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# Sodium Permeability of Dog Red Blood Cell Membranes

## I. *Identification of Regulatory Sites*

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ABSTRACT Divalent cations and group-specific chemical modifiers were used to modify sodium efflux in order to probe the molecular structure of sodium channels in dog red blood cells. $Hg^{++}$, $Ni^{++}$, $Co^{++}$, and PCMBS (parachloromercuribenzene sulfonic acid), a sulfhydryl reactive reagent, induce large increases in $Na^+$ permeability and their effects can be described by a curve which assumes 2:1 binding with the sodium channel. The sequence of affinities, as measured by the dissociation constants, reflects the reactivity of these divalent cations with sulfhydryl groups. In addition, the effects of $Hg^{++}$ and PCMBS can be reversed by the addition of dithiothreitol, an SH-containing compound, to the medium. Much smaller increases in $Na^+$ permeability are produced by $Zn^{++}$ and the amino-specific reagents, TNBS (2,4,6-trinitrobenzene sulfonic acid) and SITS (4-acetamido-4′-isothiocyano-stilbene-2-2′-disulfonic acid). The $Zn^{++}$ effect can be described by a curve which assumes bimolecular binding with the channel, and its effect on $Na^+$ permeability can be reversed by the addition of glycine to the medium. The effects of $Ni^{++}$ and SITS can be completely reversed by washing the cells in 0.16 M NaCl while TNBS binding is partially irreversible. Measurements of mean cell volumes (MCV) indicate that the modifier-induced increases in $Na^+$ permeability are not caused by shrinkage of the cells. It is concluded that the movement of sodium ions through ionic channels in dog red blood cells can be enhanced by modification of amino and sulfhydryl groups. $Zn^{++}$, TNBS, and SITS increase $Na^+$ permeability by modifying amino groups in the channel while $Hg^{++}$, $Ni^{++}$, $Co^{++}$, and PCMBS act on sulfhydryl groups.

### INTRODUCTION

The permeability characteristics of canine red blood cells are of considerable interest because of the uniqueness of the cell type. Dog erythrocytes differ from human and most other mammalian red cells in their ionic composition and transport mechanisms. Human red cells have a high potassium-low sodium content and maintain large electrochemical gradients for these ions with a ouabain-sensitive pump. In contrast, dog erythrocytes are of a low potassium-

high sodium type, and these electrolytes are more nearly in Donnan equilibrium with the plasma. No ouabain-sensitive cation flux has been measured in dog red cells (J. Hoffman, 1966; Miles and Lee, 1972; Parker, 1973 *a*), and no (Na + K)-sensitive ATPase has been found in their membranes (Chan et al., 1964).

In most mammalian red blood cells osmotic swelling is prevented by the active extrusion of sodium and accumulation of potassium by such a ouabain-sensitive pump (Tosteson and Hoffman, 1960). The apparent absence of such a mechanism in dog red cells presents a problem as to the maintenance of homeostasis in these cells. Parker and Hoffman (1965) have reported that the permeability of dog red cells is volume dependent, i.e., $Na^+$ permeability is decreased and $K^+$ permeability is increased when the cells are swollen, while the opposite is true in shrunken cells. Miles and Lee (1972) found that cation permeability in dog red cells is energy dependent, i.e., $Na^+$ permeability decreases, while $K^+$ permeability increases in ATP-depleted cells. Yet, they found cell volume to be unaffected by energy depletion.

Recently, Romualdez et al. (1972) observed that the effect of cell volume on membrane permeability is abolished by treatment with phloretin, an inhibitor of lactate production. They concluded that volume regulation in canine erythrocytes is accomplished by an energy-dependent cation carrier system. Parker (1973 *b*) appears to have identified this energy-dependent mechanism for the extrusion of sodium and maintenance of cell volume as a pump requiring external calcium. However, at normal cell volume this calcium-dependent sodium movement represents only 3% of the total sodium efflux. Therefore, much of the sodium transport in these cells is passive and presumably through ionic channels, i.e., sodium channels, and the regulation of sodium transport through these channels is of considerable interest.

The present study is an attempt to characterize the nature of the functional groups of proteins which are located within the ionic channels and which control passive sodium permeability. One approach to identifying sites within a channel is to pharmacologically modify these groups and observe the effects of such modification on ionic movement. In this investigation two different methods of pharmacological modification were used: heavy metal ions and chemical modifiers. The effects of each of these forms of protein modification on sodium efflux were measured. An abstract containing some of the results reported here has appeared elsewhere (Castranova and Miles, 1973).

## MATERIALS AND METHODS

### *Sodium Efflux Measurements*

Blood was obtained from adult dogs anesthetized with sodium pentobarbital (40 mg/kg body weight) by direct heart puncture. Sodium heparin (1,000 U/liter of blood) was used to prevent clotting. The blood was immediately centrifuged, and the plasma and buffy coat of while blood cells were removed by suction. The red cells were then washed three times by alternate resuspension and centrifugation in ice-cold 0.16 M NaCl. The erythrocytes were loaded with radioactive sodium by incubating the cells for 3 h at 37°C in a medium containing $^{22}Na$. The composition of this medium (pH = 7.4) was: $Na^+$ (167.33 mM), $Cl^-$ (152 mM), $K^+$ (5 mM), $HPO_4^=$ (9.32 mM), $H_2PO_4^-$ (1.65 mM), and glucose (5.55 mM). When loaded, the red cells were removed from the incubation medium and washed

four times with ice-cold 0.16 M NaCl as before.

