# Spin-Label Detection of Hemoglobin–Membrane Interaction at Physiological pH†

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ABSTRACT: The interaction between hemoglobin and the cytoplasmic surface of human erythrocyte membranes at physiological pH was studied by monitoring the electron paramagnetic resonance (EPR) signal of spin-labeled membrane ghosts in hemoglobin solutions of various concentrations. The EPR spectra indicate the existence of a significant hemoglobin–membrane interaction which exhibits a substantial hemoglobin concentration dependence over the concentration range 0–12 mg/mL. An equilibrium binding model yields a hemoglobin–membrane dissociation constant, $K_d$, on the order of $10^{-4}$ M, at and above physiological pH; the interaction is classified as very low-affinity binding. The interaction increases significantly when the pH is decreased. Half-saturation of the binding sites occurs at a ratio of about $10^8$ hemoglobins per cell.

As the red cells age, small but progressively increasing amounts of hemoglobin and other proteins become bound to the cell membrane (Sears et al., 1975; Sears & Lewis, 1980). Several other changes, including changes in carbohydrate composition, have also been reported to occur in erythrocyte membranes during aging in vivo. It is generally believed that in vivo erythrocyte destruction involves alterations in the cell membrane. However, the nature of the membrane alteration producing erythrocyte destruction is not clear, and little is known about the interaction between membranes and hemoglobin, especially under physiological conditions.

Early studies by radioactive tracer techniques showed complete mixing of unlabeled intracellular hemoglobin with labeled extracellular hemoglobin upon osmotic lysis at pH 7.5, suggesting that no hemoglobin was covalently bound to the erythrocyte membranes (Hoffman, 1958). In studies of hemoglobin association with membranes prepared at various pH values, it was found that about 1% of the intracellular hemoglobin molecules were bound to membranes at low pH (pH 6.15) but that no binding was observed at pH 7.65 (Mitchell et al., 1965). Thus, it is possible to prepare hemoglobin-free membrane samples with buffers at physiological pH (Dodge et al., 1963; Steck & Kant, 1974). In a reconstitution study, various concentrations of $^{14}$C-labeled hemoglobin were introduced to membrane samples at a constant membrane (protein) concentration of 1 mg/mL, and at pH 6.5. About 50 $\mu$g of hemoglobin was found to associate with the membrane after excess hemoglobin was removed by gradient centrifugation (Fischer et al., 1975). At pH 8.0, only about 20% of the amount of hemoglobin bound to membrane at pH 6.5 remained bound to the membrane (Fischer et al., 1975). These studies indicate that the electrostatic interactions between hemoglobin and the membrane surface are significant at low pH but not at neutral pH, resulting in a small amount of hemoglobin associating strongly with the membrane at low pH values. Recently, studies with a fluorescent probe embedded in the membrane showed that about 3% of the intracellular hemoglobin binds to membranes at pH 6 but not at pH 7 (Shaklai et al., 1977). The data further demonstrated that the hemoglobin binding sites on the membrane at pH 6 exhibit two different affinities. The majority exhibit a low affinity with an equilibrium dissociation constant, $K_d$,[1] of about $1.6 \times 10^{-7}$ M, while a small portion exhibits a high affinity with a $K_d$ of about $1.2 \times 10^{-8}$ M (Shaklai et al., 1977). It was later suggested that dimers of oxyhemoglobin bind to band 3 on the cytoplasmic surface of membranes under these conditions, and a new $K_d$ of about $0.2 \times 10^{-6}$ M was given (Salhany & Shaklai, 1979).

