FDNB in 10% aqueous methanol in a quartz cuvette, which was then shaken vigorously and placed in a Gilford spectrophotometer in which the temperature was maintained at 35.5–36°C. The optical density at 354 nm was read against a blank containing FDNB but no cysteine, in order to compensate for the absorption of FDNB itself, and for the increase in optical density due to the formation of 2,4-dinitrophenol (24).

The wavelength for measurement of the reaction was selected by inspection of spectra obtained for samples of S-DNP-cysteine and *N*,S-di-DNP-cysteine (Mann Research Labs., Inc., New York) suspended in the same buffered saline which was used for the experiments. Kinetics of the reaction were interpreted on the basis of the ionic equilibria of cysteine (25).

RESULTS

PCMBS, a specific sulfhydryl reagent, is known to cause a pronounced increase in cation permeability (11) but because its effect on anion permeability had not yet been tested, direct comparisons of its effects on cation and anion permeability were made. The anion permeability was measured by the efflux of radioactive sulfate from cells equilibrated with sulfate. The calculation of permeability depends not only on the assumption that sulfate is at equilibrium but also that the cation content does not change during the measurement, with concomitant changes in volume. The latter condition cannot easily be met in PCMBS-treated cells because of the large increase in cation permeability. Two sets of measurements were therefore performed, one in normal saline in which PCMBS induces a slow swelling due to the fact that the increased Na influx slightly exceeds the increased K^+ efflux (11), and a second

in which the Na of the medium was partially replaced so that K^+ loss exceeded Na^+ gain, resulting in shrinkage. In each case the net rate of K^+ efflux was increased by a very large factor, 2800%, whereas the sulfate efflux showed small changes, a decrease of only 5% when the cells were swelling and an increase of 30% when the cells were shrinking. These small changes, in the opposite directions, are parallel to volume flow. It can be suggested therefore that PCMBS has no direct effect on anion permeability and that sulfhydryl groups are not involved. Furthermore, FDNB has an entirely different action. Under the conditions of cell shrinkage (in low NaCl), in which PCMBS causes an apparent 30% *increase* in sulfate permeability, FDNB causes at least an 80% *decrease*. It can be concluded that the effects of FDNB on anion permeability are *not* due to reaction with the PCMBS-reactive sulfhydryl groups.

Both PCMBS and FDNB increase cation permeability. It is not clear, however, whether sulfhydryl groups are the target for both agents or whether amino groups are involved in the case of FDNB. In order to differentiate the two possibilities, experiments were designed to determine whether PCMBS could protect the membrane sulfhydryl groups from FDNB[2] in much the same way that PCMBS can protect them from radiation damage (26). With model compounds such as cysteine, PCMBS in a 1:1 ratio can almost fully protect the sulfhydryl group against a 100-fold excess of FDNB (Fig. 1) and at a 2:1 ratio of PCMBS to cysteine, the protection is virtually complete provided that PCMBS is added before FDNB. The correct order of addition is necessary because the PCMBS reaction, although of much higher affinity, is reversible, whereas the FDNB reaction is irreversible. The slow change in optical density in the presence of PCMBS (Fig. 1) is due to a reaction of FDNB with the α-amino group as indicated by its pH dependence. This reaction is slow at pH 7.2 because only 2.4% of the amino groups are in the reactive form $R—NH_2$ (calculated from pK's) (25) but is markedly increased in rate at higher pH.

Based on the established protective capacity of PCMBS against FDNB in the model sulfhydryl compound cysteine, experiments were undertaken with red blood cells. The cells were first exposed to PCMBS for 60 min in a high K^+ medium (to prevent K^+ loss from the cells). The amount of PCMBS used was a two and one-half-fold excess relative to the total membrane sulfhydryls titratable with this agent (28) and at least a twentyfold excess over the number of sulfhydryls involved in cation permeability (11). The time of exposure allowed a maximal binding of PCMBS to the cation-affecting sulfhydryl groups. Indeed, under similar conditions, PCMBS affords protection against a second sulfhydryl agent, radiation (26). The PCMBS-treated cells (with excess PCMBS still present) were then exposed to FDNB for 10 min. Then cysteine

[2] It was assumed but not proven that PCMB can protect against reaction of FDNB with sulfhydryl groups of the enzyme, glycogen phosphorylase (27).

was added in excess and the cells were washed and transferred to fresh choline-chloride medium.