The loaded red cells were then placed in Erlenmeyer flasks containing a buffered solution of the following composition: $Na^+$ (146 mM), $Cl^-$ (151 mM), $K^+$ (5 mM), glucose (2.77 mM), and Na PIPES [piperazine-*N*-*N*'-bis (2-ethane sulfonic acid)] (5 mM). The hematocrit was always less than 5%. Na PIPES was used rather than a phosphate buffer since PIPES has been shown to have a negligible binding affinity for divalent cations such as those used here as modifiers (Good et al., 1966). The pH of the medium was set at 6.5 for all experiments because the divalent cations used precipitate, probably as the hydroxide, at pH levels above 7.0. Treatment of the red cells with divalent cations or chemical modifiers consisted of the addition of minute amounts (final concentrations of 0.005–3 mM) of the modifier to the various flasks of PIPES buffered medium before the addition of the red cells. It was found that the sodium efflux measured for red cells incubated in PIPES buffer at pH = 6.5 is comparable to that measured in phosphate buffer at the same pH.

A measure of sodium permeability was obtained by determining the rate constant for sodium efflux. The counts per minute of the $^{22}Na$ in the medium at time $t$ ($\bar{P}_t$) was obtained by taking supernatant samples at various times and measuring the radioactivity of these samples. The counts per minute in the medium at time infinity ($\bar{P}_\infty$) was estimated from the number of counts per minute in a sample of the whole suspension. The optical densities of these samples were measured at 540 nm to correct for the counts per minute due to any hemolysis which may have occurred (Crosby et al., 1954). The results indicate that sodium efflux from dog red blood cells behaves as a three-compartment system. In such a system Sha'afi and Lieb (1967) have shown that the cell interior consists of two compartments. Efflux from the first intracellular compartment (5% of the total cell sodium) is very rapid and reaches equilibrium with the plasma within 30 min. The major portion of sodium efflux is from the second intracellular compartment (95% of the total cell sodium) and is described by the equation: $\ln (1 - (\bar{P}_t/\bar{P}_\infty) = -k_{21}t$, where $k_{21}$ is the rate constant for efflux from the major intracellular compartment. Thus, in the present study, $\ln (1 - (\bar{P}_t/\bar{P}_\infty)$ was plotted against time and the rate constant for the major portion of sodium efflux was obtained from the slope of this line after 30 min. Lee and Miles (1973) were unable to demonstrate exchange diffusion of sodium in puppy red blood cells. In addition, we have been unable to demonstrate any exchange diffusion in adult dog cells (unpublished results). Therefore, the contribution of an exchange diffusion mechanism to the sodium efflux measurements is negligible.

The rate constant has been shown to be a measure of sodium efflux by Sha'afi (1965), since $M_o^{Na} = k_{21}[Na^+]_i$, where $M_o^{Na}$ = Na efflux and $[Na^+]_i$ = internal Na concentration. It must be emphasized that these symbols refer only to the slowly exchanging component of sodium efflux. If the driving force remains constant, i.e., the cells are in the steady state, then the rate constant is also a measure of Na permeability ($P_{Na}$), since $M_o^{Na} = (P_{Na}) \times$ (driving force). In this study the sodium concentration of all cells (normal and modified) was found to remain constant during the time of the experiment. This indicates that these cells are in the steady state during the time that the flux measurements are made, and the driving force for sodium is probably constant since large changes in membrane potential are unlikely (see Discussion). Therefore, the rate constant is indeed a measure of $Na^+$ permeability. The effect of pharmacological modification was measured by comparing the rate constants for $Na^+$ efflux in the presence of modifier to the rate constants in unmodified cells (controls).

### *Mean Cell Volume Measurements*

The effect of the various divalent cations and chemical modifiers on mean cell volume (MCV) was determined. MCV's were calculated in the usual manner by dividing the

hematocrit by the cell count. Washed red cells were incubated at 37°C in PIPES buffered medium which contained either the divalent cation or chemical modifier. The hematocrit was always less than 5% as in the case of the sodium efflux measurements. These incubations were carried out for a period of 2 h which is well into the time interval during which the rate constants for sodium efflux were measured. Then the red cells were spun down and supernatant was removed until the hematocrit was approximately 30–50%. The cells were then resuspended in the remaining medium and mean cell volume measurements were made using this suspension. Hematocrits were measured and the cell counts were determined by using a Coulter Counter (model B, Coulter Electronics, Inc., Hialeah, Fla.). The MCV's were expressed in cubic microns.

