Water Distribution in Blood During Sickling of Erythrocytes

By Frank W. Fales

Plasma urea and protein determinations proved suitable for measuring changes in total diffusible water and plasma volume in whole blood. Deoxygenation by saturation with carbon dioxide at 25°C caused no change in plasma urea, but a significant increase in plasma protein concentration was induced with both normal and sickle-cell (HbSS) blood. Thus in HbSS blood there was no binding or trapping of water as a result of sickling and there was a normal influx of water into the cells (Bohr effect) despite the polymerization of the hemoglobin molecules with sickling. Consistent with this observation was the finding that the deoxygenation induced a similar increase in concentration of the plasma cations, sodium plus potassium. HbSS erythrocytes neither lost nor gained water under the more physiologic conditions of deoxygenation with a 95% nitrogen, 5% carbon dioxide gas mixture.

PERUTZ AND MITCHINSON\(^1\) in 1950 suggested the following mechanism for sickling: With deoxygenation the S-hemoglobin crystallized inside the red cell, and the change in shape was forced upon the cell partly by the habit of the crystals and partly by the loss of water from the cell that would be expected to accompany the crystallization. Since hemoglobin crystals have a much lower water content than the intracellular fluid, they theorized that 15\(^\%\) - 20\(^\%\) of the intracellular water would be released and would diffuse out of the cell due to the loss of osmotic pressure, causing the cell membrane to collapse. However, that mechanism of sickling has not been confirmed.

Rather than true crystallization with deoxygenation, it has appeared that there is a sol to gel transformation followed by organization of the gel into double-refracting concentrated masses of rodlike particles of hemoglobin with parallel alignment.\(^2\) These fibers appear to be composed of microfilaments of six or eight strands arranged in helical tubes.\(^3^4\)

There has been disagreement concerning the loss of water. Tosteson and co-workers\(^5^6\) found that sickling caused no significant loss of water from the red cells. Harris\(^7\) attributed the retention of water to a high water content of the fibers. On the other hand, Masys et al.\(^7\) reported that sickling induced a very substantial shrinkage of the cells, but Levine et al.\(^8\) reported that sickling caused a dramatic 18\(^\%\) - 35\(^\%\) increase in cell volume.

Herein is reported a study of the changes in the water distribution brought about by deoxygenation of blood from homozygous sickle-cell anemia patients (HbSS blood). The study was undertaken to answer several questions. First, is a portion of the red cell water in some way sequestered or bound during sickling so it is no longer in osmotic equilibrium with the extracellular water? Some
such occurrence would seem required if indeed there is retention of water during the polymerization of hemoglobin. Second, is there an actual uptake of water under conditions that induce a very strong Bohr effect? If this is the case, then the membrane is normally permeable and there is a normal responsiveness of the sickled cells to changes in intracellular osmotic pressure. Third, does the sickling brought about by deoxygenation at normal pH and tonicity alter the water distribution between red cells and plasma? There has been disagreement on this point, and it would seem to be of considerable importance in understanding sickling in vivo.

The data presented indicated that the deoxygenation in vitro of HbSS blood with carbon dioxide elicits a normal response in regard to water distribution in that there was no indication of sequestering of intracellular water, and indeed the deoxygenation with CO₂ induced a transfer of water from the plasma into the cells, as was the case with normal blood. Furthermore, there was no significant loss or gain of water when sickling was induced by deaeration with 95⁰ N₂ and 5⁰ CO₂.

MATERIALS AND METHODS

Experimental Design

Tosteson et al.²⁶ determined the water content of erythrocytes by centrifuging and measuring the loss of dry weight of the packed cells. They corrected for the plasma trapped in the packed cells with radioactive tracers restricted to the extracellular compartment of the blood. Trapped medium has been an especially difficult problem with deoxygenated sickled blood. With the irregularly shaped cells, the volume of trapped plasma is about ten times that of normal blood.⁶

A rather simple method for determining changes in distribution of blood water that avoids the problem of trapped plasma is to measure the changes in concentration of a material restricted to the extracellular fluid. If the concentration of the material in the plasma increases, there is an influx of water from the plasma into the cells; if the concentration decreases, there is an efflux of water from the cells. Similarly, changes in the total water that is in osmotic equilibrium can be determined by measuring the plasma concentration of a material with unrestricted distribution. Fortunately, materials having each of these distributions are present naturally in blood. Plasma protein is restricted to the extracellular medium, while highly invasive urea is distributed throughout the total water.

