Classification and Localization of Hemoglobin Binding Sites on the Red Blood Cell Membrane†

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ABSTRACT: The binding of hemoglobin to the red cell membrane was characterized over a wide range of free hemoglobin concentrations by measurement of membrane bound and supernatant hemoglobin. Scatchard analysis of the binding data revealed two classes of sites: high affinity sites with a binding constant of 1×10^8 M^{-1} and 1.2×10^6 sites per cell, and a second, low affinity class of sites with a binding constant of 6×10^6 M^{-1} and 6×10^6 sites per cell. The low affinity sites are shown to be nonspecific and appear to be a result of the ghost preparation. The high affinity sites are shown to be specific to the inner surface of the red cell membrane. The competition of hemoglobin and glyceraldehyde-3-phosphate dehydrogenase suggests band III proteins as a potential binding site for hemoglobin.

In a preceding study using a fluorescent chromophore (Shaklai et al., 1977), we provided evidence for the specificity in the hemoglobin–red blood cell membrane interaction. A comparison between the amount of hemoglobin (Hb)[1] found in our study and other estimates available in the literature showed a considerable difference between the value we obtained and the two values reported previously (Fischer et al., 1975; Mitchell et al., 1965). In the following work, we studied quantitatively the interaction of Hb with the red cell membrane and extended binding studies to include higher Hb concentrations, in an effort to clarify the quantitative differences reported from different laboratories. We then investigated the sidedness of the interaction of hemoglobin with the membrane.

For these studies, we used two independent methods: (1) fluorescence quenching of a membrane probe by hemoglobin, and (2) measurement of hemoglobin binding to the membrane by separation of the cells from the supernatant.

The interpretation of our findings was facilitated by the large amount of information already available on the interaction of different proteins with the red blood cell membrane.

Materials and Methods

Rabbit muscle aldolase, rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, and horse heart cytochrome c were purchased from Sigma. Hemoglobin was prepared from the hemolysates of the ghost preparations. Bovine serum albumin (BSA) and neuraminidase were Calbiochem products. All other chemicals were analytical grade products of Baker, Mallinckrodt, and Fisher. 12-(9-Anthroyl)stearic acid (AS) was a product of Molecular Probes, Inc. (Roseville, Minn.).

Ghosts were prepared from freshly drawn blood, according to the method of Steck (Steck and Kant, 1974) and Hanahan (Hanahan and Ekholm, 1974), as described previously (Shaklai et al., 1977). The ghosts were stored up to 2 weeks in a concentrated suspension and were counted, using a Coulter counter Model A, equipped with a 100-μm aperture.

Sialic Acid Reduced Ghosts. Sialic acid reduced cells were prepared using neuraminidase at pH 6.5, following the procedure of Singer and Morrison (1974).

Spectrophotometric Determinations. A Beckman DU spectrophotometer was used for spectrophotometric determinations. Absorbance spectra were obtained with a Cary-14 spectrophotometer. Fluorimetric titrations were carried out, as described previously (Shaklai et al., 1977).

Proteins. All proteins used were dialyzed against the buffer before the titration into ghost suspensions. Hemoglobin concentration was measured using $E_{415}^{m} = 5.36 \times 10^5$ (van Assendelft, 1970). Cyt *c* concentration was measured using $E_{410}^{m} = 1.06 \times 10^5$ (Margoliash and Frowirt, 1959). Aldolase concentrations were measured with $E_{280}^{1\%} = 9.38 \times 10^6$ (Donovan, 1964) and for G3PD, $E_{280}^{1\%} = 10.0 \times 10^6$ (Fox and Dandliker, 1956).

Results

Binding Experiments Using Membrane Separation from Solution. The ghosts (prepared as described) were diluted with solutions of hemoglobin to the desired Hb concentrations. The number of ghosts in an aliquot from each tube was counted. The tubes were then equilibrated for approximately 15 min at 22 °C, followed by centrifugation at 38 000*g* for 45 min at the same temperature. Hemoglobin concentration of the supernatant was determined. The amount of bound hemoglobin was estimated from the difference between the total and free hemoglobin concentrations. The data were treated by drawing Scatchard plots and extrapolating to infinity for the free Hb concentration to find the maximal average number of Hb molecules per cell.

