The Osmotic Fragility of Erythrocytes After Prolonged Liquid Storage and After Reinfusion

By Ernest Beutler, Wanda Kuhl, and Carol West

Although it is recognized that red cells lose membrane during storage, estimation of the osmotic fragility of erythrocytes has not previously proven to be a useful measurement of the storage lesion. Erythrocytes from blood stored in CPD-A2 were found to have a markedly increased osmotic fragility. A major portion of this increase was found to be due to accumulation of lactate, which is only slowly transported from within erythrocytes and which therefore exerts a strong osmotic effect in the usual osmotic fragility test. After an hour's incubation in a large volume of iso-osmotic buffer, the osmotic fragility curve of stored erythrocytes was much more nearly normal. Such cells were found to have a volume 5%-8% greater than that of normal cells, indicating that even after removal of lactate more osmotically active material was present in the stored erythrocytes than in fresh cells. Most of this difference can be accounted for by substitution of chloride ion for 2,3-DPG, since chloride exerts approximately 3.7 times the osmotic effect of 2,3-DPG per unit charge. In addition to the shift in osmotic fragility produced by the increased intracellular osmotically active material, a "fragile tail" of red cells was also present. Stored erythrocytes were labeled with $^{51}$Cr and reinfused into the volunteer donors. The osmotic fragility of the reinfused cells was estimated using a technique of sequential osmotic hemolysis that permitted accurate estimation of osmotic fragility of transfused cells using very small amounts of $^{51}$Cr. The osmotic fragility of the reinfused cells became less than those of fresh cells after 24 hr and was exactly the same as those of fresh cells after 4 days. The fragile tail disappeared at a rate that approximated the rate of loss of nonviable erythrocytes from the circulation as measured by $^{51}$Cr. These findings are consistent with the preferential destruction of a subpopulation of red cells with a diminished surface area.

The Osmotic Fragility is one of the oldest methods of investigating the physical state of erythrocytes. It is not actually the "fragility" of the erythrocytes that is measured when they are suspended in graded hypotonic solutions. Rather, the osmotic fragility measures the surface/volume ratio of erythrocytes as it exists when they are suspended in an isotonic medium. In other words, the osmotic fragility of red cells reflects the extent of membrane redundancy present when the red cell is in equilibrium with an isotonic salt solution.

Although many attempts have been made in the past to relate the adequacy of storage of red cells in various preservative media to their osmotic fragility, such measurements have fallen into disrepute, since they have little predictive value. As Mollison pointed out in his classic monograph, "the osmotic fragility of stored red cells is affected by many factors which do not have any influence on viability."

However, there is evidence that some loss of membrane occurs during the storage of erythrocytes. If membrane loss is the as-yet-unidentified limiting factor in the storage of erythrocytes, then the osmotic fragility of stored cells should prove to be a very valuable predictive measurement in evaluating blood storage. If a red cell lost membrane during storage, it would assume a more spherical shape, have less membrane redundancy, and would lyse at a relatively high osmotic strength. In the present study, we have measured the iso-osmotic volume and the osmotic fragility of red cells at the end of prolonged storage and then have followed the changes in osmotic fragility that occur in vivo after reinfusing the cells labeled with $^{51}$Cr sodium chromate.

MATERIALS AND METHODS

These investigations were performed as a part of the evaluation of a new red cell preservative solution, CPD-A2. The volunteer studies were carried out under a protocol approved by an institutional review committee, the General Clinical Research Center review committee, the Army Drug Review Board, and the Food and Drug Administration. Informed consent was obtained from all volunteers.

CPD-A2 contains 15.6 mM citric acid, 89.6 mM sodium citrate, 230 mM glucose, 18.5 mM monobasic sodium phosphate, and 4 mM adenine. Four-hundred fifty milliliters of blood from 15 donors were drawn into 63 ml of CPD-A2 and were held at room temperature for 7–8 hr. The samples were then centrifuged and the quantities of plasma calculated to provide a hematocrit of 80% were removed. The units were then stored at 4°C without agitation, some in the lying position and some in the standing position.

