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Study of Amino and Sulfhydryl Sites in the Sodium Pathway in Dog Red Blood Cell Membranes

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Summary. Amino reactive TNBS (2,4,6-trinitrobenzene sulfonic acid), SITS (4-acetamido-4′-isothiocyano-stilbene-2-2′-disulfonic acid), and Zn^{++}, and SH reactive Hg^{++} were employed to study sodium channels in dog red blood cells. Simultaneous modification of the membrane with both a SH and an amino modifier results in an increase in Na^+ permeability which is equal to the sum of their individual effects. This indicates that SH and amino sites are separate units. Three lines of evidence indicate that the amino sites are more superficial than the SH sites. (1) Pretreatment with an amino modifier decreases the effectiveness of subsequent SH modification. (2) SITS, a nonpenetrating amino reagent, enhances Na^+ permeability while DTNB, a nonpenetrating SH modifier, is ineffective. (3) Pretreatment of amino sites decreases the apparent affinity of Hg^{++} for SH sites. In addition, three lines of evidence indicate that TNBS and Zn^{++} modify different amino sites. First, simultaneous modification with TNBS and Zn^{++} results in an increase in Na^+ permeability equal to the sum of their individual effects. Secondly, Zn^{++} causes an increase in Na^+ permeability in cells previously treated with TNBS. Finally, the pH dependence of Zn^{++} modification is opposite that for TNBS modification. These pH experiments suggest that Zn^{++} enhances Na^+ permeability by reacting with unprotonated amino sites while TNBS modifies protonated amino sites. It is concluded that the sodium permeability of dog red blood cells is normally limited by superficial amino sites and deeper sulfhydryl sites in the sodium channels.

In an earlier paper (Castranova & Miles, 1976) we reported that sodium movement through ionic channels in dog red blood cells is affected by amino and sulfhydryl groups. Pharmacological modification of these sites with heavy metal cations and group-specific chemical modifiers results in an increase in sodium permeability. We determined that treatment of the cells with amino specific reagents, such as TNBS (2,4,6-

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trinitrobenzene sulfonic acid), MNT (2-methoxy-5-nitrotropone), and SITS (4-acetamido-4′-isothiocyano-stilbene-2-2′-disulfonic acid), as well as with the divalent cation, Zn^{++}, increases sodium permeability by interacting with amino groups in the sodium channel. The reaction, at least in the case of Zn^{++} and SITS, is bimolecular. In addition, the sulfhydryl specific reagent, PCMBS (parachloromercuribenzene sulfonic acid), and the divalent cations, Hg^{++}, Ni^{++}, and Co^{++}, increase sodium permeability by reacting with sulfhydryl groups in the channel in a manner which can be described by 2:1 binding isotherms, i.e., two molecules of modifier react with each sulfhydryl site.

The objective of this paper is to study primarily the amino sites which are present within the sodium channels. In addition, the functional relationship and relative positions of the amino and sulfhydryl sites are investigated. Such information assumes great importance when one considers that as much as 97% of sodium efflux from dog red blood cells is probably via a passive pathway (Parker, 1973). This investigation represents an attempt to resolve the following questions concerning the nature of the sodium channels and, in particular, the amino and sulfhydryl sites. (1) Do the amino and sulfhydryl sites represent two separate and distinct units which affect sodium movement? (2) What are the relative positions of the amino and sulfhydryl sites within these channels? (3) Is there more than one type of amino site which affects sodium permeability? Abstracts containing some of the results reported here have appeared elsewhere (Castranova & Miles, 1974, 1975).

Materials and Methods

Sodium Efflux Measurements

The procedures for cell preparation and measurement of Na^+ efflux were essentially the same as those described earlier (Castranova & Miles, 1976). In general, dog red blood cells were loaded with ^{22}Na in a phosphate buffered medium and then placed in flasks containing a PIPES buffered medium of the following composition: Na^+ (146 mM), Cl^- (151 mM), K^+ (5 mM), glucose (2.77 mM), and Na PIPES [piperzine-N-N′-bis(2-ethane sulfonic acid)] (5 mM). The pH of the medium was adjusted to 6.5 for most of the experiments. For experiments in which the pH of the medium differed from 6.5, HCl or NaOH was used to adjust the pH. The functional relationship between amino and sulfhydryl sites and between different amino sites in the sodium channels was investigated by subjecting these red cells to simultaneous and successive treatment with amino and sulfhydryl modifiers.

The efflux curves indicate that sodium efflux from dog red blood cells behaves as a three-compartment system. It has been shown that in such a system the cell interior consists of two compartments (Sha'afi & Lieb, 1967). Efflux from the first compartment represents only 5% of the total cell Na^+ and equilibrium with the extracellular fluid is reached within 30 min. The major portion of Na^+ efflux (i.e., 95% of the total cell Na^+) is from the second intracellular compartment. Therefore, in all of these experiments

the rate constant for Na^+ efflux was measured after one-half hour of incubation. The change in Na^+ efflux in response to chemical modification of the cell membrane was measured by comparing this rate constant in the presence of modifiers to the rate constant in unmodified cells (controls).

