The Effect of Deoxygenation on Red Cell Density: Significance for the Pathophysiology of Sickle Cell Anemia

By Mary E. Fabry and Ronald L. Nagel

We have used continuous density gradients made with Percoll-Stractan mixtures to study the effect of deoxygenation on AA and SS cell density. Red cell density is directly correlated with the intracellular hemoglobin concentration and inversely correlated with red cell volume. We find that AA cells become less dense (swell) when deoxygenated under all conditions and that SS cells either become more dense or maintain constant density (shrink or maintain constant volume) when deoxygenated. The response of SS cells is affected by the pH, the amount of potassium present, and the characteristics of the cells (that is, reversibly or irreversibly sickled cells). Irreversibly sickled cells become denser when deoxygenated under all conditions. Below pH 7.25 and at physiologic potassium concentrations (5 meq/liter), reversibly sickled cells become denser when deoxygenated. Furthermore, they remain denser than the CO control cells even after CO is added and the gradient is recentrifuged. Therefore, the increase in density is not reversible. This is in contrast to AA cells where the decrease in density after deoxygenation is reversible. Above pH 7.4 and/or in buffer mixtures with high potassium (140 meq/liter), deoxygenated reversibly sickled cells maintain constant density. We conclude that two effects occur simultaneously in deoxy reversibly sickled cells. Above pH 7.4 and/or in high potassium media, the failure of reversibly sickled SS cells to become less dense may be the result of reduced osmotic contribution due to polymerization of HbS and/or an increase in negative charge secondary to polymerization. Below pH 7.25, at physiologic potassium levels, the increase in density may be due to a loss of potassium which is not compensated by a sodium influx in addition to the effects due to polymerization of HbS.

THE INCREASE in cell volume exhibited by AA cells following deoxygenation was described in the earliest literature pertinent to the study of the osmotic properties of red cells (Van Slyke et al.¹ and Henderson et al.²). The effect of deoxygenation on SS cell volume has been more controversial. The initial report of Tosteson and coworkers³ found no significant change in intracellular water with sickling; however, their method was also insensitive to water changes during the deoxygenation of normal red cells. In 1974, Masys et al.⁴ using labeled albumin as a marker of extracellular volume, reported that SS cells shrink during sickling. They also showed that AA cells, in contrast to SS cells, swell when they are deoxygenated. Levine et al.⁵ reported in 1975 that sickled cells show a dramatic increase in volume of 18%–35% upon deoxygenation, as measured by the size distribution of unfixed blood cells. Matters were not allowed to rest here, as Fales in 1978,⁶ using the concentration of plasma urea and proteins as the measure of total diffusible water and plasma volume, reported that SS red cells neither gain nor lose water when sickling. In an effort to resolve this controversy and advance our understanding of the factors controlling SS cell volume, we report our findings with two types of isopycnic continuous density gradients that allow the separation of red cells by density. If changes in the cell water content occur with deoxygenation in either SS or AA cells, they should be reflected in changes of the density of the red cell. The use of spontaneously formed continuous density gradients has three advantages: (1) it allows detection of small changes in cell density; (2) it allows the heterogeneity of SS cells and the variation from individual to individual to be clearly displayed; and (3) it allows deoxy conditions to be maintained that would be technically difficult to achieve with preformed gradients.

