Red Cells Shrink During Sickling

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Rapid transformation of discoid hemoglobin SS erythrocytes into grossly distorted sickled forms suggests alteration of surface/volume ratio. We, therefore, examined the effect of deoxygenation on red cell volume. $^{131}$I-labeled albumin was used as a marker of plasma volume in normal (Hb AA), sickle (Hb SS), and sickle-C (Hb SC) blood. Heparinized blood was incubated for 30 min at 37°C in a tonometer under deoxy (90% N₂, 10% CO₂) or oxy (90% O₂, 10% CO₂) atmospheres. Whole blood and plasma concentrations of radioactivity were determined. The hematocrits thus obtained with $^{131}$I-albumin showed that deoxygenation caused: (1) Hb AA cells to swell (mean MCV +4.0 cu μ ± 3.0, p < 0.01), as is predicted by the Bohr effect; (2) Hb SS cells to shrink (mean MCV -9.4 cu μ ± 2.9, p < 0.001); Hb SC cells to shrink (mean MCV -4.1 cu μ ± 0.8, p < 0.02). We also measured size distributions of glutaraldehyde-fixed oxy and deoxy cells using a Coulter Model B counter. This method confirmed the results of the radioalbumin experiments. These results demonstrate loss of cell volume during sickling of intact erythrocytes. Possible mechanisms for this loss include potassium efflux with obligatory water efflux, increased intracellular hydrostatic pressure from deformation of the cell membrane, or loss of intracellular osmotic activity of hemoglobin secondary to polymerization. This dehydration enables the sickled cell to develop more extreme shape distortion without membrane disruption, may facilitate continued hemoglobin polymerization, and may play a role in the formation of "irreversibly" sickled cells.

OXYGENATED HEMOGLOBIN SS (Hb SS) erythrocytes generally appear as symmetrical biconcave discs indistinguishable from normal (Hb AA) erythrocytes. Deoxygenation of Hb SS blood results in grossly distorted, spiculated sickled cells; these spicules may be several normal cell diameters in length. This striking morphologic change suggests that sickling may force alterations in cell surface-to-volume ratio. Such a change could result from increased surface area (i.e., stretching of the red cell membrane over spicules of polymerized hemoglobin), or from loss of intracellular volume.

Evidence derived from osmotic lysis¹,² and micropipette techniques³ suggests that red cell total surface area cannot be acutely increased. The alternative hypothesis, that Hb SS red cells might lose volume during sickling, was proposed first by Perutz and Mitchison on the basis of early hemoglobin solubility studies.⁴ Tosteson and colleagues⁵ measured cell water in oxy and deoxy Hb SS red cells, but did not demonstrate significant differences.

We have studied cell volume changes accompanying deoxygenation of red cells using two techniques. In the first, $^{131}$I-labeled albumin was used as a plasma marker, so that movement of water out of or into red cells could be...
measured as a function of the resulting dilution or concentration of the radioactive label. In the second method, red cells were fixed under oxygenated or deoxygenated atmospheres, and size distribution curves obtained from a standard electronic cell counter, the Coulter Model B. In addition, we measured plasma potassium and sodium levels resulting from deoxygenation of Hb SS and hemoglobin SC (Hb SC) red cells to determine the composition of the fluid involved in cell volume changes.

**MATERIALS AND METHODS**

Approximately 35 ml of blood from Hb AA, Hb SS, or Hb SC donors was drawn by venipuncture into heparinized tubes. Hematocrit was adjusted to approximately 50% by removal of plasma from the centrifuged specimen. Isotonic \( {^{131}}I \) radio-labeled serum albumin (Abbott Laboratories) was added to give final counting frequencies of about \( 10^6 \) cpm over background with a Packard Auto Gamma Spectrometer. Blood samples were divided into two aliquots, and were equilibrated for 30 min at \( 37°C \) in a Farhi tonometer, under an atmosphere of either 90% \( O_2 \)-10% \( CO_2 \) or 90% \( N_2 \)-10% \( CO_2 \). Samples were withdrawn anaerobically. Aliquots were taken for measurements of whole blood radioactivity, red cell counts (Coulter Model F/n), and hemoglobin concentration (Coulter hemoglobinometer), and for fixation in isotonic 1% glutaraldehyde. The remainder of the whole blood sample was centrifuged, maintaining the oxy or deoxy atmosphere. Plasma aliquots then were taken for measurements of radioactivity, plasma hemoglobin by the benzidine method, and sodium and potassium by flame photometry. Finally, the fixed cells were diluted in isotonic buffer and sized on a Coulter Model B, using the method described by the manufacturer.

Using the radioalbumin method, the change of cell volume (\( \Delta MCV \)) following deoxygenation was obtained using the expression:

\[
\Delta MCV = \left( 1 - \frac{\text{oxy plasma} \ [^{131}I]}{\text{deoxy plasma} \ [^{131}I]} \right) \times \frac{(1 - \text{Hct})}{\text{Hct}} \times MCV_{oxy}.
\]

The hematocrit (Hct) used in this expression was the true oxy hematocrit, derived from the concentrations of radioactivity in oxygenated whole blood and plasma. If no movement of water into or out of cells occurs, the plasma concentration of radiolabel is equal in both samples, and the first term becomes zero, as does the change in cell volume. The values for changes in the plasma volume were converted to the corresponding changes in red cell volume through multiplication by the plasmacrit/oxy hematocrit ratio. Multiplication by the mean cell volume (\( MCV_{oxy} \)) then converted the fractional change to change in cubic microns. To correct for hemolysis, we estimated the added volume of hemolysis (\( V_h \)) in ml per ml plasma as

\[
V_h = Hct \times H_b /H_f \quad \text{where} \quad H_b \text{ is plasma hemoglobin concentration and } H_f \text{ is whole blood hemoglobin concentration. We then multiplied both oxy and deoxy plasma radioactivity by the appropriate values of } (V_h + 1) \text{.}
\]

In our experiments, actual hemolysis never exceeded 2% of total cells, and \( V_h \leq 0.02 \).

