

# Chemical Modification of Membranes

## II. *Permeation paths for sulfhydryl agents*

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**ABSTRACT** The amino-reactive reagent, 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid (SITS),<sup>1</sup> considerably reduces the uptake of the sulfhydryl agent, parachloromercuriphenylsulfonic acid (PCMBS), but does not reduce its effects on cation permeability and on cation transport. These data indicate that PCMBS enters the membrane by at least two channels, one sensitive and the other insensitive to SITS, with only the latter leading to the cation-controlling sulfhydryl groups. Substitution of phosphate or sulfate for chloride results in an inhibition of PCMBS uptake via the SITS-insensitive pathway. These and other data lead to the conclusion that the SITS-sensitive pathway is the predominant one for anion permeation, and the insensitive one for cation permeation. Parachloromercuribenzoate (PCMB), an agent that is more lipid-soluble than PCMBS, penetrates faster but has a smaller effect on cation permeability. Its uptake is less sensitive to SITS. These and other observations suggest that the cation permeation path involves an aqueous channel in the membrane.

### INTRODUCTION

The effects of chemical reagents on membranes are determined not only by their ligand specificity but also by their geographic specificity; that is, their ability or inability to reach functional sites with which they might react. Although this second type of specificity greatly complicates the interpretation of the effects of chemical modifiers, it can potentially provide a unique source of information regarding certain details of membrane organization, particularly with respect to the role, number, and location of functional ligands of membrane proteins (1).

Information concerning membrane geography has already been deduced from studies of the effects of parachloromercuriphenylsulfonic acid (PCMBS), an organic mercurial which binds reversibly to sulfhydryl groups. PCMBS

<sup>1</sup>The following abbreviations are used in this paper: BMHP, 1-bromomercuri-2-hydroxypropane; FDNB, 1-fluoro-2,4-dinitrobenzene; MNT, 2-methoxy-5-nitropropene; PCMB, parachloromercuribenzoate; PCMBS, parachloromercuriphenyl sulfonic acid; PCV, packed cell volume; SITS, 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid; TNBS, 2,4,6-trinitrobenzene sulfonic acid.

reacts rapidly with a small population of sulfhydryls at or near the surface of the red cell, resulting in inhibition of glucose transport (2). Penetration into and through the membrane is, however, relatively slow compared with other organic mercurials such as 1-bromomercuri-2-hydroxypropane (BMHP), chlormerodrin, and parachloromercuribenzoate (PCMB), presumably because of the relatively low lipid solubility of PCMB (3). As PCMB penetrates into the membrane it reaches internal populations of sulfhydryls concerned with permeability to cations (4) and active transport of cations (5). The kinetics of development of the PCMB effects, as well as the rates of reversal of its effects and binding by penetrating and nonpenetrating sulfhydryl compounds, indicate that the internal populations of functional sulfhydryl groups are separated by diffusion barriers from both the outside solutions and the cytoplasm. The rather complicated permeability and binding pattern of PCMB thus provides a means of distinguishing different classes of functionally important SH groups on the basis of their different locations within the membrane.

A second reagent, 4-acetamido-4'-isothiocyano-stilbene-2,2'-disulfonic acid (SITS), appears to bind amino groups near the outer cell surface (6, 7). Binding to these groups causes a large decrease in anion permeability, as measured by sulfate exchange at Donnan equilibrium, with only insignificant changes in cation permeability (7). Other amino-reactive reagents such as 2,4,6-trinitrobenzene sulfonic acid, 2-methoxy-5-nitrotropone, and 1-fluoro-2,4-dinitrobenzene, have similar effects on anion permeability but also cause an increase in cation permeability. In contrast to SITS, PCMB, the sulfhydryl agent, only increases cation permeability and is without effect on anion permeability (7).

Because PCMB is an anion, it might be expected to reach various populations of sulfhydryl groups by diffusing into the membrane via the same channels as do sulfate and other anions. If this is so, then other anions which compete with each other for permeation (8-10) should inhibit the permeation of PCMB. Furthermore, SITS and the other amino reagents which inhibit the permeation of anions should also inhibit the permeation of PCMB.