The binding data of Hb concentration at a range of 10^{-6} to 10^{-7} M showed two typical features: (1) the affinity seemed to be much lower than the calculated affinity from the fluorescence quenching measurements (Shaklai et al., 1977); and (2) the data varied with preparations and, even more, with time. We then extended the binding data to lower Hb concentrations. (At very low concentrations the optical path length used was 10 cm instead of the 1-cm path length used regularly for measurement of Hb concentration by optical absorption.) The Scatchard plots were not linear for the entire range studied.

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[1] Abbreviations used are: AS, 12-(9-anthroyl)stearic acid; RBC, red blood cell; Hb, hemoglobin; Cyt *c*, cytochrome *c*; BSA, bovine serum albumin; G3PD, glyceraldehyde-3-phosphate dehydrogenase; $NaDodSO_4$, sodium dodecyl sulfate; 5PBX, 5 mM phosphate buffer at pH = *X*.

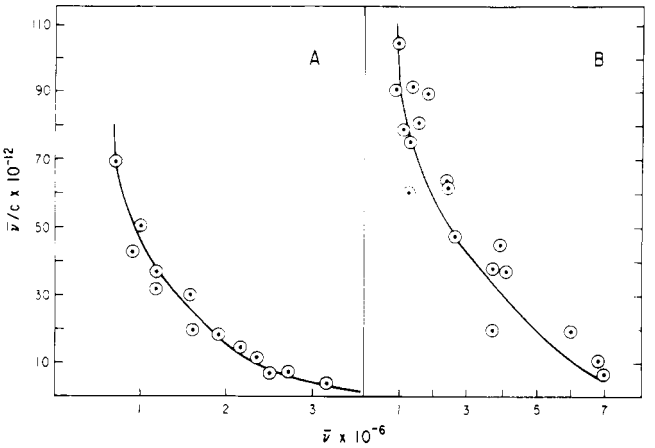

FIGURE 1: (A) The binding of hemoglobin by depleted ghosts at pH 6.2 at 5 mM phosphate buffer. $\bar{\nu}$ = the average number of molecules bound per cell. c = free molar Hb concentration. *The cells are one day old.* The curve is nonlinear. The points are experimental binding points from 3 different experiments. The curve is a theoretical binding curve of two independent classes of binding sites as explained in the text. (B) The binding of Hb by depleted ghosts at pH 6.2 at 5 mM phosphate buffer. $\bar{\nu}$ = the average number of Hb molecules bound per cell. c = free molar concentration of Hb (tetramer). The cells in this figure are *12 days old.* The points indicate the experimental data. The curve is the theoretical fit for two classes of sites as explained in the text.

In Figures 1A and 1B, two typical Scatchard plots are shown. These two experiments represent the lower and higher number of bound Hb molecules that were measured. Various numbers between these values were obtained in other experiments.

Scatchard plots resembling those of Figures 1A and 1B could result from negative cooperativity or from the presence of different independent binding sites. On the basis of data which will be presented below, the likelihood of independent binding sites appeared the more reasonable explanation for the observations.

In each experiment the data could fit within two classes of sites. The lines in Figures 1A and 1B represent the theoretical dependency of two independent classes of binding sites. In Figure 1A the theoretical curve fits two classes of binding sites: one class having a binding constant $K_1 = 1 \times 10^8\ M^{-1}$, and the number of sites per cell, $n = 1.4 \times 10^6$; the second class of binding sites with $K_2 = 5 \times 10^6\ M^{-1}$, $n_2 = 2.3 \times 10^6$. In Figure 1B the theoretical curve again fits two classes of binding sites: one with $K_1 = 1 \times 10^8\ M^{-1}$, $n_1 = 1.2 \times 10^6$ Hb molecules bound per cell; and the other class with $K_2 = 6 \times 10^{-6}\ M^{-1}$, $n_2 = 6.0 \times 10^6$ molecules bound per cell.

