The Dependence of Shape of Human Erythrocyte Ghosts on Calcium, Magnesium, and Adenosine Triphosphate

By Jiri Palek, Gwendolyn Stewart, and Fabian J. Lionetti

ATP depletion of erythrocytes is associated with discocyte-echinocyte transformation and accumulation of calcium. This study was undertaken to evaluate whether this shape transformation results from interaction of Ca\(^{2+}\), Mg\(^{2+}\), and ATP with structural proteins of the membrane. ATP, Mg\(^{2+}\), or Ca\(^{2+}\) were incorporated into ghosts during hypotonic hemolysis followed by restoration of isotonicity with NaCl and incubation. Reconstituted ghosts were examined by electron microscope after osmium tetroxide fixation followed by platinum and carbon shadowing and in-phase contrast microscope. Four ghost configurations were observed: (1) flat, discoidal electron-penetrable ghosts were produced when ghosts were prepared without any additives, or with ATP alone, Mg\(^{2+}\) alone, or EDTA; (2) biconcave and cup-shaped less electron-penetrable ghosts predominated when equimolar concentrations of Mg\(^{2+}\) and ATP (0.5-2 mM) were introduced into ghosts during hemolysis; (3) spherical, spiculated, or markedly deformed electron-dense ghosts of smaller volume were the prevalent shape forms when Ca\(^{2+}\) alone was introduced during hemolysis; (4) small, smooth, electron-dense spheroids were present when both Ca\(^{2+}\) and Mg ATP (2 mM) were added during hemolysis. The above shapes were independent of ouabain, monovalent cations (NaCl, KCl, or choline chloride), transmembrane osmotic gradient, and ghost volume. The shape effects of Ca\(^{2+}\) and Mg ATP were prevented by preincubation of red cells with SH-blocking agent N-ethylmaleimide (4 mM) and by repeated ghost washing, which was associated with removal of water-soluble fibrous proteins from membranes. It is concluded that the interaction of Ca\(^{2+}\), Mg\(^{2+}\), and ATP with membrane structural proteins exerts a significant role in the control of shape of human erythrocytes.

THE MECHANISM whereby red cells maintain their biconcave shape has been a subject of numerous studies,\(^1,13\) and several explanations for red cell biconcavity have been proposed.\(^1,5,7-11,13\) One of the critical factors for the maintenance of biconcave shape is the level of red cell adenosine triphosphate (ATP, see references 14-16). It has been suggested that the maintenance of biconcavity by ATP is mediated by ATP interaction with fibrillar contractile-like proteins.\(^14-16\) Such systems have been found in a number of cell types.\(^17,20\)

Since the original report on actomyosin-like red cell proteins by Ohnishi,\(^21\) several groups have isolated one or more fibrillar proteins from red cell membranes.\(^22,28\) These proteins differed in the presence or absence of ATPase activity,\(^22,25\) solubility and molecular sieving characteristics,\(^22,24,28\) suggesting

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that they represented several classes of membrane proteins. Although the physiologic role of these proteins requires further clarification, they have been suggested to be involved in control of cell shape, volume, viscoelastic and osmotic properties.\(^{14-16,25-29,30}\)

The present investigation was undertaken to study the shape dependence of reconstituted ghosts on calcium, magnesium, and ATP content and to see if the shape-regulating role of these substances is mediated by their interaction with fibrous proteins of red cell membranes. Preliminary results have been published.\(^{31}\)

**MATERIALS AND METHODS**

**Red Cells**

Venous blood was collected from healthy volunteers into acid-citrate-dextrose anticoagulant (ACD, NIH Formula A) and used within 48 hr of storage at 4°C. Red cells were isolated by centrifugation at 2500 \(g\) for 15 min. The supernatant and buffy coat were discarded by aspiration, and red cells were washed three times with four volumes of isotonic saline solution.

