The Cytosol-Membrane Interface of Normal and Sickle Erythrocytes

EFFECT OF HEMOGLOBIN DEOXYGENATION AND SICKLING*

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The effect of deoxygenation on the amount of hemoglobin (Hb) proximal to the membranes of intact, density-fractionated normal (AA) and sickle (SS) red cells was studied by estimating resonance energy transfer efficiencies from fluorescent probes, 12-(9-anthryloxy)stearic acid or 2-(9-anthryloxy)stearic acid, in the outer lipid layer to cytoplasmic hemes. For each density fraction, heme concentrations at the cytosol-membrane interface (hc) were derived from the probe decay rates for ghosts and intact cells, measured by front-face fluorometry, and compared with mean cell heme concentrations (hM). With AA cells, hM/hc varied little with cell density; a 33% drop in hM on deoxygenation is attributable to organic phosphate binding to deoxy-Hb. With oxy-SS cells, hM/hc increased with cell density to twice the values for AA cells, but SS ghosts showed no evidence of increased probe quenching by membrane-bound Hb. On deoxygenation, hc for each SS density fraction fell (reversibly) to one-third the oxy value. The finding that deoxy-HbS withdraws from the membrane bilipid layer much more than deoxy-HbA is consistent with evidence for an increased net negative charge on deoxy-HbS polymers and/or the suggestion that the cytoskeleton, readily penetrated by monomeric Hb, presents a barrier to polymeric HbS. Membrane-associated HbS is therefore thought to play an unimportant role in polymerization.

In recent years, many investigators have sought evidence for a possible role of Hb-membrane interactions in the structure and function of normal and abnormal red cells. In SS cells, it has been suggested that such interactions may contribute to alterations in membrane protein and lipid organization (1), to oxidative membrane damage (2), and to the sickling process itself, i.e., the intracellular polymerization of deoxy-HbS (3). There is abundant evidence of increased accumulation of denatured Hb (in hemichrome form) in SS cells (4-6) which is retained in the membrane ghosts after hypotonic lysis. It is not clear, however, whether this denatured Hb is primarily free in the cytoplasm or is membrane-bound, and its location and distribution have yet to be determined. Native HbA and HbS have shown to bind to the inner surface of membrane ghosts, probably at a site on the transmembrane protein “band 3” and to glycoporphin, under conditions of low ionic strength and low pH (7, 8). Electron micrographs of freeze-etched membranes of deoxygenated sickle cells were described as showing polymer fibers fixed to the inner membrane surface (9), suggesting that polymerization of membrane-bound HbS might play an important role in the sickling process, but there is conflicting evidence as to whether the presence of red cell membranes promotes polymerization of deoxy-HbS. One report that polymerization was accelerated by the presence of intact or fragmented membranes (10) was not supported by a subsequent study, which concluded that neither the kinetics of polymerization, nor the equilibrium solubility of deoxy-HbS was altered by the presence of ghosts or various inside-out membrane vesicle preparations (11).

We have recently developed techniques to study the cytosol-membrane interface in intact cells by determining the efficiency of RET from fluorescent probes imbedded in the lipid bilayer to heme acceptors in the cytoplasm. With the aid of an analysis based on a simple geometric model, a series of 9-anthryloxyesteric acid probes were shown to be located in the outer leaflet of the phospholipid bilayer and estimates were obtained for the distances of their fluorophores from the cytosol-Hb boundary layer and for the concentration of this layer (12). Similar techniques have been employed to demonstrate that the concentration of the boundary layer increases as the cytoplasmic pH is lowered to pH 6, presumably because as the Hb becomes more positively charged it is more attracted to the membrane phospholipid head groups or to some other more specific binding sites (e.g. band 3) (13).

In the present experiments, we used RET to study the concentration of Hb in the cytosol boundary layer in normal erythrocytes and in density-fractionated sickle erythrocytes, both in the oxygenated and in the deoxygenated (sickled) state. We found that, upon deoxygenation of normal erythrocytes, the hemoglobin layer adjacent to the membrane was depleted by about 33%. In SS cells, upon sickling, the cytosol-hemoglobin boundary layer was more severely depleted of Hb, possibly because the kinetic equilibrium in the cytoplasm was shifted toward participation in the highly concentrated polymer phase, which thus appears to be largely withdrawn from the membrane. We also found that, compared to the MCHC, there was considerably more Hb in the boundary layer of oxygenated sickle cells than in that of normal cells, and that the ratio of boundary layer Hb concentration to MCHC was

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The abbreviations used are: Hb, hemoglobin; SS, sickle anemia cells; AA, normal red blood cells; RET, resonance energy transfer; MCHC, mean cell hemoglobin concentration; ISC, irreversibly sickled cells; PBS, phosphate-buffered saline; 12-AS, 12-(9-anthryloxy)stearic acid; 9-VA, 9-vinylanthracene.
By Mo M. Jula, A. H. A. S. S.

Materials and Methods

Biochemical Methods—Heparinized blood was obtained from sickle cell anemia (homozygous SS) and normal (AA) donors, with informed consent, and experiments were generally completed within 24 h. The erythrocytes were washed three times in isotonic buffer containing 10 mM Na phosphate, pH 7.4, 0.15 M NaCl (PBS) before being loaded on a discontinuous Stractan density gradient (14) with densities ranging from 1.095 to 1.118 for SS cells. After centrifugation at 20,000 rpm for 1 h at 4 °C, four red blood cell fractions were harvested and washed 3 times in PBS to remove Stractan.