*Concentration-Effect Relationships*

Curves relating relative $Na^+$ efflux to the concentration of divalent cation or chemical modifier added to the medium were constructed by applying the following analysis. As a first approximation, it was assumed that a certain number ($n$) of modifier molecules ($Y$) bind to regulatory sites ($X$) within the sodium channel as described in the reaction:

$$nY + X \rightleftarrows Y_nX. \tag{1}$$

The dissociation constant ($K_D$) is then defined as:

$$K_D = \frac{[Y]^n[X]}{[Y_nX]}, \tag{2}$$

where the brackets denote molar concentrations. The fraction of channel sites occupied by the modifier has been designated as $\alpha$ and is given by:

$$\alpha = \frac{[Y_nX]}{[Y_nX] + [X]}. \tag{3}$$

This equation can be rearranged to give the following:

$$\alpha = \frac{1}{1 + \frac{K_D}{[Y]^n}} \tag{4}$$

The data from these experiments were plotted in two different ways. First, a saturation curve is obtained when $\alpha$, which is measured as the fraction of the maximal change in relative sodium efflux, is plotted against the concentration of the modifier added to the medium. In order to obtain the best curve the experimental points were fitted to a theoretical saturation curve, given by Eq. 4, by determining the binding type ($n$) from the procedure described below (Hill plot), and by picking a dissociation constant ($K_D$) such that the sum of the squares of the deviations between the experimental points and the theoretical curve was a minimum.

The equation for a saturation curve can be linearized to the form

$$\log\left(\frac{1}{\alpha} - 1\right) = -n \log [Y] + \log K_D. \tag{5}$$

Plotting $\log((1/\alpha) - 1)$ against $\log [Y]$ results in a straight line such that the slope ($-n$) indicates the binding type and the $y$ intercept is the log of the dissociation constant. This analysis is virtually identical to that for a Hill plot (Van Holde, 1971). In this investigation the data were fitted to the best line by least squares. The binding type and $K_D$ obtained from the Hill plots were found to be similar to that determined from theoretical saturation curves, so numbers from these two different plots are used interchangeably in the text.

In this investigation it would have been ideal to correlate the changes in $Na^+$ efflux with the fraction of channel sites modified. However, for most modifiers it would be difficult to measure actual binding to the $Na^+$ channels due to the reversibility of their effects, i.e., the modifiers used in these experiments are easily washed out. In addition, there are probably so few binding sites associated with the permeability change that non-specific binding would obscure the results.

It must be emphasized that this analysis of the concentration-effect relationships is used only as an approximation to the actual binding. We have assumed that a number ($n$) of modifier molecules react simultaneously with the $Na^+$ channel and that intermediates such as $Y_{n-1}X$ do not exist, which may be unlikely. Indeed, many kinetic models may fit our data. However, this analysis was used merely to distinguish 1:1 binding from other types of binding. It must also be pointed out that the absolute values of the dissociation constants are not important, but rather the emphasis should be placed on the sequence of apparent affinities of the modifiers for the $Na^+$ channel.

## RESULTS

### *Modification of the Sodium Channel with Divalent Cations*

The effect of certain heavy metal ions on the sodium permeability of canine red blood cells is illustrated in Fig. 1. This figure shows sodium efflux from untreated red cells (control) and from erythrocytes treated with heavy metal ions in concentrations which produce maximum changes in sodium permeability. Note

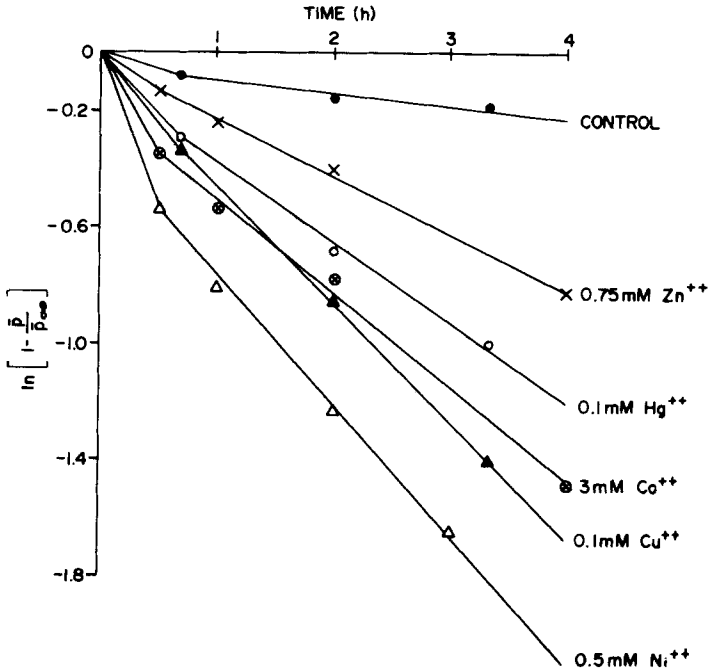


FIGURE 1. Sodium efflux measurements showing the increase in sodium permeability caused by divalent cations. The data in this figure represent results obtained in red blood cells from several different animals. The concentrations of divalent cations used were those which cause the maximum increase in sodium efflux.

that sodium permeability is increased to varying degrees by all the divalent cations listed in this figure. However, other divalent cations were found to have no effect on $Na^+$ efflux, e.g., $Ba^{++}$, $UO_2^{++}$, and $Ca^{++}$ did not alter sodium permeability. None of the divalent cations tested caused a decrease in sodium permeability. A summary of these effects is given in Table I. The relative sodium efflux shown is the maximum increase in the rate constant for sodium efflux in treated red cells over that measured for untreated erythrocytes. For example, the rate constant in the presence of 0.5 mM $Ni^{++}$ is 14.3 times greater than the rate constant with no divalent cation added. The sequence for the increase in relative sodium permeability, i.e., $Ni^{++} > Cu^{++} > Hg^{++} > Co^{++} > Zn^{++}$, is similar to but does not follow exactly the sequence for the crystal ionic radii of these ions, i.e., $Ni^{++} < Cu^{++} = Co^{++} < Zn^{++} < Hg^{++}$ (Weast and Selby, 1967).