MATERIALS AND METHODS

The method of Vettore et al.⁷ was used to prepare Percoll (colloidal silica coated with polyvinylpyrrolidone; Pharmacia, Piscataway, N.J.) Renografin-76 (66% diatrizoate meglumine and 10% meglumine sodium; Squibb, Princeton, N.J.) gradients from a mixture of Percoll: Renografin-76: water:0.9% NaCl = 3.5:1.7:4:1:0.7. The resulting osmolarity was 320 ± 5 mosmole and the pH, before the addition of cells, was 7.50 ± 0.3. Sorvall 98 × 13 mm tubes were spun for 20 min at 17,000 rpm (35,000 g) and at 37°C in a Sorvall SS-34 rotor. Renografin increases the overall density of the spontaneously formed Percoll gradient to allow red cell separation; however, a new and superior gradient can be generated if the Renografin is replaced with Stractan (an arabino-galactan polysaccharide; St. Regis Paper Co., West Nyack, N.Y.). The Stractan stock solution was prepared as described by Corash et al.,⁸ with the exception that dilution with potassium phosphate buffer was omitted. Stractan has the advantage of a lower osmotic contribution (less than 200 mosmole versus 1500 mosmole from Renografin) and forms a more linear density gradient with Percoll.⁹ The mixture that was selected as producing the best separations for AA and SS cells contained Percoll:Stractan (density 1.207):water:buffer 1 = 3.5:3.0:2.8:0.7 (Table 1). The initial pH and osmolarity were 7.25 ± 0.05 and 280 ± 5 mosmole, respectively. This methodology makes it particularly...
Because of the higher viscosity of the Stractan solution, these gradients were spun for 55 min at 37°C to reach the optimum gradient shape. Because the Stractan stock solution contains glucose, the gradient mix described above has about 3 mM glucose, which is sufficient to maintain normal metabolism during centrifugation. The effect of elevated glucose was tested in some experiments as indicated by additional glucose in the buffer added to obtain constant osmolarity and Stractan concentration. It is therefore necessary to adjust both the amount of Stractan and the amount of concentrated buffer added to obtain constant osmolarity and Stractan content. The necessary calculations will be described in a more technical paper. Red cell aggregation does not occur in either Percoll-Renografin or Percoll-Stractan gradients; however, it was found to be a problem when Percoll was used by itself to form the gradient.

Densities were determined using three different methods of calibration. (1) Pharmacia density marker beads were used, but since the exact density of these beads is sensitive to the medium in which they are suspended, two other methods of calibration were used. (2) The refractive index was measured at 1-cm intervals. Since the refractive index is known as a function of density for both the Percoll (Pharmacia Technical Bulletin) and the Stractan (St. Regis Paper Company) solutions, the refractive index of the mixture as a function of density can be calculated. (3) The main determinant of the density of a red cell is its MCHC and the relation between MCHC and osmolality is approximately linear for the narrow range around 260–350 mosmole. We can therefore use the red cells themselves at different osmolarities to calibrate the gradient. The osmolality of the gradient mixture can be varied by adding small amounts of concentrated NaCl; and by using Pharmacia density marker beads, it can be shown that the density as a function of depth in the tube is independent of the osmolality of the gradient. The osmolality of the gradient mixture was measured using a Precision Systems Micro Osmometer (Precision Systems Inc., Sudbury, Mass.). For this range of osmolarities we can use Ponder’s equation

$$d_i = 1 + (d_{i0} - 1)(V/\rho)$$

(2)

from Ponder and Hoffman 13 where $d_{i0}$ is the isotonic cell density, we can calculate red cell density as a function of osmolality or MCHC. When oxy AA cells are added to gradients of different osmolarity, their final depth in the tube can be predicted by equations (1) and (2). This final method of calibration agrees with the previous two methods, as is illustrated in Fig. 1. Both of these gradients (Percoll-Renografin and Percoll-Stractan) have been used to study the effect of deoxygenation on red cell density. The red cells were stored in plasma in the cold and used 6 hr or less to avoid 2,3-DPG depletion. Red cells in heparinized plasma

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**Table 1. Buffer Mixtures Used in Percoll-Stractan Gradients**

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<td>Glucose</td>
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*Weights are in grams; salts were dissolved in 100 ml of water.
†Used for equilibration with CO2.
‡Used for equilibration with N2.
§Used for equilibration with CO2; increasing or decreasing the amount of bicarbonate added increases or decreases the final pH after CO2 equilibration.
We have studied a total of 10 different SS patients and 5 different AA controls under both CO and deoxy conditions in the presence and absence of CO₂. Repeated experiments with the same individual are reproducible and comparison of different individuals yields consistent results.

In all cases, the results reported are for matched pairs of samples. Within these pairs, both the gradient composition and the history of the cells is identical, with the exception that half of the cells have been equilibrated with carbon monoxide. Slight differences in gradient composition and shape occur between pairs. These differences can be compensated for by use of density marker beads. The density distribution on Percoll-Stractan gradients of oxygenated AA and SS cells in the absence of dithionite is indistinguishable from that of cells first equilibrated with CO and then exposed to dithionite at the concentrations used here.

