Size Dependence of Ghosts From Stored Erythrocytes on Calcium and Adenosine Triphosphate

By Jiri Palek, William A. Curby, and Fabian J. Lionetti

Heterogeneity within ghosts prepared from stored erythrocytes was studied by multichannel particle analysis of size distributions of reconstituted ghosts. On restoration of isotonicity of hemolysates from fresh erythrocytes, ghosts were smaller than the corresponding red cells. After subsequent incubation for 15 min, they swelled to a final stable volume. The hemolysis of stored erythrocytes and restoration of isotonicity yielded ghosts similar to those from fresh cells, but, after subsequent incubation, a second population of small ghosts appeared. This population resulted from markedly diminished ghost swelling, that is attributed to decreased sodium permeability and increased membrane rigidity, causing resistance to changes in volume. Erythrocyte ATP depletion during storage of ACD blood was associated with an increase in the percentage of small ghosts. Similar ghosts were produced from fresh cells when CaCl2 (1 × 10⁻⁶ M) was introduced into ghosts during hemolysis. The erythrocyte calcium content increased during prolonged storage from 11.3 ± 6.3 μg atoms/liter of cells (stored for 1–2 wk) to 22.0 ± 7.0 μg atoms/liter of cells, after 7–9 wk of storage. The introduction of nucleotides (2 × 10⁻³ M) into ghosts during hemolysis of stored cells prevented the appearance of small ghosts. The inhibitory effect was greater than predicted from stability constants of calcium nucleotide complexes, indicating the specific interaction of nucleotides with the membrane. The percentage of small ghosts from red cells stored for various times agreed with the percentage of non-viable cells derived from the literature. It is concluded that the decrease in ATP and the accumulation of calcium in stored erythrocytes induce conformational changes of membrane fibrous (contractile) proteins that results in decreases in membrane elasticity and permeability, which is in turn reflected by formation of small ghosts.

Recent studies have suggested that the posttransfusion viability of red cells stored in ACD solution is determined by physical properties of erythrocyte membranes. This has been indicated by correlations between red cell posttransfusion viability and filterability, deformability, osmotic fragility, and shape of red cells and their ghosts. Two significant observations emerged from these studies. (1) Shape abnormalities and bimodal derivative curves of osmotic fragility of stored red cells suggested that membrane...
Fig. 1. Kinetics of swelling of reconstituted ghosts from fresh (left) and stored (right) red cells. Fresh and stored (5 wk) erythrocytes were hemolyzed in 5 volumes of 5 mM Tris-HCl buffer, pH 6.9, and the isotonicity was immediately restored. After dilution with Tris-HCl buffered isotonic saline solution, ghost-size distribution was measured kinetically at 20°C. Data are shown as plots of particle frequency against channel No. of counter. Calibration of instrument has been made with red cells and ghosts, the volumes of which have been determined from ghost counts and microhematocrits. Dotted curve represents size distribution of erythrocytes washed three times in isotonic NaCl containing 5 mM Tris-HCl buffer, pH 6.9.

Abnormalities were not randomly distributed and that two populations of erythrocytes, one with normal and the other with different membrane properties, were present. The alterations in shape and the decreases in filterability and deformability resembled analogous changes in metabolically depleted cells, which were attributed to ATP depletion and intracellular accumulation of calcium.

The incorporation of Ca²⁺ into human erythrocyte ghosts has been shown to result in a marked decrease in ghost size. The Ca²⁺-dependent shrinkage was due both to membrane “contraction” and to a decrease in solute permeability, presumably reflecting conformational changes of membrane proteins that exhibit Ca²⁺ ATPase activity. The present article reports analysis of size distributions of populations of reconstituted ghosts of stored cells. From fresh cells, a single population of ghosts was derived. The storage of red cells
in ACD at 4°C was associated with progressive increase in red cells that after osmotic hemolysis and subsequent restoration of isotonicity yielded ghosts of a distinctly smaller size than those of original erythrocytes. The smaller ghosts reflected alterations in the physical state of membranes from stored erythrocytes, as induced by a decrease in ATP and an increase in calcium content.