As reported previously (11), PCMBS-treated cells exposed to cysteine have a near normal rate of K^+ leakage (Fig. 2). Yet cells exposed to PCMBS, FDNB, and cysteine were as leaky as cells exposed only to FDNB and cysteine. PCMBS afforded no measurable protection against the effects of FDNB, indicating that PCMBS and FDNB increase cation permeability by different mechanisms and that the affected ligand in the case of FDNB cannot be the PCMBS-binding sulfhydryl groups.

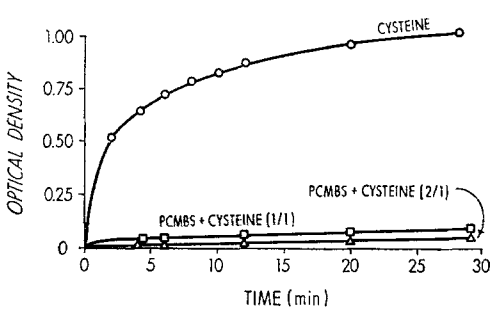

FIGURE 1

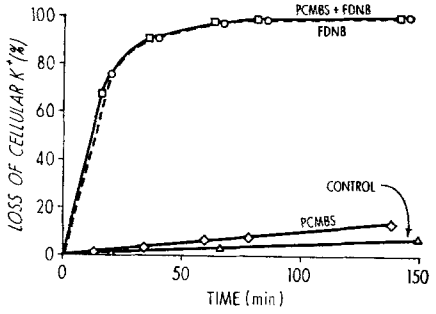

FIGURE 2

FIGURE 1. The effect of PCMBS on the reaction of FDNB with cysteine. The pH was 7.27; cysteine concentration 0.05 mM; PCMBS concentration 0.05 and 0.1 mM; FDNB concentration 5.37 mM; temperature 35.5°C. Optical density was measured at 354 nm as described in Methods.

FIGURE 2. The effect of PCMBS on the FDNB-induced K^+ efflux. The PCMBS concentration was 0.1 mM; FDNB, 5.37 mM; cysteine, 10 mM; temperature 37°C. Cells suspended in high K^+ medium were preteated with PCMBS for 60 min, then with FDNB for 10 min. The cells were resuspended in a medium containing cysteine for 30 min to reverse the effects of PCMBS. The measurement of K^+ efflux was made in choline medium.

The clear distinction between the action of PCMBS and FDNB with respect to both the anion and the cation permeability implies that sulfhydryl groups are not the target in the case of the latter agent. Potential targets are phenolic hydroxyl, imidazole, and amino groups. If amino groups are involved, then other agents more specific for amino groups should produce effects similar to FDNB and the agents should compete with FDNB for the same binding sites.

Three reagents were tested. The first, 2,4,6-trinitrobenzene sulfonic acid (TNBS), has been employed to modify amino groups in many studies of protein structure and function (29–32). No reaction is reported to occur with the imino groups of histidine or proline, nor with the hydroxyl groups of tyrosine, serine, or threonine even at temperatures ranging from 45 to 85°C, reaction times up to 3 days, and with up to a 29-fold excess of reagent (33). TNBS reacts with sulfhydryl groups (34) but the resulting products are extremely

labile at physiological pH in the presence of amino compounds with which they react to form *N*-trinitrophenylated products. At low reagent concentrations it reacts preferentially with amino groups in the presence of sulfhydryls (34, 35). According to the available literature, the second reagent, 2-methoxy-5-nitrotropone (MNT), is completely specific for amino groups (36). No reaction was observed with the phenolic hydroxyl groups of tyrosine, the imidazole group of histidine, the guanidinium group of arginine, or the SH group of cysteine (37). To date this reagent has not been used as extensively as TNBS, so its reported specificity has not been thoroughly tested.