With SS fractions, the top fraction (of five), which was found to contain nearly all of the reticulocytes and remaining leukocytes, was not included in this study. A drop of each washed fraction was put into 10% buffered formalin for morphological examination. 200 cells not included in this study. A drop of each washed fraction was put into 10% buffered formalin for morphological examination. 200 cells from each density fraction were examined with a Zeiss microscope equipped with Nomarsky optics at ×1000 magnification and were classified as normal discocytes, ISCs (elongated cells with a length/width ratio of 2 or more), and otherwise deformed cells. The last category comprised mostly dense, crumpled cells which did not meet the criterion for ISCs. The cells were then labeled with 12-AS (Molecular Probes, Junction City, OR) or with 9-VA by adding 30 μl of an ethanolic solution (5 mg/ml) per ml of packed cells suspended in PBS at a hematocrit of 20%, and incubating for 30 min at 4 °C. This procedure resulted in membranes containing approximately 104 probes/cell for each density fraction. The lifetime and yield of these probes depend weakly on their concentration in the membrane. The fluorescence yields were therefore always determined for labeled intact cells and for ghosts made from an aliquot of this sample, since the yield ratio is independent of probe concentration over a wide range.

Erythrocytes were lysed by adding 1 ml of packed cells to 50 ml of 5 mM Na phosphate buffer, pH 8 at 4 °C, the membranes were washed 3 times with this buffer, and ghost titers were determined spectrophotometrically after adding 1% sodium dodecyl sulfate as described previously (12). An aliquot of each fluorescent labeled cell fraction was washed three times with PBS and resuspended, the fluorescence decay rates of the fluorescent probes in the ghosts were determined, employing a 3-mm square cuvette.

MCHC of the density fractions was determined from the samples' hematocrits, measured on a Readacrit centrifuge (Clay-Adams, Inc.) and the hemoglobin concentrations, which were measured spectrophotometrically following conversion to hematin using Hycel 116-00 (Hycel, Inc.) containing 0.1% Triton X-100.

Data Acquisition and Analysis—In this study, as in a previous one (15), the fluorescence yields of the membrane probes in the presence and absence of acceptors were determined by measuring the normalized decay functions of the fluorophores for intact cells and ghosts, respectively. This method offers far greater accuracy than the direct determination of steady state fluorescence intensities because the samples being compared, suspensions of intact red blood cells and their ghosts, differ greatly in absorbance and scattering power. Their decay characteristics were measured by means of a light-efficient interference filter fluorometer using front face and right angle geometry for intact cells and ghosts, respectively (15). The excitation source was a high pressure hydrogen spark lamp (EYE Scientific, La Jolla, CA) and the monophoton detection system employed an Ortec 437 time-to-amplitude converter and a Tractor Northern pulse height analyzer (Model TN-1750). With 8 memories of 512 channels each to time the detection of emitted photons relative to the excitation pulse. The content of the memory was transmitted to a time-shared VAX computer for storage and eventual analysis. Before transmission, each decay profile obtained with samples of intact erythrocytes was corrected for the small (<10%) residual scattering contribution by subtracting from it the profile obtained from an equivalent sample of unlabeled erythrocytes, normalized according to the relative collection times of the samples. This subtraction, accomplished by a program built into the analyzer, was unnecessary for ghost samples. Ghost suspensions (corresponding to a hematocrit of 0.02%) were measured in 3-mm square quartz cuvettes, while red cell suspensions (25% hematocrit) were measured using either a front face cell (16) or a specially constructed anaerobic cell. The latter consists of a 2-mm path length quartz absorption cell, which contained the sample during measurement, joined to a spherical bulb (5 cm diameter) and stopcock through which gas could be flushed. While being flushed, the sample was tipped into the bulb to present a large surface area for gas equilibration. Red cell samples were deoxygenated by exposure to water-equilibrated argon for 30 min at room temperature and were reoxygenated with water-equilibrated oxygen in a similar manner. Following such deoxygenation, a drop of sickle red cell suspension was fixed in 10% formalin in isotonic PBS and was examined microscopically. Nearly all cells from each SS density fraction were found to have sickled, as judged by the presence of irregular sharp projections.

The fluorescence decay profiles were analyzed as described previously (12) by using the method of moments deconvolution procedure (17) to obtain the generally biexponential, decay function

\[ I(t) = \sum_{i} \alpha_i \exp(-t/T_i) \]

where \( \alpha_i \) is the normalized amplitude of the component with decay time \( T_i \). was then convoluted with the lamp profile obtained from a scattering experiment and was compared to the experimental fluorescence decay profile. As can be seen from Fig. 4, the fit between these two curves was excellent and the mean deviation of all channels for decay profiles, covering three decades of intensity and a time interval of 100 ns, was random with respect to time and less than 10%.