In order to investigate more fully the interaction between these heavy metal ions and the channel, the concentration-effect relationships for these divalent

TABLE I

MAXIMUM EFFECT OF DIVALENT CATIONS ON $Na^+$ EFFLUX

| Divalent cation | Relative $Na^+$ efflux (± SEM) | No. of exps. |
|---|---|---|
| $Ni^{++}$ | 14.3±2.0 | 6 |
| $Cu^{++}$ | 10.1±0.4 | 4 |
| $Hg^{++}$ | 7.6±0.6 | 20 |
| $Co^{++}$ | 6.9±0.8 | 9 |
| $Zn^{++}$ | 4.7±0.4 | 15 |
| Control | 1.0 | |

The concentrations of divalent cations used to achieve maximum increases in $Na^+$ efflux were: 0.5 mM $Ni^{++}$, 0.1 mM $Cu^{++}$, 0.1 mM $Hg^{++}$, 3.0 mM $Co^{++}$, 0.75 mM $Zn^{++}$.

cations were studied. This was done by measuring the increase in sodium efflux from red cells treated with various concentrations of each divalent cation. It was assumed that such increases in sodium efflux result from the binding of the divalent cations to groups which affect sodium permeability as described in the Methods. The objective of these experiments was to obtain a binding number ($n$), i.e., the number of divalent cations which bind to each regulatory group within the channels, and to obtain a measure of the apparent affinity of the heavy metal ions for the channel, i.e., the dissociation constant ($K_D$). An example of the type of plot used to determine $n$ and $K_D$ is shown in Fig. 2 A. In this figure $\alpha$ represents the fraction of channel sites bound, and it is measured as the fraction of maximal $Na^+$ efflux achieved. This curve is a Hill plot drawn for points representing the mean values from three experiments with $Co^{++}$. The slope of this line was used to determine the type of binding ($n$) while the $y$ intercept gives the $K_D$. The slope of $-1.7$ is taken to indicate that two $Co^{++}$ ions bind to each regulatory site in the sodium channel while the $y$ intercept gives an apparent $K_D$ of 0.3 $mM^2$. The curve in Fig. 2 B is the theoretical saturation curve of best fit for $Co^{++}$, drawn by assuming that two $Co^{++}$ ions bind to one site in the

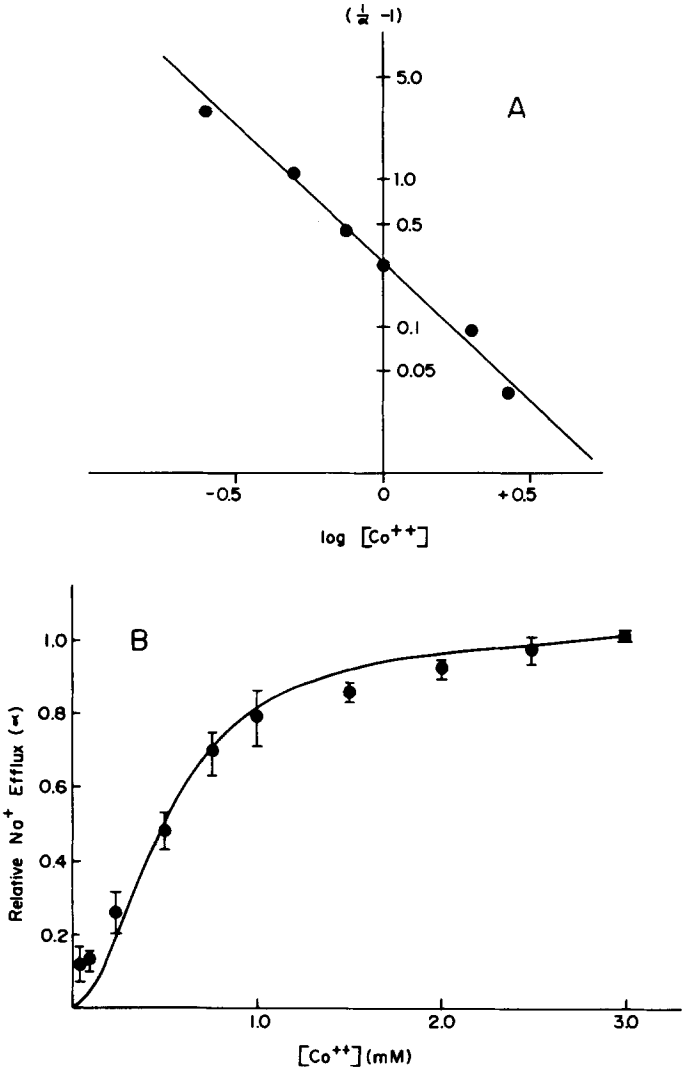


FIGURE 2. (A) Hill plot for the binding of $Co^{++}$ with the sodium channel. The scale on the abscissa is in millimolar. The points are means of three experiments and the line is a least squares fit. The slope is $-1.7$, and the $y$ intercept gives a $K_D$ of 0.30 $mM^2$ for the $Co^{++}$-channel complex. (B) Curve showing the effects of various $Co^{++}$ concentrations on sodium permeability. The points are means of three experiments ± SEM. The curve is the best fit theoretical saturation curve drawn by assuming that two $Co^{++}$ ions bind to one site in the sodium channel with a $K_D$ of 0.25 $mM^2$ and a maximum effect occurs at 3.0 mM $Co^{++}$.

sodium channel with an apparent $K_D$ of 0.25 $mM^2$. Note that the dissociation constants derived from both types of plots are similar. The binding number and the $K_D$ were determined for each heavy metal ion by using this type of analysis.

