J. Membrane Biol. 61, 199–207 (1981)

The Journal of
Membrane Biology

Time Dependence of the Effect of *p*-Chloromercuribenzoate on Erythrocyte Water Permeability: A Pulsed Nuclear Magnetic Resonance Study

David L. Ashley and J.H. Goldstein
Department of Chemistry, Emory University, Atlanta, Georgia 30322

Summary. Pulsed nuclear magnetic resonance spectroscopy is employed to determine the time dependence of the change in erythrocyte water permeability following exposure to *p*-chloromercuribenzoate (PCMB) or *p*-chloromercuribenzene sulfonic acid (PCMBS). pH variation was used to examine the environment of the sulfhydryl groups reactive to these drugs. PCMB reacted with at least two sulfhydryl groups which affect water permeability. This was shown by the double exponential character of the change in erythrocyte diffusional permeability with time after PCMB addition. However, only one inhibition rate process could be distinguished following PCMBS exposure, suggesting that one site bound by PCMB is not accessible to PCMBS. This site is postulated to be located in a hydrophobic region of the membrane, whereas the site reached by both drugs is located in the normal anion permeation channel. The effect of pH on the degree of inhibition due to each component and the inhibition rates is explained in terms of its effect on solubility of the reagents in the membrane and variation of the dissociated-to-undissociated ratio of PCMB.

Key words: Erythrocyte, water permeability, nuclear magnetic resonance, PCMB

The mechanism of water transport across biological membranes has not been fully elucidated. Various theories have been proposed and investigated, but currently a complete understanding of the controlling factors in water movement has not been achieved [2, 7, 15, 25]. The erythrocyte, due to its relative simplicity and availability, has often been the chosen object of permeability studies. A frequently used approach has been the exposure of these membranes to reagents whose reaction properties are known and which have a measurable effect on membrane permeability.

Sulfhydryl-reactive reagents are one of the most popular classes of drugs used in examination of membrane transport mechanisms. These agents exhibit a wide variety of physical properties, even though they react principally with protein sulfhydryl groups when exposed to biological systems [1]. A good deal of work has been done with these sulfhydryl-reactive reagents so that many of their properties are known [11, 13, 22]. Of special concern in clarifying transport mechanisms is the location of the binding sites of these drugs. Two drugs, often used due to their contrasting membrane permeability and lipid solubility, are the sulfhydryl-specific reagents [1], *p*-chloromercuribenzoate (PCMB) and *p*-chloromercuribenzene sulfonic acid (PCMBS). In previous studies these drugs have been shown to affect the red blood cell permeability of a wide range of substances. The changes in transport rate include an increase in cation permeability [12, 27], a decrease in glucose transport [28], and a decrease in the movement of water and nonelectrolytes across the erythrocyte membrane [6, 15, 16, 18]. Oddly enough, PCMBS has been shown not to affect anion permeability, even though it is an anion and is expected to permeate the membrane by the same paths as other anions, thus reaching any sulfhydryl groups in this pathway [13]. Detailed examination of the time course of the change in water permeability after exposure to PCMB and PCMBS can contribute to the determination of the location of the reactive sites and, hence, localization and understanding of the permeation process.

Pulsed nuclear magnetic resonance has already been shown to be a valuable technique in the measurement of the erythrocyte water permeability [5, 6, 19]. The advantages of this technique have already been discussed in detail [19]. These include speed of data collection, relative technical simplicity, measurement

0022-2631/81/0061-0199 $01.80

under equilibrium conditions, and reproducible results. With this technique the mean residence time of water within the cell, which is inversely proportional to the permeability [4], can be measured precisely approximately once per minute, allowing relatively fast reaction processes to be monitored.

In this paper the pulsed nuclear magnetic resonance technique is utilized to follow the variation in the water permeability of erythrocytes after their exposure to PCMB. The effect of pH variation on the time course of this process is examined and compared to the known properties of the drug. In addition, the inhibition of water transport by PCMBS is compared to that of PCMB.

Materials and Methods

Blood Sample Preparation

Blood was obtained from adult donors with no known hematological disorders, by venipuncture into Vacutainers with heparin used as an anticoagulant. The final heparin concentration was 10 USP units/ml. All samples were used within eight hours of collection and kept constantly mixed to prevent settling.

The pH of the blood was varied by the addition of HCl or NaOH solutions, made isotonic by the addition of the nonpenetrating solutes NaCl or KCl. Different pH values were obtained by adding varying volumes of the same acid or base solutions, or constant volumes of solutions with differing concentrations of acid and base to give stock blood samples. Both methods gave identical results within experimental error. pH was measured after the addition of drug and found not to vary throughout the course of the experiment by greater than ± 0.02. pH was measured directly in the drug-treated stock blood solution and varied only slightly between donors.

$MnCl_2$ was added directly to the blood from a 100 mM stock solution to give a final concentration of 1.7 ± 0.2 mM. The stock solution was always prepared at least one week in advance to reduce the rate at which Mn enters the erythrocyte [29]. The measured water exchange rate was found to be constant over a 5-hr period under these conditions in agreement with previous observations [6, 19]. All measurements were completed within three hours of the addition of Mn to the blood.

The sulfhydryl-reactive reagents PCMB and PCMBS were both obtained from Sigma (Sigma Chemical Company, St. Louis, Mo.). Care was taken to avoid exposure of the drugs to excessive light to prevent degradation [1]. Since it has been stated previously [28] that fresh solutions are important to achieve constant effects, the drug solutions were prepared on the day of the experiments and were made isotonic by the addition of saline. This solution was added directly to a predetermined amount of blood in the NMR sample tube and immediately placed in the spectrometer after mixing. The error in final drug concentration was ± 0.04 mmol/liter.