To test the effectiveness of plasma protein and plasma urea determinations in estimating extracellular and total blood water, analyses were made on undiluted blood as well as on blood diluted with isotonic saline containing 0.15 moles NaCl as well as 4.5 millimoles glucose per liter. The glucose was included in the diluent so as not to impair red cell metabolism by dilution of substrate. From thoroughly mixed blood containing ethylenediaminetetraacetate (EDTA) as anticoagulant, five 1.00-ml samples were transferred to test tubes. To the separate tubes the following volumes of isotonic diluent were added: 0, 0.10, 0.20, 0.30, and 0.50 ml. The tubes were stoppered and the bloods mixed for 30 min at room temperature (about 25°C) on a slowly turning laboratory rotator. After centrifugation, urea was determined by the direct method of Wybenga et al.⁹ using 20-μl plasma samples and the protein by a microaeroplas of the Wechselsbaum⁸ method. The biuret reagent was diluted with an equal volume of 0.33 moles/liter NaOH solution just before use, and 50 μl of plasma was rinsed into 3 ml of the diluted reagent. The relationship between the original pool volume $V_p$, the initial plasma concentration $C_0$, and the concentration $C_4$ after addition of a volume $V_d$ is given by

$$\frac{C_0}{C_4} = \frac{V_p + V_d}{V_p} = \frac{V_d}{V_p} + 1.$$ 

Plotting the ratio of the initial to the diluted concentration ($C_0/C_4$) against the final volume of added diluent ($V_d$) gave a straight line in which the negative intercept of the $X$ axis was the graphic estimate of the original pool volume ($V_p$).
WATER DISTRIBUTION DURING SICKLING

The data averaged for four normal blood samples are shown in Fig. 1. The pool volumes calculated from the regression formulas of the lines were 0.901 ml for the urea and 0.539 ml for the protein pool volume of 1.00 ml of blood. These volumes are given in terms of milliliters of plasma because all of the analyses were made on the basis of concentration units per unit volume of extracellular fluid. The protein pool is a measure of the plasma pool; \((1 - 0.539) \times 100 = 46.1\%\) was an independent measure of the average hematocrit of the four samples. The determined average hematocrit uncorrected for trapped plasma was 46.8\%. The water content of the two pools could be estimated by multiplying each by 0.93, the normal volume fraction of water in plasma.\(^1\)

Thus the total water per ml blood was 0.84 ml, the plasma water 0.50 ml, and the difference, the intracellular red cell water, 0.34 ml. With the normal volume of 0.71 ml water/ml red cells\(^2\) the water content of the cells calculated from the hematocrit was 0.33 ml. Within experimental error, then, the total water was in equilibrium with the plasma water in normal blood. This conclusion agrees with that of Gary-Bobo and Solomon,\(^3\) who found ethanol, 2-propanol, and glucose distributed throughout the cell water in normal erythrocytes. Thus these experiments showed that the plasma protein and urea determinations gave valid indications of changes in the distribution of water between the plasma and cells as well as changes in total water in osmotic equilibrium.

Conditions especially favorable both for sickling and osmotic swelling of red cells were required to fulfill one of the objectives of this investigation. Osmotic swelling is caused by decreased anionic ionization of hemoglobin on deoxygenation, partly owing to decreased ionization of deoxyhemoglobin as compared to oxymoglobin and partly owing to decreased pH with an increased concentration of CO\(_2\). This change would lead to a gain of small anions (i.e., HCO\(_3^-\)) that can exchange with Cl\(^-\) and hence osmotic swelling.\(^4\) Thus the passing of blood from a condition of saturation with air to saturation with CO\(_2\) at 25°C elicited close to maximal osmotic swelling owing both to the Bohr effect\(^5\) and to the substantial decrease in pH resulting from the high solubility of CO\(_2\). Fortunately these conditions were very favorable for sickling. Hahn and Gillespie,\(^6\) with their hanging drop method for microscopic observation of sickling, found that passing CO\(_2\) through the gas chamber at room temperature induced sickling in less than 2 min.

**Experimental Methods**

Because of the mechanical fragility of the HbSS erythrocytes under anaerobic conditions,\(^7\) all harsh methods of mixing were avoided. Based on the hematocrit, a quantity of plasma was removed from the HbSS blood so that the remaining blood had a hematocrit of about 45\%. This adjustment allowed a more rational comparison of water distribution of normal and HbSS blood.

