Sucrose Density Gradient Analysis of Erythrocyte Membranes in Hemolytic Anemias

By Thomas P. Flynn, Gerhard J. Johnson, and David W. Allen

To investigate the membrane abnormalities that may play a pathophysiologic role in several hemolytic anemias we determined the density distribution on sucrose density gradients of human red blood cell (RBC) membranes from patients with these disorders, from normal controls, and from incubated normal RBC. We analyzed the fractions for membrane-adsorbed hemoglobin (Hb), globin, and nonglobin cytoplasmic proteins. The relationship between the cytoplasmic proteins absorbed on the membranes and the specific gravity (SG) of the membranes was linear. An increase in SG of the entire membrane population was seen in Hb C disease due to adsorbed Hb. Subpopulations of membranes with increased SG due to adsorption of nonglobin protein were evident in the membranes from two splenectomized patients with hemolytic glucose-6-

phosphate dehydrogenase (G6PD) variants. Dense membrane subpopulations found in RBC membranes from three splenectomized patients with Hb Köln were associated with adsorbed globin, while similar subpopulations in RBC membranes from three splenectomized patients with hereditary spherocytosis demonstrated increased SG due to adsorbed Hb. Splenectomized normals had no such abnormality in membrane density. Sucrose density gradients demonstrate that membrane bound cytoplasmic protein is characteristic of the RBC membranes in several hemolytic disorders. Additionally, gradients are useful for the isolation and further analysis of those subpopulations of RBC membranes with abnormal SG and exaggerated membrane protein abnormalities.

Although extensively studied with RBC incubated under a variety of conditions in vitro, increased RBC membrane binding of cytoplasmic proteins has not been systematically analyzed in hemolytic anemias. Weed and coworkers observed that when RBC were incubated aerobically in vitro without added glucose there was an increased adsorption of both Hb and non-Hb proteins to the membrane with increased viscosity, and decreased deformability and presumably survival. Sears et al. incubated RBC in phosphate buffered saline (PBS) and showed an increase in globin content of the membrane on polycrylamide gel electrophoresis in sodium dodecyl sulfate (PAGE SDS). Numerous workers have analyzed RBC membrane polypeptides with PAGE SDS in model systems and various hemolytic anemias. A distinctive pattern of changes was observed due, in part, to increased recovery of extrinsic membrane proteins and to membrane-adsorbed cytoplasmic proteins which included globin, and bands 4.2, 4.5 (catalase), 5, and 6 (glyceraldehyde-3-phosphate dehydrogenase). However, membranes lose extrinsic membrane proteins as well as Hb on the successive washes needed for isolation. Further, there has been no clear indication of whether changes in membrane composition are uniform or confined only to subpopulations.

Sucrose density gradients have been used to analyze Hb-binding of membranes and to separate membranes containing phenylhydrazine-induced Heinz bodies. In this work, we have isolated RBC membranes from incubated RBC and several hemolytic anemias on sucrose density gradients as a reproducible method of membrane preparation and used the polypeptide composition of the fractionated membranes to determine the relationship of membrane-bound protein to membrane density.

MATERIALS AND METHODS

Both normal healthy human subjects and patients with a variety of hemolytic anemias were studied. G6PD Long Prairie and G6PD "Tomah" are previously described variants with chronic hemolytic disease. Hereditary spherocytosis patients had typical peripheral blood morphology, and abnormal unincubated osmotic fragility curves. Hb C patients had the diagnosis confirmed by isoelectric focusing. Hb Köln had characteristic isoelectric focusing, heat instability, isopropanol instability, and increased oxygen affinity.