NMR Measurements

The NMR equipment and procedure have been described previously [19]. Measurements were made at 60 MHz, employing the standard Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence [3, 17]. A continuously variable delay trigger was adjusted to sample only the tops of the peaks of the spin echos. Care was taken to properly adjust sequence and reference phases and pulse widths to obtain maximum signal. Between measurements the sample was shaken to minimize settling.

Temperature was measured by a Duo-wrap 36 gauge Cu-constantan thermocouple (Thermo Electric Co., Saddle Brook, N.J.) calibrated with a Keithley 1608 digital multimeter (Keithley Instruments, Cleveland, Oh.) against a Mettler FP-5 temperature controller (Mettler Instrument Corp., Princeton, N.J.). The error was determined to be within ± 0.2 °C. The thermocouple was inserted into a melting point capillary tube containing heat-sink compound for good conduction, and this assembly was then inserted directly into the NMR sample tube. The temperature was maintained at 21.7 ± 0.4 °C throughout the experiments.

The transverse relaxation time (T_2) of the intracellular compartment was measured on blood treated identically as previously described, except for the exposure to Mn. Following pH measurement, the blood was centrifuged at $1000 \times g$ for 10 min and the plasma and buffy coat removed. It was then centrifuged a second 10 min at $1000 \times g$, and the upper layer of loosely packed cells removed. Finally, the blood was centrifuged for 50 min at $1000 \times g$ as previously described [19]. This procedure leaves a trapped plasma volume of less than 3% [8]. Treating the blood in this way gave substantially reproducible values of intracellular T_2. The T_2 of the packed cells was found to vary with pH so that values had to be measured over the entire pH range. Intracellular transverse relaxation time values were determined to be accurate to within $\pm 5\%$, which yields an error in mean residence time of only $\pm 1.5\%$ [19]. The use of experimentally measured values of intracellular T_2 in the determination of mean residence times has already been described in detail [19].

Spectra of drug-treated whole blood exposed to Mn were time averaged to increase the signal-to-noise ratio with eight scans taken over a 16-sec period and the time of measurement considered to be the midpoint of the averaging period. Time was accurate to within ± 2.0 sec as measured directly and also compared to the internal clock of the DECsystem-10 computer (Digital Equipment Corp., Marlboro, Mass.), to which the data was transferred. Single spectra were normally taken at 1 to 2 min intervals over the first 40 min after exposure to drug. At this time 70–80% of the inhibition had taken place, so that measurements were made every 4 min, which was often enough to characterize the curve. With 4 min intervals, two spectra were taken at each measurement and these values were averaged.

Data Analysis

Data analysis was performed as described previously [19] using a DECsystem-10 computer with the nonlinear regression program NLE (B. Blumenstein, *personal communication*) employing the two-site exchange equations given by Hazelwood et al. [10]. All experimental curves consisted of greater than 300 points in order to accurately define the magnetization decay.

The mean residence time of water within the cell was determined from the nonlinear fit of the CPMG magnetization decay curve and this value used to calculate the relative permeability of the blood compared to the permeability of the initial measurement. These values were then extrapolated back to zero time, and an initial, before-drug, value was determined. The relative permeability of all the measurements was then recomputed with this new starting permeability. The extrapolated value always agreed within $\pm 5\%$ of values measured independently at identical pH without drug. This data was then analyzed with the nonlinear regression program NLE and/or DISCRETE, a program based on the Fourier convolution theorum [20]. Both programs gave results which agreed in every case.

Results

Even though the kinetics of the effect of PCMBS on the hydraulic water permeability has been investigated [18], the change in diffusional water permeability following exposure to sulfhydryl-reactive reagents as a function of time has not been examined in detail. The diffusional permeability of water through a cellular structure (P_d) is related to the mean residence time of water inside the cell by the expression [4]

$$P_d = V/A\tau_a \tag{1}$$

in which A is the surface area of the cell, V is the volume of the cell, and τ_a is the mean residence time. This expression indicates that P_d is inversely proportional to τ_a under conditions of constant volume and surface area. Rich et al. [21] has pointed out that the erythrocyte surface area remains relatively constant, even when other properties of the cell change appreciably. The volume of the red blood cell is also demonstrated not to vary significantly under the current experimental conditions according to the following argument. In addition to the value of τ_a, the fraction of water which is inside the cell, P_a, is also estimated from the nonlinear regression analysis of the CPMG decay of whole blood. P_a is easily related to the hematocrit of the sample, as has been previously discussed [19]. Because the total volume of the sample and the number of cells within the sample remain constant, the hematocrit is, therefore, directly proportional to the mean cell volume. Thus, by monitoring P_a, any change in the volume of the cell can also be observed. For the 29 experiments reported, P_a varied by an average of only 6.6 ± 0.4 (SE)%. The change in cell volume indicated by this variation is relatively insignificant when compared to the large changes in τ_a, which were usually about 100%. Therefore, because there is no significant change in the volume or area of the cell as the reaction takes place, the relative permeability (P_r) is given by the expression

$$P_r = P_d/P_d^0 = \tau_a^0/\tau_a \tag{2}$$

in which the superscript zero refers to the initial values before the addition of drug.

Initial experiments showed that the total inhibition of water transport varied widely over the drug concentration range of 1 to 3 mM. The concentration of PCMB required to give maximum inhibition was determined by successive experiments at varying drug concentrations. Concentrations of greater than 3 mM were not found to give any increased inhibition so that 3 mM was used throughout the study. The permeability was determined to decrease to approximately half its original value at maximum inhibition.