In the case of 4 units, postinfusion viability and osmotic fragility studies of blood stored for 42 days were also performed. At the end of 39 days, a sample was pressed into a satellite bag for bacteriologic culture. On the 42nd day, 3–8 μCi of $^{51}$Cr-sodium-chromate were added to 10 ml of the packed cells and agitated occasionally at room temperature for 20 min. The suspension was then diluted in 40 ml of preservative-free 0.9% sodium chloride solution at room tempera-
ture, centrifuged, and the supernatant saline removed. The packed cells were washed one more time in 40 ml of sodium chloride solution, all of the supernatant saline was removed, and a 50% suspension of the red cells in saline was injected intravenously into the volunteer donor. Samples were removed from the opposite arm at 5, 10, 15, 20, and 60 min after injection and at 1 day and 4 days after injection. The percent viability was calculated from the extrapolated time zero value based on the 5, 10, 15, and 20 min \(^{125}\text{Cr}\) counts of the blood samples.

Osmotic fragility measurements were performed using a series of dilutions of a buffer containing 18 g NaCl, 2.73 g Na\(_2\)HPO\(_4\), and 0.374 g NaH\(_2\)PO\(_4\) in 200 ml. This buffer is osmotically equivalent to 10% NaCl and was diluted with water to produce “osmotic fragility solutions” of 0.15%–0.9% sodium chloride equivalents in increments of 0.05%. A special technique was devised to make possible the estimation of the osmotic fragility of reinfused red cells. This was necessary because of the small amount of radioactivity used and the limited amount of blood that could be drawn without unduly influencing the results. In this technique, 5–7 ml of packed red cells were brought to a volume of 200 ml using the 0.9% osmotic fragility solution. The suspension was allowed to stand at room temperature for 1 hr and was centrifuged at 850 g for 10 min at 4\(^\circ\)C, the supernatant removed and saved, and the packed cells brought to a volume of 200 ml with the 0.75% osmotic fragility solution. The suspension was immediately centrifuged at 850 g for 10 min at 4\(^\circ\)C, the supernatant saved, and the process was repeated, decreasing the osmotic fragility solution by 0.05% at each step until all of the cells had been lysed. To concentrate the radioactivity in the supernatant, it was treated with 25 ml of 0.15 M barium hydroxide and 25 ml of 0.15 M zinc sulfate and filtered through Whatman no. 42 filter paper. The filter papers were dried overnight at 45\(^\circ\)C, placed into counting vials, and \(^{125}\text{Cr}\) activity determined. Experiments showed that 80%–90% of added \(^{125}\text{Cr}\)-labeled red cells as counted in the original whole blood were recovered by this procedure and that the recovery, even with quantities as low as 1 \(\mu\)l, was linear with respect to the volume of labeled cells.

To measure the mean corpuscular volume (MCV) of red cells, 5 ml of an 80% suspension of erythrocytes was added to 95 ml of 0.9% osmotic fragility solution and the red cell suspension was allowed to equilibrate at room temperature for 1 hr. A portion of the solution was then centrifuged at 500 g for 5 min and the supernatant containing stroma of any hemolyzed cells was removed. The cells were resuspended in the osmotic fragility solution at a hematocrit of approximately 50%. A \(^{125}\text{I}\)-albumin solution, which had been dialyzed against 0.9% osmotic fragility solution, was added to give approximately 0.008 \(\mu\)Ci/ml. The number of red cells in the suspension was measured using a Coulter electronic counter, while the hematocrit was measured using a microhematocrit centrifuge. The amount of trapped fluid in the red cell column, which never amounted to more than 1.15%, was determined by measuring the \(^{125}\text{I}\) radioactivity.