Blood was obtained aseptically, white blood cells and platelets were removed by centrifugation, and RBC washed with phosphate buffered saline pH 7.4, and membranes prepared as before, except that the hemolyzing and wash solutions (5 mM phosphate pH 8) contained 1 mM EDTA. RBC were quantitated by a Coulter counter and by microhematocrit. In certain experiments, washed RBC were aerobically incubated in 2 volumes of Dulbecco's PBS without glucose or calcium at pH 7.4, 37°C for 16–24 hr. In other experiments, washed RBC were incubated in 5 volumes of PBS with acetylsalicylhydrazine (1 mg/ml), 90 min, 37°C or in 9 volumes of Tris buffered saline pH 8 for 90 min, 37°C with 0.2–0.8 mM diamin. Membrane protein, Hb by the o-tolidine method, lipid phosphorus, PAGE SDS, and membrane polypeptides were analyzed as before. Membrane spectra were obtained on a Gilford 250 spectrophotometer.

Membrane SG was measured on 0.2–0.5 ml (1–3 mg protein) of

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Once washed membranes placed on 32 ml 15%-60% linear sucrose density gradients in 5 mM phosphate 1 mM EDTA, pH 8 and centrifuged 75,000 g for 16 hr at 5°C in a preparative ultracentrifuge. Density equilibrium was reached after 4 hr. One ml fractions were collected, absorbance was measured at 280 nm, and SG obtained initially by weighing 0.5 ml volumes and more recently equivalent results were obtained by using a temperature-controlled (20°C) Abbé 3L refractometer (Bausch and Lomb, Rochester, N.Y.). Measurement of the SG of 20 sucrose gradient samples both by weighing (to the nearest 0.1 mg, or 4 significant figures) and by the refractive index (to 5 significant figures) agreed within the nearest .001 SG units (4 significant figures) and are reported as such. Measurement with the refractometer did not require accuracy in filling a pipette, provided precise temperature control, and was considerably more rapid. The turbidity imparted by the membranes filling a pipette, provided precise temperature control, and was considerably more rapid. The turbidity imparted by the membranes filling a pipette, provided accurate temperature control, and was considerably more rapid.

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For the hemoglobin binding experiments membranes were prepared from normal controls and incubated with 0.2-20 μM Hb LA, S, and C in 5 mM phosphate at pH 6.2 for 15 min at 20°C prior to centrifugation and analysis. At this lower pH considerable Hb binding occurred (see below). In other experiments, such membranes were separated on sucrose gradients as above.

When at least three observations are available the results are presented as the mean ± SD. Standard statistical methods were used.  

**RESULTS**

Sucrose density gradients provided a method of isolating erythrocyte membranes in a reproducible procedure that could be used to define membrane-bound cytoplasmic protein. Proteins, such as Hb, that can be washed off the membranes were almost completely removed. Thus, when normal fresh control membranes were incubated with 1-10 μM Hb at pH 6.2 in 5 mM phosphate buffer, and then separated by centrifugation without changing the pH or Hb concentration they contained 43.3 ± 4.1% globin by PAGE SDS (which did not distinguish Hb and globin). If the same membranes with Hb bound at pH 6.2 were separated on sucrose gradients at pH 8 (see Materials and Methods), the globin content was reduced to 1.3% (n = 4). Thus, we defined adsorbed membrane protein as that additional membrane-bound protein not removed by sucrose gradient isolation of the membranes. Forty-eight sucrose gradients of 12 normal controls showed SG = 1.151 ± .003, Hb = 1.1 ± .4%.

Figure 1 demonstrates the relationship between the amount of adsorbed cytoplasmic protein to the SG of the RBC membranes from all the hemolytic anemias studied, in which such measurements are available. For simplicity, only two normal controls were plotted. The regression line drawn through the 26 points (open symbols) has an r value of 0.93 (p < .0005), indicating a highly significant positive correlation between adsorbed membrane protein and specific gravity.

The amount of adsorbed protein was calculated from the membrane composition as follows. The fraction f of adsorbed cytoplasmic protein was obtained by planimetry of the PAGE SDS analysis of the sucrose gradient fractions. The adsorbed cytoplasmic protein, x, (globin and nonglobin) was assumed added to the 500 fg of normal membrane protein per cell already present. Thus, f = x/(500 + x) or x = 500 f/(1 - f).