**Reconstituted Ghosts**

Reconstituted ghosts were prepared by osmotic hemolysis followed by restoration of isotonicity.\(^{32,33}\) One volume of washed red cells was hemolyzed in 20 volumes of 5 mM Tris-HCl buffer, pH 6.9, under constant mixing on a whirl-mixer for 2 min at 22°C. Unless otherwise stated, the tonicity was subsequently adjusted to 290 milliosmols by \(\text{NaCl}, \text{KCl}, \text{or choline chloride}.\) In some of the experiments the hemolytic buffer contained various concentrations of \(\text{CaCl}_2, \text{MgCl}_2, \text{and/or the sodium salt of adenosine triphosphate (ATP)}\). It has been established that under the above conditions, considerable amounts of ATP can be introduced into ghosts.\(^{34,35}\) This is due to the loss of the selective membrane permeability at the time of hemolysis and immediately thereafter, allowing the introduction of various nonpenetrating compounds into the cells. Careful attention has been given to pH of hemolytic solutions containing various additives, which was always adjusted to pH 6.9. Unless otherwise stated, ghosts were subsequently incubated for 1 hr at 37°C.

**White Ghosts**

White ghosts were prepared as described by Dodge et al.,\(^{36}\) except that Tris-HCl (5 mM, pH 7.15) with 2 mM EDTA was used instead of phosphate buffer. EDTA was omitted in the last ghost washing.

**Phase Contrast Microscopy**

Reconstituted ghosts were sedimented by centrifugation at 12,000 \(g\) for 15 min, and the supernatant was discarded. Ghosts were examined either untreated or fixed in 1\(^{\text{st}}\) \(\text{osmium tetroxide (see below).}\)

**Electron Microscopy**

Reconstituted ghosts were sedimented by centrifugation at 15,000 \(g\) for 30 min. The supernatant was removed and the ghost pellet resuspended in 1\(^{\text{st}}\) \(\text{osmium tetroxide in saline (290 milliosmols) buffered with phosphate (pH 7.4). The suspension was held in ice for 30-60 min. The fixed ghosts were sedimented at 1800 \(g\) for 3 min, rinsed twice with distilled water, and dehydrated with 100\(^{\text{th}}\) \(\text{ethanol. A suspension of ghosts in 100\(^{\text{th}}\) \text{ethanol was flooded on the surface of a washed air-dried glass slide and the excess drained off. The dry slides were shadowed at an angle of 25°-30° at a distance of about 4 cm from the source. After evaporating 1 cm of 8-mil platinum wire, a heavy coat of carbon was evaporated on the surface at a 90° angle. The slide was covered with a thin film of 1\(^{\text{st}}\) \(\text{Formvar in ethylene dichloride to form a laminate consisting of ghosts, platinum, carbon, and plastic. The specimens were stripped onto the surface}}\)
of distilled water from which they were picked up on 150-mesh grids and examined by electron microscopy.

**Ghost ATP, Calcium, and Volume**

Ghosts were isolated by centrifugation at 42,000 g on a Sorvall refrigerated high-speed centrifuge at 4°C for 30 min. ATP was determined enzymatically with hexokinase and glucose-6-phosphate dehydrogenase. Calcium was measured by atomic absorption spectrophotometry of ethanol-HCl extracts of dry ashed ghosts as previously described. Ghost volume has been determined from counts and microhematocrits of ghost suspensions as described.

**Extraction of Nonhemoglobin Proteins From Ghosts**

After resealing, the reconstituted ghosts were washed twice with 0.9% saline containing 2mM Mg ATP. The washed ghosts were suspended in the same medium, mixed, and divided into two aliquots. One aliquot was homogenized with a Vertis Macromiliber (Model 23) set at full speed for 5 min in an ice bath. Parts of the aliquots of both homogenized and intact ghosts were extracted overnight (4°C) with 0.6 M KCl. The fragments resulting from homogenization and the aliquot of intact ghosts were both sedimented at 48,200 g for 60 min at near 0°C. The supernatant was removed and checked for membrane contamination with light and electron microscopy and held below 5°C until assayed. Total protein in the supernatant solution was measured by the method of Lowry et al., using both albumin and hemoglobin as standards with little difference between the two. Hemoglobin was measured by the Hycel cyanmethemoglobin method and with benzidine. Because the amount of hemoglobin was small, 2 ml of the KCl extract were added to 4 ml of Hycel reagent. The sedimented fragments were fixed for electron-microscope examination before and after extraction.