MATERIALS AND METHODS

Blood

Acid citrate dextrose-collected blood (ACD, N.I.H. formula A) was stored under standard conditions and investigated at the times indicated. Some units were analyzed repeatedly at various times of storage. Repeated blood sampling was performed aseptically, as confirmed by testing for bacterial contamination. Whole blood was centrifuged at 2500 g for 15 min, the supernatant and buffy coat were discarded by aspiration, and red cells were washed three times with 2 volumes of isotonic saline.

Reconstituted Ghosts

Ghosts were prepared by osmotic hemolysis followed by restoration isotonicity. It has been established that, after restoration of isotonicity, ghosts decreased their size and volume to a magnitude predicted by their osmotic behavior.9-12 The physiologic characteristics of these ghosts have been described.13-14 One volume of erythrocytes was hemolyzed in 5 volumes of 5mM Tris-HCl buffer pH 6.9, and the isotonicity was subsequently restored with hypertonic NaCl. In some of the experiments, 1-4 × 10^-9M ethylenediaminetetra-acetate (EDTA), ethyleneglycol-bis (β-aminoethyl ether) -N, N tetraacetate (EGTA), adenosine triphosphate (ATP), adenosine di- and monophosphate (ADP, AMP), inosine triphosphate (ITP), or guanosine triphosphate (GTP) were introduced into ghosts during hemolysis. The principle of this technique is based on the finding that erythrocyte membranes lose their selective permeability at the time of hemolysis and immediately thereafter, allowing the introduction of various normally nonpenetrating compounds into the cells.

Hemoglobin-free Membranes

These were prepared as described by Dodge et al.,17 except that Tris-HCl (5mM, 7.15) with 2 mM EDTA was used instead of phosphate buffer and EDTA was omitted in the last ghost washing.

Size Distribution of Red Cells and Reconstituted Ghosts

The reconstituted hemolysates were diluted immediately with Tris-buffered, isotonic saline solution, pH 7.0, to a final ghost or erythrocyte count of 1 × 10^6/ml. Unless otherwise stated, ghosts were analyzed after 15 min of preincubation at 20°C with a multichannel particle size analyzer, designed by one of the authors (W. A. C.) as recently described.9 The instrument was calibrated with red cells or ghosts of known volumes (determined conventionally from cell counts and hematocrits of approximately 50% cell suspensions). The relation between mean corpuscular volume of red cells (MVC) or ghosts and channel number of the peak of size distribution curve was not linear (Fig. 1). This is consistent with previous results with latex spheres of a known diameter.18 In all samples of ghosts, the ghost counts per unit volume of the ghosts suspension were similar. For convenience in evaluating percentage composition of small ghosts, the curves were amplified electronically by a scale factor up to two times.

Ghost Volume and Hemoglobin Concentration

Ghost volume was computed from counts and microhematocrits of ghost suspensions.9 Ghost hemoglobin was measured as cyanmethemoglobin.19
Ghost Osmotic Behavior

This was evaluated from Boyle-Van't Hoff's relation by plotting ghost volume against the reciprocal of osmolarity.13.

Erythrocyte Shape, Volume (MCV), and Hemoglobin Concentration (MCHC)

Erythrocyte ATP depletion is accompanied by shape transformation in a sequence: biconcave discs, crenated discs, crenated and smooth spheres. We have used the method of Szasz et al., consisting of a differential count of red cells at different stages of disc-sphere transformation. The red cells of each shape were counted, and their percentage was multiplied by a factor for each cell type. The factors were as follows: discs, 1.0; crenated discs, 0.75; crenated spheres, 0.5; spheres with a few fine spicules, 0.25; and smooth spheres, 0.0. The sum of the percentage counts of different shape forms, after multiplication by the above factors, was used as an index of disc shape transformation. Shape index = Σ(percentage of each cell type × factor).