By utilizing model systems in which large amounts of membranes were available for replicate analysis we confirmed the validity of this relationship (Table 1). In these systems adsorbed cytoplasmic protein was...
Table 1. Relation of Membrane Specific Gravity to Adsorbed Cytoplasmic Protein (results ± SD, n = 4)

<table>
<thead>
<tr>
<th></th>
<th>Fresh Control</th>
<th>Aerobic Incubation (22 hr)</th>
<th>Diamide 0.8 mM Diamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total nonhemoglobin membrane protein per cell (fg)</td>
<td>495 ± 30</td>
<td>561 ± 11*</td>
<td>434 ± 57</td>
</tr>
<tr>
<td>Adsorbed nonhemoglobin cytoplasmic protein per cell (fg)</td>
<td>0</td>
<td>66</td>
<td>0</td>
</tr>
<tr>
<td>Adsorbed hemoglobin per cell (fg)</td>
<td>2.5</td>
<td>65</td>
<td>2</td>
</tr>
<tr>
<td>Total cytoplasmic protein adsorbed (fg)</td>
<td>2.5</td>
<td>131</td>
<td>2</td>
</tr>
<tr>
<td>Lipid phosphorus (fg/cell)</td>
<td>11.6 ± 1.8</td>
<td>7.8 ± 1.4</td>
<td>10.3 ± 1.4</td>
</tr>
<tr>
<td>Membrane specific gravity</td>
<td>1.150 ± .004</td>
<td>1.185 ± .008</td>
<td>1.148 ± .004</td>
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</table>

*Significantly different from appropriate control, p < 0.05.

measured by determining the difference in the protein content of membranes between incubated and control RBC. This permitted an independent estimate of adsorbed cytoplasmic protein (Fig. 1, solid symbols). An analysis of covariance showed no significant difference in the value of the adsorbed protein between the hemolytic anemias and the model systems after adjustment for the within groups regression coefficient. In Table 1, the approximately equal contribution of nonglobin and globin cytoplasmic proteins to the adsorbed protein is evident in the RBC incubated for 22 hr in PBS. With diamide treatment of the RBC, seven times more of the adsorbed cytoplasmic protein was nonglobin than globin (Table 1). Globin or nonglobin protein adsorbed on the membrane produced approximately equal increased in the specific gravity, since points representing the membranes of diamide incubated RBC (dense because of nonglobin cytoplasmic protein) and points of dense membranes from aerobically incubated RBC (containing both globin and nonglobin proteins) both lay near the regression line (Fig. 1).

Several experiments were performed to explore possible causes of increased membrane density. Fresh and aerobically-incubated normal RBC fractionated on dextran gradients into dense and light fractions, and then hemolyzed to yield membranes, demonstrated membranes of identical SG. Thus, under these conditions, denser RBC did not yield denser membranes. RBC dehydrated in 5% sodium chloride also did not result in dense membranes. Membranes from RBC incubated in sodium based Hank’s balanced salt solutions in the presence of the ionophore A 23187 were dense (SG = 1.176 ± .001, n = 4) from adsorbed globin and nonglobin cytoplasmic proteins. These changes were nearly prevented by blocking of the Gardos effect with substitution of potassium-based balanced salt solution in the incubation (membrane SG = 1.155 ± .001, n = 4). Incubation of normal RBC with increasing concentrations of diamide, a permeable oxidant that decreases RBC deformability and oxidizes sulfhydryls to disulfides, produced membranes with increasing density. Figure 2 demonstrates that the adsorption of nonglobin proteins (Table 1) was associated with a shift of the entire population of membranes towards increased SG. Unlike aerobic incubation, the diamide incubation did not significantly decrease lipid phosphorus (Table 1), indicating that membrane loss of lipids does not explain the increased membrane density with this oxidant stress. In Figure 2, the results are plotted as the cumulative percent of the total adsorbancy of the membranes, an integrated plot permitting better estimation of the SG corresponding to a median.
membrane fraction, and allowing better comparison of the density distribution under different conditions. Acetylphenylhydrazine treatment of normal RBC produced a single population of dense, Heinz-body containing globin-rich membranes (SG 1.161 ± .002, 15.1% globin, n = 4).