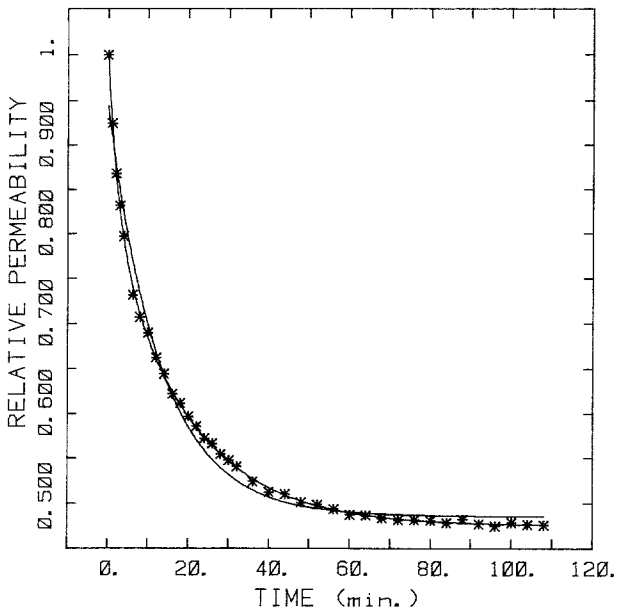

Fig. 1. Change in relative permeability of erythrocyte membranes after exposure to 3 mM PCMB with the best-fit single and double exponential curves

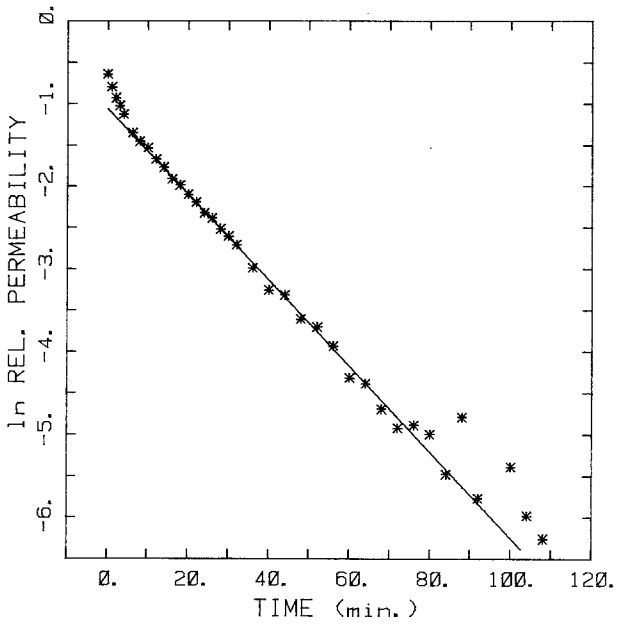

Fig. 2. Natural logarithm of relative permeability following PCMB exposure minus the final equilibrium baseline *vs.* time with the best-fit line through the slow component

PCMB Inhibition Kinetics

The time course of the change in relative permeability was measured over a 2-hr period, until the mean residence time had reached a constant value. A typical set of data is shown in Fig. 1. As can be seen, the relative permeability shows an initially rapid decrease which slows to reach a constant equilibrium value.

Table 1. Parameters of PCMB inhibition kinetics[a]

pH	P_1	k_1 (min^{-1})	P_2 (min^{-1})	k_2	B
8.06	0.066±0.013	0.352±0.137	0.413±0.006	0.0294±0.00086	0.521±0.003
7.76	0.081±0.011	0.299±0.067	0.398±0.005	0.0343±0.00075	0.520±0.002
7.52	0.149±0.010	0.352±0.046	0.336±0.005	0.0477±0.00138	0.515±0.002
7.33	0.163±0.008	0.227±0.022	0.329±0.005	0.0515±0.00152	0.508±0.002
7.14	0.179±0.007	0.386±0.030	0.347±0.004	0.0524±0.00107	0.474±0.002

[a] Errors given are 99% nonlinear confidence intervals.

Nonlinear regression analysis of double and single exponential fits of the data are also shown in Fig. 1. It is evident from these graphs that the double exponential fit characterizes the data accurately, but the single exponential fit does not adequately describe the measurements. Nonlinear regression analysis of a triple exponential fit did not improve the signal-to-noise ratio or the error in the fit appreciably, compared to the double exponential. Figure 2 is the natural logarithm of the same relative permeability data with the baseline subtracted. The graph of this quantity *vs.* time also confirms the double exponential character of the results. A single exponential response would be evidenced by the points all falling on a straight line, but the presence of an additional rapid rate causes the graph to deviate from this line for the first few points measured. The large deviation from a straight line at long time measurements is a common property of log plots of exponential data.

The results have been fit to an equation of the form

$$P_r = P_1 \exp(-k_1 t) + P_2 \exp(-k_2 t) + B \qquad (3)$$

in which P_r is the relative permeability at some time t. The parameters P_1 and P_2 are measures of the extent of inhibition due to each of the separate exponential processes. The parameters k_1 and k_2 are the rate constants for the double exponential inhibition process. B is the final equilibrium fraction permeability which is attained after the reaction has gone to completion. This quantity is equal to one minus the total fractional inhibition, which is equal to the sum of P_1 and P_2. The values of these parameters over the pH range of 7.05 to 8.05 for one donor are shown in Table 1. The accompanying data for three of these pH values with the double exponential best-fit curves are shown in Fig. 3. A notable increase in the overall rate of the inhibition as pH decreases is readily apparent from the figure. There is also evidence of an increase in the total inhibition as pH decreases.