In the present paper, the permeation of PCMB into the membrane and its effects on cation permeability were examined in normal cells and in cells pretreated with SITS and other amino-reactive reagents. The competitive effects of other anions on PCMB were also determined. From the data on uptake and from parallel observations on the effects of PCMB on both active and passive cation transport, it can be concluded that PCMB enters the membrane via at least two distinct pathways, one relatively smaller than the other. Furthermore, only one of the permeation paths for PCMB, the smaller one, leads to the functionally important populations of sulfhydryl sites within the membrane. PCMB which penetrates more rapidly than

PCMBS (2, 3) was also studied. Comparisons of uptakes and effects suggest that most of the PCMB penetrates via a third pathway that provides no access to the cation-controlling sites.

#### METHODS

*Preparation of Cell Suspensions* Fresh red cells were obtained from hematologically normal adults and were defibrinated by stirring. After centrifugation, the buffy coat was removed and the cells were washed three times in isotonic (165 mM) sodium chloride prior to use.

*PCMBS Uptake* PCMBS uptake was measured using the  $^{203}\text{Hg}$ -labeled compound (The Radiochemical Centre, Amersham, England). PCMBS at a known specific activity was added to a suspension of red cells (final hematocrit  $\cong 10\%$ ), buffered with isotonic Tris (5% v/v) at pH 7.4. At various times samples of the suspensions were centrifuged. The supernatants were removed and the cells were washed twice with ice-cold Tris-buffered saline or choline chloride. Preliminary experiments demonstrated that after two washes trapped extracellular PCMBS caused an error of less than 0.3% in the determination of counts taken up by the cells. Further washing removed insignificant amounts of bound PCMBS from the cells.

$^{203}\text{Mercury}$ -labeled PCMBS bound to the cells was determined by counting the 0.279 Mev gamma emission using a Picker NaI well scintillation counter.

*Potassium Leakage* Cells were suspended in either isotonic sodium chloride or choline chloride containing 5% (v/v) isotonic Tris buffer at pH 7.4 and 5 mg% ouabain. The suspensions were continuously shaken in a water bath at 37°C during the course of the experiment. Samples were removed at appropriate intervals, centrifuged, and aliquots of the supernatants were taken for potassium and hemoglobin determinations. The efflux of potassium into the initially potassium-free medium was expressed as per cent of the total initial cellular  $\text{K}^+$  determined by analysis of an aliquot of hemolyzed suspension (4). Some hemolysis (generally less than 5%) occurred in the cells exposed to PCMBS. Since hemolysis leads to an appearance of  $\text{K}^+$  in the medium, the efflux data were corrected to eliminate the effects of hemolysis according to the following equation:

$$\% \text{K}^+ \text{ lost} = \frac{K_{\text{med}} - h K_o}{K_o(1 - h)} \times 100$$

where  $K_{\text{med}}$  is the  $\text{K}^+$  concentration in the medium, initially zero,  $K_o$  is the  $\text{K}^+$  concentration in the hemolysate, and  $h$  is the fraction of cells which have hemolyzed. Since the hematocrit was low and only a relatively small amount of hemolysis occurred, the values obtained from this equation differed insignificantly from those calculated by more exact methods. Hemolysis was measured by hemoglobin determination using the cyanmethemoglobin procedure (11).

*Ouabain-Sensitive  $\text{Na}^+$  Efflux* Red cells were suspended at a hematocrit of about 12% in a solution containing 50 mM NaCl, 100 mM KCl, 0.1% glucose, and 17 mM Tris at pH 7.4. A small amount of  $^{24}\text{sodium}$ -labeled NaCl solution was added and the

suspension was incubated for 2 hr at 37°C. The  $^{24}\text{Na}$ -labeled cells were then washed five times, each time with 5 volumes of ice-cold isotonic magnesium chloride. Samples of the washed cells were taken for sodium and hemoglobin determinations.