In this study Hg^{++} was used to modify sulfhydryl sites while TNBS (Sigma Chemical Co., St. Louis, M.), SITS (Nutritional Biochemicals Corp., Cleveland, Ohio), and Zn^{++} were chosen as amino reagents. The reactivity of SITS, which is a labile compound, was not checked. However, SITS which was obtained from the same supplier and maintained in the same manner produces 98% inhibition of sulfate fluxes in dog and cat red cells (Castranova, Weise & Hoffman, 1976). In most experiments, the concentrations of modifier used were those which had previously been shown to cause maximal changes in Na^+ permeability, i.e., 0.1 mM Hg^{++}, 3 mM TNBS, 0.1 mM SITS, and 0.75 mM Zn^{++} (Castranova & Miles, 1976). Hg^{++} was chosen as the sulfhydryl reagent because it has a high affinity for sulfhydryl sites in the channels, yet produces an increase in Na^+ permeability which is less massive than other sulfhydryl reagents (e.g., PCMBS, Ni^{++}, and Co^{++}), i.e., the effect of Hg^{++} on Na^+ permeability is less likely to obscure the smaller effects of amino reagents.

Concentration-Effect Studies

The apparent affinity of the sodium channels for Hg^{++} was determined by measuring sodium efflux with varying concentrations of Hg^{++} in the medium. These measurements were made both in normal red cells and in cells which where preincubated for 1.5 hr in medium containing 0.1 mM SITS. The data analysis consisted of constructing Hill plots as described previously (Castranova & Miles, 1976). The equation for this type of plot is

$$\log\left(\frac{1}{\alpha}-1\right)=-n\log\left[Hg^{++}\right]+\log K_D,$$

where α is the fraction of channel sites occupied by Hg^{++}, n is the binding type, $[Hg^{++}]$ is the concentration of Hg^{++} in moles per liter, and K_D is the equilibrium dissociation constant for this reaction. A plot of $\log\left(\frac{1}{\alpha}-1\right)$ vs. $\log[Hg^{++}]$ yields a straight line such that the slope $(-n)$ indicates the binding type and the y-intercept is $\log K_D$. In these experiments α is measured as the fraction of the maximal change in Na^+ efflux (relative sodium efflux). Therefore, we have assumed that the change in Na^+ efflux is linearly related to the fraction of sites modified. It must be emphasized that this analysis of the concentration-effect relationships is used only as an approximation to the actual binding.

Mean Cell Volume Measurements

The change in mean cell volumes (MCV) was measured over the pH range from 6.0 to 7.5 both in the presence and absence of amino modifiers. The washed red cells were incubated in a medium at the given pH (with and without modifier) for about 1 hr. This time of incubation corresponded to the time during which sodium efflux measurements were made. In addition, the hematocrit used was less than 5%, which is the same as that used for flux measurements. After the incubation the cells were spun down and enough supernatant was removed so that the hematocrit was between 30% and 50%. Then the cells were resuspended and MCV measurements were made using this suspension. The MCV's were calculated in the usual manner by dividing the hematocrit by the cell count. Cell counts were determined by using a Coulter Counter (Model B, Coulter Electronics, Hialeah, Fla.). The MCV's were expressed in cubic microns.

Results

Simultaneous Modification of Amino and Sulfhydryl Sites

Pharmacological modification of either sulfhydryl or amino groups within sodium channels enhances sodium permeability (Castranova & Miles, 1976). Therefore, two possibilities exist as to the functional relationship between these sites. First, the sulfhydryl and amino groups may act as two separate and distinct units which affect sodium movement, or, secondly, these two groups may be parts of a single unit. If the two sites are separate, then simultaneous modification of both sites may cause an increase in sodium efflux equal to the sum of the effects of their separate modification. On the other hand, if the sites are not separate, i.e., if they are part of a single unit, simultaneous modification should cause an increase in Na^{+} permeability which is less than the sum of the effects of separate modification.

