Biochimica et Biophysica Acta 815 (1985) 477–485
Elsevier

BBA 72572

Effects of preparative procedures on the volume and content of resealed red cell ghosts

Gerard B. Nash and Herbert J. Meiselman *

Department of Physiology and Biophysics, University of Southern California School of Medicine, 2025 Zonal Avenue, Los Angeles, CA 90033 (U.S.A.)

(Received November 5th, 1984)

Key words: Erythrocyte membrane; Ghost preparation; Membrane resealing; Ghost volume; Osmolality; Osmotic pressure

The effects of variations in preparative procedures on the volume and content of resealed red cell ghosts have been investigated. Following hypotonic lysis at 0°C, and after a variable delay time (t_d), concentrated buffer was added to restore isotonicity; resealing was then induced by incubation at 37°C for one hour. Using this procedure, both the resealed ghost volume and the residual hemoglobin (Hb) content decreased for increasing t_d. If ghosts were maintained at 0°C (i.e., no 37°C incubation), they remained nearly spherical until isotonicity was restored. Their volume then fell abruptly, but subsequently increased toward an intermediate level. The fall in volume was greater and the final level achieved was smaller for longer delay times. At 0°C, return to isotonicity also halted the otherwise gradual loss of residual Hb from unsealed ghosts. In addition, ghosts with internal osmolality of 40 to 300 mosmol/kg were prepared by adding different amounts of concentrated buffer before resealing for one hour at 37°C. Under these conditions, the final ghost volume was inversely related to the resealing osmolality (i.e., lower osmolality yielded a larger volume). Ghost volume also increased, along with Hb content, if the quantity or concentration of the red cell suspension added to the lysing medium was increased. We conclude that resealed ghost volume is influenced by the ratio of lysate to resealing medium osmolality and by the colloid osmotic pressure of the residual ghost Hb. These data indicate methods by which ghosts with desired characteristics can be prepared, and have potential application for studies of ghost mechanical and biophysical behavior.

Introduction

Red cell membrane ghosts can be prepared by hypotonic lysis of intact cells, and can be classified as either 'white' or 'resealed' ghosts (see Schwoch and Passow [1] for review). The former are nearly hemoglobin free, are permeable to solutes and are primarily used for biochemical studies of membrane structure and function. Resealed ghosts are formed, after hemolysis, by return of salt to the lysing medium and/or by incubation at temperatures above 0°C. These ghosts regain selective solute permeability, and since they continue to be used for studies of membrane transport [2,3], it is of interest to obtain an understanding of the mechanism and dynamics of the resealing process. In addition, red cell ghosts have frequently been used in investigations of membrane mechnical properties, and of red cell rheology [4–9]. For example, they can be used to assess the relative roles of membrane and cytoplasm in determining the response of red cells to shear forces [6,7], and to study the structural basis of membrane visco-

* To whom correspondence should be addressed.

elasticity [8,9]. For such work, it is necessary to be able to prepare ghosts with intact membrane structure and with desired size, shape and content.

The process of hemolysis itself has been widely studied [10–15]. When suspended in sufficiently hypotonic media, red cells rapidly swell to a critical spherical volume and subsequently experience loss of internal ions and hemoglobin. Immediately after lysis, ghosts may shrink slightly but subsequently return toward the maximum spherical volume (V_{max}) determined by their surface area [11–13]. Subsequent resealing of the membrane proceeds at a rate dependent on temperature and salt concentration [16,17]. Resealed ghost volume has not always been found to equal the original red cell volume [9,16,18], and it is not certain how or if the volume necessarily varies during the resealing process. In previous studies of ghost volume during and after lysis, a variety of lysing media tonicities and temperatures have been used [11–13,18–21], and the stage at which resealing of the membrane actually occurs has not always been evident. The dependence of resealed ghost characteristics on variations in the resealing and hemolysing procedure thus remains to be fully elucidated.

In the present study resealed ghost volumes and hemoglobin (Hb) contents were measured for various combinations of lysate and resealing medium osmolalities. Ghost volumes were also measured at 0°C immediately following lysis and when salt was added after various delay times. Volume changes during resealing, and the final resealed ghost volume and Hb content, were strongly dependent on this delay time. Resealed ghost volumes also varied according to the ratio of lysate to resealing medium osmolalities. Our results provide a basis for understanding the dynamics of the resealing process and indicate procedures by which resealed ghosts with desired volumes and hemoglobin and ion content can be prepared for studies of their mechanical properties.