2 ml portions of the packed cells were resuspended in 12 ml of a solution containing 140 mM NaCl, 10 mM KCl, 0.1% glucose, and isotonic phosphate buffer (10% v/v) at pH 7.4. SITS (0.35 mg/ml PCV) and/or ouabain (0.1 mM) were added, and then 5 min later PCMBS was added at a final concentration of 0.17 mM. 2 ml samples were taken at 10 min intervals, centrifuged, and the supernatants were counted on a Nuclear Chicago well scintillation counter. A sample of the suspension was hemolyzed by adding Cutscum (Fisher Scientific Co., Pittsburgh, Pa.), and also counted to obtain total  $^{24}\text{Na}$  activity.

No decay corrections were necessary since each series of samples was counted within a 10 min interval. Since the efflux follows first-order kinetics, the rate constant and flux were determined from a plot of the logarithm of the fraction of  $^{24}\text{Na}$  remaining in the cells vs. time.

The ouabain-sensitive efflux was calculated as the difference between the fraction of  $\text{Na}^{24}$  efflux in the absence and presence of ouabain as proposed by Hoffman (12). If  $W$  is the counts per minute of the hemolyzed suspension and  $P(t)$  the counts per minute in the supernatant at time  $t$ , then the fraction remaining in the cells at time  $t$  is approximately  $[W - P(t)]/W$  and since the efflux is first order,  $\ln\{[W - P(t)]/W\} = -kt$ . In order to obtain the ouabain-sensitive efflux, the value for  $\ln\{[W - P(t)]/W\}$  in the presence of ouabain was subtracted from that in the absence of ouabain for each experimental point. The difference was plotted against time.

## RESULTS

The amino-reactive reagents, SITS, MNT, FDNB, and TNBS, shown previously to inhibit sulfate permeation (7, 8) also inhibited the uptake of PCMBS (Table I). The extent of the inhibition was relatively independent of the concentration of PCMBS but was considerably modified in certain of the buffers. Choline and Tris had little effect, but in the presence of sulfate and especially of phosphate, the inhibition by SITS markedly increased.

In a previous study (4) it was demonstrated that the uptake of PCMBS is followed by a slow release due to PCMBS-binding substances containing sulfhydryl groups that leak out of the cells (2, 13). In the presence of SITS the same pattern of binding and release occurred (Fig. 1), but the rate of uptake and the rate of release were reduced in this particular case by a factor of about 50%. At the time of maximal binding, approximately 50% of the available PCMBS has been taken up in the control and 25% in the SITS-treated cells. Until the time of maximal binding, much of the cellular PCMBS is in the membrane but at later times when further release of PCMBS is almost zero, virtually all of the PCMBS is in the cytoplasm bound to hemoglobin (2). It is clear from Fig. 1 that SITS not only reduces the uptake into the membrane and the efflux from the membrane, but also the amount of agent that reaches the inside of the cell as well. Thus SITS inhibits both the uptake

into the membrane and the permeation through the membrane into the interior of the cell.

In cells treated with SITS or with other amino reagents, the inhibition of sulfate permeability is incomplete, about 85% (7, 8). In the case of PCMBS the inhibition is also incomplete in cells treated with a concentration of SITS that reacts with virtually all available binding sites (7). The incomplete inhibition can be explained by assuming that a single permeation channel for anions is partially blocked by SITS, or alternatively, that two permeation