RESULTS

Validation of the Method of Performing Serial Osmotic Fragility Measurements

The osmotic fragility of a normal blood sample was measured by adding 0.1 ml of blood to 10 ml of each osmotic fragility solution and measuring hemolysis in the supernatant. Red cells from the same sample were subjected to serial lyses with 200-ml aliquots as described in Materials and Methods. Results of this study are depicted in Fig. 1. It is apparent that the osmotic fragility curves obtained through these two methods when applied to fresh blood are superimposable.

The Osmotic Fragility of Stored Red Cells

When a standard osmotic fragility test is performed on red cells that have been stored in CPD-A2 for 42 days, they manifest a marked increase in their fragility. If, however, the same cells are washed or merely suspended in saline solution for 1 hr, the osmotic fragility curve becomes more nearly normal. The effect of the suspension of red cells in 20 volumes of isotonic saline solution for up to 3 hr is shown in Fig. 2. The

Fig. 1. Comparison of osmotic fragility of red cells performed by the standard method (\(\bullet\)) and by sequential hemolysis (\(O\)) in large volumes as described in text.
OSMOTIC FRAGILITY OF STORED RBC

Fig. 2. Osmotic fragility curves performed by the standard method on fresh blood (•) and blood stored 42 days in CPD-A2. Stored blood was tested untreated (○) and treated by suspension for 1 (△) and 3.5 (□) hr in 20 volumes of 0.9% osmotic fragility solution at room temperature. Details of the method are described in the text.

change in osmotic fragility of cells after suspension of cells in saline suggests that the stored red cells have a hyperosmolar interior and that on suspension in isotonic saline, the osmotically active molecules leave the cell, with equilibrium being reached at the end of 1 hr.

Red cells produce copious amounts of lactate during storage, a concentration of over 40 mM being achieved at the end of 42 days of storage. Lactate leaves red cells only slowly through a carrier-mediated process. As shown in Table 1, after 1 hr, lactate has nearly equilibrated with the isotonic saline solution in which stored red cells have been diluted. To verify that lactate could produce this effect on osmotic fragility, concentrated lactic acid was added to a continually stirred sample of blood freshly collected in CPD-A2 until the lactate concentration reached 45 mM. After being allowed to stand for an additional 30 min, the osmotic fragility of the lactate-treated sample and of a control to which saline was added instead of lactic acid was measured. The same degree of increase in osmotic fragility was observed in the lactic-acid-treated cells as occurred in stored cells (Fig. 3).

Volume of Stored Red Cells

Stored red cells and freshly drawn red cells from the same donor were equilibrated for 1 hr with isotonic

Table 1. Lactate Loss From Stored Erythrocytes

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Lactate (mM)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Erythrocytes</td>
</tr>
<tr>
<td>0</td>
<td>46.9</td>
</tr>
<tr>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td></td>
</tr>
</tbody>
</table>

Lactate determinations were done on a PCA extract of packed stored erythrocytes. They were then diluted with 9 volumes of 0.9% sodium chloride solution, and at intervals, lactate estimations were performed on PCA extracts from the supernatant of centrifuged aliquots of this suspension.

Fig. 3. Blood was collected in CPD-A2 and immediately allowed to equilibrate for 30 min with sufficient lactic acid to provide a lactate concentration of 45 mM. Blood incubated with an equivalent volume of saline served as the control. Osmotic fragility measurements were made using a standard method. The change in osmotic fragility in the lactate-treated blood is comparable to that observed after storage for 42 days.
osmotic fragility buffer. The results of measurement of the MCV of these cells are presented in Table 2. It is apparent that stored cells equilibrated with isotonic buffer had a volume ranging from 105.6% to 108.0% of fresh cells from the same donor. Equilibrating the cells for 2 or 3 hr gave essentially identical results. The effect of restoring the 2,3-DPG levels of 49-day stored red cells by incubation for 1 hr at 37°C with a mixture containing 10 mM inosine and 10 mM pyruvate was investigated. As shown in Table 3, restoration of 2,3-DPG levels resulted in a shift toward normal of the volume of the stored red cells after incubation with isotonic buffer.