The third reagent, 4-acetamido-4′-isothiocyano-stilbene-2,2′-disulfonic acid (SITS), has the interesting property that, at least in ox erythrocytes, it does not penetrate into the cell (38). No detailed studies of its specificity have been reported, but unless its reactivity is considerably different from that of an analogue, phenylisothiocyanate, reaction with groups other than amino and sulfhydryl is unlikely (39–41) especially under the very mild conditions used for reaction of SITS with red cells. In rat liver cells it appears to react mainly with SH groups (17) but in human red cells SITS affects very few of the PCMBS-titratable SH groups on the outer surface of the membrane (as is the case with TNBS and MNT as well).

All three amino reagents studied decreased anion permeability as measured by ^{35}S-sulfate exchange at Donnan equilibrium. The effects of SITS and TNBS as compared to FDNB are demonstrated in Fig. 3. The reported inhibitory effect of MNT (12) was readily confirmed. It is more effective than TNBS but less effective than SITS and FDNB.

The differences in effects of the agents on cation permeability were far more dramatic. The pronounced effect of FDNB is demonstrated in Fig. 2, confirming observations in the literature (3, 7). The cation flux can be increased by as much as 100 times. TNBS and MNT had much smaller effects. For example, 1.44 mM of TNBS (10% hematocrit) increased K efflux an average of 2.8-fold (average of four determinations) and 2.88 mM, an average of 5.8-fold (two determinations). MNT[3] had a small, variable effect, with an average increase of 1.5-fold (three determinations). In contrast to the other agents, SITS (11 determinations) never caused an increase in cation permeability but caused a small decrease of doubtful significance. Cells treated with 0.54 μM per ml packed cell volume (PCV) leaked K^+ 86.5 $\pm$ 13.2% as fast as the controls, with a similar reduction in sodium influx. The amount of SITS used is sufficient to produce a dramatic effect on sulfate permeability (Fig. 3).

Of the amino reagents tested, SITS discriminates best between effects on anion and cation permeability, suggesting that it might prove useful in characterizing the anion-affecting sites and in distinguishing them from the

[3] MNT has a limited solubility (12). A saturated solution at 20°C is 0.53 mM. As in previous studies (12), a suspension of the agent was used containing 5.6 mmoles per liter.

cation-affecting ligands. The reason for its failure to affect cation permeability may be related to its inability to penetrate into the membrane. Even some divalent anions which are much smaller than SITS, such as tartarate and glutarate, are almost completely excluded from the red cell (1, 42). Furthermore, in ox erythrocytes, Maddy (38) demonstrated the failure of SITS to penetrate by showing that globin from hemolysates of SITS-treated cells was free of SITS fluorescence. The SITS binding to the ox cells was essentially complete in less than 5 min, and did not increase during the next 30 min. The total binding of SITS was constant over a range of SITS concentrations from 5 to 250 μM, and averaged only 12 $\pm$ 6 nmoles per ml of packed cells, or about 4.5 $\times$ 10^5 molecules per cell.

In human red cells the binding properties of SITS are similar to those reported for the ox. Almost all of the binding, about 40 nmoles per ml PCV, occurred within the first 6 min (the earliest time at which samples could be taken) with very little increase at 60 min (Fig. 4) and about a 20% increase in 5 hr (four estimates). When the temperature was raised from 25 to 37°C the binding also increased slightly, especially after long reaction times. Since hemolysis was also greater under these conditions, this effect was probably caused by increased binding to hemolyzed cells. Under similar conditions,