**RESULTS**

Figure 1 depicts the results of radioalbumin experiments with Hb AA, Hb SC, and Hb SS blood. The mean volume changes caused by deoxygenation are shown by the shaded areas; the standard deviations are shown by the brackets. Deoxygenated Hb AA cells from nine normal donors swelled 4.0 cu \( \mu \pm 3.0 \) (\( p < 0.01 \)). In contrast, deoxygenated Hb SS cells from ten donors decreased in volume by 9.4 cu \( \mu \pm 2.9 \) (\( p < 0.001 \)). Deoxygenated cells from three Hb SC donors also decreased in volume by 4.1 cu \( \mu \pm 0.8 \) (\( p < 0.02 \)).

Figure 2 shows size distributions of oxy and deoxy fixed cells from one Hb AA and one Hb SS donor. The horizontal axes of these graphs are the electrical threshold values, which on the Coulter apparatus correspond directly to particle
size. Deoxygenation of Hb AA cells shifted the size distribution curve to larger threshold values, consistent with the results of the radioalbumin method. In contrast, deoxygenation of Hb SS cells shifted the curve to smaller values, indicating cell volume loss. These graphs are representative of cell size changes found with blood from all Hb AA and Hb SS donors.

Figure 3 shows the results of plasma sodium and potassium determinations, using blood from one Hb SS donor. Prior to incubation, plasma sodium was 137 mEq/liter and plasma potassium was 4.5 mEq/liter. At 30 min of incubation, plasma sodium decreased and potassium increased, but the total cation concentration of the now-expanded plasma volume remained essentially unchanged.

If the cells had lost only water, a dilution of plasma electrolytes would have resulted. The fact that deoxy plasma total cation concentration was nearly
identical to that of the corresponding oxy sample indicated that the fluid lost from red cells was nearly isotonic with respect to plasma. At 90 min of incubation the cation sum again was not significantly different in oxy and deoxy samples, though reciprocal changes in sodium and potassium may have resulted from ion for ion exchange.

In Fig. 4, the same measurements are shown for blood from one Hb SC donor. Preincubation values were normal, and there was no significant difference between the sums of plasma sodium and potassium in oxy and deoxy samples, suggesting that the fluid lost from Hb SC red cells was also approximately isotonic with respect to plasma.

DISCUSSION

We have demonstrated red cell volume changes resulting from deoxygenation of Hb AA, Hb SS, and Hb SC erythrocytes using two independent systems of measurement. The swelling of normal red cells is consistent with the so-called "isohydric shift" accompanying the Bohr effect and results from the fact that deoxy hemoglobin is a better proton acceptor than oxy hemoglobin. Net movement of protons and concomitant anions into the deoxygenated cell occurs, the intracellular osmotic activity is increased, and water enters the cell. Cells containing hemoglobins SS and SC should swell by the same mechanism, yet we have shown that such cells lose volume with deoxygenation. This paradoxical change in the volume of sickled cells might result from several factors. First, net cation efflux may be accompanied by obligatory water efflux. Our experi-
ments demonstrated that fluid loss from the sickled red cells at 30 and 90 min of deoxygenation was isotonic with plasma. We have not determined, however, whether cation loss is the cause of cell water efflux, the effect of it, or simply a concomitant process. If, for example, increased tension on the cell membrane were induced by spicules of polymerized hemoglobin, water might be forced from the cell by increased hydrostatic pressure. Such a cell would then be relatively hypertonic, and normal internal cation concentrations might be restored by transfer of an osmotically-equivalent amount of cation to the plasma. Alternatively, a deformed cell membrane, or one whose permeability was altered by attachment of polymerized hemoglobin, might leak potassium accompanied by chloride and water. Following osmotic restabilization, further potassium efflux would occur in exchange for sodium. Another possibility involves the osmotic effects of hemoglobin polymerization. Hemoglobin has an anomalously osmotic coefficient, so that, at physiologic concentrations (about 7 m\(M\)), its contribution to the intracellular osmotic load is approximately three times (about 20 m\(M\)) that predicted by simple particle numbers.\(^{10}\) Polymerization should lead to a decrease in this disproportionate osmotic effect of hemoglobin, and may play a role in cell volume loss.\(^4\)

Regardless of the mechanism of cell water and solute loss, its occurrence during the sickling process may have several important effects. By effectively increasing intracellular hemoglobin concentration, it may facilitate further hemoglobin polymerization. More extreme distortion of cell shape may occur without development of undue membrane tension which could cause cell lysis. In addition, because the deoxygenated sickle cell has a decreased cell volume, decreased cell water, increased hemoglobin concentration, and increased sodium and decreased potassium concentrations, it resembles the "irreversibly" sickled cell.\(^11\) Thus, cell shrinkage during sickling may play a role in the formation of "irreversibly" sickled cells.

REFERENCES

7. Instruction and Service Manual, Coulter Model B, Hialeah, Fla., Coulter Electronics
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