Sodium efflux measurements from red cells treated with Zn^{++} or Hg^{++} alone and from cells treated simultaneously with both Zn^{++} and Hg^{++} are shown in Fig. 1. Note that separate treatment of red cells

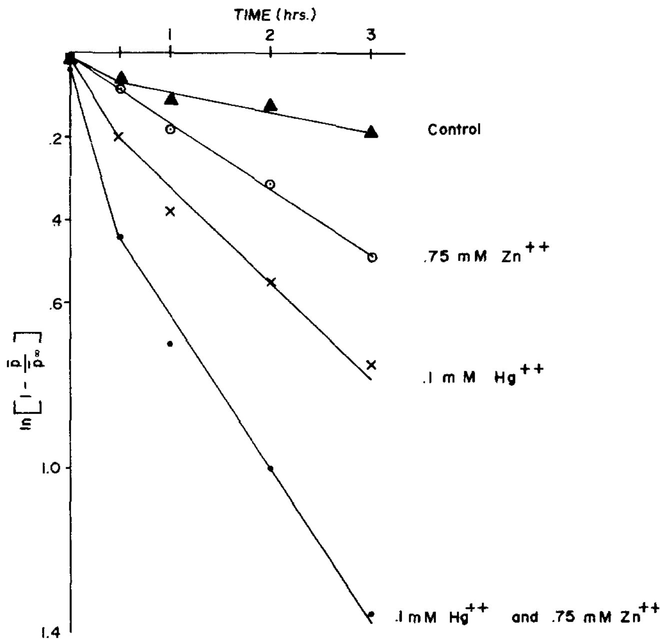

Fig. 1. Sodium efflux measurements showing the effects of membrane treatment with 0.75 mM Zn^{++} alone, 0.1 mM Hg^{++} alone, and simultaneous treatment with 0.75 mM Zn^{++} and 0.1 mM Hg^{++}. The concentrations of divalent cations used were those which cause the maximum increase in sodium efflux

Table 1. Effect of simultaneous modification of membrane amino and sulfhydryl groups

Modifiers	Relative Na^+ Efflux $\pm$ SEM	n
Hg^{++}	6.6$\pm$ 1.6	3
Zn^{++}	4.3$\pm$ 0.5	3
Hg^{++} and Zn^{++}	10.9$\pm$ 1.9	3
Hg^{++}	7.2$\pm$2.5	3
SITS	1.6$\pm$ 0.3	3
Hg^{++} and SITS	8.7$\pm$2.9	3

Relative sodium efflux values were obtained by dividing the rate constant for sodium efflux in the presence of modifier by the rate constant for efflux in control cells. These values represent mean values obtained from 3 experiments. (The values for Hg^{++} differ because two different sets of experiments were performed.)

with amino reactive Zn^{++} or sulfhydryl reactive Hg^{++} results in an increase in sodium efflux over the control level and that simultaneous treatment of cells with both Zn^{++} and Hg^{++} causes an even greater increase in efflux. Similar results are also obtained with the simultaneous addition of Hg^{++} and SITS. These results are summarized in Table 1. Simultaneous modification of the membrane with amino and sulfhydryl reagents increases sodium permeability to a level approximately equal to the sum of the effects of separate modification. These data are taken to indicate that the amino and sulfhydryl sites represent two distinct barriers for sodium movement in dog red blood cells.

Successive Modification of Amino and then Sulfhydryl Sites

In order to gain information about the relative positions of these two sites, an attempt was made to completely modify the amino site and then measure the change in sodium permeability induced by subsequent modification of the sulfhydryl site. The experimental procedure consisted of measuring sodium efflux for 1.5 hr in the presence of the amino modifier, adding the sulfhydryl modifier (the amino modifier was still present), and then measuring sodium efflux for one more hour. Relative sodium permeability for the amino modifier was obtained by measuring the rate constant for sodium efflux from 0.5 to 1.5 hr while that for subsequent modification was obtained from the rate constant during the final hr. In addition, separate controls were done in which the rate constant in the presence of the amino modifier alone was also

Table 2. Effect of successive modification of membrane amino and sulfhydryl groups

Modifiers	Relative Na^+ Efflux $\pm$ SEM	% Decrease in Effectiveness	n
Hg^{++}	6.6 ± 1.6	–	3
Zn^{++}	4.3 ± 0.5	–	3
Zn^{++} and Hg^{++} simultaneously	10.9 ± 1.9	–	3
Zn^{++} then Hg^{++} successively	5.9 ± 1.0	46%	3
SITS	1.6 ± 0.3	–	3
SITS and Hg^{++} simultaneously	8.7 ± 2.9	–	3
SITS then Hg^{++} successively	2.1 ± 0.6	76%	3

Relative sodium efflux values represent mean values obtained from 3 experiments. The percent decrease in effectiveness was calculated by dividing the value for relative Na^+ efflux during successive modification by that value obtained from simultaneous modification and then subtracting this result from one.

obtained during the final hr of the experiment. In no case did this rate constant differ from that obtained during the time period of 0.5 to 1.5 hr.