MCV and MCHC were determined by conventional methods.19

Erythrocyte ATP

Erythrocyte ATP was determined enzymatically with hexokinase and glucose-6-phosphate dehydrogenase.21

Erythrocyte Calcium

Erythrocyte calcium was measured by atomic absorption spectrophotometry of ethanol-HCl extracts of dry ashed erythrocytes, previously washed as described by Harrison and Long.22 In accord with their results, the data were unaffected by variations in cellular Na+, K+, and hemoglobin content. Calcium ion added to packed cells prior to washing was quantitatively recovered (102% ± 8%, SD).

Analysis of Size Distribution Curves

The per cent of small ghosts in bimodal size distribution curves was estimated as follows. Ghosts were prepared in a standard manner (A) or by hemolysis in 4 mM ATP (B) at otherwise identical conditions, and their size distribution curves were measured. In the latter sample (B), the formation of small ghosts was completely abolished by ATP and hence, this served as a control. The latter size distribution curve was corrected by a factor:

\[ F = \frac{f_1}{f_1'} \]

where \( f_1 \) and \( f_1' \) are relative frequencies of modes of the normal ghost population in preparations A and B, respectively. The size distribution curves of preparation A and the corrected curve B were then integrated electronically and the per cent of small ghost population computed as follows:

\[ \text{Small ghosts} (%) = \frac{A - B}{A} \times 100 \]

where A and B are ghosts counts of curve A and corrected curve B respectively. This method assumed that no cells of the second population were present in the mode of the normal ghost population (\( f_1 \)). Figure 4 shows that in the "ideal" second population, produced by incorporation of \( 2 \times 10^{-6} \) M CaCl₂ into ghosts from fresh cells, the expected frequency of small ghosts at \( f_1 \) was 10%. Therefore, the error of the calculation was less than 5% when the per cent of the second population was less than 50%. For bimodal curves where the percentage of small ghosts was greater than 50%, the corrected curve B was made by using a factor...
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Fig. 2. Size distributions of reconstituted ghosts from ACD whole blood stored at 4°C. Ghosts were prepared from units stored for 1–13 wk, and their size distributions measured after swelling, e.g., 15 min after restoration of isotonicity of hemolysate and dilution in Tris-buffered isotonic saline solution.

\[ F = \frac{f_1}{f_1' - 0.1f_2} \]

where \( f_2 \) is the frequency in the mode of the small ghost population.

Statistical Analysis

The least square regression lines for per cent of small ghosts, calcium content, ghost volume, and ghost hemoglobin concentration and the statistical significance of differences in calcium content in red cells at the beginning and after prolonged storage has been determined by standard methods.23 Values, given in the text, represent means ± SD.

RESULTS

Size Distribution of Reconstituted Ghosts

Figure 1 shows a kinetic determination of ghost size distributions. Immediately upon restoration of isotonicity, ghosts had a smaller size than that of the initial erythrocytes. Within the subsequent 15 min, ghost volume increased rapidly to approximately 150 ± 10% of initial erythrocyte volume. During the following 1 hr of incubation, the volume remained nearly constant. The initial swelling of ghosts was also observed by others24,25 and was attributed to leakiness of the membrane to monovalent cations, followed by water influx allowing the Donnan equilibrium to be established. Kinetic changes in size of ghosts from stored (5 wk) red cells are shown on the right side of Fig. 1. Immediately upon restoration of isotonicity, a single population of ghosts was again observed that was similar in size to ghosts derived from fresh cells. After swelling, however, two populations of ghosts appeared, one with a size identical to that of fresh ghosts and a second population of markedly smaller ghosts. For approximately 8–42 min the percentage ratio of both cell populations remained almost unchanged. After prolonged standing at 20°C, the second population of small ghosts gradually disappeared.

The variations in size distribution of reconstituted ghosts after the initial swelling, i.e., 15 min after restoration of isotonicity, were studied in ghosts prepared from red cells of ACD blood at different times of storage at 4°C (Fig. 2). The second population of ghosts of smaller size was noted in ghosts prepared from red cells stored 2–4 wk. This population was more pronounced
in long-stored units, while the population of ghosts of normal size decreased proportionately. In blood stored approximately 8 wk, more than 50% of ghosts were small ones. Similar alterations in ghost size were observed when the size distributions of ghosts were determined repeatedly in the same units of blood at various times of storage. Such bimodality was not observed with intact erythrocytes. Unless otherwise noted, all the size distributions shown below were measured 15 min after restoration of isotonicity, i.e., after the initial swelling phase.