Number of Sites Calculated by Changing pH or Membrane Exterior Charge. The total amount of Hb bound at saturation was measured according to the following method. Ghosts from the same 4–7 day old preparation were equilibrated with different amounts of Hb to determine the binding capacity at pH 6.0 and pH 6.8. The affinity for Hb at pH 6.8 was much lower than the affinity at pH 6.0 and, thus, more Hb was needed to obtain saturation at pH 6.8. In contrast, the binding capacity at both pH values was found to be the same. In this particular preparation the binding capacity was 5.0×10^6 Hb molecules per cell.

In a different set of experiments the cells were treated with neuraminidase to cleave sialic acid residues from the outer surface of the membrane (Singer and Morrison, 1974). These experiments were designed to ascertain the localization of the binding sites. At pH 6.0 the binding capacity for neuraminidase treated cells was found to be 4.5×10^6 Hb molecules bound per cell. The small detectable change observed following treatment with neuraminidase was within experimental error.

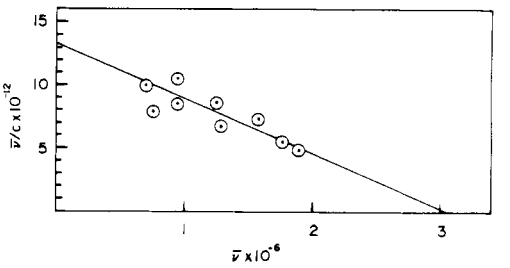

FIGURE 2: The binding of Hb by ghosts at pH 6.2 buffered by 5PB in the presence of 2×10^6 M G3PD. The data are plotted as a Scatchard plot. Temperature = 22 °C.

Binding of Hb as Shown by the Centrifugation Method in the Presence of Other Cytoplasmic Proteins. Aldolase and G3PD are two cytoplasmic enzymes that have been shown to bind specifically to the inner surface of the RBC membrane (Kant and Steck, 1973; Strapazon and Steck, 1976). The following experiments were done on the binding of Hb to the RBC membrane in the presence of these two enzymes. Hemoglobin binding was first measured by the centrifugation method. The addition of aldolase up to a concentration of 4×10^{-6} M did not change the observed Hb binding when compared with the data obtained in the absence of aldolase. Total concentrations of Hb from 1×10^{-8} M to 1×10^{-6} M were used.

On the other hand, in the presence of G3PD less Hb was bound than in the absence of G3PD. By the addition of G3PD up to a concentration of 6×10^{-6} M a fraction of hemoglobin was still bound even at lower total Hb concentrations (approximately 1×10^{-8} M). These data could be explained by the competition of G3PD for the two different binding sites of Hb, shown in Figure 1. In the presence of G3PD the observed affinity of membranes for Hb would be decreased so that less Hb would bind to both sites. In such a case the Scatchard plot would remain nonlinear but the two observed binding constants would be lower as compared with the constants in the absence of G3PD. Alternatively, G3PD might compete only for one class of binding sites while the other class retained its binding characteristics. The Scatchard plot would then become a straight line. A Scatchard plot of the quantitative data obtained in the presence of excess G3PD is shown in Figure 2; the Scatchard plot is linear. The binding constant calculated from the slope is $K = 4.5 \times 10^6\ M^{-1}$ with the number of sites equal to 3.0×10^6 per cell. These data were obtained using a 5-day old preparation of ghosts in which (as explained before) the total number of bound Hb molecules in the absence of G3PD was around 5×10^6 per cell. The linearity of the curve indicated that G3PD competed for only one kind of site. The measured binding constant and the number of sites indicated that only the high affinity sites of hemoglobin compete with G3PD for membrane binding.