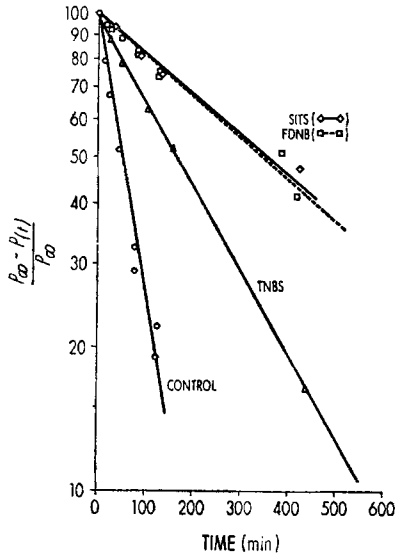

FIGURE 3

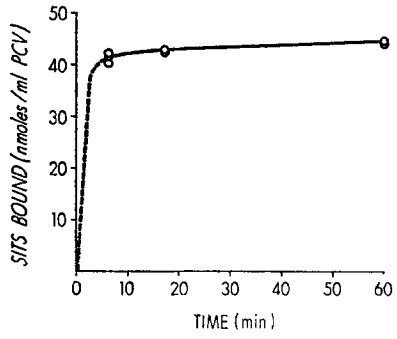

FIGURE 4

FIGURE 3. The effects of amino agents on sulfate efflux. Treatment with agents was as follows: (*a*) 2.9 mM TNBS for 2 hr at 37°C; (*b*) 5.37 mM FDNB for 11 min at 23°C; (*c*) 0.1 mM SITS for 9 min at 23°C. The pH was 7.4 and the temperature for measurement of the efflux was 37°C.

FIGURE 4. Time course of SITS binding. The total amount of SITS added amounted to 60 nmoles/ml PCV. The temperature was 23°C.

cell hemolysates rapidly bound over 3500 nmoles of SITS per ml of original packed cell volume.

When a series of data on SITS binding are compared (Table I), the results from a single blood sample agree very well, but there is more scatter among values obtained with blood from different donors. This is due probably to biological variations in binding capacity, similar to that observed in ox erythrocytes (38).

The mean binding of SITS corresponds to 1.8×10^6 molecules per cell (or 3×10^{-18} moles per cell), which is about four times the value for ox erythrocytes. Even this is a very small number of sites compared to the total number of amino groups available in the membrane (Table II). The SITS sites represent 2.5% of the total phosphatidyl serine and phosphatidyl ethanolamine in the red cell membrane, less than 2% of the lysine amino groups in the membrane, and a far smaller percentage of the total number of

TABLE I

BINDING OF SITS TO THE HUMAN RED CELL

Date	Donor	Binding	N	SD
		(*nmoles/ml PCV*)		
10/10/68	AG	37.43	3	0.99
11/68	MW	33.85	4	2.24
11/68	MW	34.40	3	0.80
2/21/69	AMP	32.18	9	4.25
2/17/69	BD	42.82	7	1.42
2/25/69	—	18.95	2	2.82
Total		34.64	28	5.33

amino groups available inside the cell. This selectivity of binding may be ascribed to the size and charge of SITS which restrict it to a small number of the potentially available reactive sites, presumably those located near the outside surface of the membrane. Lipid chromatography of ghosts from SITS-reacted cells revealed no SITS fluorescence except at the origin, where the protein remains, so SITS presumably reacts only with superficial ligands of proteins. In the ox red cell, SITS reaction was also limited to protein ligands (38).

Since SITS contains two negative charges per molecule, and since it binds near the outside surface of the red cell, it was of interest to determine whether the addition of negative charge to the membrane might be involved in the inhibition of anion permeability by SITS. Assuming that the SITS charge is randomly distributed on the cell surface, it should produce an effect similar to that of the negative charge which is normally present. The SITS molecules represent about 6.8% of the total number of sialic acid molecules (Table II), and since each SITS molecule has two charges, about 12.6% of the total

charge (assuming that sialic acid molecules contribute 94% of the charge) (43). In order to evaluate the role of surface charge, cells were treated with neuraminidase to remove their sialic acid. If the normal surface charge is indeed important in limiting anion permeability, removal of this charge should increase the anion flux. Also, if the normal surface charge is reduced, SITS treatment should cause a greater percentage increase in charge and so the SITS effect would be enhanced.