Methods

Ghost preparation

Blood was obtained from healthy laboratory personnel via venipuncture into heparin (5 IU/ml) and used immediately. Red blood cells were washed three times via $2000 \times g$ centrifugation in phosphate-buffered saline (PBS, 0.122 M NaCl/0.030 M $KH_2PO_4 + Na_2HPO_4$/2 mg · ml^{-1} glucose (pH 7.44), 300 mosmol/kg), and the buffy coated was discarded after each centrifugation. Finally, the packed red blood cells were suspended at 12.5% (v/v) in phosphate-buffered saline and cooled to 0°C. Concentrated phosphate buffer (5 × PBS) was prepared using five times the above NaCl, KH_2PO_4, Na_2HPO_4 and glucose concentrations.

The basic method for preparation of resealed ghosts was similar to that recommended by Schwoch and Passow [1]. The lysing medium was 0°C dilute phosphate buffer (0.007 M $KH_2PO_4 + Na_2HPO_4$ (pH 7.4)) containing 0.004 M $MgSO_4$, 0.5 mg/ml adenosine triphosphate (ATP) and penicillin and streptomycin (100 U/ml each). One volume of red blood cell suspension was added to 30 volumes of lysing medium (final lysate was 40 ideal mosmol/kg (pH 7.0)). After a variable delay time (t_d, from 30 s to 120 min) at 0°C, the lysate was returned to 300 mosmol/kg by addition of 5 × PBS. After 5 min on ice, this suspension was incubated at 37°C for 1 h. The ghosts were harvested by centrifugation at $20000 \times g$ for 10 min and washed once in a large excess of phosphate-buffered saline. They were finally suspended in phosphate-buffered saline containing 0.2 g% human serum albumin and their morphology assessed by phase contrast microscopy.

The above method for preparation of 'standard' ghosts (i.e., red blood cell suspension volume/lysing medium volume = 1/30, red blood cell dilution in the lysate 1/248 (v/v), resealing medium 300 mosmol/kg) was varied for some experiments. In one series, ghosts were resealed at four different osmolalities (40, 100 and 200 as well as 300 mosmol/kg) by adding different volumes of 5 × PBS to the standard lysate. These ghosts were washed and finally suspended in phosphate buffers with osmolalities equal to the four resealing media. In another set of experiments, different ratios of red blood cell suspension to lysing medium volume were used (1 : 2.5, 1 : 10 as well as 1 : 30) before resealing at 300 mosmol/kg. At these dilution ratios, ghosts were prepared using concentrated red blood cell suspensions of nearly 100% cells (i.e., centrifuged packed cells) as well as the standard 12.5% suspension. When the concentrated

red cell suspensions were used, or when hemoglobin measurements were to be made, extra washes of the final ghosts were carried out until the supernatant was visually free of hemoglobin.

Measurement of ghost volumes

Mean ghost volumes and volume distributions were measured electronically using a computerized Electrozone Celloscope orifice system (Particle Data, Inc, Elmhurst, IL, model 112 LA/ADCW); suspensions were diluted to approximately 50 000 ghosts/ml with media of appropriate osmolality. This system has an adjustable normalizer which compensates for changes in suspending medium conductivity and temperature. Measurements indicated that for inert test particles (i.e., latex beads and fixed red blood cells), this normalization provided volume data that were unaffected by the range of conductivities and temperatures used in the present study. The Celloscope was operated using a pulse width editor which eliminated from the pulse height analysis the widest 20% of the pulses. This editing procedure removes the artifact caused by non-axial transit of some cells through the orifice; such cells experience greater electric field strengths in the aperture and smaller deforming flow forces, and yield inappropriately large pulse heights [22].

The height of the electronic pulse generated as a cell passes through the sizing orifice of the Celloscope or similar devices is proportional to the cell volume multiplied by a shape factor, S [23]. Thus, pulse height analysis allows measurement of cell volume only when S is known. Experimentally, calibration of the Celloscope was carried out using latex spheres with known volume (Dow Chemical Co., Indianapolis, IN), and by assuming a shape factor of 1.50 for the spheres and 1.05 for the cells (a value considered appropriate for normal intact red blood cells). However, measured ghost volumes had to be corrected, since this assumed cell shape factor of 1.05 will not be valid for all cases (e.g., swollen ghosts).