The results obtained when cells are pretreated with the amino reagents, Zn^{++} or SITS, and then exposed to sulfhydryl reactive Hg^{++} are summarized in Table 2. It can easily be seen that subsequent treatment of amino pretreated cells with Hg^{++} does not increase Na^+ efflux nearly as much as when the cells are modified simultaneously, i.e., the effectiveness of Hg^{++} is decreased by amino pretreatment. Furthermore, the Hg^{++} effect is blocked to a greater extent by pretreatment with SITS than with Zn^{++}. This may mean that the interference with the effect of Hg^{++} is related to the size of the pretreating particle since SITS is larger than Zn^{++}. One way to explain these results is to assume that pretreatment of the amino sites physically blocks the movement of Hg^{++} to the sulfhydryl sites. This would be the case if the amino sites were located more superficially than the sulfhydryl sites. The fact that SITS, an amino reagent which cannot penetrate the membrane (Maddy, 1964; Knauf & Rothstein, 1971), enhances Na^+ permeability also indicates that the amino sites are located near the outer surface of the cell membrane. In addition, DTNB (5,5′-dithiobis-[2-nitrobenzoic acid]), a sulfhydryl reagent which seems too large to penetrate the membrane (Ellman, 1959), has little effect on Na^+ efflux. Indeed, 1.0 mM DTNB increases Na^+ efflux only 1.2 times control as compared to the 11.6-fold increase in Na^+ permeability induced by 0.1 mM PCMBS, a sulfhydryl reagent which can penetrate the membrane (Knauf & Roth-

Table 3. n and K_D for Hg^{++} in normal and SITS pretreated red cells

	Binding Number (n)	K_D ($\times 10^3$)	r
Normal Cells	−2.0	1 mM^2	0.95
SITS Pretreatment	−2.3	18 mM^2	0.99

These data for Hg^{++} binding were obtained from Hill plots by using the mean values from 3 experiments in normal and in SITS pretreated red cells. r is the correlation coefficient for the line. *See* text for further explanation.

stein, 1971). However, the possibility that DTNB fails to react with accessible SH sites cannot be excluded. These data then indicate that the sulfhydryl sites are probably relatively deep within the membrane.

If amino pretreatment decreases the accessibility of the sulfhydryl site, one may expect a decrease in the apparent affinity of Hg^{++} for the sulfhydryl sites in amino pretreated cells. The dissociation constants and the type of binding between Hg^{++} and the sulfhydryl sites in normal and in amino pretreated cells are listed in Table 3. These data were obtained from Hill plots. The binding number (n) and dissociation constant were obtained from the slope and y-intercept respectively of a plot of log $(\frac{1}{\alpha}-1)$ *vs.* log (Hg^{++}) where α is the fraction of the maximal increase in Na^+ efflux produced by Hg^{++}. Note that the apparent affinity of Hg^{++} for the channel decreases some 18-fold after amino pretreatment, i.e., the dissociation constant is 18-fold greater in amino pretreated cells than in normal cells. Therefore, modification of the superficial amino sites does seem to block the movement of Hg^{++} to the deeper sulfhydryl sites. Table 3 also shows that the slope of the Hill plot, which indicates the binding type, does not change significantly in amino pretreated cells, i.e., binding is still 2:1. The results thus far then suggest that amino and sulfhydryl sites which affect Na^+ permeability are separate and distinct and that the amino groups are probably located more superficially.

Simultaneous and Successive Modification of Amino Sites

Although modification of amino sites within the sodium channel with three different amino modifiers, Zn^{++}, TNBS, and SITS, results in an increased Na^+ efflux, the extent of the permeability change varies with the modifier used. These results, which were published previously (Castranova & Miles, 1976), are summarized in Table 4. Zn^{++} causes

Table 4. Simultaneous modification with amino modifiers

Modifier	Relative Na^+ Efflux ±SEM	n
Zn^{++} (0.75 mM)	4.7±0.4	15
TNBS (3 mM)	3.5±0.2	12
SITS (0.1 mM)	2.6±0.3	18
TNBS+ Zn^{++}	7.7±0.7	5
Control	1.0	

Relative sodium efflux values were obtained by dividing the rate constant for sodium efflux in the presence of modifier by the rate constant for efflux in control cells. The number of experiments in each case is shown at the right (n).

the greatest increase in Na^+ efflux, TNBS the next greatest, and SITS is the least effective of the amino modifiers. Two possibilities exist to explain the different maximal effects of the modifiers: either they react with different amino groups, or they all react with the same amino sites but each of the modifier-amino site complexes yields a different maximal increase in Na^+ permeability. The remaining experiments presented here were designed to distinguish between these possibilities.

If the modifiers react with different amino groups, then simultaneous treatment of the cells with two different modifiers may yield an increase in Na^+ efflux equal to the sum of the effects of the modifiers when they are used alone. The effect of simultaneous treatment with Zn^{++} and TNBS is shown in Table 4. Note that the value obtained for relative Na^+ efflux is approximately equal to the sum of the effects of their separate application. These results suggest that Zn^{++} and TNBS increase Na^+ permeability by acting on different amino sites. The effects of simultaneous application of SITS with TNBS or Zn^{++} are not shown because it was suspected that SITS reacts with the other two modifiers in free solution.