The results of an experiment with neuraminidase-treated cells[4] are shown in Fig. 5. Removal of the sialic acid from the cell surface caused no increase in the rate of sulfate exchange. The sulfate exchange flux of cells treated with

TABLE II

COMPARISON OF THE NUMBER OF POTENTIAL MEMBRANE BINDING SITES WITH THE NUMBER OF SITES AFFECTED BY VARIOUS REAGENTS

Nature of membrane sites	Moles per cell $\times 10^{18}$	Source
Inorganic mercury sites (presumably sulfhydryl groups)	182.0	(28)
PCMBS sites	38.2	(28)
Chlormerodrin sites	39.8	(28)
Amino lipids (phosphatidylserine and phosphatidylethanolamine)	146.0	(58)
Protein ϵ-amino groups	224.0	(58, 59)
Protein α-amino groups	4.1	(58, 59)
Sialic acid	39.9	(43)
	44.1	(58, 59)
Surface chlormerodrin sites	2.1	(28)
Surface PCMBS sites	2.3	(28)
SITS binding sites	3.1	Table I

neuraminidase and then with SITS was reduced by 61%, vs. 70% for cells treated only with SITS. Even this small change is in the opposite direction from that to be expected if SITS were reducing anion permeability by increasing the negative surface charge of the cell.

Since SITS does not inhibit anion permeability by forming a random network of negative charge on the surface, it must produce this effect by binding to specific but superficial sites which are identical with or near to those molecules that determine the rate of anion flow through the membrane. An attempt was made to determine the location of SITS binding by subjecting the membrane to partial enzymatic digestion. Pronase removed more than

[4] Neuraminidase-treated cells were supplied by Dr. Marshall Lichtman. Removal of surface charge was checked by cell electrophoresis and by sialic acid assay. The cells were incubated for 60 min in Hanks' balanced salt solution at hematocrit 25%, pH 7.2, with 1000 units of neuraminadase; temperature, 37°C.

80% of the sialic acid within 15 min, but even after more than an hour only a small fraction (less than 10%) of the SITS was released. These results were confirmed by measurements of anion transport (Table III). Pronase itself produced a small decrease in anion permeability but even when this difference in control fluxes is taken into account, the SITS inhibition of anion permeabil-

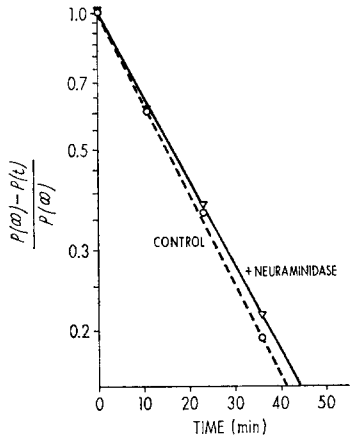

FIGURE 5. The effect of neuraminidase treatment[4] on sulfate efflux. The pH was 7.1 and temperature 37°C.

TABLE III

EFFECTS OF PRONASE AND SITS ON SULFATE EXCHANGE

Procedure: Cells were exposed to SITS (60 nmoles/ml PCV) for 13 min and were then treated with pronase (2.4 mg/ml PCV) for 100 min. The hematocrit was 8.4% and the temperature 37°C. Conditions as in Cook and Eylar (44). Efflux of ^{35}S-sulfate at pH 7.16 was measured as described in Methods.

Treatment	$t_{1/2}$ (min)	Reduction in rate	
		% of appropriate control	
Control	18	0	
Pronase	26	31	0
SITS	43	58	
SITS + pronase	48	62	46

ity is decreased only from 58 to 46%. In view of the complicating effect of pronase alone, this difference may be insignificant.

Unless SITS in some way inhibits the activity of pronase in its vicinity, these results indicate that very little SITS is bound to the surface glycopeptides which are removed by pronase treatment and which contain sialic acid and the M and N blood group antigens (44).

SITS, FDNB, MNT, and TNBS all inhibit anion permeability, but it is

unclear whether they bind to the same or different sites. In order to answer this question, red cells were first reacted with amino reagents and then SITS binding was measured (Table IV). SITS binding was partially reduced by each of the agents, the degree of reduction being 20, 34, and 27% for MNT, TNBS, and FDNB. The lack of more complete overlap is not surprising in light of the different selectivities of these chemically dissimilar reagents for some amino groups in preference to others. Even the most reactive agent, FDNB, for example, binds to less than 10% of the membrane protein amino groups (45).