Percoll-Renografin gradients are less linear than Percoll-Stractan gradients. Stractan has the additional advantage of being nontoxic to the red cell, cells tolerate prolonged exposure (24 hr) at room temperature to the Percoll-Stractan gradient mixture without change in cell volume. Otherwise the results obtained with both gradients are the same and the results of the Percoll-Renografin gradients will not be illustrated. Similarly, most of the experiments described were done in duplicate with gradient media equilibrated with 5.6% CO₂–94.4% N₂ and with media equilibrated with N₂ only. Both SS and AA cells equilibrated with physiologic levels of CO₂ are slightly more dense (equivalent in an increase in MCHC of about 1 g/dl) in both the oxy and deoxy state. The results illustrated are only for cells equilibrated with CO₂.

**Effect of Deoxygenation on AA Cells**

We find that on both gradients, deoxy AA cells are less dense than the paired control of carbon-monoxy AA cells (Fig. 2). This difference persists over a pH range of 6.8–7.6 and an osmolarity range of 260–370 mosmole. Deoxy AA cells are less dense than CO AA cells in gradients equilibrated with 5.6% CO₂–94.4% N₂ (Fig. 2), in gradients equilibrated with N₂ only (data not shown), and in gradients containing low (5 mM, physiologic, Fig. 2) and high (140 mM) levels of potassium (data not shown).

Another important feature of this phenomenon is that the density changes in AA cells are reversible. That is, if CO is subsequently added to both the CO and deoxy tubes and the contents of the tubes are mixed and then recentrifuged, the difference in density disappears. This observation also supports the conten-
Fig. 2. Typical results for matched pairs of samples at two different pHs. Percoll-Stractan equilibrated with 5.6% CO₂-94.4% N₂, centrifuged for 55 min at 37°C. 280 ± 10 mosmole. (A) AA and SS cells at pH 7.25; (B) AA and SS cells at pH 7.55.

Fig. 3. Matched pairs of SS cells in physiologic (5 mM) and high (140 mM) potassium medium. Percoll-Stractan, pH 7.20 ± .05, 275 ± 5 mosmole, equilibrated with 5.6% CO₂-94.4% N₂. (A) Centrifuged 55 min at 37°C; (B) CO added to all tubes, samples mixed, recentlyfuged 30 min at 37°C.

tion that the Percoll-Stractan gradient mix is relatively benign to the red cell.

Response of SS Cells to Deoxygenation

In contrast, deoxygenated SS cells either maintain constant density or become denser than the paired control of CO equilibrated cells. The effect observed depends on the pH (Fig. 2), the amount of potassium present (Fig. 3), and the subtype of cells studied [reversibly sickled cells (RSC or fraction 2) and the irreversibly sickled cells (ISC or fraction 4)]. ISC become denser when deoxygenated under all conditions. The behavior of the RSC is more complex. At physiologic concentrations of potassium (5 mM) the
RSC become denser at pH of 7.25 and below and show only small changes in density at pH of 7.4 and above. In high potassium medium (140 mM) (buffer 3, Table 1), the RSC maintain constant density even at pH 7.25 and below (Fig. 3). In high potassium medium, the RSC density was slightly decreased; however, since our major concern was the effect of deoxygenation, the origin of this effect was not explored.

The density changes in RSC at pH 7.25 and below are irreversible (Fig. 3); however, in high potassium medium, the RSC do not shrink and also maintain constant density after addition of CO and centrifugation, even at pH below 7.25 (Fig. 3). Above pH 7.4, at both physiologic and high potassium levels, RSCs maintain constant density (volume) after addition of CO.

To compare our results with previous observations by Roth et al., buffers 4 through 7 were used, which contain 0.1 mM ouabain. The results in the presence of ouabain at pH 6.9 and 7.6 and in low (5 mM) and high (140 mM) potassium were similar to those illustrated in Fig. 3. A large increase in deoxy cell density was observed in the pH 6.9 low potassium sample and none in the others. This is consistent with our previous results. The effect of high (10 mM) glucose was also tested, and it was found not to prevent the density increase below pH 7.25.

When deoxy SS cells become denser, as shown in Fig. 2, cells in the lightest density bands (at the top of the tube) move to higher densities. This could occur in one of two ways, either the lightest cells become more dense and move to the bottom of the tube, or all of the cells could become somewhat more dense. That the latter possibility is true for SS cells can be demonstrated by first fractionating the cells into four fractions by use of a gradient, and then subjecting the fractions to CO and deoxy conditions. We find that small shifts in density occur in all fractions (Fig. 4).

Using the calibration curve in Fig. 1, we can estimate the change in MCHC that occurs when AA cells are deoxygenated. This curve was redetermined for each batch of Stractan by use of density marker beads. The average density decreases 2 g/dl, which corresponds to a 6% increase in volume and compares favorably with the 5% increase in volume found by Masys and with the original reports by Van Slyke and Henderson of volume increases of 4% on partial deoxygenation.