We have shown previously that part of the binding of TNBS to dog red cell membranes is covalent, i.e., the effect of TNBS on sodium permeability is partially irreversible (Castranova & Miles, 1976). Therefore, modification with both TNBS and SITS, as well as with TNBS and Zn^{++}, is possible by adding SITS or Zn^{++} to cells previously treated with TNBS. In these experiments red cells were pretreated with TNBS for 20 min. The pretreated cells were then separated by centrifugation and the medium containing TNBS removed by suction. These pretreated cells were then added to a fresh medium containing no modifier or

Table 5. Effect of Zn^{++} and SITS on TNBS-pretreated cells

Modification	Relative Na^+ Efflux ±SEM	n
TNBS pretreatment	1.9 ± 0.3	5
TNBS+SITS	1.7 ± 0.3	5
TNBS+ Zn^{++}	4.8 ± 1.0	5

TNBS pretreated cells were incubated for 20 min in medium containing 3 mM TNBS. The cells were then separated by centrifugation and placed in fresh medium with no TNBS present. TNBS+SITS represents TNBS pretreated cells which have been placed in medium containing 0.1 mM SITS while TNBS+ Zn^{++} represents TNBS pretreated cells placed in medium containing 0.75 mM Zn^{++}. Relative Na^+ efflux values are expressed relative to the control with no TNBS pretreatment. n is the number of experiments in each case.

to a TNBS-free medium containing SITS or Zn^{++} and the rate constant for sodium efflux was measured.

The results of the experiments with TNBS pretreated cells are shown in Table 5. The relative Na^+ efflux obtained in TNBS pretreated cells is 1.9, which represents a much smaller increase than that of 3.5-fold obtained in cells with TNBS in the medium. This is probably due to the fact that some of the TNBS is removed during centrifugation. The addition of Zn^{++} to TNBS pretreated cells causes an increase in sodium permeability. This finding is in agreement with results from the simultaneous modification experiments and suggests that Zn^{++} and TNBS affect different amino sites. The fact that SITS does not increase sodium permeability in TNBS pretreated cells is taken to suggest that TNBS and SITS act on the same amino sites.

pH Dependence of Amino Site Modification

Another possible way to determine whether or not these modifiers react with different amino sites, i.e., protonated or unprotonated, is to study the pH dependence of the modifier effects. Since these are presumably ionizable sites, the pH dependence of the modifier-induced effects should be the same if the modifiers interact with the same amino sites. Aliquots of loaded cells were placed in media of different pH's either with or without modifier. Then the rate constant for Na^+ efflux for each of these suspensions was measured after one-half hr of incubation. Experiments with SITS and TNBS were performed over the pH range of 6.5 to 7.5, and the Zn^{++} experiments were done between a

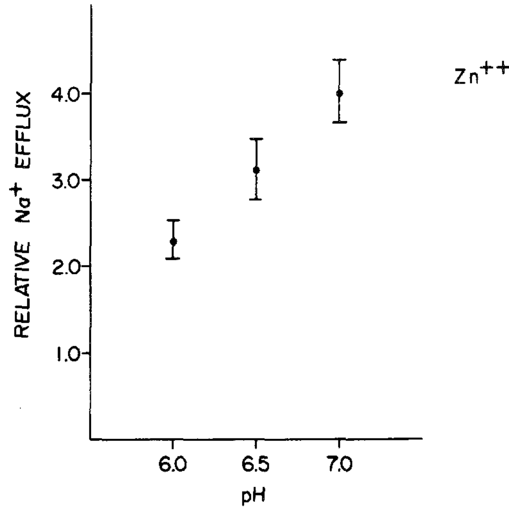

Fig. 2. Effect of pH on the Zn^{++}-induced changes in relative Na^{+} efflux. Relative Na^{+} efflux was obtained by dividing the rate constant for Na^{+} efflux in the presence of Zn^{++} by the rate constant obtained at the same pH with no Zn^{++} in the medium. The concentration of Zn^{++} used was 0.75 mM. Each point represents the mean value from 5 experiments and the vertical lines are standard errors of the mean

pH of 6.0 and 7.0 due to the tendency of Zn^{++} to precipitate, probably as the hydroxide, above pH=7.0.