The time course of inhibition of water permeability after exposure to PCMB was measured on five subjects at five different pH values for each. Figures

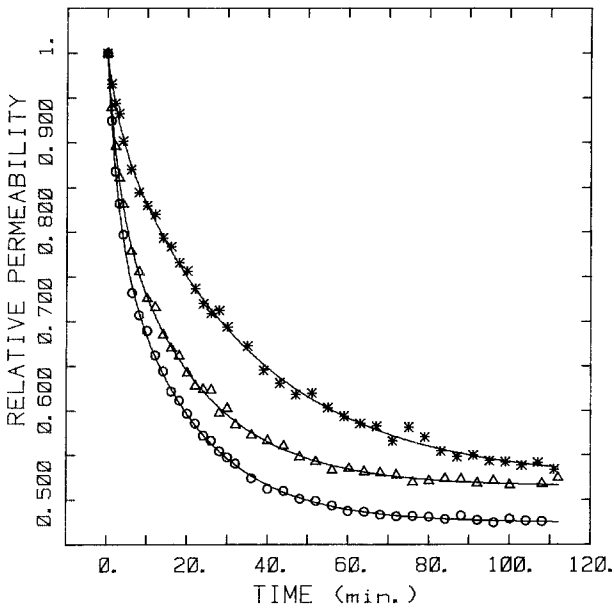

Fig. 3. Change in relative permeability after PCMB exposure with double exponential fits at different pH values. pH values are denoted: 8.06, asterisks; 7.52, triangles; 7.14, circles

4–7 show the variation with pH of each of the four kinetic parameters. A number of results are evident from these graphs and the values in Table 1. Figure 4 shows that P_1, which is the fraction of inhibition due to the reaction which occurs in the first few minutes after exposure to PCMB, decreases approximately linearly as the pH is increased. This response continues until pH reaches a value greater than about 8.10. Above this value, the nonlinear regression analysis would not converge for a double exponential equation, but a single exponential fit characterized the data adequately. The graph of k_1 *vs.* pH is given in Fig. 5. This plot illustrates that the rate of the fast component of the inhibition reaction does not change as the pH is varied. The relatively large scatter in this graph is mainly due to the fact that this reaction occurs very quickly. Because the majority of the process is complete within five or six minutes, this exponential is described by only four or five data

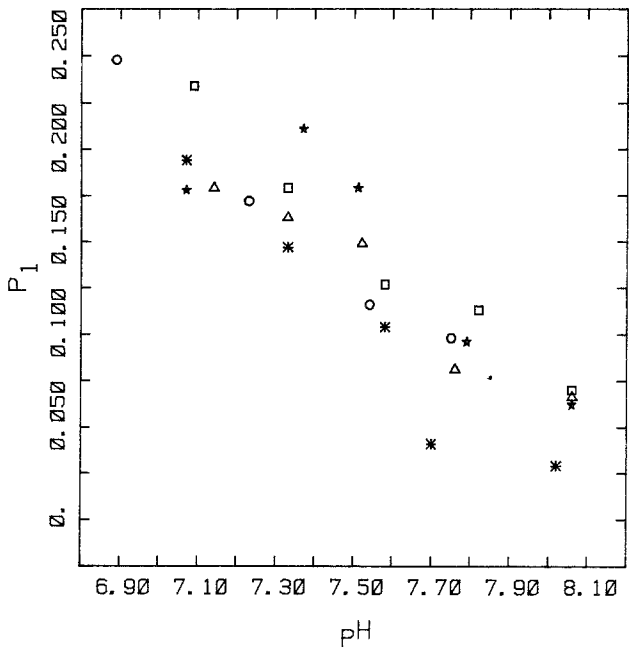

Fig. 4. Extent of inhibition due to the rapidly reacting component of PCMB inhibition *vs.* pH. Different donors are indicated by different geometric figures

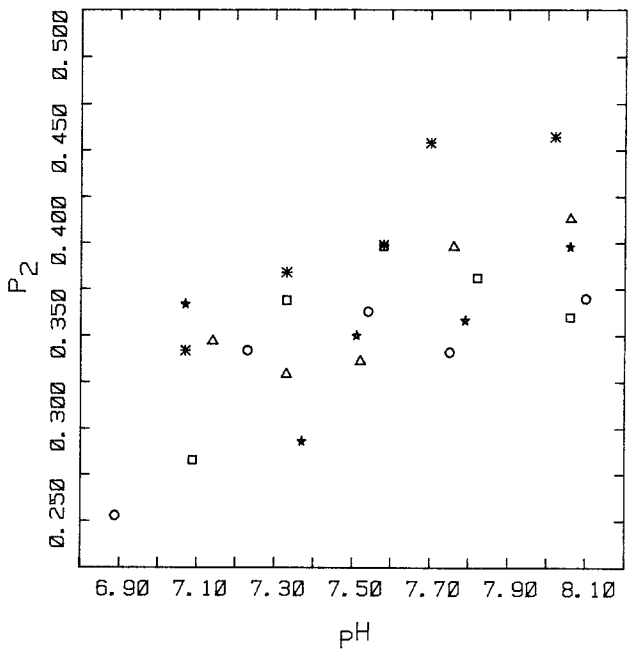

Fig. 6. Extent of inhibition due to the slowly reacting component of PCMB inhibition *vs.* pH. Donors are indicated as in Fig. 4