**RESULTS**

**Morphologic Responses of Reconstituted Ghosts to Mg²⁺, ATP, and Ca²⁺**

Figure 1A shows the shape of control ghosts prepared by osmotic hemolysis of 1 volume of fresh erythrocytes in 20 volumes of 5 mM Tris-HCl buffer, pH 6.9, followed by restoration of isotonicity by NaCl and incubation. These ghosts were flat with a coarsely granulated surface. Their shape was unchanged when EDTA (2 mM) was present in the hemolyzing solution.

The effect of Mg ATP on ghost shape is shown in Fig. 1B. Mg ATP ghosts were prepared in an identical fashion as the control preparation, except that the hemolytic solution contained 2 mM Mg ATP. The ATP concentration in such ghosts was 1.2 ± 0.2 μmoles/ml of ghosts. Mg ATP ghosts consisted of a variable proportion of biconcave and cup-shaped ghosts, which were both counted as concave ghosts (86% ± 12%, mean ± SD). Only a few ghosts lacked the central concavity (8% ± 3%). The surface texture of Mg ATP ghosts was finely granular, although ghosts with coarsely granular surfaces were occasionally noted. The characteristic concave shape of Mg ATP ghosts required both Mg²⁺ and ATP. Either Mg²⁺ or ATP alone (2 mM) produced ghosts similar to the control preparation.

The shape of ghosts prepared when Ca²⁺ (2 mM) was present in the hemolytic solution is shown in Fig. 1C. The most frequent shape (84% ± 6%) was that of small, dense, and extensively spiculated spheres. Occasional ghosts were flat, with a finely granular surface and smooth edges (6% ± 4%). The effect of calcium did not require the simultaneous presence of ATP. On the contrary, high concentrations of ATP were inhibitory (Fig. 3). The simultaneous addition of 2 mM Ca²⁺ and Mg ATP to the hemolytic solution produced relatively
Fig. 1. Transmission electron micrographs of fixed, shadow-cast preparations of reconstituted ghosts prepared from fresh human red cells. These ghosts were prepared by exposing 1 volume of washed, packed red cells to 20 volumes of 5 mM Tris-HCl buffer, pH 6.9, with or without divalent cations and ATP at 22°C. The ghosts were resealed by restoring the isotonicity by the addition of 9% NaCl and incubation at 37°C for 1 hr. (A) Control—Neither divalent cations nor ATP were added to this sample. The ghosts are flat with a coarsely granular surface. They are much thicker (as indicated by electron density) than the serially washed ghosts shown in Fig. 7A. 4800 x. (B) Mg ATP—Mg ATP was incorporated into ghosts by adding 2 mM MgCl₂ and sodium salt of ATP to the hemolyzing solution. Most of the ghosts have regained some degree of curvature which most likely reflects an attempt to return to the biconcave form. The surface of some is extremely finely granular while that of others is moderately granular. 4800 x. (C) Ca²⁺—Calcium (2 mM) was incorporated into ghosts during hemolysis. Most of the structures are roughly spherical and extensively spiculated while a few are flat with only a few protrusions. 4800 x. (D) Ca²⁺ Mg ATP—Calcium and magnesium ions as well as ATP were incorporated into ghosts. They have regained more curvature and become more dense than the others. 3400 x, inset 4800 x.

homogenous small spheroidal ghosts, some of which contained a small central concavity (Fig. 1D).

The above-described shape effects of Mg ATP and Ca²⁺ were also observed when these compounds were introduced into ghosts after hemolysis (not shown), suggesting that they did not result from a mere inhibition of the egress of membrane structural proteins during hemolysis.

During the course of the studies, it was noted that ghosts acquired the above shapes immediately after restoration of isotonicity. Therefore, in the subsequent studies ghost incubation after restoration of isotonicity was shortened or omitted, as specified below.
Shape Dependence on Concentration of Mg ATP and Ca\(^{2+}\)

Figure 2 shows the relationship between the concentration of Mg ATP in the hemolytic buffer, amounts of ATP introduced into ghosts, and the percentage of ghosts which achieved concave shape. The lowest ATP concentration in the hemolytic solution at which 80% of ghosts were concave discs was 0.5 μmoles/ml of hemolytic solution. This resulted in intracellular ghost ATP content of
Fig. 4. Inhibition of ghost shape response to Mg ATP and Ca\(^{2+}\) by N-ethyl-maleimide (NEM). Phase-contrast micrographs of ghosts prepared from fresh erythrocytes preincubated as 20% suspension with Tris-HCl-buffered isotonic saline solution (pH 7.4) containing NEM (4 mM) for 30 min at 37°C. Ghosts were prepared as outlined in Fig. 1.
ouabain (2 mM) was added to the hemolytic buffer. In contrast, shape responses to Mg ATP or Ca\(^{2+}\) were completely abolished by preincubation of red cells with the SH-blocking agent N-ethylmaleimide (NEM) (Fig. 4).