If we assume that the four agents exert effects on anion permeability by binding to common sites, the data of Table IV indicate that only about 20%

TABLE IV

EFFECTS OF PRETREATMENT WITH AMINO REAGENTS ON SITS BINDING

Reagent	No. of estimates	Mean binding	Control
		nmoles/ml PCV	%
Control	3	34.4	100
MNT	3	27.6	80.3
TNBS	3	22.7	66.3
Control	5	42.1	100
FDNB	2	30.6	72.7

The concentrations of agents were 0.95, 2.4, and 5.4 mM for MNT, TNBS, and FDNB, and the times of exposure, 60, 60, and 10 min, respectively. The hematocrit was 8% and temperature 37°C. The cells were washed five times and the SITS binding was measured as described in Methods. The experiment on MNT and TNBS involved blood from one donor and the experiment on FDNB from another donor.

of the total number of SITS binding sites are responsible for the entire inhibition of anion permeability. Experiments using a variety of SITS concentrations may support this assumption since small amounts of SITS are proportionately more effective in reducing sulfate efflux (Fig. 6). For example, the binding of 6 nmoles/ml PCV reduced the anion permeability by 40%. The maximal effect of 85% reduction (Fig. 3) is produced by the maximal binding of 36 nmoles (Table I). Thus about half of the maximal effect is associated with less than 20% of the binding sites. These data are compatible with the assumption that only a small fraction of the SITS binding sites, those with a relatively high affinity (or greater accessibility), is associated with changes in anion permeability. The data were, however, obtained by use of the fluorometric procedure described in Methods. It is the only procedure sufficiently sensitive to determine the small amounts of SITS, but it is not highly reliable.

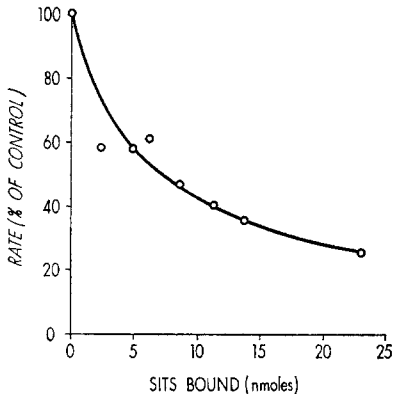

FIGURE 6. The relationship between SITS binding and sulfate permeability. The cells were exposed to various concentrations of SITS for 10 min at 23°C. The packed cell volume was 0.81 ml for each determination. The pH during measurement of sulfate flux was 6.9 and the temperature 37°C.

DISCUSSION

The use of a variety of chemical modifiers of ion permeability allows conclusions to be drawn relating to the chemical nature of the ligands involved in the permeability barrier, their number, their location, and to a limited degree, their function.

In the case of anion permeability the evidence is strong that sulfhydryl groups play no role. PCMBS in high concentrations has no effect; yet PCMBS, itself an anion, would be expected to reach ligands in the anion permeation path. This expectation is supported by the findings that the permeation of PCMBS, although slow (11), is inhibited by other anions and that it is also blocked by SITS (16). Further evidence that sulfhydryl groups exert no influence on anion permeability is the finding that X-irradiation of red cells also caused no detectable change in anion permeability (46) even though radiation converts sulfhydryl to disulfide in the membrane, reacting with many of the same sulfhydryl groups as does PCMBS (26).

In contrast, the importance of amino groups is clearly indicated by the fact that each of the four amino reagents tested caused dramatic decreases in the sulfate exchange permeability. As pointed out in describing each agent, none except perhaps MNT is absolutely specific for amino groups. For example, several will react with sulfhydryl groups. Thus the effects of any one might be ascribed to reactions at sites other than amino groups. It is highly improbable, however, that lack of specificity could account for the similar effects of each of four different reagents, especially if sulfhydryl groups can be removed from consideration. Furthermore, preliminary studies indicate that maleic anhydride, which reacts specifically with amino groups (47), also decreases anion permeability (A. L. Obaid, personal communica-

tion). Additional evidence includes the fact that reversible inhibitors of anion permeability such as iodide, nitrate, and thiocyanate exert an effect in rough proportion to their affinity for amino groups (48); that the effects of pH on anion permeability can be explained on the basis of the proton dissociation of amino groups (2, 3, 7); and that the permeability sequence of the red cell for halides (49) fits the calculated selectivity properties of positively charged groups such as amino (50).