**Osmotic Fragility of Stored Red Cells After the Reinfusion**

The osmotic fragility of red cells from 4 donors was determined at intervals after reinfusion. The preinfusion osmotic fragility curve was determined by sequential hemolysis of a mixture of donor blood and the chromated preinjection sample. The results of these studies are presented in Figs. 4–7. The fragility curve of unstored donor red cells was obtained from measurements of hemoglobin release in one of the serial osmotic fragility curves, since the hemoglobin, in contrast to the 51Cr label, was virtually entirely derived from the patients own unstored red cells. Not shown in the figures, these curves were identical with the 51Cr curves obtained at 4 days. The curves obtained after 24 hr consistently showed a small increase in osmotic resistance. The “fragile tail” of the stored cells was defined as those erythrocytes lysing in the osmotic fragility solutions in which the unstored erythrocyte lysis was 1% or less. In 3 subjects this was the 0.55% osmotic fragility solution. In the other subject it was the 0.50% osmotic fragility solution because this subject had a slightly leftward shifted osmotic fragility curve (Fig. 7), possibly due to mild iron deficiency; the prestudy MCV was 83 cu mm. Figure 8 shows the relationship between the disappearance of the “fragile tail” on the one hand, and the disappearance of labeled cells from the circulation on the other. After 60 min the disappearance of the fragile tail closely approximated the loss of cells from the circulation. After 24 hr, the number of cells lost approximated the original size of the fragile tail.

**DISCUSSION**

Red cells that have been stored for prolonged periods of time manifest a markedly increased osmotic fragility when measured in the usual manner. This abnormality is not due to membrane loss, but rather to

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### Table 2. MCV Determinations on Fresh Blood and Blood Stored for 42 Days in CPD-A2

<table>
<thead>
<tr>
<th>Donor No.</th>
<th>Iso-osmotic Volume</th>
<th>Stored as Percent of Fresh</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh Blood</td>
<td>Stored Blood</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>93.2</td>
<td>100.7</td>
</tr>
<tr>
<td>2</td>
<td>87.7</td>
<td>94.1</td>
</tr>
<tr>
<td>3</td>
<td>97.8</td>
<td>103.4</td>
</tr>
<tr>
<td>4</td>
<td>98.8</td>
<td>104.4</td>
</tr>
</tbody>
</table>

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### Table 3. Effect of 49-day Storage and of Regeneration of 2,3-DPG on the Volume of Red Cells Equilibrated With Iso-osmotic Buffer. The mean ± 1 S.E. values of samples from 6 donors are presented.

<table>
<thead>
<tr>
<th>Fresh Blood</th>
<th>Stored Blood</th>
<th>Stored Blood With Regenerated 2,3-DPG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iso-osmotic volume (% of Fresh)</td>
<td>2,3-DPG (\mu mole/g Hb)</td>
<td>Iso-osmotic Volume (% of Fresh)</td>
</tr>
<tr>
<td>100</td>
<td>14.3 ± 0.6</td>
<td>106.1 ± 2.0</td>
</tr>
</tbody>
</table>

The mean ± 1 SE values of samples from 6 donors are presented.
the presence of osmotically active substances within the red cell that render the interior hyperosmolar with respect to isotonic saline. The chief substance responsible for this hyperosmolar state is probably lactate, and we have been able to closely approximate the osmotic fragility of stored cells merely by equilibrating freshly drawn red cells with concentrations of lactate equal to those found after storage. Other substances that are transported slowly from red cells such as phosphate and glucose may also play a role in establishing the reversible hyperosmolar state that exists in stored erythrocytes.