**Ghost Osmotic Behavior**

Figure 5 shows differences in ghost osmotic behavior expressed as a plot of ghost volume against the reciprocal of osmolarity. Ca\(^{2+}\) ghosts and, in a lesser magnitude, Mg ATP ghosts exhibited considerable shrinkage in solutions of increasing tonicities, indicating decrease in membrane ion permeability. Since the magnitude of osmotic shrinkage of Ca\(^{2+}\) ghosts was higher than that of Mg ATP ghosts, experiments were conducted in order to establish whether the Ca\(^{2+}\)- and Mg ATP-dependent shape changes resulted from differences in transmembrane osmotic gradient and volume between Ca\(^{2+}\) and Mg ATP ghosts or from direct interaction of Ca\(^{2+}\) and Mg ATP with structural components of the membrane. Figure 6 compares shapes of Ca\(^{2+}\) and Mg ATP ghosts suspended in solutions of different osmolarities, the volumes of which are shown on Fig. 5. It can be seen that Mg ATP ghosts suspended in solutions of increasing osmolarities exhibited concave shape at approximately 120 milliosmols. Further increase in osmolarity accompanied by concomitant decrease in volume was associated with a more pronounced concave shape. Ca\(^{2+}\) ghosts, on the other hand, exhibited a distorted, echinocytic shape within a wide range of tonicities, and they did not resemble Mg ATP ghosts at any of the osmolarities tested. Thus, both the Mg ATP-dependent discocyte configuration and the
Fig. 6. Phase-contrast micrographs of ghosts at various tonicities of the hemolysate. Ghosts, prepared as described in Fig. 5, were centrifuged and fixed with 1% osmium tetroxide, the osmolarity of which was adjusted to that of the original hemolysate. Left, MgATP ghosts; right, Ca$^{2+}$ ghosts. The individual micrographs correspond to volumes and tonicities indicated by opened symbols in Fig. 5 and follow a sequence from the lowest to the highest osmolarities.

Ca$^{2+}$-dependent echinospherocytic shape were independent of cell volume, consistent with observations of others that human red cells undergoing disc-sphere transformation may maintain a constant volume.32

The Site of Mg ATP Effect on Ghost Shape

Earlier studies have shown that red cell membranes were highly permeable to ATP at the time of hemolysis. The ATP permeability markedly decreased after subsequent restoration of isotonicity and incubation.33-35 On the basis of this observation, we designed an experiment in which we compared shape and ATP content of the following ghost preparations: (1) ghost made with Mg ATP (0.5 mM) present during hemolysis, and (2) ghosts into which Mg ATP (0.5 mM) was added after hemolysis and restoration of isotonicity, when partial ghost resealing took place. Results are shown in Table 2. It can be seen that in
Table 2. Comparison of Effects of Internal and External ATP on Ghost Shape*

<table>
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<tr>
<th>Additives</th>
<th>Ghost ATP (μmoles/ml)</th>
<th>Concave Ghosts (%)</th>
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<tr>
<td>Mg ATP during hemolysis</td>
<td>0.42 ± 0.06</td>
<td>92 ± 6</td>
</tr>
<tr>
<td>Mg ATP after hemolysis</td>
<td>0.12 ± 0.04</td>
<td>22 ± 4</td>
</tr>
<tr>
<td>No additives</td>
<td>0.07 ± 0.02</td>
<td>4 ± 2</td>
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*One volume of red cells was homolysed by 20 volumes of 5 mM Tris-HCl buffer (pH 6.9). The isotonicity was restored by the addition of 9% NaCl and samples incubated for 15 min. at 37°C. Mg ATP (final concentration in the homolysate 0.5 mM) has been added during hemolysis or 5 min after restoration of isotonicity. Average results of four experiments.