The pH dependence of the Zn^{++}-induced change in Na^{+} efflux is shown in Fig. 2. Relative sodium permeability was taken as the rate constant in the presence of modifier divided by the rate constant at that given pH with no modifier added. Note that Zn^{++} becomes more effective in increasing Na^{+} permeability as pH is increased. The pH dependence of the TNBS-induced change in sodium permeability is shown in Fig. 3*A*. The pH range used here is slightly different from that used in the Zn^{++} experiments, but there is overlap in the range of 6.5 to 7.0. Note that unlike Zn^{++}, TNBS becomes less effective in increasing Na^{+} permeability as pH is increased. The pH dependence of SITS-induced changes in Na^{+} efflux is similar to TNBS, and this effect is shown in Fig. 3*B*. Thus, TNBS and SITS exhibit a pH dependence different from that of Zn^{++}. These data again suggest that Zn^{++} affects different amino sites than either TNBS or SITS. The directions of these changes in Na^{+} efflux with pH suggest that Zn^{++} reacts with an unprotonated amino site while TNBS and SITS react with a protonated amino group.

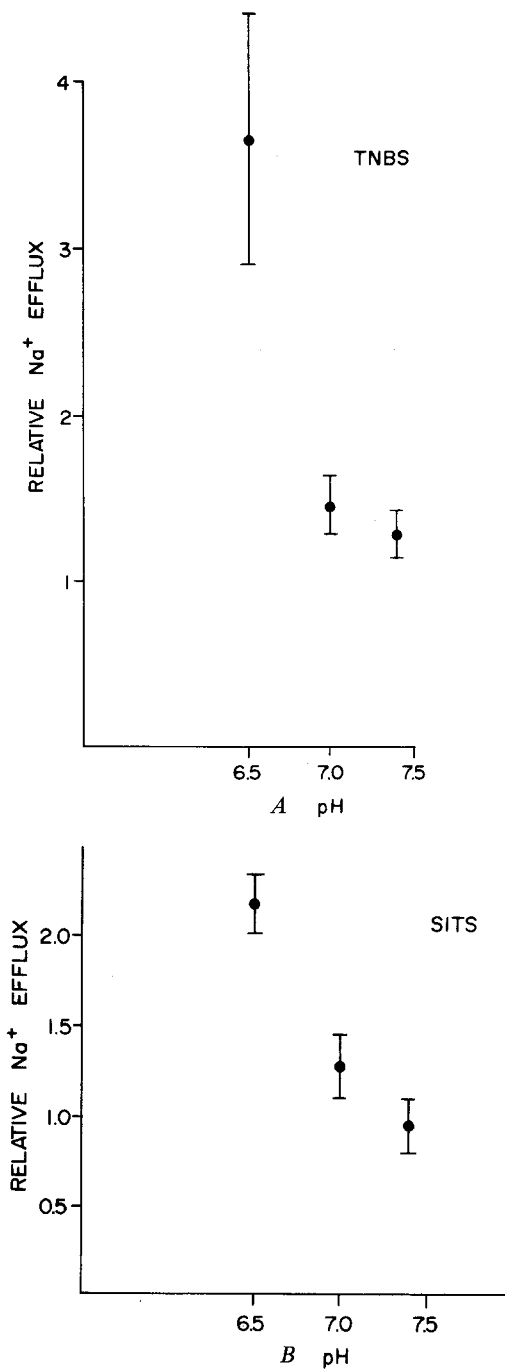

Fig. 3. Effect of pH on the TNBS-induced (*A*) and the SITS-induced (*B*) changes in relative Na^+ efflux. Relative Na^+ efflux was obtained by dividing the rate constant for Na^+ efflux in the presence of TNBS or SITS by the rate constant obtained at the same pH with no TNBS or SITS in the medium. The concentration of TNBS used was 3 mM and the SITS concentration was 0.1 mM. Each point represents the mean value from 5 experiments and the vertical lines are standard errors of the mean

Effect of Modifiers and pH on MCV's

Parker and Hoffman (1976) have shown that sodium permeability in dog erythrocytes is volume dependent. Sodium permeability increases as the cells shrink and decreases as the cells swell. Therefore, we measured mean cell volumes (MCV) to determine if the change in effectiveness of the modifiers with increasing pH is due to a change in effect of the modifiers on cell volume. These volume measurements were made at times which correspond to those during which flux measurements were made. We have shown in a previous study (Castranova & Miles, 1976) that the increases in Na^+ permeability caused by amino modification at pH = 6.5 are not due to cell shrinkage.

The results of the MCV experiments are summarized in Table 6. The top part of the table shows the percent change in the MCV in the presence and absence of Zn^{++}. The control cells, i.e., cells with no Zn^{++} in the medium, shrink by about 14% as the pH is increased from 6.0 to 7.0. Over the same pH range cells with Zn^{++} in the medium actually swell by about 4%. These data indicate that the volume effect in the case of Zn^{++} is one which would tend to decrease Na^+ permeability over the control level. Yet the effectiveness of Zn^{++} actually increases over this pH range, indicating that the Zn^{++} effect is not related to a change in cell volume.