The situation with SS cells is more complex, at pH 7.4 and above, the RSCs in both sodium and potassium buffers maintained nearly constant volume. As the pH is lowered from 7.4, subtle increases in density occur that can be demonstrated by densitometry (data not shown). At pH 7.25 and below, the change in SS cell density on deoxygenation varies from patient to patient but is readily apparent (Fig. 2). The average density change is about 2 g/dl (from 33 to 35 g/dl). In the ISC fraction, the change is more difficult to estimate because the shape of the gradient compresses the relation between depth and density at the bottom of the tube (Fig. 1). However, we can estimate an increase in the average value from about 44 to 48 g/dl. As the pH

Fig. 4. The cells of one SS patient were separated into four fractions (F1–F4) on a Percoll-Stractan gradient and then exposed to CO and deoxy conditions on a Percoll-Stractan gradient, pH 7.20 ± 0.05, 270 ± 5 mosmole, and 5.6% CO2-94.4% N2 at 37°C for 55 min.
is lowered from 7.25, the density changes become larger, and at pH 6.8, a very large density change is observed for the RSCs, where the average density increases from about 33 to 37 g/dl.

**Comparison With Previous Results**

Our finding that AA cells decrease in density (increase in volume) when deoxygenated, while SS cells increase in density (decrease in volume) when deoxygenated below pH 7.25 agrees with the earlier work on AA cells\(^1,2\) and with the work of Masys et al.\(^4\) on AA and SS cells. The latter used dilution of \(^{131}I\)-albumin to detect water movement. Tosteson et al.\(^3\) and Fales\(^6\) reported no change for either AA or SS cells following deoxygenation. In both of these cases, there is an apparent lack of sensitivity to the known effect of deoxygenation of AA cells. Furthermore, at the pH used by Tosteson et al. (pH 7.40 \(\pm\) 0.04) we find that SS (but not AA) cells maintain constant volume when deoxygenated. Fales does not report the pH for his work, and it is possible that the pH was 7.4 or higher. Levine et al.\(^5\) found a 4% increase in AA cell volume and a drastic increase in SS cell volume as measured by Coulter counter. Fales has an extensive discussion of the errors resulting from use of the Coulter counter to determine volumes. The study of Mohandas et al.\(^15\) on this subject is particularly relevant, since it emphasizes the systematic errors that arise when the Coulter counter is used to measure volume on high MCHC cells. The major sources of discrepancies appear to be: lack of sensitivity, failure to recognize the importance of pH, and systematic errors introduced by the Coulter counter.

**DISCUSSION**

The increase in density of deoxy SS cells observed under physiologic conditions should be contrasted to the clear decrease in density observed for AA cells under the same conditions. This implies that even though the increase in density of SS cells is relatively small, the phenomena that are responsible for the shrinkage are quite significant.

In the earliest literature\(^1,2\) the water shift into AA red cells following deoxygenation was attributed exclusively to the change in the “base binding properties” of the hemoglobin. In more modern usage, this would be called proton uptake by deoxyhemoglobin and be described in terms of the Bohr and Haldane effects. These effects are important to the analysis of cell volume because the conformation changes associated with the deoxygenation of hemoglobin affect its net ionization and in turn affect the number of counter ions needed to preserve electrical neutrality. The total number of particles present ultimately determines the osmotic pressure and water content of the cell. Deoxygenation-dependent factors that affect hemoglobin ionization can be broken down as follows. (1) The protonation of the groups responsible for the Bohr effect, which changes the pI of HbA from 7.05 to 7.12 after it is deoxygenated.\(^16\) (2) The binding of 2,3-DPG adds negative charges and reverses the previous effect. As demonstrated by Benesch and Rubin,\(^17\) DPG binding is accompanied by the uptake of protons in addition to the Bohr protons; however, the net effect is that the pI of the complex is still lowered with respect to that of oxyhemoglobin.\(^18\) (3) Finally, in the presence of CO\(_2\), carbamate formation occurs, which adds more negative charge to the hemoglobin. It is possible that some of this charge is also neutralized by additional proton uptake. The magnitude of the first effect, the change of pI in the absence of DPG or CO\(_2\), is known and would lead to an increase in deoxy cell volume. The magnitudes of the other two effects are less well known, particularly in the intact red cell, but both would tend to decrease cell volume and offset the first effect.