Effects of Competing Proteins on the Quenching of AS by Hb and Cyt c. To complete comparisons of binding data derived by the two different methods, the effects of aldolase and G3PD in the fluorescence experiments were studied. While the addition of aldolase did not affect the Hb quenched ghosts (Figure 3a), the addition of salt following aldolase reversed the hemoglobin effect by releasing Hb from the membrane. In Figure 3b experiments on Hb quenched ghosts using G3PD are shown. The addition of G3PD reversed the Hb effect. The addition of salt after G3PD did not lead to additional changes since the Hb molecules were already expelled from the membrane. The reversibility of G3PD–hemoglobin competition was demonstrated in the behavior of ghosts preincubated with

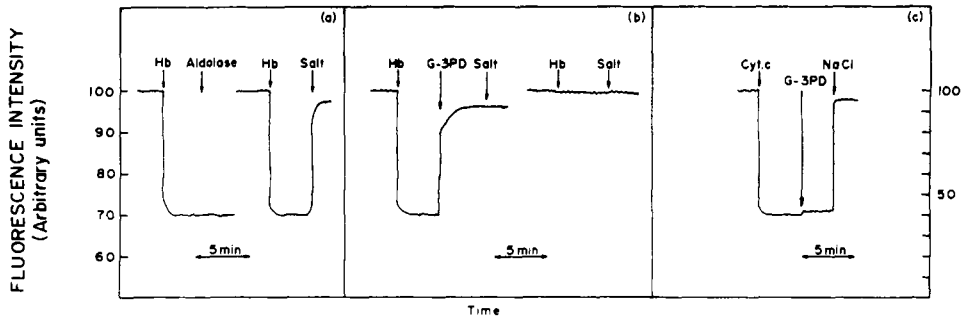

FIGURE 3: (a and b) The effect of aldolase and G3PD on the quenching of AS ghosts by hemoglobin. The cell concentrations: 5×10^6 per mL. [Hb] = 2×10^{-7} M; [aldolase] = 6×10^{-6} M; [G3PD] = 5×10^{-6} M; [NaCl] = 0.15 M. The proteins or salt were added in the order shown on the time scale. The solutions were stored at 22 °C in 5PB6. (c) The effect of G3PD on Cyt *c* quenched ghosts. [Cyt c] = 1×10^{-6} M; [G3PD] = 5×10^{-6} M; temperature = 22 °C in 5PB at pH 6.0. Cell concentration = 5×10^6 cells/mL. The right ordinate scale refers only to section c of the figure; the ordinate for sections a and b is on the left.

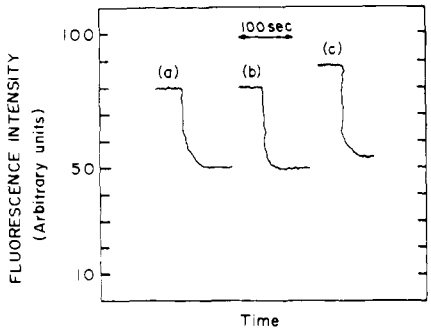

FIGURE 4: The reduction of fluorescence quantum yield by Hb. Cell concentration = 5×10^6 cells/mL. Hb = 2×10^{-7} M; pH 6.0 and 5 PB. Temperature = 22 °C. (a) Negative charges on the outer surface are partially neutralized by neuraminidase treatment. (b) Nontreated ghosts. (c) Ghosts in the presence of BSA; BSA = 1×10^{-6} M.

TABLE I: The Binding of Hemoglobin to the RBC Membrane in Different Preparations.[a]

Type of membrane and conditions of the binding expt	Av No. of Hb molecules bound per cell
Regular ghosts, pH 6.0	5.0×10^6
Sialic acid reduced, pH 6.0	4.5×10^6
Regular ghosts, pH 6.8	5.0×10^6

[a] The ghosts in the different experiments are from the same preparation and are 4–7 days old. The binding in each case is under low ionic strength conditions. 5PB is used to buffer the solutions in each case.

G3PD: such ghosts did not change in their fluorescence intensity as a result of the addition of Hb (left part of Figure 3b). The absence of any effect of the addition of salt indicated that no Hb was bound to the membranes.

In Figure 3c the effect of addition of G3PD to Cyt *c* quenched ghosts is shown. (Note the difference in quenching scale.) G3PD had a negligible effect on bound Cyt *c*. That Cyt *c* was still membrane bound following the addition of G3PD was demonstrated by the elevation of the fluorescence quantum yield by the addition of salt.