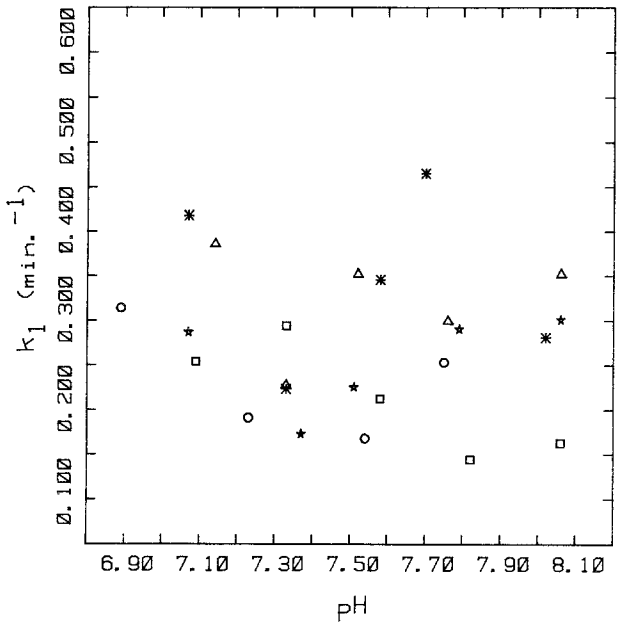

Fig. 5. Rate constant of the rapid reaction *vs.* pH. Donors are indicated as in Fig. 4

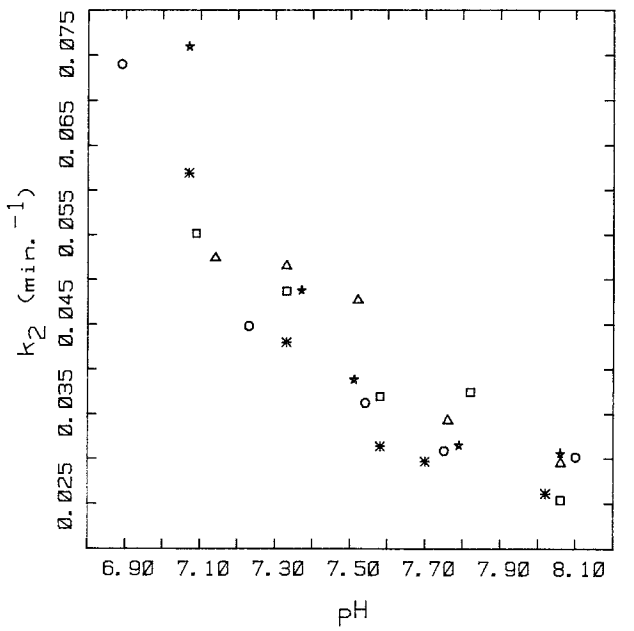

Fig. 7. Rate constant of the slow reaction *vs.* pH. Donors are indicated as in Fig. 4

points. Small errors in mean residence time at these short time measurements have a significant effect on this reaction rate.

Figure 6 shows the variation of P_2, the fraction of inhibition due to the slower reaction, as a function of pH. As can be seen in the graph, this parameter shows a tendency to increase as the pH increases, except at pH values greater than 7.70. At high pH, the values no longer increased but reached a fairly constant value. The rate constant for this slower process, k_2, clearly decreased as pH was increased, as demonstrated in Fig. 7. The rate constant, in the same

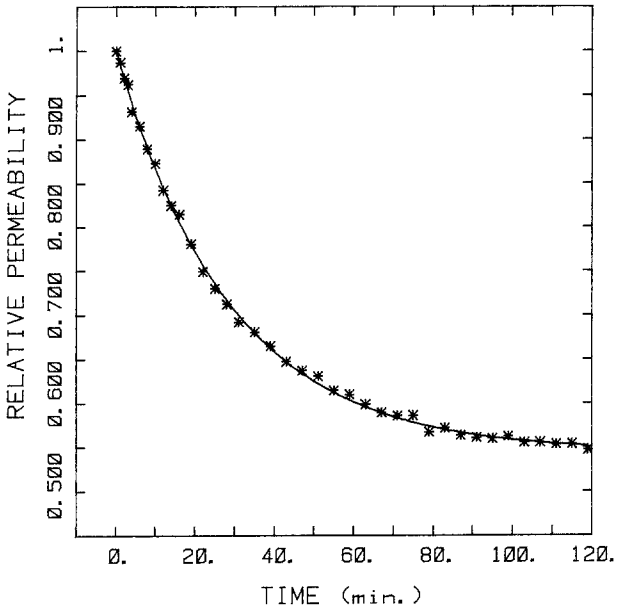

Fig. 8. Change in relative permeability of erythrocyte membranes after exposure to 3 mM PCMBS with the best-fit single exponential curve

Table 2. Parameters of PCMBS inhibition kinetics[a]

pH	P_1	k_1 (min^{-1})	B
7.61	0.416±0.007	0.0303±0.00097	0.584±0.003
7.34	0.456±0.004	0.0346±0.00064	0.544±0.002
7.06	0.490±0.006	0.0412±0.00106	0.510±0.003
6.84	0.501±0.005	0.0710±0.00136	0.498±0.002

[a] Errors given are 99% nonlinear confidence intervals.

manner as the fraction of inhibition for this process, reaches a value approaching constancy at high pH.

PCMBS Permeability Effects

The time course of the reaction of PCMBS with a single exponential fit through the data is shown in Fig. 8. In contrast to the results with PCMB, inhibition of water transport permeability by PCMBS could be characterized by a single exponential. A double exponential fit either did not converge to give a best least-squares fit, or did not appreciably improve the signal-to-noise ratio of the fit. This response was observed over the entire pH range considered. The overall rate of the reaction was much slower with PCMBS than with PCMB.

The variation of the fractional permeability with time was fit by nonlinear regression according to the equation

$$P_r = P_1 \exp(-k_1 t) + B \tag{4}$$

in which the variables are the same as those in Eq. (3). The variation in these values as pH changes are given in Table 2. As can be seen in this table, the total inhibition increased dramatically as pH was decreased. The rate of the reaction also decreased as the pH became more basic. The interpretation of these results is presented below.