The dissociation constants and the type of binding between each divalent

cation and the sodium channel are listed in Table II. $Hg^{++}$, $Ni^{++}$, and $Co^{++}$ exhibit 2:1 binding, i.e., two heavy metal ions modify one regulatory site in the sodium channel, while $Zn^{++}$ binding follows a 1:1 relationship. Since $Hg^{++}$ is known to react strongly with sulfhydryl groups (Weed et al., 1962; Vallee and Walker, 1970), and since $Ni^{++}$ and $Co^{++}$ also exhibit second-order binding, it is not unreasonable to assume that these three cations bind to SH groups in the channel. Further evidence for this is that the sequence of apparent affinities for the divalent cations exhibiting 2:1 binding, i.e., $Hg^{++} < Ni^{++} < Co^{++}$, is identical to the sequence of affinities for the binding of these divalent cations with cysteine (Martell and Sillén, 1964) and is unrelated to the sequence of crystal ionic radii for these cations. $Zn^{++}$, on the other hand, seems to increase $Na^+$ permeability by binding with some other ligand in the channel since it exhibits 1:1 binding even though it is a smaller ion than $Hg^{++}$. One possibility is that the site of $Zn^{++}$ modification may be at an amino group within the sodium channel. The following experiments were designed to test this hypothesis.

TABLE II

$n$ AND $K_D$ FOR $Me^{++}$-MEMBRANE BINDING

| Divalent cation | $K_D$ (× $10^3$) | Slope (type) | $r$ |
|---|---|---|---|
| $Hg^{++}$ | 1 $mM^2$ | −2.0 (2:1) | 0.95 |
| $Ni^{++}$ | 12 $mM^2$ | −1.8 (2:1) | 0.96 |
| $Co^{++}$ | 300 $mM^2$ | −1.7 (2:1) | 0.99 |
| $Zn^{++}$ | 59 mM | −1.1 (1:1) | 0.94 |

The dissociation constants and binding types were determined from Hill plots of the mean values from three experiments for each divalent cation. $r$ is the correlation coefficient between the fitted line and the data. (The affinities, which were calculated from the concentration of modifier added to the medium, are apparent affinities.)

*Modification of the Sodium Channel with Amino and Sulfhydryl Reagents*

Another approach to identifying functional groups within the sodium channel involves the chemical modification of these sites with group-specific reagents. PCMBS is a reagent which binds specifically to sulfhydryl groups (Sutherland et al., 1967), while TNBS, SITS, and 2-methoxy-5-nitrotropone (MNT) are used as amino-specific reagents (Satake et al., 1960; Maddy, 1964; and Tamaoki et al., 1967). It should be noted that TNBS and SITS reactions with other groups (e.g., sulfhydryl groups) may be possible.

A summary of the effects of these group-specific reagents on $Na^+$ permeability is given in Table III. As in Table I, the concentration of modifier used is that necessary to produce a maximal effect, and relative $Na^+$ efflux indicates the maximum increase in the rate constant for sodium efflux in treated red cells over the control level. Note that sodium permeability is increased to varying degrees by all of these reagents indicating that both sulfhydryl and amino groups are involved in regulating the movement of sodium through the channel. However, the MNT effect is a small one and may not be significant due to a possible effect of this reagent on cell volume, which was not measured (see

TABLE III
MAXIMUM EFFECT OF CHEMICAL MODIFIERS ON $Na^+$ EFFLUX

| Chemical modifier | Relative $Na^+$ efflux (± SEM) | No. of exps. |
|---|---|---|
| PCMBS | 11.3±1.0 | 9 |
| TNBS | 3.5±0.2 | 12 |
| SITS | 2.6±0.3 | 18 |
| MNT | 1.2±0.1 | 3 |
| Control | 1.0 | |

The concentrations of chemical modifiers used to achieve maximum increases in $Na^+$ efflux were: 0.1 mM PCMBS, 3.0 mM TNBS, 0.1 mM SITS, and 3.0 mM MNT.

section on cell volume). This small effect may also be due to limitations in the solubility of MNT.

The sulfhydryl-reactive PCMBS is more effective in increasing $Na^+$ efflux than the amino reagents, TNBS and SITS. These data tend to support the previous conclusion that $Ni^{++}$, $Co^{++}$, and $Hg^{++}$, which are the most effective heavy metal ions, increase $Na^+$ permeability by interacting with sulfhydryl groups. PCMBS also exhibits 2:1 binding with the channel as do $Ni^{++}$, $Co^{++}$, and $Hg^{++}$. This suggests that the thiol regulatory unit consists of two sulfhydryl groups. On the other hand, $Zn^{++}$, which exhibits 1:1 binding with the channel and is less effective than the sulfhydryl reactive heavy metal ions, is in the same range of effectiveness as the amino reactive reagents, TNBS and SITS. Experiments indicate that the binding of SITS is most closely described by 1:1 binding, while that of TNBS is 2:1.