The 1- or 2-ml samples of thoroughly mixed blood were placed in separate, round-bottomed, 40-ml centrifuge tubes having restricted mouths. The tubes were closed with rubber serum-bottle stoppers. A 20-gauge hypodermic needle was inserted through the stopper of the tube that served for the oxymoglobin sample, allowing the maintenance of atmospheric conditions without significant evaporation. This sample was saturated with air at room temperature (about 25°C) for at least 30 min on a slowly turning laboratory rotator. Two hypodermic needles were inserted
through the stopper of the other tube. The air in the tube was displaced by either $100\%$ CO$_2$ or a $95\%$, N$_2$ $5\%$ CO$_2$ gas mixture.

Deaeration was accomplished by way of a train. The replacement gas entered the train at a rate of about 50 ml/min; it was first bubbled through isotonic saline solution (humidifier), then passed into the sample tube through one needle, the displaced gas passing out through the second needle, and finally bubbled out through a water valve. The whole train was maintained in a water bath at 25°C. Deaeration was continued for 30 min while the tube was occasionally rotated by hand in order to spread the blood over the inner surface of the large tube. This procedure allowed a large surface for gas exchange with a minimum of agitation. The hypodermic needles were then removed and the samples contained in the stoppered tubes were centrifuged. The plasma was quickly removed with a minimum of agitation. Plasma urea and protein concentrations were determined as previously described, and potassium and sodium concentrations were determined with a flame photometer.

The use of the train allowed the rapid and complete deoxygenation of the blood without danger of evaporation. In trial runs it was found polarographically$^{14}$ that the pO$_2$ of the gas train and manometrically$^{15}$ that the oxygen content of the blood both reached zero within ten min. These direct measurements agreed quite well with visual estimates that oxyhemoglobin was always completely converted to purple deoxyhemoglobin after 7-12 min deaeration. Complete sickling was observed with HbSS blood containing EDTA as anticoagulant when deoxygenated as described after fixing the cells by injecting glutaraldehyde solution into the tube.$^{16}$

RESULTS

The results of this investigation are shown in Table 1. All analyses of each sample were carried out in triplicate. There was no indication of a change in the total water in osmotic equilibrium, since the oxygenated to deoxygenated (O/D) ratio of the plasma urea concentration was not significantly different from unity. This result was true with both HbSS and normal blood regardless of the mode of deoxygenation. On the other hand, there was an influx of water into the cells when the blood was deoxygenated with $100\%$ CO$_2$. Under these conditions favoring a near-maximal intracellular anionic osmotic pressure, the influx of water caused a very significant decrease in the O/D ratio of the plasma protein both in HbSS ($p < 0.01$) and normal ($p < 0.001$) blood and with their O/D ratios not significantly different from each other. Deoxygenation with a gas mixture of $95\%$, N$_2$ $5\%$, CO$_2$ appeared to cause a small influx of water with

<table>
<thead>
<tr>
<th>Blood Sample</th>
<th>Plasma Constituent</th>
<th>Deoxygenation Gas</th>
<th>No. of Samples</th>
<th>Concentration Ratio $^*$ (Oxygenated/Deoxygenated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Urea</td>
<td>100% CO$_2$</td>
<td>21</td>
<td>$1.004 \pm 0.007$</td>
</tr>
<tr>
<td>Sickled</td>
<td>Urea</td>
<td>100% CO$_2$</td>
<td>8</td>
<td>$1.005 \pm 0.020$</td>
</tr>
<tr>
<td>Normal</td>
<td>Urea</td>
<td>95% N$_2$ $5%$ CO$_2$</td>
<td>6</td>
<td>$1.000 \pm 0.200$</td>
</tr>
<tr>
<td>Sickled</td>
<td>Urea</td>
<td>95% N$_2$ $5%$ CO$_2$</td>
<td>6</td>
<td>$0.996 \pm 0.23$</td>
</tr>
<tr>
<td>Normal</td>
<td>Protein</td>
<td>100% CO$_2$</td>
<td>21</td>
<td>$0.957 \pm 0.010$</td>
</tr>
<tr>
<td>Sickled</td>
<td>Protein</td>
<td>100% CO$_2$</td>
<td>8</td>
<td>$0.965 \pm 0.014$</td>
</tr>
<tr>
<td>Normal</td>
<td>Protein</td>
<td>95% N$_2$ $5%$ CO$_2$</td>
<td>6</td>
<td>$0.995 \pm 0.012$</td>
</tr>
<tr>
<td>Sickled</td>
<td>Protein</td>
<td>95% N$_2$ $5%$ CO$_2$</td>
<td>6</td>
<td>$0.990 \pm 0.012$</td>
</tr>
<tr>
<td>Normal</td>
<td>Na + K</td>
<td>100% CO$_2$</td>
<td>8</td>
<td>$0.970 \pm 0.011$</td>
</tr>
<tr>
<td>Sickled</td>
<td>Na + K</td>
<td>100% CO$_2$</td>
<td>5</td>
<td>$0.953 \pm 0.026$</td>
</tr>
<tr>
<td>Normal</td>
<td>Na + K</td>
<td>95% N$_2$ $5%$ CO$_2$</td>
<td>5</td>
<td>$0.987 \pm 0.015$</td>
</tr>
<tr>
<td>Sickled</td>
<td>Na + K</td>
<td>95% N$_2$ $5%$ CO$_2$</td>
<td>6</td>
<td>$0.995 \pm 0.005$</td>
</tr>
</tbody>
</table>