TABLE I  
EFFECTS OF AMINO AGENTS ON UPTAKE OF  
PCMBS INTO THE MEMBRANE

Agent	Temperature	PCMBS concentration	Buffer	Inhibition of PCMBS uptake*
	°C	moles/liter		
SITS	23	$1 \times 10^{-5}$	Tris-saline	59
		$2 \times 10^{-5}$	Tris-saline	57
		$2 \times 10^{-5}$	Tris-choline	59
		$1 \times 10^{-4}$	Tris-choline	62
	37	$5 \times 10^{-5}$	Tris-choline	50 (3)
			Tris-saline	59 (2)
			Tris-sulfate	71 (2)
			Phosphate-saline	91
		$1.7 \times 10^{-4}$	Phosphate-saline	94 (4)
TNBS	23	$5 \times 10^{-5}$	Phosphate-saline	84 (3)
MNT	23	$5 \times 10^{-5}$	Phosphate-saline	37 (3)
FDNB	23	$2 \times 10^{-5}$	Tris-saline	63
	23	$5 \times 10^{-5}$	Tris-saline	57 (2)
	23	$5 \times 10^{-5}$	Phosphate-saline	67 (3)

All buffers are isotonic at pH 7.4. Tris-saline and phosphate-saline contain 5% isotonic Tris or phosphate by volume. Tris-choline and Tris-sulfate contain 5% isotonic Tris in isotonic choline or sulfate. The concentrations of agents were as follows: SITS, 0.3 mg/ml PCV; TNBS, 2.9 mM; FDNB, 1.4 mM; MNT, 5.6 mM.

\* Inhibition of PCMBS uptake is expressed as per cent of control. Numbers in parentheses are the numbers of determinations.

channels exist, one of which is completely blocked by SITS, while the other is not affected. The two possibilities were explored by measuring the effect of PCMBS on  $K^+$  leakage in normal and in SITS-treated cells (Fig. 2). As demonstrated previously, SITS itself had no effect on  $K^+$  leakage (7), whereas after a delay, PCMBS produced a rapid  $K^+$  loss (4). At the time that PCMBS uptake slowed down and reached a maximum value (Fig. 1), the rate of  $K^+$  loss also decreased and returned to normal. In SITS-treated cells, despite the fact that the PCMBS uptake was considerably reduced, the onset of  $K^+$  leakage was just as rapid as in the untreated cells. In fact at later stages of the

experiment, the extent of  $K^+$  leakage was considerably greater in the treated cells. These results are in sharp contrast to those in which the uptake of PCMBs is reduced by decreasing the external concentration of PCMBs (4). When PCMBs uptake is reduced in this manner, the lag period before  $K^+$  leakage is prolonged, the maximal rate of  $K^+$  efflux is reduced, and the extent of  $K^+$  loss is decreased. On this basis a reduction of 50% in uptake of PCMBs caused by SITS should noticeably delay and decrease the PCMBs effect if PCMBs enters the membrane by a single pathway partially blocked by SITS. The results in Fig. 2 are completely contrary to this prediction, and lead to the conclusion that two permeation channels exist for PCMBs, and

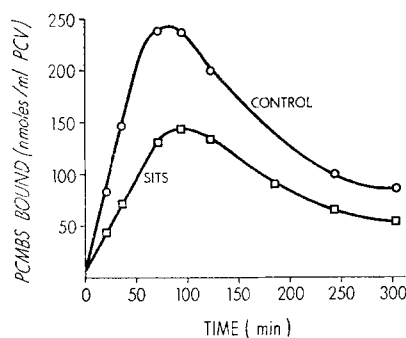


FIGURE 1

FIGURE 1. Effect of SITS on PCMBs uptake. Cells were exposed to SITS (0.3 mg/ml PCV) for 30 min at 23°C, centrifuged, and resuspended in Tris-buffered choline chloride containing 0.05 mM PCMBs. Temperature, 38°C; pH 7.4.

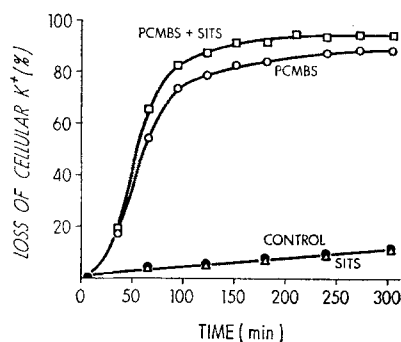


FIGURE 2

FIGURE 2. Effect of SITS on the potassium efflux induced by 0.05 mM PCMBs. Reaction with SITS was carried out as described in Fig. 1. Efflux of potassium into Tris-buffered choline chloride at 38°C and pH 7.4 was measured as described in Methods.

that the channel which is insensitive to SITS is the one that contains the SH groups which control cation permeability.