The bottom part of Table 6 shows the percent change in the MCV in the presence and absence of TNBS and SITS. The control cells shrink by about 13% as the pH is increased from 6.5 to 7.5. Over the same pH range cells treated with TNBS shrink by about 16%, which means the cells are actually smaller than control. This means that the volume

Table 6. Modifier – induced changes in MCV with pH

Treatment	% Change in MCV	No. of expts.
Control (pH = 6.0 → pH = 7.0)	↓ 13.8% ± 2.0%	6
0.75 mM Zn^{++}	↑ 4.0% ± 3.4%	6
Control (pH = 6.5 → pH = 7.5)	↓ 12.7% ± 1.7%	7
3 mM TNBS	↓ 16.0% ± 1.6%	6
0.1 mM SITS	↓ 2.8% ± 3.2%	6

The % change in MCV (± SEM) is expressed as a change relative to the volume at the lower pH, i.e., it represents the change which occurs when the pH is increased from 6.0 to 7.0 or from 6.5 to 7.5. Arrows in the downward direction indicate cell shrinkage and upward-directed arrows indicate cell swelling. The far right hand column shows the number of experiments in each case.

effect would tend to increase Na^+ permeability even more in the presence of TNBS. However, the effectiveness of TNBS actually declines over this pH range, which again indicates that a change in cell volume is not the reason for this decline. Therefore, the pH dependence of both the Zn^{++} effect and the TNBS effect, which indicates that these two modifiers act on different amino groups, is not due to an effect of these modifiers on cell volume. On the other hand, cells treated with SITS only shrink by about 3% over this pH range, which indicates that they are actually larger than control. Thus, the decrease in Na^+ permeability seen during SITS treatment could be due at least partially to a change in cell volume. The data in this paper then show that there are two types of amino sites which affect sodium permeability in dog red blood cells: one which is modified by Zn^{++} and another which is modified by TNBS, and probably SITS.

Discussion

Relative Positions of Amino and Sulfhydryl Sites

The results of these experiments show that the amino and sulfhydryl sites which affect sodium permeability are separate and distinct. Furthermore, data from successive modification experiments suggest that the amino sites are located superficially in the membrane. However, such an interpretation requires further explanation to resolve differences which result from simultaneous and successive modification, i.e., Na^+ permeability in amino pretreated cells subsequently exposed to Hg^{++} is actually less than that obtained during simultaneous modification experiments in which additive effects are observed. The following mechanisms have been proposed to explain these differences.

Pretreatment of amino sites with Zn^{++} or SITS diminishes the effect of Hg^{++} in increasing sodium permeability. We have suggested that the amino reagents bind to the superficial amino sites which decreases the accessibility of the interior sulfhydryl sites to subsequent Hg^{++} treatment. One alternate explanation for this diminished effect of Hg^{++} is that the amino reagents cause some hemolysis and the hemoglobin released from the cells binds with Hg^{++} leaving less to react with the channels. This seems unlikely since hemolysis in the presence of SITS is slight, yet the effectiveness of Hg^{++} after SITS pretreatment is greatly diminished. Another possibility is that other SH-containing compounds which are smaller than hemoglobin leak from the cells during amino

pretreatment and cause the diminished effect of Hg^{++}. However, this does not seem likely because the effect of Hg^{++} does not depend upon the time of its addition, i.e., untreated red cells respond in the same way to Hg^{++} after 1 hr incubation at 37 °C as they do with no previous incubation.

Although the model of superficial amino and deep SH sites explains the results of amino pretreatment, it creates problems in explaining the additive results obtained when amino and sulfhydryl reagents are added simultaneously, i.e., why isn't the Hg^{++} effect decreased during simultaneous modification experiments? One explanation for this would be that Hg^{++} reacts more rapidly with the sodium channel than does Zn^{++} or SITS. Indeed, evidence is presented later in this paper to indicate that Hg^{++} does react rapidly with red cell membranes. In simultaneous modification studies this difference in reaction rates would enable Hg^{++} to enter the channel and have access to interior sulfhydryl sites before Zn^{++} or SITS completely binds to the superficial amino sites. Thus, with simultaneous modification, blockage of the channel would occur too late to decrease the potency of Hg^{++}, and the result would be additivity of the effects of the two modifiers.

We have assumed that the superficial amino sites which block the entrance of Hg^{++} are the ones which are located in the sodium channels. However, there is at least one other explanation. It is possible that Hg^{++} does not enter the cell via the sodium channels but rather via anion channels since in the presence of chloride, Hg^{++} forms anionic complexes (Webb, 1966). Once inside the cell Hg^{++} could then enter the sodium channels to modify the SH sites. Anion channels are blocked by SITS in human red cells (Knauf & Rothstein, 1971) and by SITS and to a lesser extent by Zn^{++} in dog red cells (V. Castranova, *unpublished results*). Entrance of Hg^{++} via anion channels is also indicated by the very short period of time (less than 3 min) which it takes for Hg^{++} to equilibrate in human red cells (Weed, Eber & Rothstein, 1962). In addition, this rapid equilibration of Hg^{++} could explain the results of the simultaneous modification experiments, i.e., the results are additive because Hg^{++} reacts more rapidly with the channel than does SITS or Zn^{++}. Therefore, the results from amino pretreatment studies can be interpreted in either of these two ways, i.e., amino pretreatment hinders Hg^{++} movement through either cation or anion channels, but in either case we conclude that the sulfhydryl groups which affect sodium permeability are deep within the membrane.