The background gained from the study of model systems was used to analyze the membranes of hemolytic anemias. The averaged density distribution of fresh membranes from 12 normal controls, and from 5 incubations of normal RBC for 22 hr, are compared with the density distribution of membranes from fresh RBC of a patient with G6PD “Tomah” in Fig. 3. This plot (percent maximum absorbancy) allows better analysis of distinct peaks or subpopulations, since any decrease in absorbancy between subpopulations is recorded as such. The membranes of the hemolytic G6PD mutant contain a dense subpopulation between SG of 1.165 and 1.19. When fractions from this subpopulation were analyzed by PAGE SDS, 2% of the total membrane protein was globin and 4% was nonglobin cytoplasmic proteins including bands between 4.2 and 5, bands 5, 6, 7 and a new band before globin (Fig. 4). Thus, this subpopulation of G6PD membranes like the entire population of membranes from aerobically incubated normal RBC owes its increased density to adsorbed proteins, both globin and nonglobin. Membranes from the fresh RBC of the hemolytic mutants G6PD “Tomah” and G6PD Long Prairie have been analyzed eight times with an average percent of total membranes of the subpopulation, SG > 1.165, of 9.0 ± 2.2% (fresh normal RBC membranes 1.6 ± 1.4% p < 0.005).

Figure 5 shows the density distribution of RBC membranes from a splenectomized patient with hereditary spherocytosis. A total of 6 such determinations on three splenectomized patients with this disease have been done. The average percent of the shoulder, SG > 1.165, is 8.3% ± 1.2%, significantly different from normal control membranes (0.2% ± .3%) (p < 0.005). Unlike the hemolytic G6PD mutants, the increased membrane adsorption of globin alone accounts for the increased density of this subpopulation. Three nonsplenectomized patients with hereditary spherocytosis (one the son of the patient shown) have no
Evidence of a dense subpopulation of cells. The dense membranes of hereditary spherocytosis have the spectrum of the adsorbed oxyhemoglobin. The presence of Hb is confirmed by the near equivalence of measurement of adsorbed Hb by the o-tolidine test\textsuperscript{14} and of adsorbed globin by PAGE SDS.\textsuperscript{5}

When the membranes from three splenectomized patients with Hb Köln were analyzed on sucrose gradients two membrane peaks were present. The denser of the two peaks (SG = 1.211 ± .003, \( n = 6 \)) contained Heinz bodies by phase microscopy. The second peak had a normal density (SG = 1.150 ± .005, \( n = 6 \)) and no Heinz bodies. The entire increase in density of the Heinz body-containing membranes was due to their content of globin. The color of the Heinz body-containing membranes was tan rather than pink and the spectrum was that of a ferrihemichrome as previously described.\textsuperscript{23} Globin assayed by PAGE SDS exceeded Hb measured by the o-tolidine method by a factor of 8–10, an observation which implies heme loss.\textsuperscript{24} Three affected relatives of the Hb Köln patients with intact spleens had no Heinz bodies and no dense subpopulations of membranes.

The RBC membranes of two hematologically normal individuals who had been splenectomized were studied and found to be not significantly different from normal (SG 1.151 ± .002, and SG 1.147 ± .001, \( n = 4 \)), with no subpopulations of dense membranes. One patient had his spleen removed after trauma (splenectomized normal, Fig. 5), and one during the course of gastric surgery. Both had no spleen by liver-spleen scan, and peripheral blood findings of the hyposplenic state.