Once the red cells have been equilibrated with isotonic saline, their mean cell volume is greater than that of freshly drawn red cells and their osmotic fragility is therefore still slightly increased. The greater size of the equilibrated stored cells indicates that even after lactate has been removed, the amount of osmotically active solute within the red cells is increased. A major determinant of the higher osmolarity of the stored cells may be the substitution of chloride for 2,3-diphosphoglycerate (2,3-DPG) during the course of storage and subsequent equilibration with isotonic saline. At pH 7.2, 2,3-DPG has 3.67 negative charges. Therefore, 3.67 moles of chloride must be substituted for each mole of 2,3-DPG that has been hydrolyzed to maintain electroneutrality. Normal red cells contain approximately 5 mM 2,3-DPG. If this were replaced charge-for-charge with chloride, an additional chloride content exerting 18.35 mosmole would be present in the cell. Subtracting the 5 mosmole exerted by the 2,3-DPG anion that has been hydrolyzed, the 13.35 mosmole increase of solute represents an increase of approximately 4.5% of the osmolarity of the red cell. This change in osmotically active solute in the red cell would account for most of the increased red cell volume observed when the red cells were equilibrated with an iso-osmotic solution containing largely chloride as the anion. Indeed, restoring 2,3-DPG levels to preinfusion concentrations was shown to restore to nearly normal the iso-osmotic volume of stored erythrocytes.

Since the osmotic fragility curve of stored erythrocytes turns out to be a complex of several different effects, it is not surprising that analysis of fragility
Fig. 7. Osmotic fragility curves of 42-day stored blood determined by sequential hemolysis of chromated red cells before and after reinfusion. T = 0 (\(\Phi\)) was performed on a mixture of 0.1 ml of the chromated cells for infusion and 5–7 ml of the donor’s fresh blood drawn just prior to infusion. Postinfusion time points were at 5 min (\(\bigtriangleup\)), 60 min (\(\Delta\)), and 24 hr (\(\square\)), at which times the percentage of circulating chromated cells were 99.8, 93.9, and 78.9, respectively. Details of methods given in the text.

curves has not previously been helpful in predicting the postinfusion viability of erythrocytes. Removing lactate by equilibrating the cells with an isotonic buffer removes the major perturbing influence. The swelling of the cells in isotonic medium because of substitution of chloride for 2,3-DPG is presumably quite uniform in the red cell population, since even 21-day storage in acid citrate preservatives results in total depletion of red cell 2,3-DPG. A uniform shift in the fragility of the red cell population is expected as a result, and this was observed. This shift was rapidly corrected in vivo in a manner consistent with the rapid regeneration of 2,3-DPG that has previously been documented.\(^8\)\(^9\) The apparent “overshoot” observed 24 hr after reinfusion is unexplained, but presumably is the result of adjustment of ion concentrations, which is known to occur after reinfusion of stored cells.\(^10\) A tail of osmotically fragile cells was found in stored erythrocytes equilibrated with an iso-osmotic solution. The fragile tail of stored cells presumably consists of the cells that have lost the greatest amount of membrane.

Cooper and Jandl\(^11\) first used red cells labeled with 300 \(\mu\)Ci of \(^{51}\)Cr as a means to study osmotic fragility of red cells after infusion into patients with liver disease. We have modified this ingenious technique by performing sequential hemolysis and concentrating the hemolysate with Ba(OH)\(_2\)-Zn SO\(_4\) after each step. This enabled us to make repeated measurements of osmotic fragility, using small blood samples and only 5–8 \(\mu\)Ci, a level of radioactivity that is acceptable for use in normal volunteers. When studied using this technique, the fragile tail disappeared from the circulation at a rate similar to the rate of removal of nonviable cells as shown in Fig. 8. An alternate possibility, viz., that the cells were not removed but rather gained membrane surface in the circulation, was also considered. However, such conversion to cells with greater osmotic resistance would have been associated with a corresponding increase in the amount of radioactivity released when the cells were treated with solutions of lower osmolarity. No such increase was observed.

The range of viabilities observed in the current study was relatively narrow. Thus, our data do not provide a critical test of the question of whether membrane loss is a major determinant of the viability of stored red cells. A large number of studies involving poorly preserved as well as well preserved erythrocytes will be required to test the usefulness of properly performed osmotic fragility studies in red cell preservation.
REFERENCES

The osmotic fragility of erythrocytes after prolonged liquid storage and after reinfusion

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