From the decay function in Equation 1, the fluorescence intensity, \( I \), was determined according to the following expression:

\[ I(t) = \int_0^t I(t) \, dt \]

where the subscripts c and g refer to intact cells and ghosts, respectively, and the average efficiency of energy transfer for donors embedded in the membrane is then given by

\[ T = 1 - I_c/I_g \]
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excited state (D*) and an acceptor (A),

\[ D^* + A \rightarrow D + A^* \]

takes place at a rate, \( k_2 \), which is proportional to the lifetime of \( D^* \) and inversely proportional to the sixth power of the \( DA \) separation, \( R \) (18). The transfer rate is therefore conveniently written as

\[ k_T = k_2 (R_0/R)^6 \]

(4)

where \( k \) is the donor decay rate in the absence of acceptors and \( R_0 \) is the so-called Förster distance. \( R_0 \) is proportional to the orientation factor \( x^2 \), which depends on the appropriately averaged relative orientations of the \( D \) and \( A \) transition dipoles, and to the energy overlap of the \( D \) emission and \( A \) absorption spectra. According to Equation 4, the donor’s fluorescence yield is halved when the \( DA \) separation is \( R_0 \) and drops rapidly with increasing \( R \). For \( R \) greater than \( \sim 2R_0 \), the rate of energy transfer is usually negligible compared to \( k \).

In order to interpret the quenching of membrane probes by hemoglobin inside the erythrocyte, a simple geometric model, for transverse RET (i.e. across the membrane) was employed (cf. Fig. 2). A semi-infinite continuum of acceptors is assumed to be bounded by a plane, whose normal distance from the donor is \( d \). It is then possible to derive an exact expression for the rate of RET from \( D \) to the acceptors continuum (12)

\[ k_T = \frac{\pi \rho}{6x^3} \]

where \( x \) is the dimensionless distance \( d/R_0 \), and \( \rho \) is the acceptor density per volume element \( R_0^3 \).

The quenching parameter for a particular \( DA \) pair, defined as

\[ q = \frac{k_T}{k} \]

may then be expressed in terms of the RET efficiency

\[ T = \frac{k_T}{(k + k_T)} \]

(7)

and the experimental parameters \( I_T \) and \( I_0 \) (cf. Equation 2)

\[ q = \frac{6}{x^3} \left( \frac{T}{1 - T} - \frac{6I_T}{xI_0} - 1 \right) \]

(8)

In the present experiments we employed 2-AS, 12-As, and 9-VA as donors. The Förster distances for transfer from them to oxyhemoglobin, assuming both donor and acceptor to have isotropic orientational distributions (\( K^2 = \frac{3}{5} \)), were calculated to be 33.4, 36.1, and 37.9 Å respectively (12). The \( R_0 \) for transfer from 12-AS and 9-VA to deoxyhemoglobin were 37.4 and 39.8 Å, or slightly larger than for any oxyhemoglobin, reflecting the somewhat greater overlap between the donor fluorescence and acceptor absorption spectra (cf. Fig. 1). The Förster distances for all \( DA \) pairs are therefore of the order of 35–40 Å and since the half-thickness of the phospholipid bilayer is of the same magnitude, it is clear that hemes beyond about 65 Å (the approximate size of a Hb molecule) to the membrane endosurface contribute negligibly to the quenching of the donor probes. If the heme concentration in this boundary layer is \( h_0 \) (millimolar), \( R_0 \) is expressed in Angstroms

\[ \rho = 10^{-3}N d^2 h_0 \]

(9)

where \( N \) is Avogadro’s number. If \( d \) is also in Angstroms, the ratio \( h_0/d^2 \) in terms of experimental parameters, is according to Equations 6 and 8

\[ \frac{h_0}{d^2} = \frac{3.17 \times 10^{-12} (I_T - 1)}{R_0^6} \]

(10)

It follows from Equation 10 that if \( h_0 \) remains unchanged while RET from two different membrane donors, 1 and 2, is measured,

\[ \frac{d(1)}{d(2)} = \left( \frac{I_T(2)/I_T(1) - 1}{I_T(1)/I_T(2) - 1} \right)^{7/6} \]

(11)

Equation 10 is presented graphically in Fig. 3. The two shaded regions correspond to heme concentrations greater than 35 mM, the maximum obtainable in osmotically shrunken cells and to values less than 30 Å, the expected minimum for 12-AS in the outer leaflet of the bilayer and a heme in hemoglobin adjacent to the membrane (12).

The association of Hb with the membrane is characterized by \( h_0 \) in this work. Its value is derived with the aid of a model in which the boundary of the acceptor domain is sharp (cf. Fig. 2), while in real erythrocytes the heme concentration is expected to rise with distance from the membrane until it levels off at \( h_0 \). Because of the inverse cubic dependence of \( q \) on \( x \) (cf. Equation 6), the hemes which are beyond a boundary layer of an approximate thickness of 65 Å, the diameter of a Hb molecule, make a negligible contribution to the quenching of a membrane donor whose Förster distance is 30–40 Å, as is the case here. The \( h_0 \) values derived here represent therefore an average value for the heme concentration in a Hb layer adjacent to the membrane’s endosurface. Without knowledge of how the Hb concentration rises towards the cell’s interior, a more precise definition of \( h_0 \) is not possible. In evaluating absolute values of \( h_0 \), \( d \) was assumed to have the values derived in a previous study (12). It should be noted that \( h_0 \) is proportional to the cube root of \( d \), so that an uncertainty in \( d \) will not affect the value of \( h_0 \) profoundly.