Recognition of the importance of 2,3-DPG as an allosteric effector of hemoglobin by Chanutin et al.\(^19\) and Benesch et al.\(^20\) and its role as an impermeant anion and its effect on the cell water content by Duhm\(^21,22\) leads to the suggestion that DPG binding to deoxy hemoglobin should play as important a role in the red cell volume changes associated with deoxygenation as that played by the hemoglobin itself. This is because 2,3-DPG contributes to electrical neutrality in the oxygenated red cell as a polyanion; however, when it is bound to hemoglobin, it must be replaced by a larger number of monovalent anions. The magnitude of the effect measured by Duhm suggests that if all DPG were bound to deoxy hemoglobin, the cell volume should increase by about 5%. This effect will offset the decrease in volume discussed above, which is the result of increased negative charge on hemoglobin due to 2,3-DPG binding; however, the two opposing effects will not necessarily result in a zero net effect.

Deoxy hemoglobin S has a similarly decreased pI at low Hb concentrations\(^19\) and binds DPG both in solution\(^23\) and in the polymer;\(^24\) therefore, the factors that would increase cell volume are present in SS cells as well. The cell will not swell or shrink unless water movement occurs. One or a combination of four factors could account for the difference observed. (1) A change in the number of osmotically active hemoglobin molecules will occur due to hemoglobin polymerization. (2) To this point we have assumed that the major determinant of red cell volume, the potassium and sodium content, is unaffected by deoxygenation. Tosteson\(^25\) has demonstrated that this is not true for the homozygous S cell. Water loss would occur if the
potassium leak observed by Tosteson were not completely compensated by sodium influx at all pHs. (3) A change in the amount the osmotically active water may occur. (4) A change in the number of counterions needed to maintain electrical neutrality may occur if deoxy HbS is differently charged in the polymer than it is in solution.

Following the arguments of Freedman and Hoffman, the concept of osmotically inactive water has no experimental support and hence is not useful in describing the changes observed. Furthermore, although the density of the polymer is high, there is no evidence that the water of hydration associated with the hemoglobin is decreased in the polymer.

Clearly, there are two separate effects of deoxygenation on RSCs. The first effect, which is readily apparent at pHs below 7.25, can be prevented by high external potassium and is irreversible when CO is added to the deoxy cells (Fig. 3). These observations suggest that the potassium efflux observed by Tosteson and others occurs under our conditions and that it is indeed balanced by a sodium influx at pH 7.4 and above. At pHs below 7.25 and at physiologic potassium levels (5 mM), an incompletely compensated efflux of potassium may occur, which results in water loss and an increase in cell density. The second and more subtle effect occurs at all pHs and is insensitive to the presence of potassium. It prevents a decrease in density analogous to that observed in AA cells when they are deoxygenated.

The potassium-insensitive effect may be more directly related to HbS polymerization, which could affect red cell water content in one of two ways. (1) Polymerization reduces the number of osmotically active particles by removing some hemoglobin from the solution phase. The total hemoglobin concentration is 5 mM, and if we assume an empirical activity coefficient such as the factor of 3 used by Hoffman, the impact of hemoglobin polymerization on cell water content via the Donnan equilibrium is expected to be small but potentially detectable. (2) The number of counterions required to balance the hemoglobin charge in the solution and polymer phase may be different. If the polymer has an increased net negative charge, the chloride and hence the cation and water content of the cell would be reduced.

Interestingly, this property of sickled cells must be considered in the pathophysiology of the disease. Within the physiologic pH range found in plasma (pH 7.2–7.4), both effects can occur. The effects observed at pHs below 7.25 are particularly serious, since they result in increased MCHC. HbS polymerization is highly dependent on Hb concentration. Hofrichter et al. have calculated that, after nonideality is taken into consideration, the rate of polymerization varies with the tenth power of the initial concentration of HbS. An increase in the intraerythrocytic concentration of HbS will accelerate the kinetics of gelation and the sickling process. We have shown that volume change on deoxygenation is exhibited to a different degree by different classes of cells present in the sickler’s blood—an effect that is further magnified by low pH or high osmolarity, situations that may be encountered in vivo. This is another reason by which acidosis in the sickler may have particularly serious consequences. As sicklers vary in the distribution of cells among these classes, the heterogeneity may contribute to the diversity in clinical severity.

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