*Reversal of Binding*

It has been assumed that divalent cations and chemical modifiers increase $Na^+$ permeability by binding with either amino or sulfhydryl groups within the channel. If this binding is electrostatic, as seems likely for divalent cations, then it should be possible to reverse the effects of modification by dissociation of this reagent-channel complex. This type of experiment should contribute to the identification of the groups involved. One way to attempt uncoupling of the modifier from the channel is to add to the medium an excess of the functional group with which the modifier reacts. For example, the binding of PCMBS to red cell membranes has been reversed by adding either the sulfhydryl containing amino acid, cysteine (Sutherland et al., 1967), or dithiothreitol (DTT) (P. Hoffman, 1969), to the medium. In this study the reagents used to dissociate the modifier-channel complex were either an excess of sulfhydryl-containing DTT or amino-containing glycine.

The experimental procedure was modified slightly for the reversal experiments. Dog red cells were incubated in a medium containing the divalent cation or chemical modifier for 1 h. Then either 5 mM DTT or 5 mM glycine was added to the medium. A half hour later the cells were washed three times in ice-cold 0.16 M NaCl and placed in a fresh medium free of any type of modifier. Sodium efflux was then measured in the normal manner. Since the cells are

washed free of all modifiers and reagents before sodium efflux is measured, it is necessary to look also at the effect of simply washing the cells free of chemical modifiers and divalent cations.

The results of the reversal experiments are summarized in Table IV. The effect of washing alone, DTT and washing, or glycine and washing on relative sodium efflux is given as the relative control value. Note that DTT treatment increases relative $Na^+$ efflux 1.2 times while glycine causes a decrease in efflux to 0.90 of that value for untreated cells. However, these are small changes and are unimportant in this type of experiment, since each column of relative $Na^+$ efflux values is related to its own control value which has been corrected to unity. Note

TABLE IV

REVERSIBILITY OF MODIFIER-CHANNEL BINDING: THE EFFECT OF WASHING, DTT, AND GLYCINE ON RELATIVE $Na^+$ EFFLUX

| Modifier | Relative $Na^+$ efflux (modifier alone) | Relative $Na^+$ efflux (washing with 0.16 M NaCl) | Relative $Na^+$ efflux (5 mM DTT) | Relative $Na^+$ efflux (5 mM glycine) |
|---|---|---|---|---|
| Control | 1.0 | – | – | – |
| Control + washing | – | 1.0±0 | – | – |
| Control + DTT | – | – | 1.19±0.07 | – |
| Control + glycine | – | – | – | 0.90±0.08 |
| Corrected control | 1.0 | 1.0 | 1.0 | 1.0 |
| 0.75 mM $Zn^{++}$ | 4.70±0.40 | 1.86±0.12 | 1.90±0.10 | 1.20±0.05 |
| 3.0 mM MNT | 1.20±0.10 | 1.20±0.02 | 1.30±0.10 | 1.10±0.04 |
| 0.1 mM PCMBS | 11.30±1.00 | 2.20±0.03 | 0.86±0.02 | 2.18±0.24 |
| 0.1 mM $Hg^{++}$ | 7.60±0.60 | 2.00±0.20 | 1.10±0.15 | 2.73±0.43 |

All values for relative $Na^+$ efflux are means of three experiments ± SEM. See text for further explanation.

also that washing the cells causes some dissociation of the modifier-channel complex, i.e., removal of modifier from the medium causes a decrease in the effects of all the modifiers on Na efflux. Treatment with DTT causes complete reversal in the case of PCMBS and $Hg^{++}$, but does not cause a change in the binding of MNT or $Zn^{++}$. Glycine, on the other hand, causes a reversal of the $Zn^{++}$ effect but does not affect PCMBS or $Hg^{++}$. It should be pointed out that the effect of washing with glycine on the MNT-induced increase in Na efflux is not significant. These data are in agreement with that mentioned previously, indicating the involvement of both amino and sulfhydryl groups in the regulation of sodium movement. It also confirms that PCMBS and $Hg^{++}$ affect $Na^+$ permeability by modifying sulfhydryl groups while $Zn^{++}$ acts on amino groups in the sodium channel.

Reversal of the effects of SITS, $Ni^{++}$, TNBS, and $Co^{++}$ was also attempted with the following results. SITS and $Ni^{++}$ effects are completely reversed by washing with ice-cold 0.16 M NaCl, suggesting very weak electrostatic binding with the channel, or incomplete bond formation in the case of SITS due to the inability of SITS to penetrate into the membrane (Maddy, 1964; Knauf and Rothstein, 1971). Therefore, in these cases the action of DTT or glycine treatment has no meaning. The TNBS-channel complex is not reversed by either DTT or glycine, indicating that the binding is very strong and possibly covalent. Covalent binding of TNBS to amino groups has been previously reported (Barker, 1971). $Co^{++}$ data are not given in Table IV because when DTT and $Co^{++}$ are used together the cells seem to clump.

### *Effect of Divalent Cations and Chemical Modifiers on Cell Volume*

Parker and Hoffman (1965) have shown that the cation permeability of canine red blood cells is dependent upon cell volume. They have reported that sodium permeability increases and potassium permeability decreases when dog red cells shrink while the opposite is true in swollen cells. One possibility then is that these modifiers increase sodium permeability by causing the cells to shrink rather than by directly modifying a site within the channel. In order to investigate this possibility MCV's were measured for erythrocytes which were incubated with either a divalent cation or a chemical modifier in the medium for 2 h, i.e., duplicating actual experimental conditions. The results were expressed as percent change in the MCV of chemically modified cells from that of control cells.