\( I_T \) and \( I_0 \) were measured with a reproducibility of less than ±2%, leading to an error of ±3% in \( T \). Systematic errors arising from uncertainties in the model employed in the analysis affect the absolute values of \( h_0 \) and \( d \) and are discussed elsewhere (12), but they do not contribute to errors in the ratios of \( h_0 \) or ratios of \( d \) (cf. Tables II and IV).

RESULTS

Before investigating the effect of sickling on the hemoglobin in the cytoplasm’s boundary layer, we studied the effect of
dependence of subtle change in the heme's absorption spectrum upon deox-
cytes. The data of Table I also show that the deoxygenation effect on
Hb in the boundary layer in deoxygenated normal erythro-
cytes is as good as that illustrated for ghosts. These decay profiles show
clearly that the donors suffer less quenching when Hb is deoxygen-
ad, even though $R_0$ is greater for RET from 12-AS to deoxy-Hb than to $O_2$-Hb.

<table>
<thead>
<tr>
<th>Donor and acceptor</th>
<th>$R_0$ (Å)</th>
<th>$I^<em>$ (%), $T^</em>$ (ns)</th>
<th>$q$</th>
<th>$h_b/d^3$</th>
<th>$h_{b(deo)}/h_{b(oxy)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>12-AS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxy-Hb</td>
<td>36.1</td>
<td>7.31</td>
<td>0.31</td>
<td>0.83</td>
<td>6.23</td>
</tr>
<tr>
<td>Deoxy-Hb</td>
<td>37.4</td>
<td>7.73</td>
<td>0.31</td>
<td>0.68</td>
<td>4.14</td>
</tr>
<tr>
<td>9-VA</td>
<td>36.1</td>
<td>7.27</td>
<td>0.31</td>
<td>0.85</td>
<td>6.34</td>
</tr>
<tr>
<td>Oxy-Hb</td>
<td>38.3</td>
<td>6.09</td>
<td>0.47</td>
<td>1.72</td>
<td>9.05</td>
</tr>
<tr>
<td>Deoxy-Hb</td>
<td>39.8</td>
<td>6.54</td>
<td>0.44</td>
<td>1.47</td>
<td>6.15</td>
</tr>
</tbody>
</table>

* Average values for duplicate determinations. Variance was less than 2%.
* $I^*(12-AS) = 10.49$ ns; $I^*(9-VA) = 11.58$ ns.
* In units of $10^{-4}$ mM/Å² (cf. Equation 10).

hemoglobin deoxygenation on the transverse RET in normal red blood cells. As can be seen from the decay curves of Fig. 4, we observed significantly less quenching of two membrane donors when Hb was deoxygenated even though $R_0$ is greater for transfer to deoxyhemoglobin than to oxyhemoglobin! From the fluorescence intensities $I$ and $I^*$, which are listed in Table I, and from Equation 10 using the appropriate $R_0$ values for oxy-
and deoxyhemoglobin Hb, we estimate that there is some 33% less Hb in the boundary layer in deoxygenated normal erythrocytes. The data of Table I also show that the deoxygenation effect on $h_b$ is reversible with reoxygenation and that its magnitude is the same when determined by two different donor probes (Fig. 5), whose distances from the cytosol hemoglobin have previously been estimated to be 31 Å for 12-AS and 26 Å for 9-VA (12). In view of the strong dependence of $h_b/d^3$ on $R_0$ (cf. Equation 10), and the relatively subtle change in the heme’s absorption spectrum upon deox-
generation, it is gratifying to note that the change in $h_b/d^3$ was independent of the particular probe employed for the RET experiments.

We separated SS cells into four density fractions and determined for each fraction its relative yield, MCHC, and the proportion of irreversibly sickled or otherwise deformed cells as measured by microscopic observation. Table II gives these morphological and other data along with the measured donor (12-AS) fluorescence intensities for labeled intact cells and ghosts made from them ($L$, $I_q$), as described under “Materials and Methods.”

Since $d$ is unchanged by the oxygenation of Hb, the $h_b/d^3$ results may be used to obtain the ratio of $h_b$ values for deoxy-
and oxyhemoglobin. This ratio is seen to be of the order of one-third for all density fractions (cf. Table II). This means that the hemoglobin concentration in the boundary layer is reduced by two-thirds upon sickling, which is considerably greater than the reduction of $h_b$ observed in the deoxygenation of normal cells (cf. Fig. 6).

It has been pointed out that absolute $h_b$ values may be derived from the transverse RET experiments by making use of the dependence of the quenching factor on osmotic strength and hence the volume of the cell (12). It was found that $q$ for 12-AS approaches 1.4 asymptotically at high osmotic strengths, the corresponding asymptotic value of $L_q/L$, being 1.73. If at maximal osmotic shrinkage (20) $h_b$ approaches 35 mM (cf. Table III) and $d$ is independent of osmolarity and MCHC, the $L_q/L$ measured under physiological conditions, 1.4, leads to an $h_b$ of about 17 mM or about 20% less than the average heme molarity in the cell corresponding to the normal MCHC (12). With $h_b$ determined in this manner, $d$ was obtained from Equation 10. It should be borne in mind, however, that the parameters which are derived directly from experiment, $q$ and $h_b/d^3$, as well as their ratios, e.g. $h_b(deo)/h_b(oxy)$, are probably more accurate that the absolute $h_b$ and $d$.
The heme molarity of the Hb boundary layer is plotted as a function of the MCHC for oxygenated SS cell (solid symbols) and for deoxygenated (sickled) SS cells (open symbols). The circles and squares refer to samples obtained from two different donors. The solid and open triangles give \( h_b \) values for oxygentated and deoxygenated normal (unfractionated) cells.