Fluorescence Measurements for Different Treatments. The binding to sialic acid reduced ghosts was also studied by fluorescence quenching measurements. In Figure 4 the data are shown. Sialic acid reduced ghosts and nonreduced ghosts behaved similarly. Bovine serum albumin has been shown to bind with high affinity to the outer surface of the RBC membrane (Rehfeld et al., 1975); the quenching of Hb appeared independent of the presence of BSA (Figure 4).

Binding of Hb Compared with Cyt c to Fresh Red Blood Cells. Cytochrome *c* has been used as a model for a protein which has been demonstrated to be nonspecifically bound to both the inner and outer surfaces of red cell membranes (Kant and Steck, 1973). For these experiments the red cells of fresh blood were washed as usual with isotonic phosphate buffer. The cells were then suspended in isotonic sucrose solution (0.265 M) including 5 mM phosphate buffer at pH 8.0 and were washed two or three times until no hemoglobin was observed in the supernatant. Aliquots of cell suspensions were then suspended in two sets of isotonic sucrose solutions: one at pH 8.0 and the other at pH 6.0. Hemoglobin was added and equilibrated with the cells for 15 min. In a set of controls no additional Hb was added to either the pH 8.0 or pH 6.0 solutions. The cells were then centrifuged and the Hb concentration of the supernatant was determined. Cell concentrations in these experiments were in the order of 10^8 to 10^9 cells per mL, which is higher than concentrations used in the binding of Hb to ghosts. These conditions permitted the measurement of very small quantities of bound Hb. Since Hb is not bound to the RBC membrane at pH 8.0 (Dodge et al., 1963; Shaklai et al., 1977), any Hb bound to the external face of the membrane at pH 6.0 should be seen in the difference between Hb concentration of the supernatant at pH 8.0 and that, at pH 6.0, or between Hb concentration at pH 6.0 and that of the controls.

The same procedures were carried out for Cyt *c*. In this case, the binding at pH 8.0 is about the same as at pH 6.0. The binding of Cyt *c* at pH 6.0 and pH 8.0 was therefore compared with the controls to which no Cyt *c* had been added. Concentrations of Cyt *c* were measured spectrophotometrically using a molar extinction coefficient of $E_m = 90 \times 10^{-3}$, at a wavelength of 405 nm (Margoliash and Frowirt, 1959). At this wavelength the ratio of the Hb and Cyt *c* extinction coefficients is minimal and thus the effect of hemolysis on absorbance is reduced. Our experiments indicated that, by immediate analysis of freshly drawn cells, the effects of hemolysis were minimal and could be ignored. The findings (at pH 6.0) are summarized for the two proteins in Table I. The data are compared with the Hb bound by ghosts under the same conditions of pH and ionic strength. While considerable amounts of Cyt *c* were attached to the outer surface of the membrane (about 30% of total binding of Cyt *c* to ghosts), Hb binding to the outer surface was negligible.

A Comparison of the Relative Quenching of Hb and Cyt c. A comparison of the quenching ability of cytochrome *c* and hemoglobin is shown in Figure 5. The effect of Hb and Cyt *c*

TABLE II: The Binding of Hemoglobin and Cyt c to Intact RBC and Open Ghosts.[a]

	Hemoglobin	Cytochrome *c*
RBC	$(0.0\text{-}0.2) \times 10^3$	$(7\text{-}10) \times 10^6$
Ghosts	$(3\text{-}7.5) \times 10^6$	$(27\text{-}30) \times 10^6$

[a] The data represent maximal number of molecules bound per single cell on the average.

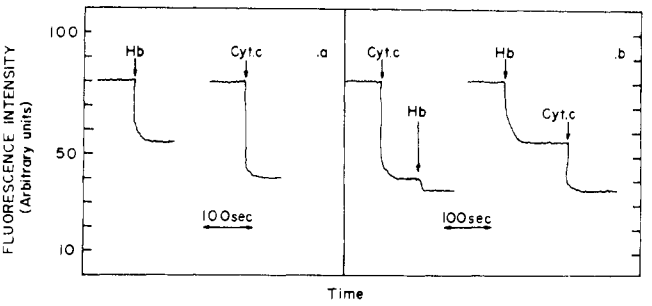

FIGURE 5: (a) The reduction of fluorescence quantum yield by Hb and Cyt c at pH 6.0, buffered by 5PB. [Hb] = 2×10^{-7} M; [Cyt *c*] = 1×10^{-6} M; 5×10^6 cells/mL. Temperature = 22 °C. (b) Fluorescence intensity changes created by both Hb and Cyt *c*. The conditions are the same as in (a). The proteins are added in the order shown on the time scale, each to give the same concentration as in (a).