The two-channel model is further supported by experiments in which the PCMBs influx was compared in cells preequilibrated for 2 hr in either isotonic sodium chloride or sodium sulfate. Anions compete with each other for permeation (7) so it seemed profitable to compare the competitive effects of  $Cl^-$  and  $SO_4^{--}$  on the two proposed channels. The uptake in the sulfate medium was considerably reduced over that in chloride, either in the presence or absence of SITS (Fig 3). If, however, the decrease in uptake due to SITS is calculated, it is virtually the same in sulfate as in chloride (Fig. 4). These data indicate that the two channels can be differentiated with respect to sulfate and chloride. Uptake of PCMBs via the SITS-sensitive channel is equally influenced by  $Cl^-$  and  $SO_4^{--}$ . In fact the total inhibitory effect of  $SO_4^{--}$  can be attributed to its effect on the SITS-insensitive channel. Phosphate probably

has a similar effect. For example, the SITS inhibition of PCMBs uptake is increased from 50–60% to over 90% in phosphate-buffered saline (Table I).

The enhancement of PCMBs-induced  $K^+$  leakage by SITS (Fig. 2) was an unexpected result, particularly in view of the fact that SITS alone had no effect. The phenomenon was explored by adding SITS at different times relative to PCMBs. In order to maximize the enhancement, the experimental medium was changed from choline chloride (used in the experiments of Figs. 1 and 2) to NaCl.<sup>2</sup> The degree of enhancement depended entirely on the relative times of addition of SITS and PCMBs. Thus in Fig. 5, if SITS

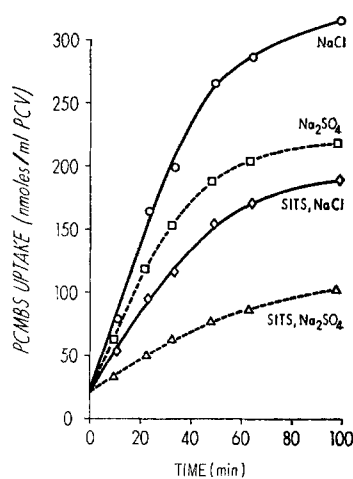


FIGURE 3

FIGURE 3. Effects of chloride and sulfate on PCMBs uptake in the presence and absence of SITS. Cells were reacted with SITS as described in Fig. 1 prior to the addition of 0.05 mM PCMBs. Temperature, 37°C; pH 7.4.

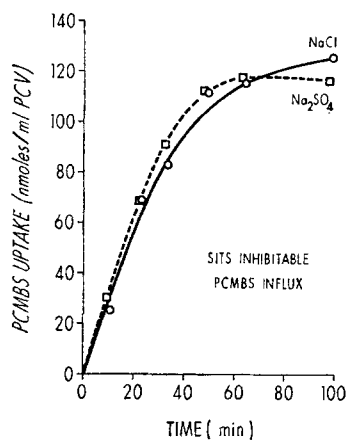


FIGURE 4

FIGURE 4. Effects of chloride and sulfate on the SITS-inhibitable PCMBs uptake. Data points calculated from Fig. 3 by subtracting the uptake in the presence of SITS from the total uptake.

was added before PCMBs, the enhancement was considerable. If it was added 30 min after PCMBs, it was less and if it was added 45 min or more after the PCMBs, no enhancement was observed. If the effect of SITS were due to some direct action on the cation permeability pathway that becomes patent in the presence of PCMBs, SITS should increase the effect of PCMBs regardless of when it is added to the cells. The results shown in Fig. 5, on the other hand, indicate that SITS is effective only if added before the PCMBs