Values obtained by the method outlined above.

The experimental MCHC in grams/dl is readily converted into the heme molarity averaged over the volume of the cell:

\[
M_b = \left( \frac{40}{M_{Hb}} \right) M_{CHC}
\]  

(12)

where \( M_{Hb} \) is the molecular weight of hemoglobin. With \( M_b \) determined as outlined in the preceding paragraph, the ratio \( h_b/h_n \) can then be evaluated to obtain a comparison of the Hb concentrations in the boundary layer and its average over the volume of the cell. Values for \( h_b/h_n \) for oxygenated SS cell fractions are given in the last column of Table II and are plotted as a function of MCHC in Fig. 7, which also shows values of \( h_b/h_n \) for density-fractionated normal cells. It can be seen that even the least dense discocyte SS cell fractions have an \( h_b/h_n \) near unity, some 30% above the value for normal cells. This ratio rises with increasing MCHC to approximately 1.5 for the densest 10-20% of the SS cells, which consist primarily of ISCs and deformed cells.

The analysis presented so far assumes that the location of the 12-AS membrane probe is the same in the membrane of normal red blood cells and the SS-red blood cell factions. With this assumption, Equation 8 yields an \( h_b \) value of about 39 mM (~63 g/dl) for the densest oxygenated fraction of SS erythrocytes, which consists almost entirely of ISCs. Since this value exceeds the greatest heme concentration obtainable by osmotic shrinkage by about 10%, a value comparable to the experimental uncertainty, the assumption of \( d \) being the same for all density fractions was examined in some detail. It
The results presented here may be considered in the most general way by recalling that the efficiency of RET increases with acceptor concentration and decreases with distance. For the geometry of our model, these effects are lumped in the quenching factor \( q \) for which a 10% decrease in the normal distance \( d \) is equivalent to a 50% increase in acceptor density. While the model employed is an idealized one, this conclusion will be at least approximately correct as the membrane retains its usual structure and dimensions. This has been demonstrated for normal red cells by the dependence of the quenching parameter on the mean cell hemoglobin concentration, varied by the osmotic strength of the suspending media, for a series of membrane probes (12).

The variations in quenching measured in these experiments were interpreted in terms of varying concentration of hemoglobin proximal to the membrane \( (h_b) \). It is, however, conceivable that the membranes possess specific Hb-binding sites in sufficient numbers \((\sim 10^8/cell)\) and of sufficient strength to cause the hemoglobin molecules to be polarized. This would alter the effective average orientation factor for energy transfer from the membrane donors, an effect which would be indistinguishable from a change in \( h_b \). The magnitude of these putative orientational effects are discussed in the Appendix.

Absolute values for the distances and heme concentration are more prone to errors than is \( q \). The boundary layer Hb concentrations \( (h_b) \) listed in Table II are maximal estimates and represent averages over the membrane area. At the same time it can be argued that, for the measured RET efficiencies, the actual \( h_b \) values cannot be much smaller without having to decrease the donor-acceptor separation to a value which is inconsistent with the dimensions of the lipid bilayer. The error in \( h_b \), including experimental errors and uncertainties related to the model used in the analysis, is of the order of \( +10 \) and \(-30\%\) (12).

The experiments presented here permit us to draw the following conclusions. 1) There is an appreciable \((\approx 33\%)\) decrease in the hemoglobin concentration of the boundary layer when normal erythrocytes are deoxygenated. The magnitude of \( h_b \) is a measure of the attraction or repulsion between hemoglobin and the negatively charged endosurface of the membrane. It has been shown that, in the presence of organic phosphates (2,3-diphosphoglycerate or ATP), Hb becomes more negatively charged upon deoxygenation (21). The reversible reduction in \( h_b \) upon deoxygenation of normal cells is consistent with this observation.

2) While the quenching parameter, \( q \), for 12-AS in the lighter fractions of oxygenated SS cells was almost the same as for normal red cells, it was found that \( q \) increased by about a factor of 2 in the densest SS fractions. The ratio of estimated hemoglobin concentrations in the boundary layer to the average cell hemoglobin concentrations, \( h_b/h_b \), increased with the cells' MCHCs, particularly for the densest 15% of the sickle erythrocytes which contain most of the ISCs and deformed cells. If it is assumed that the probes' location in the membrane is the same for cells in all density fractions (cf. conclusion 4, below), two possible explanations for these
findings, not mutually exclusive, are: 1) a closer proximity or “binding” of native oxy-HbS than oxy-HbA to the cytosolic membrane surface, and/or 2) association of denatured HbS with the membrane, particularly in ISCs.

The first of these possibilities could result from the smaller net negative charge of HbS, due to its glutamic acid → valine amino acid substitution, and as a result of the lower intracellular pH of SS cells, particularly ISCs (22). In normal cells, the greater negative charge of HbA could result in electrostatic repulsion from the membrane. This notion is consistent with observations in this and other laboratories of the pH dependence of Hb association or binding to the membrane (7, 13, 23).