It is important to determine whether the majority of the intracellular hemoglobin molecules interact with membrane components and to study the interaction at physiological pH. However, all the reported findings suggest that it is difficult to monitor this interaction, probably due, in part, to the insensitivity of the techniques used. We have spin-labeled the proteins which are mainly on the cytoplasmic side of the erythrocyte membranes and have found the electron paramagnetic resonance (EPR) technique to be a very sensitive method for monitoring the effects of hemoglobin molecules on membrane proteins by measuring the mobility of spin-labels attached to the membrane protein molecules. Specific interactions at physiological pH values are observed. The EPR data show that the hemoglobin concentration for half-saturation ($C_{1/2}$) is about 4 mg/mL ($2.4 \times 10^7$ hemoglobins/cell) at pHs 7.4 and 8.0 and about 1 mg/mL at pH 6.7. The $K_d$ values are about $10^{-4}$ M. In view of the earlier fluorescence approach where interactions are classified into high-affinity and low-affinity interactions, we classify the interaction observed in these studies to be a very low-affinity interaction.

## Experimental Procedures

*Hemoglobin (Hb) Solutions.* Blood cells (from the Detroit Red Cross Blood Bank, less than a week after being drawn) were washed 3 times with an isotonic solution of 5 mM sodium phosphate buffer, pH 8, containing 0.9% sodium chloride. The resulting packed cells were lysed with cold distilled water at a 1:2 volume ratio. The hemolysate was stirred gently for 30 min before addition of neutral, saturated ammonium sulfate solution (4 parts of hemoglobin solution to 1 part of ammonium sulfate solution). After being gently stirred for an additional 20 min, the solution was centrifuged at 12000 rpm (17000*g*) for 30 min.

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[1] Abbreviations used: $K_d$, equilibrium dissociation constant; EPR, electron paramagnetic resonance; $C_{1/2}$, half-saturation concentration; Hb, hemoglobin; CO, carbon monoxide; M, membrane; Mal-6, *N*-(1-oxy-2,2,6,6-tetramethyl-4-piperidinyl)maleimide; BSA, bovine serum albumin; W, weakly immobilized component; S, strongly immobilized component.

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The hemoglobin solution (supernatant) was obtained, gassed with carbon monoxide (CO), and dialyzed against a CO-gassed 5 mM sodium phosphate buffer at the appropriate pH overnight. The pH was further adjusted to within 0.1 pH unit, if necessary, with buffer solutions. Samples were concentrated, when needed, with an ultrafiltration cell under nitrogen pressure. The concentration was determined spectrophotometrically at 540, 569, and 577 nm (Antonini & Brunori, 1971). The hemoglobin solution was used within 1 week.

*Spin-Labeled White Membrane Ghosts.* Membrane ghosts were prepared by the method of Dodge (Dodge et al., 1963; Steck & Kant, 1974). The fresh blood cells (less than a week after being drawn) were washed with saline phosphate buffer at pH 8, as described above. The cells were lysed with 20 volumes of 5 mM sodium phosphate buffer at pH 8 and washed 3 times to give hemoglobin-free white membrane ghosts. The membrane samples were spin-labeled with *N*-(1-oxy-2,2,6,6-tetramethyl-4-piperidinyl)maleimide (Mal-6) (Syva Research Chemicals). An aliquot of the spin-label, stored in acetonitrile, was added to a vial, and the acetonitrile was evaporated under $N_2$ gas before the addition of the membrane sample (30–50 μg of Mal-6/mg of protein). After a 1-h incubation in the dark, the membrane samples were washed with buffer to remove excess spin-label. After the third wash, EPR spectra were taken; washing was terminated when the EPR spectrum of a sample was the same as the one taken after the previous wash. The membrane samples were then dialyzed overnight against the phosphate buffer of appropriate pH and used immediately. The protein concentrations of the spin-labeled membrane samples were determined by the modified Lowry method (Peterson, 1977).

*Membrane–Hemoglobin Samples.* The membrane and hemoglobin samples were diluted with appropriate buffers to 2 and 18 mg/mL, respectively. Various amounts (0–400 μL) of hemoglobin samples were added to 200 μL of membrane samples, which were stirred gently for 30 min to allow equilibration, and then centrifuged with an airfuge (Beckman Instruments). The pellet was introduced to a 50-μL glass capillary (nonheparinized microhematocrit tube). One end of the capillary was sealed, and the sample was packed to the bottom of the capillary tube with a table-top centrifuge.