<sup>2</sup> For reasons that are not clear, the effect of PCMBs on  $K^+$  leakage is greater in choline than in  $Na^+$  media. This is not due to any effect on PCMBs uptake. The enhancement by SITS as seen in Fig. 2 is not large because the cells lost most of their  $K^+$  in PCMBs alone. In NaCl medium, the same concentration of PCMBs produced a small effect, but the enhancement by SITS was greater (as in Fig. 5).

uptake into the membrane has become appreciable. These findings are consistent with the two-channel model. Normally up to 60% of the inflow of PCMBS would enter the SITS-sensitive channel and be bound to sulfhydryl groups that are not involved in cation transport, and as little as 40% of the PCMBS inflow would enter the second channel and reach the cation-controlling sites. In the presence of SITS the amount of PCMBS entering the second channel would be the same initially, but it would be greater at later times because none would have been diverted to "insensitive" sites. Of the total PCMBS entering the membrane, more will reach the "sensitive" sites.

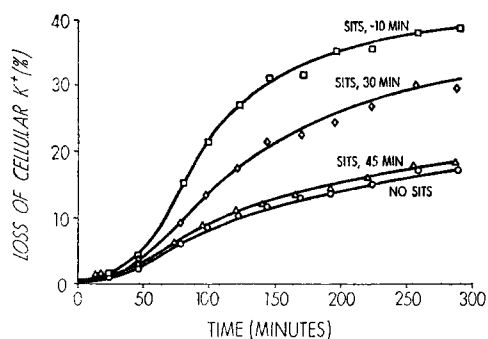


FIGURE 5

FIGURE 5. Effect of the time of addition of SITS on the PCMBS-induced potassium efflux. Cells were suspended in Tris-buffered saline at 37°C. PCMBS (0.05 mM) was added at time zero. SITS (0.3 mg/ml PCV) was added at the times indicated.

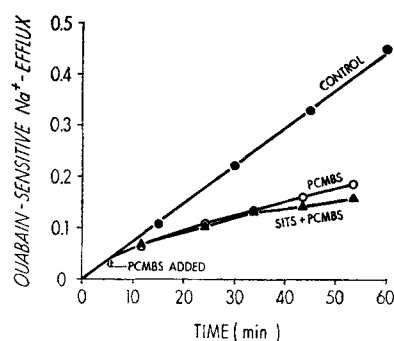


FIGURE 6

FIGURE 6. Effects of SITS on the PCMBS-induced inhibition of ouabain-sensitive sodium efflux. Experimental conditions and units for  $\text{Na}^+$  efflux are described in Methods. Results with SITS-treated cells were virtually identical to the control.

The active transport of cations is inhibited by the reaction of PCMBS with sulfhydryl groups within an inner compartment (5). Presumably a different population of sites is involved than those controlling permeability because active transport is more sensitive to PCMBS. The mechanism of inhibition is presumably related to the effect of the agent on the  $\text{Na}^+\text{-K}^+$ -activated ATPase (14), which is an essential part of the transport system. In order to gain more insight into the membrane pathways by which PCMBS reaches the sulfhydryl groups involved in active transport, experiments were carried out with SITS similar to those described for the study of cation permeability. The transport, measured in terms of the ouabain-sensitive  $\text{Na}^+$  efflux, is given in Fig. 6.

SITS itself had no effect on cation transport but as reported previously (5), PCMBS causes a considerable inhibition. As is the case for permeability (Fig. 2), SITS did not slow down or reduce the inhibitory effect of PCMBS



even though the uptake of PCMBS in this experiment was reduced more than 90% (Table I). It must be concluded that the active transport sites as well as the permeability sites are within a SITS-insensitive channel.

Comparison of the rates of uptake of PCMBS and PCMB and of their effects on cation leakage suggests that an additional pathway through the membrane is available to the latter agent. PCMB permeates much more rapidly than does PCMBS (2, 3), a finding that has been explained by the suggestion that a small amount of PCMB may be undissociated and penetrate by virtue of its lipid solubility (2). The more rapid penetration of PCMB was readily confirmed. Its uptake at pH 7.4 and 37°C is over five times as rapid.