$^*$Fiducial limits at 95% confidence level defined by mean < $t_{0.05} \times SE$. 

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both normal and HbSS cells, but in neither case was the O/D ratio different ($p > 0.05$) from unity.

The O/D ratios of the sum of the plasma sodium and potassium concentrations were compared because of the exchange of extracellular sodium for intracellular potassium that occurs during sickling. Deoxygenation with both gases caused changes in the plasma cations strikingly similar to those in plasma protein concentration. Again, the O/D ratio was significantly less than unity with both normal ($p < 0.01$) and HbSS ($p < 0.01$) blood when deoxygenated with $100\%$ CO$_2$, but in neither case was the change significant with $95\%$ N$_2$–$5\%$ CO$_2$. This observation indicates that there was no substantial net shift of cations either into or out of the cells with either normal or HbSS blood regardless of the mode of deoxygenation. These data do not support the hypothesis that an efflux of water from the cells is a direct and necessary consequence of sickling, nor do they confirm that sickling causes a very substantial swelling.

**DISCUSSION**

In this study it was found that deoxygenation with a $95\%$ N$_2$–$5\%$ CO$_2$ gas mixture caused no significant change in distribution of water in either normal or HbSS blood. This finding agrees with those of Tosteson et al. Apparently, neither study was of sufficient sensitivity to distinguish the $\sim 1\%$ swelling induced by the Bohr effect in normal cells. However, the HbSS cells were normally responsive to changes in intracellular osmolality in that the increased osmolality induced by deoxygenation with $100\%$ CO$_2$ caused a significant influx of water into the cells comparable to the influx observed with normal cells. The changes in plasma cation (Na $+$ K) concentration mirrored the changes in plasma protein, indicating that there was no net transfer of cations. Contrary to these findings, Masys et al. reported that sickling induced by deoxygenation with $90\%$ N$_2$–$10\%$ CO$_2$ caused about a $10\%$ shrinkage of the cells, but a like treatment of normal cells caused a $3\%$ swelling. At the other extreme, Levine et al. reported that sickling in vitro brought about by deoxygenation induced a drastic $18\%$–$35\%$ swelling of the cells.

Masys et al. calculated shrinkage in the volume of the cells during sickling from measurements of the dilution of added $^{131}$I-albumin, a measurement similar in principle to that used in the present study. To accompany the supposed large efflux of water during sickling they found no significant change in the concentration of the plasma cations (Na $+$ K) suggesting a leakage of isotonic fluid from the cells. A driving force for such a dehydration was difficult to postulate. As supporting evidence they compared the size distribution of fixed red cells in oxygenated and deoxygenated states with the Model B Coulter cell counter. Deoxygenation of normal cells shifted the size distribution curve to higher threshold values, but deoxygenation of HbSS blood shifted the distribution to lower values. However, the Coulter method gives a spurious estimate of the relative volumes of the fixed, oxygenated, and deoxygenated red cells from HbSS blood because of the different shapes of the cells. When a cell passes through the orifice of the counter, the reduction in conductivity depends not only upon its volume but also upon its shape and orientation with respect to the electric field. Shank et al. found that the mode of the distribution of
Fluorescence measurements were made on a Perkin-Elmer MPF-2A fluorescence spectro-photometer equipped with a thermostated cell holder and a stirring apparatus. The sample was excited at 620 nm (slit, 6 nm) and the emission monitored at 670 nm (slit, 6 nm). The signal was first measured in the absence of probe: then the diS-C3-(5) (final concentration 2.0 x 10^(-6) M) was added. After the fluorescence had stabilized, the appropriate perturbant was added and the fluorescence monitored until no further change occurred (usually 20 min). The results are reported as ratios F/F₀ of observed (F) to initial (F₀) fluorescence intensities.