*Membrane–Bovine Serum Albumin Samples.* Bovine serum albumin (BSA) (Sigma Chemical Co.) solutions of 18 mg/mL in phosphate buffers were used to prepare membrane–BSA samples, with procedures similar to those used in membrane–hemoglobin samples.

All procedures described above were carried out at 4 °C in a cold room or on ice. pH values were controlled to ±0.1 pH unit for all samples.

*EPR Measurements.* For each EPR measurement, the sample in the capillary was placed in a 3-mm quartz tube filled with a small amount of silicon oil to give thermal stability. A Varian E109E EPR spectrometer equipped with a TM cavity and a variable temperature controller was used for all measurements. The temperature of the sample was monitored by a digital readout device connected to a copper–constantan thermocouple. A Nicolet 535 time averager was interfaced with the EPR spectrometer to improve signal to noise ratios. EPR measurements were carried out at 20 ± 0.5 °C immediately after the samples were prepared. Standard procedures used in this laboratory were followed (Fung, 1981).

## Results

The low-field region of the EPR spectrum, shown in the inset of Figure 1, exhibits two types of spin-label signals: one component is a weakly immobilized (W) component, and the other a strongly immobilized (S) component. The ratio of the signal amplitudes, $W/S$, is very sensitive to such experimental conditions as temperature, ionic strength, and pH, as well as to other factors that affect membrane states (Fung, 1981). The $W/S$ ratio for several independent hemoglobin-free membrane preparations ($n$ = 23) in 5 mM phosphate buffer at pH 8 and 20 °C is 4.03 ± 0.56. The standard deviation for the $W/S$ ratio is about 0.2 for samples prepared in parallel, using the same blood sample and buffer solutions. Thus, much of the variance in the absolute values of the $W/S$ ratio appears to be due to individual differences between the membranes of different blood samples.

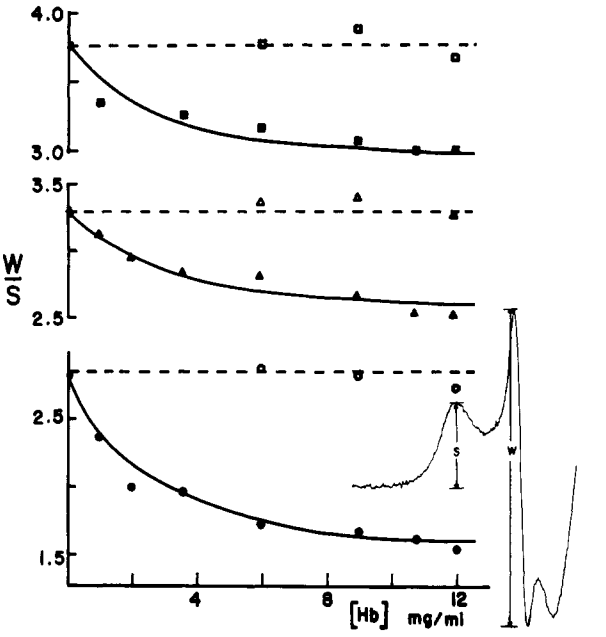


FIGURE 1: $W/S$ values of membranes, in 5 mM phosphate buffer, as a function of hemoglobin concentration at pH 8.0 (●), 7.4 (■), and 6.7 (▲) at 20 °C. Bovine serum albumin (BSA) was used as control, and the $W/S$ values of membranes in the presence of BSA are given in the open symbols. The lines shown through the data are spline fits and have no theoretical significance. A portion of the EPR spectrum showing the W and S components is given in the inset.