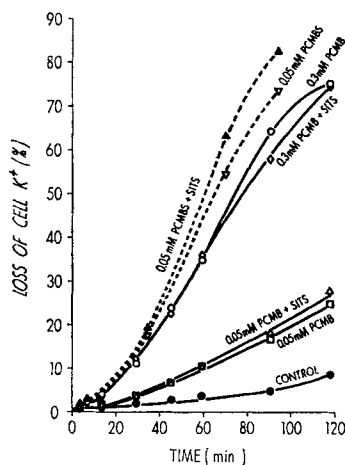


FIGURE 7. Effect of PCMB on  $K^+$  loss in the presence and absence of SITS. Cells were treated with SITS as described in Fig. 1.  $K^+$  efflux was measured as described in Methods. Temperature, 37°C; pH 7.4. PCMBS data taken from Fig. 5 for comparison.

The rapid penetration does not proceed, however, through the SITS-sensitive channel. In contrast to a SITS inhibition of 60% in the case of PCMBS uptake, the inhibition was less than 10% in the case of PCMB uptake, about that expected if the "extra" uptake proceeded by a SITS-insensitive channel or channels.

Although the uptake of PCMB via SITS-insensitive routes is about 10 times that for PCMBS, its effect on  $K^+$  leakage is much smaller. Thus in Fig. 7, at equal concentrations (0.05 mM), the induced  $K^+$  leakage was only about 25% as high although the kinetic pattern was similar, with a delay followed by a linear rate of loss. SITS had no influence on the PCMB-induced  $K^+$  leakage. It can be concluded that most of the PCMB traverses the membrane by a route that is neither SITS-sensitive, nor associated with the cation-controlling proteins.

## DISCUSSION

The use of chemical modifiers in membrane studies has not only demonstrated the functional importance of ligands such as amino and sulfhydryl groups, but also has provided information concerning their location at various levels within the membrane. Sugar transport, for example, is affected by a small population of SH groups located near the outer surface (2), whereas other sulfhydryl groups deeper within the membrane play a role in cation permeability (4) and active cation transport (5). A population of amino groups, probably located inside the membrane, is also involved in cation permeability, while another small population, located more superficially, affects anion permeability (7).

The relative location of surface amino groups and intramembrane sulfhydryl groups is further confirmed by the finding that amino-reactive reagents, especially SITS, can inhibit PCMBS entry into the membrane. Since the inhibition of PCMBS uptake by SITS caused no corresponding decrease in the PCMBS-induced effects on cation permeability or on active cation transport, it is necessary to conclude that PCMBS enters the membrane by at least two different routes, one of which is subject to inhibition by SITS and the other of which provides access to the intramembrane sulfhydryl ligands which affect both active and passive cation transport. The anion specificities of the two pathways are also different, in that replacement of chloride by sulfate or phosphate inhibits uptake of PCMBS via the second channel with little effect on the first.

Although the PCMBS results define the means whereby a toxic organic agent gains access to functionally important sites, it is important to ask what relevance these findings have to the transport process for inorganic ions. In the case of anions, it seems clear from the similar effects of inhibitors that the SITS-inhibitable pathway for PCMBS entry is also the route through which most sulfate flows under normal conditions.<sup>3</sup> It is already well established that the sulfate pathway is the same as that for other anions (8-10). PCMBS is also capable of entering the cell via the second pathway. Since sulfate and phosphate can enter this channel and affect the rate of uptake of PCMBS through it, it seems probable that part of the flux of these ions (some of that fraction which is not inhibited by SITS) also passes through this channel. If this is the case, it might provide an explanation for Passow's observation (15) that the maximum inhibition by high FDNB concentrations is not the same for each anion. Values range from 83.6% for sulfate to only

<sup>3</sup> The mercuric ion and the mercury moiety in organic mercurials form complexes with anions including  $\text{Cl}^-$ . Thus in saline solutions these compounds are polyanions. The interactions of mercurials with anions and with sulfhydryl groups are reviewed in Webb, J. L. 1966. *Enzyme and Metabolic Inhibitors*. Academic Press, Inc., New York. 2:739-985.