The second possibility is supported by the observations of increased retention of denatured Hb in SS cell ghosts, as noted earlier. Our finding that there was no difference in fluorescent intensity (Iₜ) of probes in SS and normal ghosts, however, indicates that the increased Hb concentration in the boundary layer of SS cells cannot be attributed to hemo-containing material which is irreversibly bound to the membrane. It is possible, however, that such membrane-associated material exists as aggregates whose quenching efficiency is greatly reduced compared to dispersed heme-containing material or is detached during hypotonic lysis, despite retention (trapping) within the ghosts. Observations of such SS ghosts by phase contrast microscopy showed many dense inclusion bodies which probably represent insoluble denatured Hb; while some of these appeared attached to the membrane, most moved about freely within the ghosts.

3) The findings that upon deoxygenation and sickling the quenching parameter q for each SS density fraction dropped by about a factor of 3, reversibly rising upon reoxygenation, indicate that the intracellular polymerization of deoxy-HbS produces a marked depletion of Hb in the cytosol boundary layer. This is contrary to the notion that polymerized HbS fibers become fixed to the internal membrane surface on sickling (9) and also does not support the suggestion of polymerization of membrane-bound Hb (3). Rather, it appears that, as the polymers form, Hb tends to withdraw from the membrane. This does not imply that the polymer fibers maintain much distance from membrane skeletal proteins. It is possible that the reduced quenching on deoxygenation represents withdrawal of Hb molecules which are otherwise intercalated within the cytoskeleton and adjacent to the endomembrane surface, as was demonstrated for normal red blood cells (12). It seems likely that individual Hb molecules, whose diameter is approximately 65 Å, can freely penetrate the spectrin network which extends from the membrane proper into the cytoplasm, while the polymerized HbS fibers cannot (cf. Fig. 2). Comparisons of transmembrane pH differences in oxygenated and deoxygenated SS and normal red cells, moreover, suggest that deoxy-HbS polymers exhibit a greater net negative charge than deoxy-HbA, which could contribute to repulsion from the membrane. While the present results do not exclude the possibility that widely separated membrane foci could initiate polymers which extend away from the membrane, they seem more consistent with the findings of Goldberg et al. (11) that the presence of membrane preparations has no effect on polymerization delay time or solubility of deoxy-HbS, and with the recent report of Coletta et al. (24) that the range of delay times of polymerization in single SS red cells correlates well with the delay times in solutions of deoxy-HbS with concentrations comparable to those in SS cells.

4) The modest decrease in the d(2-AS)/d(12-AS) ratio for the densest fraction of SS cells can be explained by 20% of the probes having been translocated to the inner bilayer leaflet, or by some other disturbance of the bilayer structure which permits invagination by hemoglobin. Evidence of alterations in the membrane structure of ISCs has recently been reported (1). More experiments of this type will be needed before the nature of this disturbance of the bilayer structure can be characterized.

In summary, these experiments have shown that the cytoplasmic hemoglobin in concentrations comparable to the MCHC is in close proximity to the phospholipid portions of the membrane of oxygenated sickle cells, as is the case for normal cells, that the cytoskeleton is permeable to hemoglobin molecules, and that the Hb concentration of the cytosol boundary layer is modulated by the oxygenation state of the hemoglobin. It is not clear whether this modulation has any physiological significance. Inasmuch as the boundary layer is severely depleted of Hb when SS cells sickle, it appears unlikely that the intrinsic components of the membrane, phospholipid or protein, play a part in the intracellular polymerization and gelation of HbS. The present data do not, however, exclude the possibility that HbS polymers are adjacent to the endo side of the cytoskeleton, at a distance from the donors in the lipid layer which exceeds the effective range of energy transfer (210 nm).

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APPENDIX: Orientational Effects in Transverse Resonance Energy Transfer

We wish to investigate the range of statistically averaged orientation factors, which obtain in energy transfer between an acceptor and a uniform distribution of donors confined to a plane.

Consider an acceptor, A, on the z axis, whose normal distance from the donor plane is unity and the donors within an annulus in the donor plane and centered on D, as indicated in Fig. 8. The DA separation vector is ρ and the orientation factor can be expressed as

\[ k^2 = (\sin \theta_D \sin \theta_A \cos \phi - 2 \cos \theta_D \cos \theta_A)^2 \]  

(A1)

where \( \theta_D \) and \( \theta_A \) are the angles between \( \rho \) and the \( D \) and \( A \) transition moments \( \rho \) and \( \phi \) is the angle between the \( (\rho, D) \) and \( (\rho, A) \) planes.

The principal orientations for \( D \) and \( A \) are along the triad formed by the \( z \) axis, the projection of \( \rho \) on the donor plane and the axis orthogonal to these two directions, and they are identified by the subscripts \( z, u, \) and \( v \) (cf. Fig. 8). Table V lists the \( \theta_D, \theta_A, \) and \( \phi \) values for each of the nine combinations of \( D \) and \( A \) orientations and the \( k^2(\theta) \) derived with the aid of Equation A1. The table also presents the \( k^2 \) values obtained by setting \( \theta = 0. \) Note that the average of the nine \( k^2(0) \) values is \( 5\% \), the correct average for a \( DA \) pair with isotropic orientational distributions.