Upon addition of CO-hemoglobin to spin-labeled membrane samples, the $W/S$ ratio decreases with increasing hemoglobin concentration as shown in Figure 1 for three representative samples at the pH values 6.7, 7.4, and 8.0. Data for membrane to which equivalent amounts of BSA have been added at pH 6.7, 7.4, and 8.0 are also shown in Figure 1. From these data, it can be seen that, in contrast to the behavior of the hemoglobin–membrane systems, the $W/S$ ratios for membranes in the presence of BSA remain essentially constant. This suggests that the change in $W/S$ values observed upon addition of hemoglobin to the membranes is the result of a specific hemoglobin–membrane interaction rather than a general protein–membrane interaction.

A more useful parameter than the absolute value of the $W/S$ ratio, for studying the Hb–membrane interaction, is $\Delta(W/S)_{Hb}$, the difference between the $W/S$ ratios in the absence and presence of Hb, i.e., $\Delta(W/S)_{Hb} = (W/S)_0 - (W/S)_{Hb}$, where $(W/S)_0$ is the membrane $W/S$ ratio in the absence of Hb and $(W/S)_{Hb}$ is the ratio in the presence of Hb. This difference parameter eliminates most of the variation due to different membrane preparations, permitting more sensitive detection of the effects of extrinsic agents upon the membrane (Fung, 1981). The $\Delta(W/S)_{Hb}$ values for several different runs, as well as the mean values, $\overline{\Delta(W/S)_{Hb}}$, are shown in Figure 2 as a function of hemoglobin concentration at the three pH

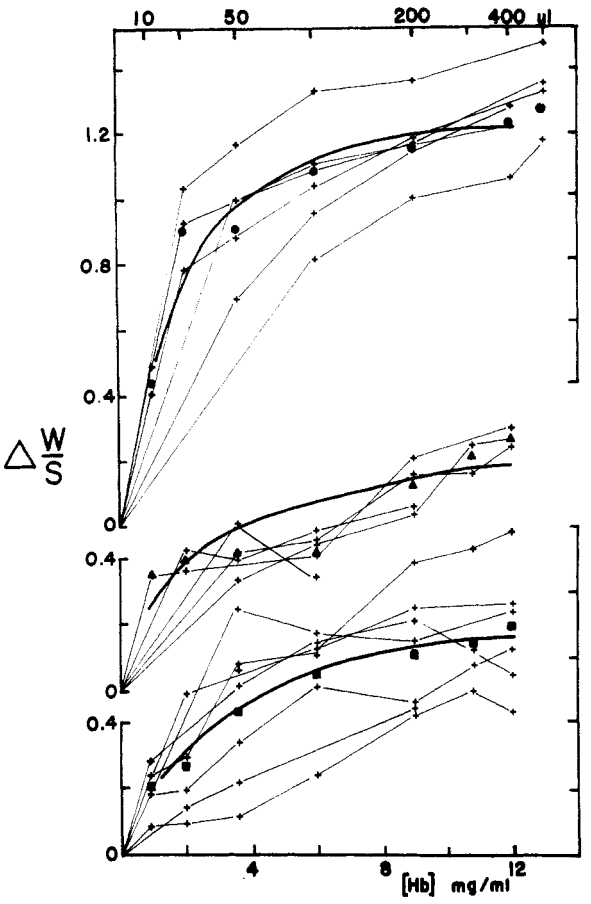


FIGURE 2: Change in the $W/S$ ratio of membranes upon addition of hemoglobin molecules of several experimental runs at three pH values. The mean values at pH 8.0 (●), 7.4 (■), and 6.7 (▲) are analyzed with the nonlinear regression method, and the calculated binding curves at each pH value are given. The $K_d$ values obtained from these curves are given in Table I. The volumes of the 18 mg/mL hemoglobin solutions added to 200 μL of membranes at 2 mg/mL to give the indicated concentrations are also shown as the upper scales in the plots.

values, 6.7, 7.4, and 8.0. Hb was added to the membrane systems from a stock solution at a concentration of 18 mg/mL; the volumes of Hb solutions added to give the indicated concentrations are also shown as the upper scales in the plots of Figure 2. The data shown in these plots indicate that the $W/S$ ratio changes rapidly upon initially adding small amounts of Hb to the membrane systems. As increasing amounts of Hb are added, however, the rate of change in $W/S$ decreases, and the ratio appears to go asymptotically toward a constant value.