In all samples of ghosts, irrespective of the age of red cells from which they were prepared, the ghost counts per unit volume of the ghost suspension were similar. This indicates that the population of small ghosts resulted from ghost shrinkage and not from cell multiplication due to formation of membrane fragments of small size. This can be also concluded from the kinetics of changes in ghost size (Fig. 1).

**Effects of Nucleotides and EDTA**

The population of small ghosts found in stored blood was abolished by introducing ATP (2 mM) into ghosts of stored red cells during hemolysis (Fig. 3). The same inhibition was seen with other nucleotide triphosphates having similar stability constants of divalent cation–nucleotide complexes. ADP and AMP were least effective. Inhibition by nucleotides did not require the simultaneous presence of Mg²⁺, while the introduction of Mg²⁺ alone into ghosts was almost ineffective.

The inhibition of small ghost formation by nucleotides required their introduction into ghosts during hemolysis. The membranes of red cells undergoing osmotic hemolysis are transiently permeable to nucleotides. High intra-
cellular nucleotide concentrations, therefore, could be achieved by addition of nucleotides to the hemolyzing solution. At a final ATP concentration during hemolysis of 2 mM, ghost ATP was 1.2 ± 0.2 μmoles/ml, and complete inhibition of small ghost formation was observed (Fig. 3). On the other hand, the addition of ATP 2 min after hemolysis (when membrane impermeability was partly restored) did not substantially increase the intracellular ghost ATP concentration (0.3 ± 0.1 μmoles/ml) and failed to inhibit the small ghost formation (Table 1).

Nucleotides form complexes with divalent cations. Therefore, the reversal of the bimodal size distribution of ghosts from stored cells to that of fresh cells by nucleotides could be attributed to nucleotide chelation of divalent cations (principally Ca²⁺), which accumulated in stored cells. Figure 3 compares the inhibitory effect of 2 mM nucleotides to that of 2 mM EDTA and EGTA. In contrast to the marked inhibition by nucleotides, the small ghost formation was less reduced by introducing either EDTA or EGTA into ghosts, although the stability constants of these complexing agents with divalent cations are much higher than those of nucleotides. An increase in EDTA concentration to 6 mM, however, gave the same inhibition of small ghost formation as 2 mM ATP.

Table 1. Site of Nucleotide Inhibition of Ghost Shrinkage

<table>
<thead>
<tr>
<th>ATP Addition (2 mM)</th>
<th>Ghost ATP Concentration (μmoles/ml)</th>
<th>Small Ghosts* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>—</td>
<td>0.01</td>
<td>55 ± 4</td>
</tr>
<tr>
<td>During hemolysis</td>
<td>1.2 ± 0.2</td>
<td>0</td>
</tr>
<tr>
<td>After hemolysis†</td>
<td>0.3 ± 0.1</td>
<td>44 ± 6</td>
</tr>
</tbody>
</table>

* Per cent of small ghosts from total ghost count after swelling.
† ATP added immediately after hemolysis, and isotonicity subsequently restored.

Fig. 4. Comparison of size distribution of ghosts from 9 wk old ACD blood and fresh red cell ghosts, into which 1 × 10⁻⁶ M CaCl₂ has been introduced during hemolysis. The size has been measured 15 min after the restoration of isotonicity (see Figs. 1 and 2 for additional details).
Calcium

We have previously demonstrated a decrease in size of ghosts into which calcium ion was incorporated during or after hemolysis. The decrease in size was prevented by nucleotides. Figure 4 compares the size distribution of ghosts from red cells stored for about 8 wk to that of fresh red cells into which Ca\textsuperscript{2+} (1 \times 10^{-6}M) was introduced during hemolysis. An apparent identity of both size distribution curves is evident.