Discussion

Inhibition of Water Transport by PCMB

The sulfhydryl-reactive reagents PCMB and PCMBS have been shown to have drastic effects on the sulfhydryl groups of proteins including those associated with the erythrocyte membrane [9]. Blockage of membrane sulfhydryl groups has been implicated as the underlying cause of the effects of PCMB on erythrocyte permeability. These changes are not the result of any variation in metabolism or the binding of drug to hemoglobin [11]. Structural modification of membrane proteins has been suggested to be the resulting membrane alteration [9]. Recent studies on water transport before and after treatment with sulfhydryl blocking agents have suggested that the protein pathways for water movement are closed by the drug, forcing the water to pass through the lipid region of the cell. This conclusion has been ascertained from the disagreement of hydraulic and diffusional permeability rates before treatment and their agreement after treatment [15, 25], in addition to the increase of the activation energy of water transport following drug exposure to a value similar to that of lipid bilayer vesicles [5, 16]. Unpublished studies in our laboratory have confirmed these results. Therefore, the protein sulfhydryl groups are the likely site of action of PCMB and PCMBS, resulting in the inhibition of water transport, although this response may be direct or indirect.

The results in Fig. 1 show that after about an hour's incubation at room temperature, PCMB reduces the diffusional permeability of the red cell membrane to approximately one half its original value. These results are in good agreement with previous results. Conlon and Outhred [5], using an NMR technique similar to the one used in this paper, determined that at 22 °C treatment of blood with 2 mM PCMB reduced the relative permeability by a factor of 0.54. Fabry and Eisenstadt [6] also found a decrease in permeability by about one half when exposed to 2 mM PCMBS. Other studies, including those by Naccache and Sha'afi [18] and Macey and Farmer [15], have found a greater decrease in the hydraulic water permeability after reaction with PCMBS in comparison with the above-mentioned diffusional studies. This is not unusual in light of the different conditions employed in the measurement of hydraulic

and diffusional permeability rates. Because of the differing measurement conditions, these methods may determine different aspects of the properties of water movement.

Time Dependence of Inhibition

The reaction of a drug with a cellular or vesicular structure can be influenced by a number of factors. These factors include (1) the solubility of the agent in the membrane; (2) the location of the reaction site in relation to the drug penetration pathways; (3) the reactivity of the site, including a number of factors which may influence it (i.e., pH, hydrogen bonding, etc.); and (4) the magnitude of the perturbation induced by the reaction [9, 22].

The results in Figs. 1 and 2 demonstrate that the time dependence of water transport inhibition by PCMB exhibits double exponential behavior. This finding suggests that there are at least two sites in distinctly different environments which are accessible to PCMB and which have an influential effect on water permeability. This is not an improbable result in view of previous studies done using sulfhydryl-reactive reagents [9, 22]. Through studies done using PCMBS, the reactive sulfhydryl group which causes cation permeability to increase upon mercaptide formation has been located in the interior of the membrane [12, 13]. PCMBS, which permeates the membrane slowly, is separated from this reactive site by a diffusion barrier when the drug is added to the extracellular medium [27]. Conversely, glucose transport is decreased immediately upon exposure to PCMBS, thereby demonstrating that these sites are located on the outside of the membrane, exposed to the exterior fluid [28]. Electron paramagnetic resonance studies have also confirmed the presence of at least two sulfhydryl groups located in distinctly different environments [24]. This study indicated the presence of one group in a hydrophilic compartment and the other in a lipid-like milieu.

PCMB, which is an organic acid, can exist in a number of forms, depending on the conditions of the solution. These conditions include the chloride ion concentration and the pH of the solution [1]. Because it is an acid, PCMB can be in either a charged dissociated form or an uncharged undissociated state. The actual fraction in either state varies with the hydrogen ion concentration. Because of the organic part of the molecule and the presence or absence of a negative charge, the two species will have drastically different properties when exposed to a membrane system. Because of its organic moiety, the undissociated molecule will be readily dissolvable in lipid or hydrophobic regions of the membrane. It has already been noted that undissociated PCMB is readily soluble in lipid [22], which accounts for the fact that it penetrates the membrane more quickly than PCMBS [28]. Because of the substitution of a sulfonic group for the carboxylic moiety, PCMBS is in a charged state within the pH range examined, preventing it from penetrating the lipid of the membrane. The dissociated acid will be insoluble in hydrophobic areas, but will be attracted to hydrophilic or positively charged regions of the membrane. The fraction of PCMB which is dissociated is similar in properties to the PCMBS molecule and must enter the cell by a slower aqueous route, accounting for the fact that PCMB is taken up by the cell for over 3 hr [28].

The results in Figs. 1 and 2 are in agreement with the presence of at least two routes for PCMB to enter the cell and encounter sulfhydryl groups which affect water permeability. One of these groups is encountered as the drug passes quickly through a hydrophobic region and is observable by the presence of a very rapid initial change in permeability. This site is not on the exterior of the membrane, otherwise this change would also be present when the cell is exposed to PCMBS. As will be explained, this response is not seen with PCMBS. The second sulfhydryl group reacts with PCMB as it penetrates through an aqueous route giving the slower permeability change.