If one assumes that the changes in the $W/S$ ratio observed upon addition of Hb to the membrane are the result of Hb binding to the membrane, these data can be used to obtain values for the Hb–membrane dissociation constant, $K_d$. Assuming that the observed $W/S$ ratio of membrane in the presence of hemoglobin, $(W/S)_{Hb}$, is the sum of the $W/S$ ratios for spin-labeled membrane sites without bound hemoglobin, $(W/S)_0$, and those with bound hemoglobin, $(W/S)_b$, it is possible to write the following expression:

$$(W/S)_{Hb} = f_b(W/S)_b + (1 - f_b)(W/S)_0 \quad (1)$$

where $f_b$ is the fraction of spin-labeled membrane sites in the sample that have hemoglobin associated with them. Since $\Delta(W/S)_{Hb} = (W/S)_0 - (W/S)_{Hb}$, eq 1 becomes

$$\Delta(W/S)_{Hb} = f_b[(W/S)_0 - (W/S)_b] \quad (2)$$

Let $(W/S)_0 - (W/S)_b = \Delta(W/S)_\infty$, the change in $W/S$ of membranes from no hemoglobin to infinite hemoglobin concentration; then eq 2 becomes

$$(W/S)_{Hb} = f_b\Delta(W/S)_\infty \quad (3)$$

We can also express $f_b$ in terms of the equilibrium dissociation constant, $K_d$, as

$$f_b = \frac{[M\cdot Hb]}{[M\cdot Hb] + [M]} = (1 + K_d/[Hb])^{-1} \quad (4)$$

where [M·Hb], [M], and [Hb] are respectively the concentrations of membrane associated with hemoglobin, of membrane, and of free hemoglobin species, and $K_d$ is the equilibrium dissociation constant of M·Hb ⇄ M + Hb. Substituting eq 4 into eq 3 yields

$$\Delta(W/S)_{Hb} = \Delta(W/S)_\infty(1 + K_d/[Hb])^{-1} \quad (5)$$

where $[Hb] = [Hb]_0 - [M\cdot Hb]$ and $[Hb]_0$ is the concentration of hemoglobin added. When the area of the cytoplasmic surface of the erythrocyte membrane is considered to be about $140 \times 10^{-12}$ m$^2$ and the cross section of a hemoglobin molecule to be about $(50 \times 10^{-10})^2$ m$^2$, the number of hemoglobin molecules required to cover the surface in regular, closely packed arrays is about $6 \times 10^6$ hemoglobin molecules per cell. This value can be considered as the maximum [M·Hb], concentration of the membranes bound with Hb. Since $[Hb]_0$ is about $10^8$ hemoglobin molecules per cell in our experiment, it is reasonable to assume that $[Hb] \simeq [Hb]_0$. Therefore, eq 5 becomes

$$\Delta(W/S)_{Hb} \simeq \Delta(W/S)_\infty(1 + K_d/[Hb]_0)^{-1} \quad (6)$$

Equation 6 could be linearized by using the reciprocal variables $\{\Delta(W/S)_{Hb}\}^{-1}$ and $\{[Hb]_0\}^{-1}$. However, the inversion overweighs values of $\Delta(W/S)_{Hb}$ at low $[Hb]_0$ and underweighs values at high $[Hb]_0$ whereas a nonlinear regression method, while more complex to use, probably yields more reliable parameter estimates. Thus, nonlinear regression methods were used to obtain values for $K_d$ and $\Delta(W/S)_\infty$ for the three data sets at pH 6.7, 7.4, and 8.0, using the data for Hb concentration greater than 2.0 mg/mL. The lowest Hb concentration data were excluded from the calculations since they could be affected by the high-affinity binding previously observed (Shaklai et al., 1977), whereas the behavior at high Hb concentrations should primarily reflect the very low-affinity binding behavior. Values of $K_d$ and $\Delta(W/S)_\infty$, along with their uncertainties, are given in Table I.