ghosts into which ATP was introduced during hemolysis, the ATP concentration (0.4 ± 0.06 μmoles/ml of ghosts) was close to an equilibrium with the external fluid. Consequently, such ghosts were predominantly concave. In contrast, the addition of 0.5 mM ATP to the ghost suspension after partial ghost resealing resulted only in small introduction of ATP into ghosts, which was below concentration requirements for biconcavity shown in Fig. 2. As a result, only a few ghosts achieved concave shape. The external ATP concentration at

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Fig. 7. Transmission electron micrographs of fixed, shadow-cast preparations of serially washed ghosts prepared from fresh human red cells. These ghosts were prepared by three washings, each with 20 volumes of 5 mM Tris-HCl buffer with 2 mM EDTA followed by restoration of isotonicity with NaCl and incubation (30 min, 37°C). EDTA was omitted during the last washing. Ca²⁺ or Mg ATP (final concentration 2 mM) were introduced into ghosts by adding them to the ghost suspensions, the pH of which was adjusted to 6.9 before restoration of isotonicity. Control—No divalent cations or ATP were incorporated into ghosts. They are extremely thin, being so electron transparent that several thicknesses are visible in the area of the folds. The small aggregates of material may correspond to precipitates of hemoglobin or membrane non-hemoglobin protein. 4800 ×. Ca²⁺—The incorporation of calcium into ghosts caused some irregular aggregation of material giving rise to areas with increased electron density. The very minimal response here is in a marked contrast to the extensive spiculation exhibited by reconstituted ghosts in the presence of Ca²⁺ (Fig. 1C) and indicates that the material responsible for the response of thick ghosts was removed during subsequent washings. 4800 ×. MgATP—The incorporation of MgATP into thin ghosts caused no response in some and only aggregation of a small amount of material in others. 4800 ×.
both conditions was identical, indicating that the formation of concave ghosts was related to ATP concentration inside ghosts.

The Lack of Response of Washed (White) Ghosts to Ca\(^{2+}\) and Mg ATP

The shape of white ghosts prepared by three washings in 20 volumes of 5 mM Tris-HCl, pH 7.15, with 2 mM EDTA followed by restoration of isotonicity with NaCl is shown in Fig. 7. Such ghosts were thinner and more electron penetrable than the control preparation of reconstituted ghosts. They contained less than 0.05\% of the original hemoglobin. The addition of Ca\(^{2+}\) or Mg ATP (2 mM) into the suspension of such ghosts prior to restoration of isotonicity by NaCl failed to produce the morphologic changes seen in reconstituted (thick) ghosts, although the content of ATP and Ca\(^{2+}\) in such ghosts was 1.1 ± 0.2 and 1.6 ± 0.3 μmoles/ml ghosts, respectively. Occasional areas of increased density and granular appearance were seen within the ghosts; these were interpreted as precipitates of unknown intracellular substances, presumably traces of membrane structural proteins, retained by such ghosts (see below).

Extraction of Nonhemoglobin Proteins From Ghosts

Overnight extraction of intact Mg ATP reconstituted ghosts with 0.6 M KC1 removed considerable hemoglobin and a small amount of nonhemoglobin protein. However, extraction of fragments resulting from homogenization of an aliquot of the same suspension removed much more total protein, of which most was nonhemoglobin. The proportion of hemoglobin to nonhemoglobin protein varied somewhat, but always more than 55\% of the protein was nonhemoglobin. A typical example is shown in Table 3. Before extraction, the fragments were thick like reconstituted ghosts (Fig. 8A). After extraction, they had become thin but had not disintegrated (Fig. 8B).

The amount of total protein extracted from white ghosts was much lower than that of reconstituted ghosts. Aliquots of the same suspension of intact and homogenized structures yielded the same amount of protein (Table 3). This indicated that the nonhemoglobin protein, extracted by KC1 from homogenized reconstituted (thick) ghosts, was absent in white ghosts, presumably due to its removal during washing. Both intact and homogenized white ghosts underwent extensive fragmentation during extraction.