**Interrelations Between Red Cell ATP, Calcium, and Shape and Size Distribution of Ghosts**

The decrease in size of ghosts by Ca\textsuperscript{2+} and the inhibition by nucleotides suggested that the small ghost population was due either to an increase in erythrocyte calcium content during storage or to an increase in cellular calcium–ATP ratio. Figure 5 shows the kinetics of changes in red cell ATP and calcium content, shape index, and ghost size distribution during storage. The small ghost population, expressed as per cent of small ghosts, increased progressively during prolonged storage, while the red cell ATP content and shape index decreased. A considerable variation in calcium content was present among individual red cell samples. A progressive increase, however, was observed during storage. After 7–9 wk of storage, the average calcium content (22.0 ± 7.0 µg atoms/liter of cells) was 195% of the mean level in the first 2 wk of storage (11.3 ± 6.3 µg atoms/liter, p <0.01).

The increase in the percentage of ghosts of smaller size during storage was accompanied by an increase in ghost hemoglobin concentration and a decrease in the mean ghost volume (Fig. 6), further suggesting that the population of small ghosts reflected shrinkage and not formation of membrane fragments of small size. The regression line of ghost volume as a function of storage on Fig. 6 indicates that the mean volume of ghosts decreased from 158 cu µ (ghosts from erythrocytes at the onset of storage) to 113 cu µ (ghosts from red cells stored for 8 wk), e.g., to 71% of the prestorage volume. Similar regression line of increases in hemoglobin concentration shows an increase from 5.8% to 8.5% and is close to a predicted value computed from ghost.

**Fig. 5.** Red cell ATP, shape index, calcium, and the percentage of small ghosts during storage of ACD blood. Small ghosts are expressed as the percentage of the total ghost count. The calcium content is expressed as µg-atoms per liter of initial cell volume (e.g., red cell volume at the beginning of the storage period). In the plots of calcium and ghost size, the least squares regression lines (solid lines) and standard errors (dotted lines) are represented.

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Fig. 6. Hemoglobin concentration and volume (cu ml) of ghosts prepared from red cells stored for 1–9 wk in ACD at 4°C. Ghosts prepared as in Fig. 2. The solid line is the least squares regression line and the dotted ones the standard error.

Ghost Osmotic Behavior

The incorporation of calcium into ghosts was previously shown to produce a decrease in passive permeability, as indicated by increased osmotic shrinkage of ghosts in solutions of different molarities. Therefore, the osmotic behavior of ghosts from erythrocytes stored for 9 wk was determined. Three blood units were analyzed. The size distribution of ghosts from these cells is shown in Fig. 4. Their osmotic behavior is shown in Fig. 7. The linearity of plots of ghost volume against the reciprocal of osmolarity indicated that the Van’t
Hoff Boyle relation applied, e.g., ghosts acted as osmometers. The magnitude of volume decrease of ghosts derived from cells after prolonged storage was higher, indicating an increased transmembrane NaCl gradient and hence, decreased NaCl permeability.

Heterogeneity in ghost size was critically dependent on the method of ghost preparation. Preincubation of the hemolysate prior to restoration of isotonicity for 2–5 min markedly reduced the quantity of the small ghost population. Washing of ghosts in 2 mM Tris-HCl (pH 6.9) buffer gave similar effects suggesting that washing caused calcium elution from the membranes. This agrees with recent observations that the recovery of calcium from membranes prevented the formation of small ghosts was supported by comparison of the size distributions and calcium content of standard reconstituted ghosts and hemoglobin-free membranes isolated from stored cells. While the standard reconstituted ghosts from red cell stored 6 wk exhibited a typical bimodal distribution, the hemoglobin-free membranes were represented by a single population of cells (not shown). In the latter, almost no calcium was detected in hemoglobin-free membranes. In triplicate assays of four samples of hemoglobin-free ghosts from units stored for 4–6 wk the calcium content was 1.2 ± 0.4 μg atoms/liter ghosts, while the corresponding ghosts reconstituted as usual contained 13.2 ± 6.3 μg atoms/liter ghosts.