15.5% for lactate. Such differences could result from the particular selectivities of the two proposed pathways for anion permeation.

With regard to cation permeability, two kinds of evidence suggest that the PCMBS effects are related to sulfhydryl groups located within the normal permeation channel. First, the increased permeability is not a general effect. It is specific to the extent that the flows of  $\text{Na}^+$  and  $\text{K}^+$  are increased by PCMBS to a much greater degree than that of choline (4). Sulfate permeability is not affected (7) and water permeability is decreased (16). Second, the cation-controlling sulfhydryls are located inside the membrane in an aqueous compartment. The evidence is twofold. By using a free-radical scavenger, *p*-aminobenzoic acid, Sutherland and Pihl (17) demonstrated that the indirect effects of X-irradiation on the cation-affecting SH groups are caused by hydroxyl radicals. They concluded that the sulfhydryl groups must be in an aqueous compartment within the membrane. This conclusion is supported by the finding that the rapidly penetrating lipid-soluble mercurials have much less effect on cation permeability than the slowly penetrating, water-soluble PCMBS. The extreme case is 1-bromomercuri-2-hydroxypropane (BMHP), one of the most rapidly penetrating agents, that has no effect on cation permeability at concentrations below  $3 \times 10^{-4}$  M (3). Another is PCMB that penetrates more than three times as fast as PCMBS but has much less effect on cation permeability than PCMBS (Fig. 7). The extra uptake of PCMB, presumably via lipid solubility, does not increase the reaction with the cation-controlling sulfhydryl groups, suggesting that they are not located in a hydrophobic region.

It seems unlikely that an existing nonspecific aqueous channel would become a new low resistance pathway specific for cations after reaction of its sulfhydryl groups with PCMBS. It seems more likely that the normal pathway for cation flow is an aqueous channel containing sulfhydryl groups and that its resistance to  $\text{Na}^+$  and  $\text{K}^+$  is decreased after reaction with PCMBS. On this basis the SITS-sensitive pathway would be the predominant channel for anion permeation and the SITS-insensitive pathway would be the predominant channel for cation permeation. The proposed separate routes for anion and cation flow would be consistent with the observation that anion and cation permeability are regulated by different rate-limiting barriers (7, 18).

Active transport of cations also involves an internal sulfhydryl compartment, reached by PCMBS via a SITS-insensitive pathway (Fig. 6). At the present time it is not possible to distinguish the pathway through which ions reach the pump sites from that through which passive cation flows occur. It is clear, however, that the transport path is distinct from that which accounts for most of the anion flow.

The information obtained from studies with chemical modifiers such as SITS and PCMBS emphasizes the complex mosaic structure of the red cell

membrane in both circumferential and radial directions. In the radial direction various small, functionally distinct, populations of sites may be distinguished on the basis of their different accessibilities to reagents added from the outside. Using this information, it has been possible to detect further organization in the plane of the membrane. Organic mercurial reagents reach different sites through at least two distinct pathways in addition to passing through the lipid (in the case of BMHP and PCMB). Other anions also seem to enter by two routes, only one of which is shared by cations. These results clearly indicate that the membrane, far from exhibiting a relatively homogeneous structure, displays a high degree of spatial organization and specialization which enables it to carry out its functions.

The technical assistance of the following is gratefully acknowledged: Suzanne Sauda, Steven Schonfeld, Bruce Gordon, and Jeffrey Rabek.

This paper is based on work performed in part with the assistance of the US Public Health Service Training Grant No. 5 T1 GM1088 from the National Institute of General Medical Sciences, and in part under contract with the US Atomic Energy Commission at The University of Rochester Atomic Energy Project. It has been assigned Report No. UR-49-1381.

Received for publication 21 December 1970.

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