The maintenance of normal levels of cation permeability depends on the integrity of both sulfhydryl groups and amino groups. The case for sulfhydryls is unequivocal. Organic mercurials such as PCMBS, with an extremely high affinity for sulfhydryl as compared to other ligands (51), increase the cation permeability by as much as 100-fold (11). Furthermore, PCMBS reacts with the same ligands as does X-irradiation and the latter increases cation permeability by formation of disulfides in the membrane (52). The case for amino groups is also strong. FDNB, the agent with the greatest effect (3, 7), does not discriminate well between sulfhydryl and amino groups in membranes (8). The effect on cations does not, however, seem to be due to sulfhydryl groups, as demonstrated by the failure of PCMBS to protect against FDNB (Fig. 2). The attribution of the effect to amino groups is reinforced by the finding that increases in cation permeability are produced by the more specific amino reagents, TNBS and MNT, and by higher values of pH at levels compatible with discharge of protons from amino groups (1, 2, 4). Furthermore, the temperature coefficient of the efflux at low ionic strength is virtually the same as that for dissociation of amino groups (4).

The amino groups involved in control of anion permeability are probably protein ligands, even though amino lipids are present in large amounts (Table II). For example, lipid chromatography revealed no SITS bound to lipids, confirming previous observations on ox cells (38). In the case of the amino groups involved in cation permeability, the attribution to protein ligands cannot be made with any certainty, particularly in view of the finding that 30% of the membrane-bound FDNB is attached to lipids (12). Phosphatidyl serine which binds no FDNB is clearly not involved. The involvement of sulfhydryl groups in cation permeability, on the other hand, clearly implicates protein.

The number of ligands involved in the control of permeation is small compared to the total number of similar ligands in the membrane. Thus the maximal SITS binding is 3×10^{-18} moles per cell (1.8×10^{6} sites per cell) compared to a total of 374×10^{-18} moles of amino groups per ghost (Table II). Of the SITS sites only 30% or less are common sites for binding of other agents that also influence anion permeability. Thus the maximal number of permeability-controlling sites for anions is 1×10^{-18} moles per cell (6×10^{5} sites per cell). The amino sites involved in cation permeability

have not been titrated, but the sulfhydryl sites constitute a small fraction of the total sulfhydryl, about 5×10^{-18} moles per cell out of 182×10^{-18} moles per cell (11, 52, 53). This small number is in agreement with other evidence that permeation of water-soluble molecules takes place through specialized regions which constitute a small fraction ($< 0.01\%$) of the membrane surface (49, 54).

Several observations point to a different location in the membrane of the anion and cation permeability barriers. The characteristics of SITS binding—rapid interaction with a small but finite population of sites (Fig. 4)—suggest that it reacts with superficial sites, confirming the earlier observations of Maddy on ox cells (38). Furthermore, on fractionation of SITS-treated cells, the agent is found primarily in a fraction containing A-B-O antigens and sialic acid, ligands associated with the outer surface (22). On the other hand, the anion-controlling sites may not be at the outermost boundary. Pronase, for example, cleaves sialic acid–containing peptides from the surface, with only a small decrease in the amount of bound SITS. Furthermore, electrophoretic mobility which measures the most superficial groups indicates that the predominant charge is due to sialic acid, with very little contribution from amino groups (43).

The conclusion that the cation-controlling barrier is deeper in the membrane than the anion-controlling barrier is based primarily on effects of PCMBS. This reagent reacts rapidly with superficial sulfhydryl groups, but it increases cation permeability only after slow penetration into an internal compartment (11, 53). The case for the internal location of the cation-controlling amino groups is not as clear. If a single barrier is limiting to cation flow under all conditions, then it must be assumed that the amino and sulfhydryl sites are both located at the same place in the membrane in an internal compartment. The concept of a single barrier, although attractive, is not necessarily correct. In the case of anions, for example, evidence suggesting the existence of two permeation pathways has been presented (16).