Table I: Hb-Membrane Binding Parameters at pH 6.7, 7.4, and 8.0 in 5 mM Phosphate Buffer at 20 °C

| pH | $\Delta(W/S)_\infty$ | $K$ ($\times 10^5$ M) | $N^a$ | $F^b$ | $P^c$ |
|---|---|---|---|---|---|
| 6.7 | 1.33 ± 0.09[d] | 1.8 ± 0.6[d] | 5 | 482 | <0.001 |
| 7.4 | 0.98 ± 0.21 | 7.1 ± 4.0 | 6 | 126 | <0.001 |
| 8.0 | 0.92 ± 0.04 | 6.6 ± 0.8 | 6 | 2622 | <0.001 |

[a] $N$ is the number of points used in the regression calculations. [b] $F$ test of the regression significance. [c] $P$ is the probability of obtaining $F$ values as large or larger than those given here. [d] Listed uncertainties are the standard errors of estimate as obtained from the regression calculations.

## Discussion

The data described above demonstrate that the $W/S$ ratio is a very sensitive parameter for studying the very low-affinity hemoglobin–membrane interaction. With our method of labeling the erythrocyte membrane, about 20% of the membrane sulfhydryl (SH) groups (25 nmol/mg of protein) are alkylated by Mal-6, with about 80% of the spin-label intensity arising from sites at the inner membrane surface (Fung & Simpson, 1979). The EPR spectra of membranes exhibit a broad,

slow-motion component, S, and a minor, weakly immobilized component, W. The weakly immobilized labels have an apparent rotational correlation time of about $10^{-9}$ s while the rotational correlation times of the strongly immobilized labels are greater than $10^{-7}$ s. At pH 8 and 20 °C, the W component comprises about 10% of the integrated total intensity (Fung, 1981). Since the W component gives rise to a narrow line, and the S component to a broad line, the conversion of one component to another will result in a large change in the amplitude of W and little change in that of S. Thus, the $W/S$ ratio is very sensitive to any interconversion between W and S components, despite the fact that the W component is only a minor portion of the total integrated signal intensity. Therefore, one is able to follow the decrease of the $W/S$ ratios [or the increase in $\Delta(W/S)$ values] in membranes due to the presence of hemoglobin molecules which interact with membrane components to cause some small changes in protein label mobilities. This immobilization of the spin-labels that are alkylated to membrane proteins is hemoglobin specific. Another protein, BSA, which has a molecular weight similar to that of hemoglobin, does not cause the $W/S$ ratio of membranes to decrease. Since our labels are mainly on the cytoplasmic surface, we suggest that the interaction sites are on the inner surface of the membrane. We have also prepared right-side-out vesicles from the spin-labeled membranes and find that the $W/S$ ratio remains essentially the same upon addition of hemoglobin molecules, providing further experimental evidence that the hemoglobin molecules interact mainly with the cytoplasmic membrane components.

Based on our EPR data, we suggest that the hemoglobin molecules that interact with the membrane surface may act as perturbants to the dynamic structure of the cell membrane which, in turn, may affect the structural and functional properties of membranes with pathological consequences. The interaction between membrane and hemoglobin molecules may also affect the functional properties of hemoglobin molecules. In sickle cell hemoglobin studies, it has been found that the presence of membranes promotes deoxyhemoglobin polymerization, and it has been suggested that the membrane surface may act as a template for hemoglobin filament formation in deoxygenated sickle cells (Shibata et al., 1980). Subsequently, permanent injury in the membrane produces cells which remain irreversibly sickled even after reoxygenation (Lessin et al., 1978). Recently, it has been shown that membrane ghosts enhance the rate of polymerization by only about 3-fold (Goldberg et al., 1981).