We are now in a position to calculate the \( k^2 \) values for planar donor distributions. We integrate the appropriate \( k^2(\theta) \) for each annular ring over the \( D \) plane, the contribution of each ring (of radius \( \rho \sin \theta \) and width \( \rho \sec \theta d\theta \)) being weighted by \( \rho^{-4} \), to take into account the energy transfer rates from
TABLE V  
Orientation factors for an acceptor and a planar distribution of donors
The first third of the table (lines 1-9) gives the $\theta_D$, $\theta_A$, and $\phi$ values for the nine principal orientations of the $D$ and $A$ (linear) transition moments, as well as $x'(\theta)$, $x'(0)$ and the value of $x'(\theta)$ averaged over the donors plane, as discussed in the Appendix. The next nine lines give the orientation factors of linear $D$ transition moments and the principal planar $A$ transitions and lines 19-27 give the same orientation factors for the case of planar $D$ and $A$ transitions. Note that for each of the three cases considered the average of $x'(\theta)$, $x'(0)$, and $(\langle x' \rangle)$ is $\frac{1}{2}$.

<table>
<thead>
<tr>
<th>Orientation of</th>
<th>$D$</th>
<th>$A$</th>
<th>$\theta_D$</th>
<th>$\theta_A$</th>
<th>$\phi$</th>
<th>$x'(\theta)$</th>
<th>$x'(0)$</th>
<th>$\langle x'^2 \rangle$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$u$</td>
<td>$u$</td>
<td>$(\pi/2) - \theta$</td>
<td>$(\pi/2) - \theta$</td>
<td>0</td>
<td>$(3 \cos^2 \theta - 2)^2$</td>
<td>1</td>
<td>1/2</td>
<td></td>
</tr>
<tr>
<td>$u$</td>
<td>$v$</td>
<td>$(\pi/2) - \theta$</td>
<td>$\pi/2$</td>
<td>$\pi/2$</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>$u$</td>
<td>$z$</td>
<td>$(\pi/2) - \theta$</td>
<td>$\theta$</td>
<td>$\pi$</td>
<td>9 $\cos^2(1 - \cos^2 \theta)$</td>
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<td>3/2</td>
<td></td>
</tr>
<tr>
<td>$v$</td>
<td>$u$</td>
<td>$\pi/2$</td>
<td>$(\pi/2) - \theta$</td>
<td>$\pi/2$</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>$v$</td>
<td>$z$</td>
<td>$\pi/2$</td>
<td>$\theta$</td>
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<td>0</td>
<td>0</td>
<td>0</td>
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<td>$u$</td>
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<td>$(\pi/2) - \theta$</td>
<td>$\pi$</td>
<td>9 $\cos^2(1 - \cos^2 \theta)$</td>
<td>0</td>
<td>3/2</td>
<td></td>
</tr>
<tr>
<td>$z$</td>
<td>$v$</td>
<td>$\theta$</td>
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<td>$\pi/2$</td>
<td>0</td>
<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>$z$</td>
<td>$z$</td>
<td>$\theta$</td>
<td>$\theta$</td>
<td>0</td>
<td>$(1 - 3 \cos^2 \theta)^2$</td>
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</tr>
<tr>
<td>$u$</td>
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<td>$-$</td>
<td>$-$</td>
<td>$(3 \cos^2 \theta - 2)^2/2$</td>
<td>1/2</td>
<td>1/4</td>
<td></td>
</tr>
<tr>
<td>$u$</td>
<td>$vz$</td>
<td>$-$</td>
<td>$-$</td>
<td>$-$</td>
<td>$9 \cos^2(1 - \cos^2 \theta)/2$</td>
<td>0</td>
<td>3/4</td>
<td></td>
</tr>
<tr>
<td>$u$</td>
<td>$zu$</td>
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<td>$-$</td>
<td>$-$</td>
<td>$(4 - 3 \cos^2 \theta)/2$</td>
<td>1/2</td>
<td>1/2</td>
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</tr>
<tr>
<td>$v$</td>
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<td>$-$</td>
<td>$-$</td>
<td>$1/2$</td>
<td>1/2</td>
<td>1/2</td>
<td>1/2</td>
</tr>
<tr>
<td>$v$</td>
<td>$vz$</td>
<td>$-$</td>
<td>$-$</td>
<td>$-$</td>
<td>$1/2$</td>
<td>1/2</td>
<td>1/2</td>
<td>1/2</td>
</tr>
<tr>
<td>$v$</td>
<td>$zu$</td>
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<td>$-$</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>$z$</td>
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<td>$-$</td>
<td>$-$</td>
<td>$9 \cos^2(1 - \cos^2 \theta)/2$</td>
<td>0</td>
<td>3/4</td>
<td></td>
</tr>
<tr>
<td>$z$</td>
<td>$vz$</td>
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<td>$-$</td>
<td>$-$</td>
<td>$(3 \cos^2(1 - \cos^2 \theta))/2$</td>
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<td>3/4</td>
<td></td>
</tr>
<tr>
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<td>$zu$</td>
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<td>$-$</td>
<td>$-$</td>
<td>$(3 \cos^2 \theta)/2$</td>
<td>2</td>
<td>3/2</td>
<td></td>
</tr>
<tr>
<td>$uv$</td>
<td>$uv$</td>
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<td>$-$</td>
<td>$-$</td>
<td>$[5 - 3 \cos^2(4 - 3 \cos^2 \theta)]/4$</td>
<td>1/2</td>
<td>3/8</td>
<td></td>
</tr>
<tr>
<td>$uv$</td>
<td>$vz$</td>
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<td>$-$</td>
<td>$-$</td>
<td>$[1 + 9 \cos^2(1 - \cos^2 \theta)]/4$</td>
<td>1/4</td>
<td>5/8</td>
<td></td>
</tr>
<tr>
<td>$uv$</td>
<td>$zu$</td>
<td>$-$</td>
<td>$-$</td>
<td>$-$</td>
<td>$(4 - 3 \cos^2 \theta)/4$</td>
<td>1/4</td>
<td>1/2</td>
<td></td>
</tr>
<tr>
<td>$vz$</td>
<td>$uv$</td>
<td>$-$</td>
<td>$-$</td>
<td>$-$</td>
<td>$[1 + 9 \cos^2(1 - \cos^2 \theta)]/4$</td>
<td>1/4</td>
<td>5/8</td>
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<td>$vz$</td>
<td>$vz$</td>
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<td>$[2 - 3 \cos^2(2 - 3 \cos^2 \theta)]/4$</td>
<td>5/4</td>
<td>5/8</td>
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</tr>
<tr>
<td>$vz$</td>
<td>$zu$</td>
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<td>$-$</td>
<td>$-$</td>
<td>$3 \cos^2(\theta + 1)/4$</td>
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<td>3/4</td>
<td></td>
</tr>
<tr>
<td>$zu$</td>
<td>$uv$</td>
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<td>$-$</td>
<td>$-$</td>
<td>$(4 - 3 \cos^2 \theta)/4$</td>
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<td>1/2</td>
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</tr>
<tr>
<td>$zu$</td>
<td>$vz$</td>
<td>$-$</td>
<td>$-$</td>
<td>$-$</td>
<td>$(3 \cos^2 \theta + 1)/4$</td>
<td>1</td>
<td>3/4</td>
<td></td>
</tr>
<tr>
<td>$zu$</td>
<td>$zu$</td>
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<td>$-$</td>
<td>$-$</td>
<td>5/4</td>
<td>5/4</td>
<td>5/4</td>
<td>5/4</td>
</tr>
</tbody>
</table>