RBC membranes from Hb C patients were uniformly increased in density (SG = 1.163 ± .006, \( n = 4 \)) (Fig. 6). This increase in density was due to the presence of adsorbed Hb C as demonstrated by PAGE SDS and isoelectric focusing of the hemoglobin from the membranes. The Hb C on the membranes had the spectrum of oxyhemoglobin, with maxima at 540 and 580 nm. Recombination experiments with normal membranes demonstrated that the increased adsorption of Hb C was a property of the Hb. With 0.2–20 \( \mu M \) Hb A or C and normal membranes on the average \( 2.3 \times 10^6 \) more Hb C molecules than Hb A molecules bound per RBC membrane (\( p < 0.005 \)).

\textbf{DISCUSSION}

Despite the multiple etiologies of the hemolytic anemias studied, this investigation demonstrates that, in each, increased membrane density is proportional to the adsorption on the RBC membranes of cytoplasmic proteins (Fig. 1). Thus, increased membrane density correlates with increased membrane binding of protein...
whether the protein is oxidized, denatured heme-poor globin as in Hb Köln, or oxyhemoglobin in Hb CC disease and hereditary spherocytosis, or non-Hb cytoplasmic protein as in G6PD mutants. We hypothesize that membrane adsorption of protein may indicate membrane damage in these hemolytic anemias and be expressed in increased membrane density as well as decreased RBC deformability, filtration, and survival.

In hemolytic G6PD mutants and in the two model systems of oxidant stress, aerobic incubation or diamide treatment of normal RBC, the increase in membrane protein is largely due to increased nonglobin cytoplasmic proteins adsorbed to the membrane. In all three there is decreased reduced glutathione, increased membrane disulfide-linked polypeptide aggregates, decreased deformability, and decreased survival in vivo. How the membrane sulfhydryl oxidation is related to increased membrane protein adsorption is not clear.

That sucrose gradients provide an efficient method of separating Heinz body-rich membranes has been observed before. Thus, it is not surprising that they are also successful in separating the Heinz body-containing membranes from RBC of splenectomized patients with Hb Köln. Although oxidant stress-induced damage occurs in membranes in Hb Köln disease (decreased glutathione, increased disulfide bonds and aggregates) any increase in membrane density due to adsorbed nonglobin proteins is negligible compared to the massive membrane adsorption of globin-containing Heinz bodies.

The observation that subpopulations of dense membranes are only evident in splenectomized patients with Hb Köln and hereditary spherocytosis and not in nonsplenectomized relatives with these diseases, suggests that RBC containing such dense membranes might be sequestered and destroyed in patients with intact spleens.

Increased binding of Hb C to membranes has been noted by other investigators. The affinity of membranes for Hb S, C and A increases with the increasing positive charge of the Hb. The fact that increased membrane-bound proteins characterize the other hemolytic anemias suggests that the membrane-binding of Hb C may play an etiologic role in that disease, as an explanation for previous observations concerning the rheologic properties of the Hb CC RBC.

In the model systems and hemolytic anemias studied here, the adsorbed membrane protein responsible for increased membrane protein density correlates closely with decreased RBC deformability and survival observed by many investigators. Thus, decreased RBC deformability is seen in aerobically incubated RBC and RBC treated with A 23187 and Ca in sodium-rich isotonic buffers but not in potassium-rich isotonic buffers. Decreased RBC deformability and survival are both seen in diamide treated dog RBC, in G6PD mutants with chronic hemolytic disease, hereditary spherocytosis, Hb Köln, and Hb CC disease. Sucrose density gradients are a sensitive method of detecting membranes with adsorbed cytoplasmic protein. It is not known whether this adsorbed protein directly inhibits membrane fluidity and decreases RBC deformability and survival, or whether the adsorption of cytoplasmic protein is an epiphenomenon reflecting other more significant changes in the configuration of membrane proteins. Despite this current limitation of our understanding, sucrose gradients detect small populations of membranes in which large changes have occurred, and provide a method for the isolation and further study of such membranes. That these subpopulations belong to effete, prehemolytic RBC seems likely, but remains to be proven.

REFERENCES

Sucrose density gradient analysis of erythrocyte membranes in hemolytic anemias

TP Flynn, GJ Johnson and DW Allen