Volume corrections were calculated using a procedure similar to that presented by Richieri et al. [24]: (a) we assumed that in the sizing aperture, ghosts deform to a prolate ellipsoidal shape [22,25]; (b) for a given surface area and volume there is only one possible axial ratio for the ellipsoid, which can be calculated from simple geometry; (c) the shape factor for such an ellipsoid can be determined from previously derived formulae [23]. Thus, for a given cell surface area and true volume (V_t), the true shape factor (S_t) can be predicted. The measured volume (V_m, which would be obtained under the assumption $S = 1.05$) is related to V_t via the expression $V_m = V_t \cdot S_t/1.05$. Fig. 1 shows predicted plots of V_m vs. V_t for various constant cell surface areas. Essentially, the plots show the deviation of S from the value of 1.05 (depicted by the dashed line $V_m = V_t$) as a function of volume at constant surface area. Measured ghosts volumes were corrected using this figure; their membrane surface area was assumed to be 135 μm^2 [26,27] and not to change during lysis or resealing [14]. Note that in Fig. 1, $V_t \sim V_m$ in the region 85–95 μm^3 for surface areas between

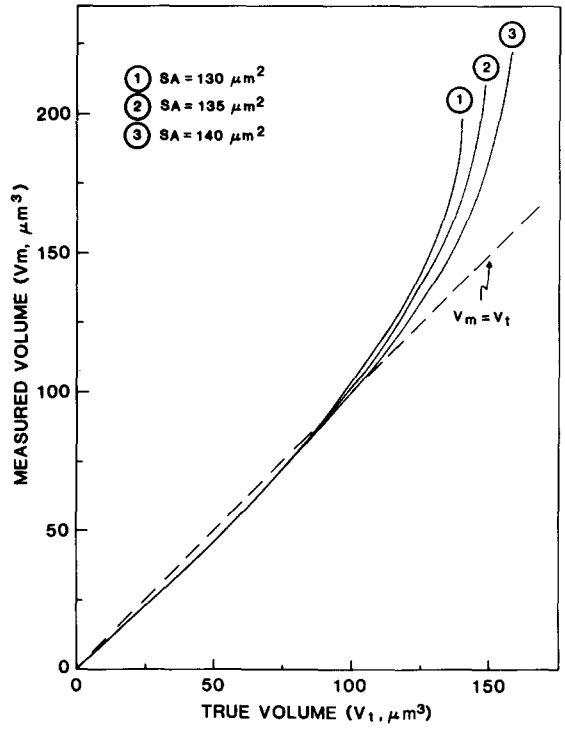

Fig. 1. Theoretically predicted relations between experimentally measured volume (V_m) and true volume (V_t) for cells with a constant surface area (SA) of either 130, 135 or 140 μm^2. The measured volume would be obtained using an orifice-type system (i.e., Celloscope) by assuming the cell shape factor to be 1.05. The dashed line indicates equality between V_m and V_t, which would be correct if the shape factor was 1.05 over the entire volume range.

130–140 μm^2. This agreement indicates that a shape factor of 1.05 is, in fact, a reasonable approximation for normal red cells whose volumes and areas fall in these ranges.

In one series of experiments, ghost volumes were meaured at intervals immediately after lysis at 0°C and after 5 × PBS was added to the cold lysate. The Celloscope sample container and sizing orifice were also ice cooled. In these studies, a more dilute red blood cell suspension was added to the lysing medium in order to keep the final ghost concentration within the limits necessary for sizing in the Celloscope. Nevertheless, the volume ratios of lysing medium, sample suspension and 5 × PBS were kept as described above for the standard ghost preparation.

Miscellaneous techniques

The quantity of residual Hb present in ghosts after incubation and washing was analyzed by the pyridine hemochromagen technique of Dodge et al. [28]. Calibration was carried out using red blood cells whose Hb had independently been determined by the cyanomethemoglobin method. Sodium and potassium contents of lysates and of resealed ghosts washed in isotonic choline chloride solution were measured by flame photometry. Solution osmolalities were measured using a freezing point osmometer (Model 2007, Precision Systems, Inc., Sudbury, MA).

As a test for ghost resealing, ghost suspensions were placed on cushions of dense sucrose solution (43% w/w in half-strength phosphate-buffered saline) and centrifuged at 27000 × g for 1 h. Unsealed ghosts, permeable to sucrose, pass to the bottom of the cushion, whilst resealed ghosts remain at its top [1]. The upper and lower halves of the centrifuged ghost/sucrose suspensions were separated and diluted, and the number of ghosts present in each portion were counted with the Celloscope.