on the same ghosts preparation is shown in Figure 5a: Cyt *c* reduced the quantum yield more than did Hb. (The maximal quenching effect is shown for each protein.) The data of Figure 5b indicate that a small additional change in quenching resulted from the addition of Hb to Cyt *c* quenched ghosts. (The concentration of the added proteins was the same.) Cytochrome *c* added following Hb quenching showed an additional significant quenching effect. The quantum yield was not dependent on the order in which the proteins were added.

The findings in Figure 5 can be explained on the basis of our data in the preceding paper as well as those of other workers (Vanderkooi et al., 1973). Cytochrome *c* has been shown capable of quenching AS molecules via energy transfer (Vanderkooi et al., 1973). R_0 for the AS-Cyt *c* pair is 39 Å (49 Å is reported by printing error). The heme is located at the center of Cyt *c* about 15 Å from the surface of the molecule (Dickerson et al., 1971). If AS is located 15 Å from the exterior surface (Waggoner and Stryer, 1973) and the membrane width is 65-75 Å (Weistein and McNutt, 1970; Peters, 1973), the donor-acceptor distance becomes 30 Å for cyt *c* molecules bound on the outside surface and about 70 Å for molecules bound to the inner surface of the membranes. Therefore the quenching effect is dominated by the cyt *c* molecules bound to the outer surface.

The case of hemoglobin is different. The R_0 (46 Å for each heme) is larger than the Cyt *c* R_0. The hemes are located at the surface of the molecule (Muirhead et al., 1967). As a result, the quenching potential of hemoglobin molecules is larger and Hb molecules bound on the inner surface are able to quench AS molecules. (The donor-acceptor distance for the close hemes is 60 Å for the inner bound molecules and 20 Å for molecules capable of binding to the exterior surface.) The fact that only 35% quenching occurs in the case of Hb indicates that there is probably little or no exterior binding. This conclusion is supported by the data of Figure 5b. While Cyt *c* has a lower average binding affinity (Shaklai, unpublished results; Kant and Steck, 1973), it can bring about extra quenching because of binding to the outer surface. Hemoglobin molecules compete with Cyt *c* molecules only on the inner surface. Thus when Hb was added following Cyt *c* only a small reduction in fluorescence of the Cyt *c* quenched ghosts was observed.

Discussion

The present studies support the following view of hemoglobin-RBC interaction. The binding consists of high affinity (which will be called class I) and low affinity (class II) binding sites. The data for class I sites are consistent within experimental error with the data from fluorescence quenching described in the previous study. The number of high affinity sites is constant and is independent of time or type of membrane preparation. However, the number of low affinity sites tends to increase with time. At different pH values the same number of sites is observed, although the observed affinity declines dramatically with pH. A specific number of sites appears to become available with elapsed time.

Location of Binding Sites. Red cell ghosts treated with neuraminidase, which would be expected to affect the electrostatic interaction of hemoglobin with the outer cell surfaces, exhibited a negligible reduction in the amount of hemoglobin bound as compared with untreated control ghosts. Neither high nor low affinity sites appeared to have been affected by neuraminidase treatment.

In fresh red cells, little or no interaction was observed between hemoglobin and the outer cell surface (the observed value of 0.2×10^3 molecules per cell is within the experimental error of these experiments, Table I). Yet control experiments with Cyt *c* demonstrated that a considerable amount of this protein is bound by the outer cell membrane.

The results shown in Figures 4 and 5 are consistent. Bovine serum albumin is bound to the outer surface and could not affect Hb quenching despite its reported higher affinity for the membrane (Rehfield et al., 1975).