The strong sensitivity of the $W/S$ ratio also implies a significant degree of fluctuations and uncertainties in the $W/S$ measurements of similar samples, producing difficulties in obtaining precise quantitative information about the hemoglobin–membrane interaction. Previously we have found the $W/S$ ratio to be very sensitive to such experimental conditions as the temperature of the EPR measurements and the ionic strength and the pH of the samples, all of which are known to affect the dynamic and structural properties of the proteins in membranes (Fung, 1981). In the measurements described in this work, in addition to having all of these experimental factors that affect the membrane state carefully controlled, we are examining the $\Delta(W/S)$ values, rather than the absolute $W/S$ ratios, of samples prepared at the same time by using the same blood samples and buffer solutions in order to eliminate some of the uncertainties and to minimize the run-to-run fluctuations.

Using this approach, we find the $K_d$ value for the Hb–membrane interaction at pH 6.7 to be about $(1.8 \pm 0.6) \times 10^{-5}$ M, while at both pHs 7.4 and 8.0 the value is about $(7.1 \pm 4.0) \times 10^{-5}$ M. $F$ tests of the regression calculation indicate that the results are highly significant at all three pH values. The uncertainties in $K_d$ values also indicate that the change in binding affinity in going from pH 6.7 to pH 8.0 is statistically significant. Furthermore, the half-saturation concentration, $C_{1/2}$, at which $(W/S)_{Hb} = {}^1/_2\Delta(W/S)_\infty$ is about 1 mg/mL at pH 6.7 and about 4 mg/mL at both pH 7.4 and pH 8.0. Thus, the findings described here clearly indicate that there is a significant Hb–membrane interaction, both at and above physiological pH, and that as the pH decreases the strength of the interaction increases. The pH dependence of the interaction also suggests that the interaction may be electrostatic in nature. However, the electrostatic energy of the interaction between two hemoglobin molecules is likely to fall off rapidly with increasing intermolecular distance (Ross & Minton, 1977), and the total charge on hemoglobin between pH 7 and pH 8 is less than 5 (Antonini & Brunori, 1971). Thus, we suggest that the very low-affinity interactions between hemoglobin and the cytoplasmic surface of the erythrocyte membrane are probably at least, in part, short-range attractive electrostatic interactions. We should also indicate that in our data analysis we have not taken the thermodynamic nonideality of the hemoglobin (Ross & Minton, 1977) into consideration. The activity, rather than the concentration, of hemoglobin should be used when the hemoglobin concentration is high (Ross & Minton, 1977). Detailed thermodynamic consideration may change the $C_{1/2}$ values slightly.

It is interesting to speculate that as the pH decreases (as would occur, for example, in sickle cells after anoxia) the increasing strength of the Hb–membrane interaction may lead to further membrane alteration by hemoglobin molecules. However, despite the potential importance of this suggestion, it should be considered very tentative, because it is difficult to define and prepare normal and "unaltered" membrane samples, and without the unaltered membrane, it is difficult to evaluate the pathological consequences of the hemoglobin–membrane interaction.

A better understanding of the labeling site(s) and a physical interpretation of label and protein motions in the membrane may provide a further understanding of this very low-affinity interaction between hemoglobin and membrane components at physiological pH. Further studies of systems at physiological ionic strength, or membrane obtained from cells at different ages, for example, may provide additional insights toward the understanding of some of the molecular details of erythrocyte destruction in circulation.

In summary, the EPR data described here indicate that hemoglobin interacts with the cytoplasmic surface of the erythrocyte at and above physiological pH. The equilibrium dissociation constant at physiological pH is around $10^{-4}$ M but decreases significantly upon decreasing the pH. Half-saturation of the binding sites occurs at a ratio of about $10^8$ hemoglobin molecules per cell. (The erythrocyte contains about $2.5 \times 10^8$ hemoglobin molecules per cell.)