these donors. With the appropriate normalization, therefore,

$$\langle x'^2 \rangle = \frac{\int_{-\pi/2}^{\pi/2} \rho^{-4}(2\pi \sin \theta) x^2(\theta) \rho \sec \theta d\theta}{\int_{-\pi/2}^{\pi/2} \rho^{-4}(2\pi \sin \theta) \rho \sec \theta d\theta}$$  \hspace{1cm} (A2)

Since

$$\rho = \sec \theta$$  \hspace{1cm} (A3)

Equation A2 reduces to the integrable expressions

$$\langle x'^2 \rangle = 4 \int_{-\pi/2}^{\pi/2} \cos^2(\theta) x^2(\theta) d(\cos \theta)$$  \hspace{1cm} (A4)

Equation A4 is used to evaluate the values of $x'^2$ averaged over the $D$ plane which are listed in the first nine rows of Table V. Note that its average, for $D$ and $A$, each with isotropic orientational distributions, is $\frac{1}{2}$, the same as for an isotropic $D,A$ pair.

Table V also gives expressions for the $x'(\theta)$, $x'(0)$, and $\langle x'^2 \rangle$ for the case in which the donor's transition moment is linear and the acceptor's is planar (lines 9-18) and for the case of planar transitions for both donor and acceptor (lines 19-27). Note that, for each of the three cases considered, the average of the set of nine values of $x'(\theta)$, $x'(0)$, and $\langle x'^2 \rangle$ is equal to $\frac{1}{2}$, the "isotropic" orientation factor.

The transition moments of the $n$-AS donors used in the present experiment have been shown to lie preferentially in the membrane plane (26) and since all orientations of $D$ in that plane are equally likely, the effective $x'$ for any $A$ orientation is obtained by averaging the $x'$ values for $D$ aligned in the $u$ and $v$ directions. Similarly, the transition moment of the heme acceptor may be considered to be a superposition of two orthogonal dipoles in the heme plane. Thus, if the heme is parallel to the $(u,v)$ plane, its effective orientation for any $D$ orientation is the average of the $x'$ values for dipoles with orientations $u$ and $v$. In this way, one obtains $\langle x'^2 \rangle$ values (listed in Table V), averaged over all donors in the donor plane, with the subscripts before and after the comma defining the donor orientation and the heme plane, respectively.

$$\langle x'^2 \rangle_{u,v} = \frac{1}{2}$$
$$\langle x'^2 \rangle_{u,v} = \frac{1}{2}$$
$$\langle x'^2 \rangle_{u,v} = \frac{1}{2}$$  \hspace{1cm} (A5)
This discussion shows that, in general, measurable effect can be expected in transverse RET if the orientation of the acceptors (or donors) is polarized as a result of site-specific binding. Estimates of the magnitude of these effects for particular $D$ and $A$ orientations may be obtained from the $(k^2)$ values listed in Table V. For RET from n-AS donors in the membrane to the hemes of cytosol hemoglobin, the orientational effects are seen to be small, even for polarized hemoglobin molecules. It should be borne in mind, however, that there is no evidence that strong and site-specific binding of Hb to the erythrocyte membrane occurs under physiological conditions. Until such evidence is obtained, it appears preferable to interpret changes in RET efficiency as being due to $h_0$ modulation.

REFERENCES