Results

Ghosts prepared by the above mentioned procedures had smooth, regular outlines if ATP and magnesium were present in the lysing medium, but if either were omitted, numerous spiculed, echinocytic shapes occurred. The volumes and shapes of ghosts were greatly influenced by the period of time allowed to elapse between lysis (i.e., addition of red blood cells to the 0°C lysing medium) and return to isotonicity (i.e., addition of concentrated buffer solution to the lysate). As this delay time, t_d, was increased, the final, post-incubation mean ghost volume (MGV) decreased. For standard ghosts (Fig. 2), the mean ghost volume fell from approximately 135 μm^3 to only 40 μm^3 as t_d was increased from 30 s to 120 min. Microscopic observations revealed that for this range of t_d, the morphology of incubated ghosts changed from ellipsoidal and rounded shapes to thin, flattened discs and cups. Note that with $t_d = 2$ min, standard ghosts had shapes and volumes very close to those for intact erythrocytes. For any given t_d, ghost shapes were relatively uniform and their volume distribution was unimodal. Thus, changes in mean ghost volume with t_d did not result from formation of a gradually increasing proportion of small ghosts, but from changes in volume of the whole population.

Centrifuged ghost pellets were much pinker for shorter delay times ($t_d < 2$ min). Measurement of residual Hb content confirmed that ghost Hb decreased with increasing t_d, from approx. 9% of that present in intact cells for $t_d = 30$ s, to less than 1% for $t_d = 45$ min (Fig. 2). Microscopically, the 30 s ghosts appeared to contain populations of

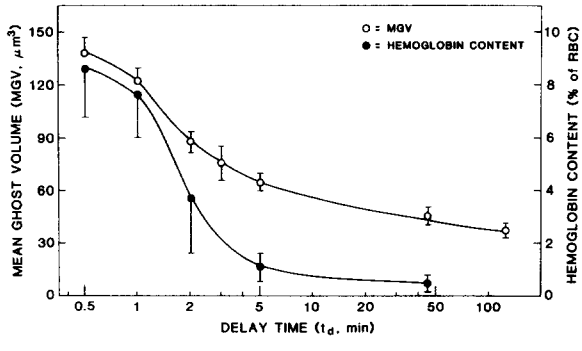

Fig. 2. Mean ghost volume (MGV) (○) and hemoglobin content (●) of resealed ghosts plotted as a function of the delay time (t_d) between lysis and addition of concentrated buffer (i.e., 5 × PBS) to restore isotonicity. Ghosts were resealed via incubation at 37°C for one hour after addition of the buffer. Points represent mean ± S.D. of two or more measurements for blood from two or three donors (MGV) or of three measurements for three donors (hemoglobin content).

relatively 'dark' and 'light' ghosts; as t_d was increased, less dark ghosts were observable. Note that the dependence of ghost shape, volume and pellet color on t_d was very similar if the lysate was returned to isotonicity by addition of concentrated NaCl solution rather than concentrated buffer.

To better understand the dependence of mean ghost volume on delay time, ghost volumes were measured at 0°C immediately following lysis and at various times after their return to isotonicity. Fig. 3 shows the variation of ghost volume as a function of the time elapsed after lysis and also after addition of concentrated buffer. Ghost volume in the 0°C lysate alone was nearly constant from 30 s up to 2 h. A slight increase in volume appeared to occur in the first 5 min, but otherwise, the ghosts had volumes only a few percent below V_{max} (the maximum volume enclosable by a surface area of 135 μm^2). Microscopic observation of ghosts, fixed by addition of 0°C glutaraldehyde to the lysate, confirmed that they were nearly spherical. However, when concentrated phosphate-buffered saline was added to the lysate, ghost volume fell rapidly, and was at a minimum within the 30 s required to make the first measurement. Subsequently, volume increased slowly over a period of about 30 min, until a final intermediate level was reached. The extent of the initial drop, and the final volume attained, depended strongly on the delay time (i.e., t_d, the time at addition of buffer); the initial fall in volume was less and the final volume was greater for shorter t_d. In addition, for a given t_d, if concentrated sucrose solution was added to the cold lysate instead of 5 × PBS, ghost volume fell further and final ghost volume was less (Fig. 3).