Characterization of the Low Affinity Sites. As shown in the previous work (Shaklai et al., 1977) low affinity sites with a concentration of 6×10^{-8} M free Hb quenched the AS intensity by not more than 5% of total quenching of 35%. Under such free Hb concentrations, 0.2% of the total class II sites were saturated. On the average 0.5×10^6 Hb molecules are then bound per cell. (The exact values are time and preparation dependent.) The fact that these bound molecules created negligible effects on the fluorescence intensity indicates that their energy-transfer abilities are poorer than those of molecules bound to high affinity sites.

The ghosts were prepared under conditions of low ionic strength. Ghost preparations were stored in a concentrated suspension of about 1:1 volume of cells to buffer. After sedimenting the cells from a suspension stored for 2 weeks, a large quantity of membrane protein was demonstrated in the supernatant. Spectrin and actin are the peripheral proteins which are released from the RBC membrane at low ionic strengths (Fairbanks et al., 1971). It has been shown by $NaDodSO_4$ gel electrophoresis of the supernatant that 70% of the available amounts of these two proteins may be released from the membrane (Elgsaeter et al., 1976). In addition, another portion of the spectrin is known to be released by the procedures of ghost preparation (Marchesi et al., 1970). Altogether, at this stage the RBC membrane is almost entirely spectrin free.

The total number of Hb molecules that could be bound in these experiments is 7.5×10^6. This number is sufficient to totally cover the membrane face, the area of which is taken to be 147 μm^2 (Gul and Smith, 1974). Potential binding sites for such electrostatic binding are the negatively charged heads of the phosphatidylserine lipids (PS). Located at the interior membrane face, these face the inner side of the red cell mem-

brane. Phosphatidylserine lipid vesicles have been shown to be able to bind positively charged proteins (Papahadjopoulos and Kimelberg, 1973). It may be that these potential binding sites for Hb are uncovered by the release of spectrin.

The inability of G3PD or aldolase to compete with Hb for the low affinity sites is explained either by a total inability of those proteins to interact with such sites or by their interaction with much lower affinity. Such a low affinity interaction was reported for G3PD (McDaniel et al., 1974).

The present studies indicate that most of the weak sites are created by the procedures of preparing Hb-free membranes. However, the possibility remains that a small fraction of these sites is available under physiologic conditions.

Characterization of the High Affinity Sites. The data of Figure 4 show that G3PD is able to compete only for class I sites. The findings presented in Figure 3 add further evidence for the binding of G3PD to class I sites. Glyceraldehyde-3-phosphate dehydrogenase is capable of restoring the fluorescence intensity quenched by Hb high affinity sites only.[2] The failure of G3PD to change the degree of Cyt *c* quenching, although by its binding characteristics the G3PD is able to displace Cyt *c* from the inner surface of the membrane (Kant and Steck, 1973), provides additional evidence that Cyt *c* molecules bound to the inner surface of the membrane do not influence the intensity of fluorescence in the present studies.

The number of high affinity sites found in this work is the same as the number of band III polypeptides per ghost reported by other workers (Fairbanks et al., 1971). It has been shown that band III provides the site of G3PD binding on the inner surface of the membrane (Kant and Steck, 1973). The affinity of G3PD for band III sites is high [from $K = 10^7$ (Kant and Steck, 1973) to $K = 10^8$ (McDaniel et al., 1974)]. The competition between G3PD and hemoglobin for the hemoglobin high affinity sites ($K = 10^8$) reported in this work suggests that band III proteins may provide the natural site for hemoglobin binding as well. The failure of aldolase to compete with Hb for these sites is explained by its relatively weak binding to these sites (Solti and Friedrich, 1976).

While the weaker sites appear to result from preparation of ghosts, the high affinity sites of the red cell membrane for hemoglobin may have a significant physiological role in membrane structure or function.

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[2] One possible explanation for the low number of Hb binding sites observed in fluorescence experiments in comparison with the other technique might be an overlap in quenching volume of the extra bound Hb. However, if this were the case, no fluorescence change would be seen when part of the bound Hb was displaced by G3PD since 2×10^6 bound Hb molecules would be sufficient to cause total quenching. The findings in Figure 5 rule out such a possibility.

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