Relation to Post-transfusion Viability

An attempt was made to correlate the percentage of small ghosts derived from cells at different times of storage to erythrocyte posttransfusion viability, as reported by others (Fig. 8). It can be seen that the regression line of the percentage of small ghosts was similar to that of nonviable cells, e.g., cells that were removed from circulation within 24 hr after transfusion.
DISCUSSION

The storage of red cells in ACD at 4°C was associated with a progressive increase in the number of cells that, after hemolysis, restoration of isotonicity and incubation yielded ghosts of distinctly smaller size than that of ghosts from fresh erythrocytes. The following data suggest that the population of erythrocytes yielding small ghosts resulted from erythrocyte ATP depletion and calcium accumulation. (1) An increase in the percentage of cells yielding small ghosts paralleled a decrease in red cell ATP and an increase in calcium content during storage. (2) The formation of small ghosts from red cells stored for prolonged time was abolished by introducing nucleotides into ghosts during hemolysis. (3) The population of small ghosts derived from stored erythrocytes could be mimicked in fresh cells when Ca\(^{2+}\) was present in the hemolytic solution. Our data disagree with those of Haradin et al.\(^1\) who failed to detect increases of calcium during 8 wk of storage in ACD. They did observe, however, an increase in calcium content during in vitro incubation of red cells in serum.\(^7\)

The mechanism of calcium accumulation in stored erythrocytes is not completely understood. Red cell membranes are nearly impermeable to this divalent cation.\(^{38,34}\) Calcium concentration in red cells is about 200 times lower than in plasma, and almost all is recovered from the membranes.\(^9,22\) Therefore, a calcium ion concentration gradient between the plasma and cell interior is high even when most of the plasma calcium in ACD blood is present as calcium citrate. Since the concentration requirements for calcium-dependent alterations in membrane physical properties are of the order of \(1 \times 10^{-6}\) M,\(^9\) physiologically significant amounts of calcium can accumulate in red cells during storage even though the membrane calcium ion permeability is very low. ATP depletion during blood storage presumably favors calcium accumulation in membranes of stored erythrocytes for two reasons. (1) ATP is utilized as an energy source for the “calcium pump,” by which an active calcium extrusion from erythrocytes against a concentration gradient occurs.\(^{29,32}\) Although the physiological significance of this system in red cells has not been clearly established, ATP depletion can cause the failure of calcium extrusion from stored red cells. (2) Nucleotides prevent the interaction of calcium ion with the membrane due to formation of calcium nucleotide complexes that, in contrast to calcium ion, do not affect membrane volume properties.\(^9\)

The inhibition of small ghost formation by nucleotides was markedly higher than that of more potent complexing agents such as EDTA or EGTA. Since during hemolysis the red cell membranes are permeable in both directions even for large molecules such as albumin or ferritin,\(^{15,33}\) differences in the incorporation of complexing agents into cells could not presumably explain the observed differences in inhibition. This suggests a more specific interaction of nucleotides with the interior of the membrane, rather than a simple chelation of calcium ion.