The results of the MCV measurements are summarized in Table V. Most of the divalent cations and chemical modifiers actually cause the cells to swell rather than shrink, and this should cause a decrease rather than an increase in sodium permeability. Therefore, it can be concluded that most of these modifiers cause an increase in $Na^+$ permeability which does not result from a change in cell volume. One would probably expect swelling of the cells to be the result of large increases in $Na^+$ permeability, i.e., sodium and water should enter the cells.

TABLE V

EFFECT OF CHEMICAL MODIFICATION ON MEAN CELL VOLUME (MCV)

| Modifier | Change in MCV (± SEM) | No. of exps. |
|---|---|---|
| | % | |
| $Ni^{++}$ | +4.2%±5.3% | 5 |
| $Cu^{++}$ | −5.1%±3.6% | 4 |
| $Co^{++}$ | +10.7%±5.4% | 5 |
| $Hg^{++}$ | −0.9%±3.1% | 4 |
| $Zn^{++}$ | +0.4%±1.8% | 5 |
| PCMBS | +5.8%±3.5% | 5 |
| TNBS | +33.5%±3.0% | 6 |
| SITS | +10.5%±3.7% | 6 |
| Control | 0 | |

The percent change in the MCV for each modifier is expressed relative to that with no modifier present. Minus signs indicate cell shrinkage and plus signs cell swelling.

However, there seems to be no correlation between the change in permeability and the volume change. TNBS is a conspicuous example in that it causes a large increase in MCV and yet only a 3.5-fold increase in $Na^+$ permeability. The reason for this exceptionally large increase in volume is not yet understood.

Two divalent cations, $Cu^{++}$ and $Hg^{++}$, cause the cells to shrink. However, in the case of $Hg^{++}$ this shrinkage is very slight and probably has no significant effect on $Na^+$ permeability. Romualdez et al. (1972) have reported that a 3% decrease in cell volume would result in an increase in sodium uptake equivalent to a relative sodium permeability of less than 1.5. Such a change is far too small to account for the 7.6-fold increase in $Na^+$ permeability in response to $Hg^{++}$ treatment. Part of the increase in $Na^+$ permeability caused by $Cu^{++}$ can probably be attributed to cell shrinkage. For this reason $Cu^{++}$ was not used in most of the experiments reported here. Finally, in all of the modified cells the intracellular sodium concentration was approximately the same as that in control cells so the driving force on sodium was approximately the same in each case. In summary, the results of the MCV measurements indicate that the enhancement of sodium permeability caused by the modifiers used in this study cannot be attributed to cell shrinkage.

## DISCUSSION

### *Other Possible Effects of Pharmacological Modification*

A basic assumption in this study is that the pharmacological modifiers act to increase sodium permeability by combining with sites within the sodium channels. It is imperative, therefore, that one eliminate any other actions of these modifiers that may have an effect on sodium flux. One possibility is that the modifiers cause a decrease in cell volume and in this manner increase $Na^+$ permeability. However, the MCV measurements shown in Table V indicate that the enhancement of sodium permeability caused by these modifiers cannot be attributed to cell shrinkage. In addition, preliminary studies in our laboratory have also shown that potassium efflux is increased by the same kinds of pharmacological modification of the cell membrane which enhance sodium efflux. However, Parker and Hoffman (1965) have reported that the sodium and potassium permeabilities vary in opposite directions in response to a given change in cell volume. Therefore, these findings also indicate that membrane modification in this study does not increase permeability by changing cell volume.

PCMBS and $Hg^{++}$ have been reported to inhibit glucose transport in human red blood cells (Weed et al., 1962). Thus, a second possibility is that the modifier-induced increases in sodium permeability reported in the present study are secondary to energy depletion due to the inhibition of glucose transport by these modifiers. However, Miles and Lee (1972) have studied transport in energy-depleted canine red blood cells and have reported that sodium permeability is decreased. Yet, PCMBS, $Hg^{++}$, and the other modifiers used in the present study increase rather than decrease sodium permeability. Therefore, an effect on glucose transport cannot explain the observed changes in sodium permeability.

Another possibility is that pharmacological modifiers increase sodium permeability by accelerating some active transport process. Indeed, PCMBS has been reported to affect ATPase activities (Godin and Schrier, 1972) and to alter the ouabain-dependent active transport of sodium and potassium in human erythrocytes (Rega et al., 1967). But these reported effects of PCMBS are inhibitory, i.e., PCMBS causes a decrease in ATPase activity and a decrease in active transport. Recently, Parker (1973 *b*) has identified a ouabain-insensitive sodium pump in dog red cells which extrudes sodium. One might suggest that cell modification may be affecting this mechanism. Yet preliminary studies from our laboratory (not reported here) indicate that cell modification induces a bidirectional increase in sodium flux, i.e., sodium influx as well as sodium efflux is enhanced. Increases in potassium efflux and influx have also been found. These bidirectional increases in sodium and potassium fluxes could not be expected if alteration of an active pump were involved since active pumps are usually unidirectional.