Protonated and Unprotonated Amino Sites

The results presented here indicate that different amino sites are modified by Zn^{++} and TNBS. Furthermore, the data seem to indicate that SITS modifies the same amino site as does TNBS. Evidence for the different amino sites comes from the simultaneous and successive modification experiments as well as from the pH dependency studies. The interpretation of the results given here depends on Zn^{++} being specific for amino groups in the sodium channels. However, it is well known that divalent cations are reactive with all functional groups in proteins and, in particular, with sulfhydryl groups. If the reaction of Zn^{++} is with a sulfhydryl site, then additivity with TNBS would be expected. However, there are three lines of evidence to show that this is probably not the case. (1) Simultaneous modification of the channels with Zn^{++} and SH reactive Hg^{++} increases Na^+ permeability to a level equal to the sum of the individual effects of these two modifiers. (2) The increase in Na^+ permeability caused by Zn^{++} can be reversed by washing the cells with glycine, but the effect cannot be reversed by washing with cysteine (Castranova & Miles, 1976). (3) In the successive modification experiments Zn^{++} pretreatment causes the same type of effect on the potency of subsequent Hg^{++} treatment as pretreatment with SITS, another amino reagent. Therefore, Zn^{++} probably reacts with amino sites in the sodium channel and interpretations of the data based on this finding are probably valid.

The direction of the Na^+ permeability change with pH in the case of both TNBS and SITS indicates that the interaction is probably with protonated amino sites. There are conflicting reports in the literature concerning proposed reaction mechanisms for TNBS. Some indicate that the protonated form of the amino group is the reactive species (Means & Feeney, 1971; Barker, 1971) while others favor reaction with the unprotonated form (Freedman & Radda, 1968; Goldfarb, 1970). In our case, reaction seems to be with the protonated species. The reaction of SITS with the channel seems to be electrostatic since simply washing the cells with NaCl will reverse its effect (Castranova & Miles, 1976). Therefore, it is not surprising that the reaction is with the protonated form of the amino site since SITS is an anion. Cabantchik and Rothstein (1972) also found that part of the SITS binding was reversible in human red cells, although the washing solution contained albumin in their case. The direction of the change in Na^+ permeability with pH in the case

of Zn^{++} indicates that the reaction is probably between Zn^{++} and the unprotonated form of the amino site. This type of reaction mechanism for Zn^{++} has been suggested by Vallee and Wacker (1970). In addition, Poensgen and Passow (1971), working with human red cells, interpreted similar results obtained for the pH dependency of DNFB in the same way.

The Sodium Channel

The results of this study along with the results from a previous investigation (Castranova & Miles, 1976) have yielded information about the molecular nature of sodium channels in dog red blood cells. Sodium permeability is normally affected by the presence of amino and sulfhydryl groups which act as two distinct barriers for sodium movement. The amino sites are superficial while the sulfhydryl sites are located deeper in the membrane. It is proposed that the amino sites limit permeability through electrostatic repulsion of sodium ions by virtue of the positive charge associated with these sites. Such electrostatic repulsion explains how TNBS and SITS increase sodium permeability since the pH dependency experiments indicate that they react with protonated amino sites and, thus, eliminate the positive charge. We have suggested that Zn^{++} reacts only with un-ionized amino groups. However, a decrease in the concentration of uncharged amino sites due to Zn^{++} modification could shift the equilibrium which exists between charged and uncharged amino groups. Thus, treatment with Zn^{++} could also result in a decrease in the number of positive charges in the channels.

In summary, we have referred to this channel as a sodium channel. But preliminary studies (not reported here) performed in this laboratory indicate that the various sulfhydryl and amino modifiers also enhance potassium permeability. This suggests that this channel may, in fact, be a generalized cation channel. It is possible that anions also move through this channel. To date the effect of midifiers on anion permeability has not been extensively studied in dog red cells, but there is evidence to suggest that there is a separate channel for anions since the order of effectiveness of TNBS, SITS, Zn^{++}, and PCMBS in inhibiting sulfate efflux is different from that for the enhancement of cation movement (V. Castranova, *unpublished experiments*). This is in agreement with evidence available from human erythrocytes which indicates that cation and anion channels are separate (Knauf & Rothstein, 1971).

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