The physical nature of red cell abnormalities resulting in formation of small ghosts is not clear. Our results imply that the heterogeneity in size of ghosts from stored erythrocytes was due to differences in swelling of ghosts after restoration of isotonicity (Fig. 1). Immediately after restoration of isotonicity,
the size of ghosts from fresh cells was smaller than that of initial erythrocytes. Within the subsequent 15 min, ghosts swelled to a constant stable volume, as also observed by others.\textsuperscript{24,25} The swelling has been attributed to cation influx followed by water movement into leaky ghosts, allowing the Donnan equilibrium to be established.\textsuperscript{24,25} The size of ghosts from stored cells, determined immediately after restoration of isotonicity, was similar to that of ghosts from fresh cells. The second ghost population became apparent after subsequent incubation, due to markedly decreased swelling of these ghosts. The diminished swelling could result variously from such causes as (1) changes in the osmotic coefficient of hemoglobin; (2) increased rigidity of the membrane, thus resisting changes in volume, and (3) decrease in ghost passive permeability, preventing the establishment of Donnan equilibrium.\textsuperscript{40,46} Changes in osmotic coefficient of hemoglobin, induced by intracellular calcium accumulation, were unlikely as indicated by previous studies from this laboratory.\textsuperscript{9} These have shown that ghosts of small volume have been produced when Ca\textsuperscript{2+} has been introduced into ghosts by adding to the hemolysate after hemolysis, when hemoglobin equilibrium has been achieved between external and internal side of the membrane. Alterations in osmotic coefficient of hemoglobin induced by in vitro aging, unrelated to erythrocyte calcium accumulation, were also not a probable cause of diminished ghost swelling. This was indicated by the inhibition of small ghost formation by complexing agents such as nucleotides or EDTA. Furthermore, cells depleted in ATP by incubation in Ca\textsuperscript{2+}-free medium did not yield ghosts of small size, unless Ca\textsuperscript{2+} has been subsequently added to the system.\textsuperscript{9} It has been also reported by others\textsuperscript{7} and confirmed in our preliminary studies that calcium, which accumulates in ATP-depleted red cells, is recovered (>60%) from the ghosts. Our results indicate that the diminished ghost swelling was not due to retention of hemoglobin by membranes, since the increase in hemoglobin concentration of ghosts derived from stored cells was proportional to the decrease in ghost volume, e.g., no net increase in hemoglobin content occurred (Fig. 6).

On the other hand, increased membrane rigidity and decreased passive permeability due to calcium accumulation were predictable causes of small ghosts formation, since calcium was shown to cause decreased membrane viscoelasticity\textsuperscript{7} and passive permeability.\textsuperscript{9} Decreased passive permeability of ghosts was further suggested by ghost osmotic behavior (Fig. 7) that demonstrated that ghosts derived from cells after prolonged storage or fresh erythrocyte ghosts exposed to calcium responded to increases in tonicity of the medium by a greater decrease in volume, indicating an increased NaCl gradient and hence, decreased permeability. This observation is in agreement with findings of decreased cation permeability of erythrocytes after exposure to calcium.\textsuperscript{48-50} Although others found that erythrocyte calcium accumulation during metabolic depletion is associated with potassium efflux.\textsuperscript{16,51,52} This apparent discrepancy requires further clarification. Perhaps other factors in addition to membrane permeability determine volume behavior, such as conformational state of membrane fibrous proteins and membrane rigidity. Alternatively, the K\textsuperscript{+} efflux from ATP-depleted cells can reflect contraction of cell membrane
and cell volume, resulting from calcium-mediated conformational changes in membrane fibrous proteins.

Previous studies suggested that ghost size and membrane deformability depend on the conformational state of membrane fibrous proteins, which is controlled by intracellular ATP and calcium content. The isolation and some characteristics of these proteins have been described. Our data are compatible with the conclusion that the formation of small ghosts reflects increased rigidity and decreased permeability of red cell membranes due to ATP-dependent conformational changes (contraction) of membrane fibrous proteins. This is further substantiated by our findings that small ghost formation resembles an “all or none” phenomenon. That is, the accumulation of calcium and the decrease in ATP in red cells during storage was associated with a progressive increase in number of erythrocytes yielding population of markedly smaller ghosts than those of fresh erythrocytes, while no ghosts of intermediate size were present. Such a phenomenon and its reversibility is compatible with a model of reversible changes in conformation of membrane fibrous proteins, possibly of contractile nature, dependent on erythrocyte ATP and calcium content. Thus, in addition to changes in red cell membrane lipid content, ATP-dependent alterations in physical properties of red cell membrane fibrous proteins are important determinants of injury to the erythrocyte membrane during storage.

The increase in rigidity of red cells in stored blood was found to be proportional to the decreases in cell filtrability in vitro and cell viability in vivo. We have, therefore, compared the percentage of small ghosts at various times of storage with viability data as reported in the literature (Fig 8). A good correlation between the percentage of small ghosts and that of nonviable cells was observed, suggesting that small ghosts derived from rigid, “contracted” red cell membranes are related to nonviable cells.

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