The bidirectional increase in sodium flux induced by these modifiers could be caused by an exchange diffusion mechanism. Even though we could not demonstrate exchange diffusion in normal cells it is possible that the modifiers are capable of turning on such a mechanism. However, in two experiments (not reported here) we found that $Zn^{++}$, $Hg^{++}$, PCMBS, TNBS, and SITS increase $Na^+$ efflux to the same extent in $Na^+$-free medium as in a medium of normal $Na^+$ concentration. These experiments indicate that the modifiers do not cause an accelerated exchange diffusion process. Therefore, it is likely that pharmacological modifiers are altering the movement of sodium through some passive permeation pathway.

Hoffman and Laris (1974) have accurately measured the membrane potential of erythrocytes by using a dye whose fluorescence is proportional to membrane potential. They have reported that the membrane potential of human and amphiuma red cells is some combination of the Nernst potentials for chloride and potassium with chloride being the dominant ion. Knauf and Rothstein (1971) have reported that amino reagents decrease anion permeability. As mentioned earlier, it has been found in our laboratory that membrane modification enhances potassium permeability. These shifts in ionic permeabilities could alter the membrane potential and alter the driving force on sodium. This would result in a change in sodium flux which would not require a corresponding change in sodium permeability, i.e., sodium channels need not be modified. Using medium and cell electrolyte concentrations for dog erythrocytes reported by Parker (1973 *a*), the Nernst potential for chloride is $-11.3$ mV while that for potassium is $-14.0$ mV. Therefore, taking the most extreme case of a shift from a totally chloride-dominated membrane to a totally potassium membrane, one would expect only a 1.24-fold change in sodium flux. This change is too small to explain the increases in sodium flux reported in the present study. Thus, it seems that the sodium channels are being modified, and that pharmacological modifiers can be used to identify ligands within these channels.

One final possibility is that rather than affecting normal sodium channels these modifiers open up nonspecific holes in the membrane, especially since potassium permeability is also increased. It should be mentioned that although

TNBS, SITS, and PCMBS increase sodium and potassium permeability, each of them causes a decrease in sulfate permeability (V. Castranova, unpublished results). This would not be expected if these reagents formed holes in the membrane. Thus, we take this to mean that these modifiers increase $Na^+$ permeability by acting on normal cation pathways although formation of new pathways has not been absolutely eliminated.

*Possible Modes of Modifier Action*

Sulfhydryl-specific PCMBS, $Hg^{++}$, $Ni^{++}$, and $Co^{++}$ all seem to enhance $Na^+$ permeability by affecting sulfhydryl groups. Furthermore, these modifiers all follow second-order kinetics. We take this to mean that there are two SH sites which must be modified in order to cause an increase in $Na^+$ permeability. It is believed that these sulfhydryl groups are involved in maintaining the structure of the sodium channel. Indeed, sulfhydryl groups have been reported to be capable of forming hydrogen bonds (Barker, 1971), and modification of sulfhydryl groups with PCMBS, $Hg^{++}$, $Ni^{++}$, or $Co^{++}$ can result in the removal of the hydrogen ion from the sulfur (Means and Feeney, 1971; Vallee and Walker, 1970) and thus, the elimination of these hydrogen bonds. This could result in some distortion of the sodium channel which causes an increase in sodium permeability. Sulfhydryl groups have also been shown to be involved in hydrophobic interactions of proteins (Heitmann, 1968). Such interactions have been suggested by Carter (1973) as a mechanism for the maintenance of membrane structure in human erythrocytes and may indeed be involved here.

One could also imagine that these sulfhydryl reagents react with disulfide groups to increase $Na^+$ permeability, especially since disulfide groups are of great importance in the maintenance of many protein structures. For example, $Hg^{++}$ has been shown to react with such disulfide groups (Vallee and Walker, 1970). Yet it is unlikely that these groups are involved in the regulation of sodium permeability since DTT, a reagent specific for disulfides, has little effect on sodium flux. Finally, the results in Table I show a great variability in the maximal effects of SH-reactive agents. The reason for this variability is not yet understood, but experiments are currently in progress to study this phenomenon.

The proposed reaction mechanisms for amino reagents (Barker, 1971) indicate that the increase in sodium permeability resulting from amino modification is associated with the loss of the positive charge from the amino sites. Thus, it is possible that amino groups may normally limit sodium permeability by the electrostatic repulsion of sodium ions. Such a mechanism for ionic regulation, i.e., the limitation of sodium movement by the presence of positive amino groups in a channel, has been proposed for human red cells by Passow (1969). These amino sites are thought to lie deep within the channel since SITS, a very large molecule, has no effect on $Na^+$ permeability (Knauf and Rothstein, 1971). This is not the case in dog red cells, however, where the amino sites seem to be located superficially since SITS is effective.

In conclusion, pharmacological modification of the sodium channels with heavy metal ions and chemical modifiers increases the sodium permeability of

dog red blood cells. Reversibility and binding studies as well as the relative effectiveness of each modifier indicate that PCMBS, $Hg^{++}$, $Ni^{++}$, and $Co^{++}$ act on sulfhydryl sites while TNBS, SITS, and $Zn^{++}$ enhance sodium permeability by affecting amino sites within the sodium channel. Thus, these amino and sulfhydryl sites normally act as barriers to limit the movement of sodium. The amino barrier seems superficial and may be due to the electrostatic repulsion of sodium ions while sulfhydryl groups may limit sodium movement by physically constraining the channel.

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