PCMBS has been shown to have a greater aqueous solubility than PCMB and to be virtually insoluble in a lipid environment. It has been suggested that PCMBS penetrates the membrane through the normal anion permeation channel [12] and that the effects of PCMBS are due to sulfhydryl groups encountered in this pathway [13]. The results shown in Fig. 8 and given in Table 2 are consistent with the presence in this pathway of at least one sulfhydryl group which has an effect on water permeability. PCMBS is excluded from the second site due to its lack of lipid solubility.

pH Effect on Inhibition Kinetics

The net effect of pH on the reaction of organic mercurials with sulfhydryl groups and the manifestation of this reaction as altered permeability is controlled by a number of factors. The solubility of PCMB and PCMBS in the erythrocyte membrane has been demonstrated to be affected by variation of pH [22]. It has also been shown that pH changes the physical properties of red cells and the conformation of certain constituent proteins [14, 26]. These changes in protein conformation can change the accessibility or reactivity of membrane sulfhydryl groups, or change the extent of permeability inhibition following reaction. pH

may also change the ratio of charged-to-uncharged species of PCMB [1]. Current studies underway in our laboratory have shown that pH has an appreciable effect on diffusional water permeability. This result is in agreement with a discernible change taking place in the protein pathway which water encounters as it traverses the membrane.

The pH dependence of the reaction rate constants for PCMB and PCMBS are in agreement with the above assessment of the drug reactive sites. A decrease in pH from 8.0 to 7.0 has been shown to increase the rate at which sulfate ions [12] and PCMBS [23] permeate the normal anion pathway. The results in Fig. 7 for PCMB and Table 2 for PCMBS imply that the slow reaction component and drug movement through this channel are correlated. The increase in drug permeation accompanying decreased pH is not fully understood, but the same response has already been noted in reference to the effect of PCMBS on cation movement [23]. By contrast, the lack of variation of the rate constant for the rapid reaction of PCMB, shown in Fig. 5, agrees with the present proposal that this site is not located in the anion channel but in a hydrophobic pathway for drug movement.

The results in Fig. 6 for PCMB and Table 2 for PCMBS demonstrate that the extent of inhibition due to the slow reaction has a different pH response for the two drugs. The increase in the extent of inhibition with decreasing pH for PCMBS can be accounted for by a protein conformational change which increases either the number of sulfhydryl groups accessible in the anion pathway or the effect of binding on water transport. On the other hand, the opposite result occurs for the slow reaction component of PCMB, suggesting the presence of some factor other than the two sited above. Rothstein et al. [23] discuss this same phenomenon as it relates to the effect of sulfhydryl-reactive reagents on cation permeability. They show that at pH 7.0, PCMBS increases K^+ loss to a greater extent than PCMB, even though the uptake of PCMB is 10 times larger. These authors propose that this effect results from the fact that, at this pH, most of the PCMB enters through a lipid pathway which does not expose the drug to the sulfhydryl groups in the anion channel that affect cation permeability. The pH effect on the slow reaction inhibiting water transport in our study is in agreement with this proposal. Because there is a decrease in the charged-uncharged ratio of PCMB with decreasing pH, a smaller amount of the drug moves through the anion pathway, while more enters the erythrocyte through the lipid. This change decreases the number of PCMB-bound sulfhydryl groups in the anion channel and thus, decreases the extent of inhibition due to this component. (Of course, this argument does not apply to PCMBS which is present only in a charged form.)

In the other case, the extent of inhibition due to the fast component, P_1, increases from approximately 0 at pH 8.1 to 0.25 at pH 6.9, as shown in Fig. 4. Even at pH 8.1, an appreciable amount of PCMB enters the erythrocyte through the membrane lipid [23], so that this effect cannot be solely due to variation in the charged-uncharged ratio of drug. The pH effect on P_1, therefore, probably reflects a variation in either the accessibility of sulfhydryl groups to the lipid permeation path or the effect of this reaction on water permeability. Current results cannot distinguish between these possibilities.

At lower pH values the permeability was sometimes observed to reverse its trend and actually increase at the end of the experiment. This observation, which has been reported previously [18], was always accompanied by a noticeable lysing of cells. It has been shown that the reaction with PCMB causes some cell breakdown and the release of hemoglobin and other intracellular constituents into the drug accessible extracellular fluid [27]. The possible explanations for this response have already been discussed [28].

The results presented in this paper disagree to some extent with the work previously reported by Naccache and Sha'afi [18]. They reported the discovery of at least two sulfhydryl sites reactive to PCMBS which affected the hydraulic water permeability. Because their technique differs from that used here, there are two possible reasons for this discrepancy. The first is that hydraulic and diffusional permeability are affected by formation of mercaptides in ways that differ. This is not unlikely, because it has been shown previously that hydraulic and diffusional permeability are inhibited to different degrees upon sulfhydryl binding. One sulfhydryl group that affects hydraulic permeability may not have any effect on diffusional permeability. This reaction would not be observed when diffusional permeability is measured after drug exposure. The second reason is that, under the different reaction conditions employed in the hemolysis technique, a second sulfhydryl group may take on distinctive properties, which enable its differentiation from the others. Because the hemolysis technique involves lysing of the erythrocyte membrane in the measurement procedure, it is important to note the increased osmotic fragility of red cells after exposure to sulfhydryl-reactive reagents [11]. This increased fragility may affect the rate of hemolysis in addition to variation in hemolysis from bulk water flow.

Using a nuclear magnetic resonance technique, it has been possible to distinguish between at least two sites which are accessible to PCMB and have a perturbing effect on water diffusional permeability.

The results of pH variation have suggested that one of these sites is located in a hydrophobic region of the cell and the other is located in the normal anion channel. The different properties of PCMB and PCMBS were used to explain that because of its negative charge PCMBS cannot reach the hydrophobic site and thus inhibits water diffusional permeability with a single reaction rate. Further understanding of the location of these sites can help determine the factors which control water movement.