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# Selective Labeling of the δ Subunit of the Acetylcholine Receptor by a Covalent Local Anesthetic†

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ABSTRACT: A radioactive photoaffinity derivative of the potent local anesthetic trimethisoquin, 5-azido[$^3$H]trimethisoquin, was used to label the acetylcholine receptor from *Torpedo marmorata* electric organ. The product labeled the 66 000-dalton (δ) subunit of the receptor with the selectivity expected for an affinity label of the site for noncompetitive blockers. That is, the labeling was enhanced by cholinergic agonists and inhibited by other noncompetitive blockers. The 40 000-dalton (α) subunit of the receptor was labeled in a manner consistent with the attachment of 5-azido[$^3$H]trimethisoquin to an acetylcholine binding site as the incorporation of radioactivity into the α chain was inhibited by cholinergic agonists and antagonists, such as carbamylcholine, *d*-tubocurarine, and α-bungarotoxin. The reversible binding of [$^3$H]phencyclidine, a potent noncompetitive blocker, to acetylcholine receptor rich membranes resembled qualitatively and quantitatively the 5-azido[$^3$H]trimethisoquin labeling of the δ subunit and was inhibited by the prior covalent labeling of the membranes with nonradioactive 5-azidotrimethisoquin. Thus, 5-azido[$^3$H]-trimethisoquin labels at least a portion of the binding site for noncompetitive blockers at the level of the δ subunit. The functional significance of this site and the use of 5-azidotrimethisoquin in the study of acetylcholine receptor structure and function are discussed.

The physiological response to AcCh[1] of the subsynaptic membrane from the neuromuscular junction or the electromotor synapse is blocked by two distinct classes of pharmacological agents: (1) the competitive, nicotinic antagonists (e.g., *d*-tubocurarine and flaxedil) which decrease the apparent affinity for AcCh without changing the maximal response and which interact directly with the AcCh binding site [for reviews, see Nachmansohn (1955), Changeux (1975, 1980), and Heidmann & Changeux (1978)] and (2) the noncompetitive blockers which include the aminated local anesthetics (Weber & Changeux, 1974; Heidmann & Changeux, 1979; Krodel et al., 1979; Cohen et al., 1980a,b), perhydrohistrionicotoxin (Daly et al., 1971; Eldefrawi et al., 1980a), phencyclidine (Kloog et al., 1980; Albuquerque et al., 1980a,b; Eldefrawi et al., 1980b), amantadine (Tsai et al., 1978), and various detergents [for a review, see Changeux (1980)] cause a major decrease of the maximal response with little effect on the apparent dissociation constant. A number of physiological observations based on noise (Katz & Miledi, 1975), voltage jump (Adams, 1977), and single channel (Neher & Steinbach, 1978) analysis and the nonlinear current–voltage relationships noticed for the end plate current in the presence of these agents (Albuquerque et al., 1980a,b; Eldefrawi et al., 1980a,b) led

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[1] Abbreviations used: AcCh, acetylcholine; AcChR, acetylcholine receptor; 5AT, 5-azidotrimethisoquin; 5A[$^3$H]T, 5-azido[$^3$H]trimethisoquin; BrAcCh, bromoacetylcholine; α-[$^{125}$I]BGT, α-[$^{125}$I]bungarotoxin; DAPA, bis(3-azidopyridinium)-1,10-decane perchlorate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; MBTA, [4-(*N*-maleimido)benzyl]-trimethylammonium; MPTA, [4-(*N*-maleimido)phenyl]trimethylammonium; $NaDodSO_4$, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; TEA, tetraethylammonium ion; Temed, *N,N,N',N'*-tetramethylethylenediamine; TDF, trimethylammonium diazonium fluoroborate; Tris, tris(hydroxymethyl)aminomethane.

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