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<th>Table 3. Protein Extracted From Reconstituted and Washed Ghosts by 0.6 M KC1 Before and After Homogenization*</th>
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<tr>
<td><strong>Ghosts</strong></td>
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<td>-------------</td>
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<tr>
<td>Reconstituted, intact</td>
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<tr>
<td>Reconstituted, homogenized</td>
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<tr>
<td>White, intact</td>
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<td>White, homogenized</td>
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*Reconstituted (unwashed) ghosts and white (serially washed) ghosts were prepared as described in Materials and Methods. Both intact and homogenized ghosts were extracted overnight with 0.6 M KC1.
Fig. 8. Transmission electron micrograph of membrane fragments from reconstituted ghosts before and after extraction with KCl. (A) Before extraction the fragments were thick over most of the surface. The background is essentially free of particulate matter. 20,000 x. (B) The same fragments after 24 hr at 0°C in the NaCl-KCl extracting solution. The membranes have become thin, and there is a high concentration of roughly spherical particles in the background. 20,000 x.

DISCUSSION

The present studies demonstrate that Mg ATP and Ca$^{2+}$ exhibited direct effects on membrane configuration of reconstituted ghosts when introduced during hemolysis. These effects were independent of monovalent cations, ouabain, or transmembrane osmotic gradient and volume. In contrast, Ca$^{2+}$ and Mg ATP effects on ghost shape were completely abolished by serial washing of
ghosts. The absence of shape responses of washed (thin) ghosts paralleled the loss of nonhemoglobin protein from such ghosts (Table 4). In previously reported studies of one of the authors, it has been shown that this protein superprecipitated with Mg\(^{2+}\) and ATP and exhibited ATPase activity which was inhibited by EDTA but not by ouabain. It differed from spectrin in solubility and molecular sieving characteristics.

Both the Mg ATP-dependent discocyte configuration and Ca\(^{2+}\)-linked echinocytic shape were inhibited by SH-blocking agent, NEM. This finding further suggested that both the Mg ATP-dependent concave ghost configuration and the Ca\(^{2+}\)-induced echinospherocytic shape reflected changes in conformation of membrane fibrillar proteins, resulting from their interaction with Mg ATP and Ca\(^{2+}\). A qualitative or quantitative defect of these proteins has been found in red cells in hereditary spherocytosis. The spherocytic shape can also be produced in intact red cells by their exposure to SH-blocking agents. However, the effects of SH-blocking agents are probably more complex since red cell membrane contains a number of classes of sulfydryl groups.

The distinctly different shape changes induced by Ca\(^{2+}\) and Mg ATP likely reflect different types of interactions of the structural proteins of the membrane. Such diversity in shape response is consistent with observations that red cell membranes contain several classes of fibrous proteins which differ in solubility, molecular sieving characteristics, and ATPase activity. It should also be noted that the changes in shape are only one of several physical changes occurring as a consequence of Ca\(^{2+}\), Mg ATP membrane interactions which include changes in membrane permeability, viscoelastic properties, and microvesicle formation.

Although there was a similarity between the shape of ghosts reconstituted with Mg ATP and biconcave shape of normal erythrocytes, ghosts exhibited several differences from intact erythrocytes. They had lower ATP requirements for concave shape, probably due to different relative concentrations of Mg ATP, hemoglobin-ATP, and "free" ATP in intact red cells. There was also a heterogeneity of shapes within each group of ghosts and limited return toward typical biconcavity with variable percentage of cup-shaped forms. This could indicate that hemolysis produced some disorganization or loss of structural proteins, the degree of which depended on the age of cells or inequalities in exposure to the hemolyzing solution. The relatively low pH of the hemolytic buffer, which was chosen to facilitate the introduction of ATP into ghosts, could in part account for the formation of cup-shaped ghosts, as the decrease in pH was associated with discocyte-stomatocyte transformation.

The factors influencing discocyte-echinocyte equilibrium previously described include ATP, calcium, pH, lysolecithin, and several others. The present studies support the conclusion that Mg ATP and Ca\(^{2+}\) control discocyte-echinocyte equilibrium through their interaction with internally located fibrillar (contractile-like?) proteins, which are involved in maintenance of the typical shape of red cells. This system appears to be organized as a layer associated with, but not an integral part of, red cell membranes.
ACKNOWLEDGMENT

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