References

1. Benesch, R., Benesch, R.E. 1962. Determination of SH groups in proteins. *In:* Methods of Biochemical Analysis. D. Glick, editor. Vol. 10, pp. 43–70. Interscience, New York
2. Brown, P.A., Feinstein, M.B., Sha'afi, R.I. 1975. Membrane proteins related to water transport in human erythrocytes. *Nature (London)* **254**:523–525
3. Carr, H.Y., Purcell, E.M. 1954. Effects of diffusion on free precession in nuclear magnetic resonance experiments. *Phys. Rev.* **94**:630–638
4. Chien, D.Y., Macey, R.I. 1977. Diffusional water permeability of red cells. Independence on osmolality. *Biochim. Biophys. Acta* **464**:45–52
5. Conlon, T., Outhred, R. 1978. The temperature dependence of erythrocyte water diffusion permeability. *Biochim. Biophys. Acta* **511**:408–418
6. Fabry, M.E., Eisenstadt, M. 1975. Water exchange between red cells and plasma. *Biophys. J.* **15**:1101–1110
7. Forster, R.E. 1971. The transport of water in erythrocytes. *Curr. Top. Membr. Trans.* **2**:41–98
8. Giberman, E. 1973. Determination of the trapped volume in a pellet of red blood cells. *Experientia* **29**:1083–1085
9. Godin, D.V., Schrier, S.L. 1972. Modification of the erythrocyte membrane by sulfhydryl group reagents. *J. Membrane Biol.* **7**:285–312
10. Hazelwood, C.F., Chang, D.C., Nichols, B.L., Woessner, D.E. 1974. Nuclear magnetic resonance transverse relaxation times of water protons in skeletal muscle. *Biophys. J.* **14**:583–606
11. Jacobs, H.S., Jandl, J.H. 1962. Effects of sulfhydryl inhibition on red blood cells. I. Mechanism of hemolysis. *J. Clin. Invest.* **41**:779–792
12. Knauf, P.A., Rothstein, A. 1971. Chemical modification of membranes. I. Effects of sulfhydryl and amino reactive reagents on anion and cation permeability of the human red blood cell. *J. Gen. Physiol.* **58**:190–210
13. Knauf, P.A., Rothstein, A. 1971. Chemical modification of membranes. II. Permeation paths for sulfhydryl agents. *J. Gen. Physiol.* **58**:211–223
14. Liu, S., Fairbanks, G., Palek, J. 1977. Spontaneous, reversible protein cross-linking in the human erythrocyte membrane. Temperature and pH dependence. *Biochemistry* **16**:4066–4074
15. Macey, R.I., Farmer, R.E.L. 1970. Inhibition of water and solute permeability in human red cells. *Biochim. Biophys. Acta* **211**:104–106
16. Macey, R.I., Karan, D.M., Farmer, R.E.L. 1972. Properties of water channels in human red cells. *In:* Passive Permeability of Cell Membranes. F. Kreuzer and J.F.G. Slegers, editors. pp. 331–340. Plenum, New York
17. Meiboom, S., Gill, D. 1958. Modified spin-echo method for measuring nuclear relaxation times. *Rev. Sci. Instr.* **29**:688–691
18. Naccache, P., Sha'afi, R.I. 1974. Effect of PCMBS on water transfer across biological membranes. *J. Cell. Physiol.* **83**:449–456
19. Pirkle, J.L., Ashley, D.L., Goldstein, J.H. 1979. Pulse nuclear magnetic resonance measurements of water exhange across the erythrocyte membrane employing a low Mn concentration. *Biophys. J.* **25**:389–406
20. Provencher, S.W. 1976. A Fourier method for the analysis of exponential decay curves. *Biophys. J.* **16**:27–41
21. Rich, G.T., Sha'afi, R.I., Romualdez, A., Solomon, A.K. 1968. Effect of osmolality on the hydraulic permeability coefficient of red cells. *J. Gen. Physiol.* **52**:941–954
22. Rothstein, A. 1970. Sulfhydryl groups in membrane structure and function. *In:* Current Topics in Membranes and Transport. F. Bronner and A. Kleinzeller, editors, Vol. 1; pp. 135–176. Academic Press, New York
23. Rothstein, A., Takeshita, M., Knauf, P.A. 1972. Chemical modification of proteins involved in the permeability of the erythrocyte membrane to ions. *In:* Passive Permeability of Cell Membranes. F. Kreuzer and J.F.G. Slegers, editors. pp. 393–413. Plenum Press, New York
24. Sandberg, H.E., Bryant, R.G., Piette, L.H. 1969. Studies on the location of sulfhydryl groups in erythrocyte membranes with magnetic resonance spin probes. *Arch. Biochem. Biophys.* **133**:144–152
25. Sha'afi, R.I. 1977. Water and nonelectrolyte permeation in red cells. *In:* Membrane Transport in Red Cells. J.C. Ellory and V.L. Lew, editors, pp. 221–256. Academic Press, New York
26. Smith, B.D., Lacelle, P.L. 1979. Parallel decrease of erythrocyte membrane deformability and spectrin solubility at low pH. *Blood* **53**:15–18
27. Sutherland, R.M., Rothstein, A., Weed, R.I. 1969. Erythrocyte membrane sulfhydryl groups and cation permeability. *J. Cell. Physiol.* **69**:185–198
28. VanSteveninck, J., Weed, R.I., Rothstein, A. 1965. Localization of erythrocyte membrane sulfhydryl groups essential for glucose transport. *J. Gen. Physiol.* **48**:617–632
29. Weed, R.I., Rothstein, A. 1960. The uptake of divalent manganese ion by mature normal human red blood cells. *J. Gen. Physiol.* **44**:301–314

Received 3 September 1980; revised 12 February 1981