Several independent lines of evidence are suggestive of an internal location for cation-controlling amino groups. The nonpenetrating amino reagent, SITS, which has a large effect on anion permeability (85% reduction) produces, under the same conditions, no increase in cation permeability, suggesting that the cation-controlling sites may be within the membrane inaccessible to the agent. The amino reagent, MNT, has a large effect on anion permeability but a smaller effect on cation permeability. Addition of 7.5% alcohol does not influence the MNT inhibition of anion permeability, but greatly enhances its effect on cation permeability (12), perhaps by increasing the penetration of the agent to the cation sites. Furthermore, FDNB at low concentrations affects primarily anion permeability, whereas at higher concentrations it affects both anion and cation permeability (3, 7).

These observations indicate that two distinct populations of amino groups control anion and cation permeability. Although the differences in the properties of the two populations could be attributed to steric factors and chemical reactivity, it seems more likely that they are due to differences in location with the anion-controlling groups being located closer to the outside of the membrane.

In addition to providing information on location, the chemical agents can give some indication of the local environment in which the responsive ligands are located. For example, ligands located in a region of positive fixed charge should react very rapidly with negatively charged reagents. The fact that SITS reacts in a few minutes with the anion-affecting sites is consistent with the hypothesis that these sites form part of a positive fixed charge region. FDNB also produces its effects very rapidly, with binding to the membrane being 80% complete after only 2 min, and virtually complete after 5 min of exposure to 1 mM FDNB (R. Juliano, personal communication). Protein amino groups which react rapidly with FDNB often lie near positive charges (24, 55) which lower the pK of the amino groups and therefore facilitate the reaction of FDNB with the uncharged form. The rapid binding of FDNB to the membrane provides further evidence that the affected sites lie in a positive fixed charge region where pK's are lower and the local pH is high.

In considering the mechanism of the effects of the chemical agents and the conclusions that can be drawn relating to the nature of anion and cation permeability, it is important to decide whether the agents act directly on the normal physiological system or whether they have created an "unnatural" mode of ion flow. It is difficult to draw unequivocal conclusions, but the weight of the evidence favors the concept that the agents are perturbing the normal permeation paths. In the case of PCMBS, for example, the effect is specific for cations. Anion permeability is not altered and water permeability is actually decreased (56). Furthermore, the effects on cation permeability are largely restricted to ions of small size such as Na^+ and K^+, with the permeability to choline ion affected to a much smaller degree (11). The agent is not opening new nonspecific channels.

In the case of the amino reagents, the evidence is even more convincing, particularly in the case of anion permeability. If the agents were, for example, establishing a new rate-limiting barrier of lower anion permeability and independent of the normal barrier, it might be expected that binding of small amounts of agent, insufficient to establish the barrier, would have a minimal effect and that the effect would increase rapidly as the barrier became more complete. The data, however, are in the opposite direction. Low concentrations of SITS (Fig. 6) and of FDNB (3, 7) are proportionately more effective than higher concentrations. Neither are the effects related to a negative charge barrier. FDNB which forms an uncharged complex has as

much effect as SITS which introduces two negative charges. Furthermore, the removal of the general negative charge barrier of the membrane by neuraminidase has minimal effects on permeability or on the action of SITS. It seems far more likely that the amino reagents produce their effects by removing fixed positive charges that are directly involved in controlling anion and cation flow as proposed by Passow (1–3, 7), to explain the anion-cation discrimination and the reciprocal effects of pH and of FDNB on anion and cation permeability.

In summary, the data presented here suggest that clusters of superficially located, positively charged amino groups enhance anion permeation, whereas the amino and sulfhydryl groups which affect cation permeability are located deeper within the membrane. These might be arranged sequentially in the same pathway as the anion-affecting groups, or they might be located in a parallel pathway. Data supporting the parallel arrangement have been obtained from studies of the effects of SITS on both PCMBS penetration and PCMBS effects on cation permeability. They have been reported briefly (57), and will be reported more fully in the following paper (16).

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