Serotonin release was measured by the method ofierushalmy and Zucker.

Determination of K⁺

Intracellular and extracellular concentrations of potassium were determined with an Instrumentation Laboratory 243 flame photometer. 3H-inulin was added to the platelet suspension to allow the determination of extracellular water, and the platelets were then sedimented through silicone oil. To determine extracellular K⁺, the supernatant was diluted fiftyfold with LiCl (5 meq/liter) before measuring. To determine intracellular K⁺, the platelet pellet was lysed with acetone and resuspended in 2 ml LiCl (15 meq/liter) using a vortex mixer.

Collagen Fibril Formation

For experiments where preformed collagen fibrils were added to the platelets, the fibrils were formed by adding collagen stock solutions to 0.05 M Tris, 0.05 M CaCl₂, pH 7.6 (final collagen concentration 0.1 mg/ml) warmed to 30°C. The solution was stirred for 3 min, the time necessary to form fibrils of sufficient size to cause immediate platelet aggregation, and an aliquot of the resulting suspension of collagen fibrils was added to the platelet suspension.

Materials

The cyanine dyes were the kind gift of Dr. Alan Waggoner. ADP, valinomycin, creatine phosphate, creatine phosphokinase, and bovine serum albumin were obtained from Sigma Chemical, St. Louis. Thrombin (topical) was obtained from Parke-Davis. 3H-inulin was obtained from New England Nuclear. Collagen was prepared as previously described. Apyrase was prepared by the method of Molnar and Lorand. All other chemicals were reagent grade.

All human experimentation was done in accordance with the Helsinki Declaration and with approval of the Human Experimentation Committee.

RESULTS

Dye Concentration

The optimal dye concentration was determined by monitoring the change in fluorescence induced by 2 x 10^(-6) M valinomycin in the presence of various concentrations of dye (Table I). The optimal concentration, i.e., the concentration of dye that caused the greatest change in fluorescence, was found to be 2 x 10^(-6) M.

<table>
<thead>
<tr>
<th>diS-C3-(5) (x 10^(-6) M)</th>
<th>F/F₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.89</td>
</tr>
<tr>
<td>1.0</td>
<td>0.81</td>
</tr>
<tr>
<td>2.0</td>
<td>0.78</td>
</tr>
<tr>
<td>4.0</td>
<td>0.80</td>
</tr>
</tbody>
</table>

Changes in fluorescence recorded after addition of valinomycin (final concentration 2 x 10^(-6) M) to cell suspensions equilibrated with diS-C3-(5) in a medium containing 5.4 mM K. Changes are expressed as the ratio of the fluorescence observed after the addition of valinomycin (F) to that observed before the addition of valinomycin (F₀).

REFERENCES

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The concentration at which the largest change was observed when valinomycin was added, was $2.0 \times 10^{-6} \text{M}$.

DiS-C$_3$-(5) did not bind to the platelets, as shown by the fact that less than 5% of the dye added could be extracted from platelets isolated after incubation with the dye.

Valinomycin was added to the suspension to show that the dye responded to changes in the membrane potential of the platelet and to calibrate the fluorescence change in terms of a change in the membrane potential. The resulting change in the membrane potential was a function of the K gradient before valinomycin was added and therefore of the external K concentration (Fig. 1).

The relationship between the change in fluorescence and that in membrane potential was estimated using the method of Hoffman and Laris. It was assumed that the membrane potential of the platelet, like that of the red cell, could be described by the constant field equation:

$$E = \frac{RT}{P_{Na}\left[Na^{+}\right] + P_{K}\left[K^{+}\right] + P_{Cl}^{-1}}$$

where $R$ is the gas constant, $T$ the absolute temperature, $F$ Faraday’s constant, $P_{Na}$, $P_{K}$, and $P_{Cl}$ the permeability constants of the respective ions; $\text{o}$ and $\text{i}$ subscripts refer to the concentrations outside and inside the cell, respectively. We also assumed that the relative values of the permeability constants for platelets were equivalent to those for the erythrocyte. If this assumption is valid, Na is much smaller than K or Cl and the Na term can therefore be neglected, resulting in

$$E = \frac{RT}{P_{K}\left[K^{+}\right] + P_{Cl}^{-1}}$$

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