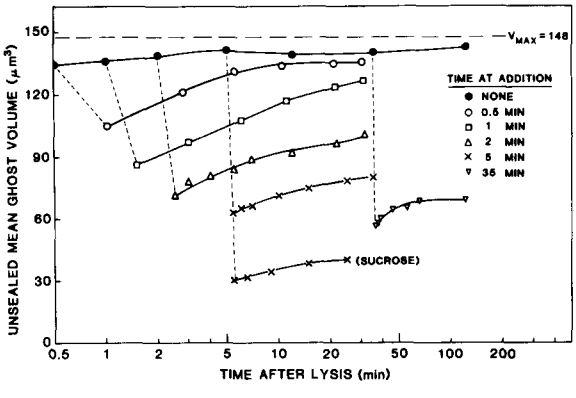

Fig. 3. Unsealed mean ghost volume plotted as a function of time after lysis; data are shown for ghosts in 0°C lysate both before and after addition of concentrated buffer (i.e., 5 × PBS) to restore isotonicity. In this series of experiments, no 37°C incubation was carried out. Dashed lines link the volume at the time of addition of concentrated buffer to the volume measured 30 s later, and thus indicate the magnitude of the rapid volume decrease consequent to the addition of this buffer. In one case data are shown for addition of concentrated sucrose solution to restore isotonicity instead of buffer. The points representing volumes measured without addition of buffer (●) are means of two to four measurements on two or three donors. The other curves are typical data from individual experiments, and are reproducible.

Although ghost membranes are expected to remain relatively permeable at 0°C (i.e., unsealed), Fig. 3 indicates that addition of solutes (e.g., 5 × PBS) causes their volume to fall. When different quantities of salt were added (i.e., resealing medium osmolality was altered), the final, post-incubation mean ghost volume also varied. Fig. 4A shows incubated ghost volumes as a function of t_d for resealing media having osmolalities of 40, 100, 200 and 300 mosmol/kg. As before (Fig. 2), the mean ghost volume generally fell for greater t_d, although the decrease in volume with increasing t_d appeared delayed for ghosts resealed at 100 mosmol/kg. Note that ghosts subjected to smaller increases in osmolality before resealing had higher volumes; ghosts incubated in lysate with no added phosphate-buffered saline (40 mosmol/kg) retained volumes just below V_{max}. Thus the final ghost volume depends on the resealing medium osmolality as well as t_d.

To study the effect of salt concentration further, the volume of red blood cell suspension added to the lysing medium was varied, thereby changing the lysate osmolality before addition of the 5 × PBS. Fig. 4B shows the post-incubation mean ghost volume as a function of t_d for ratios of red blood cell suspension to lysing medium of 1 : 2.5, 1 : 10 and 1 : 30. The corresponding osmolalities of the lysates would be approximately 110, 55 and 40 mosmol/kg; all ghosts were returned to 300 mosmol/kg for resealing. Again, final ghost volume fell as t_d was increased, but higher lysate osmolalities yielded larger mean ghost volumes for each t_d. Thus independently changing either lysate (Fig. 4B) or resealing medium

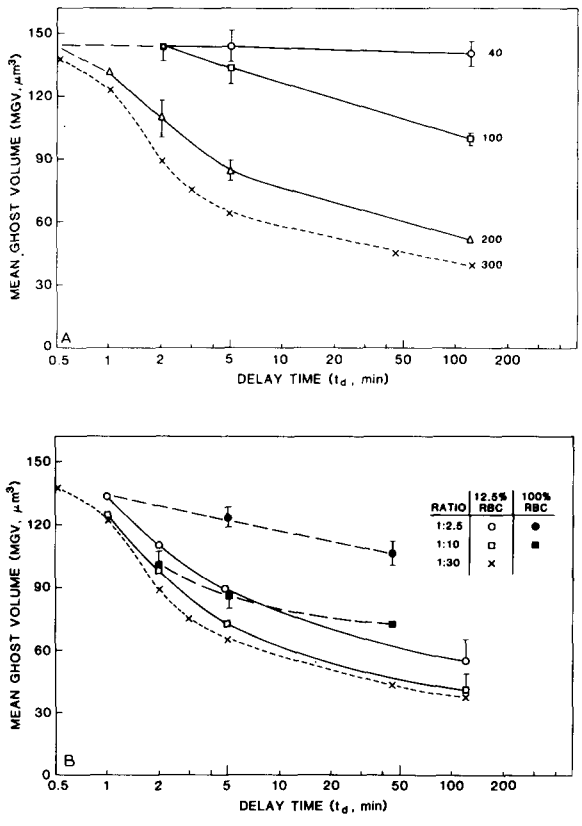

Fig. 4. Mean ghost volume (MGV) of resealed ghosts plotted as a function of delay time (t_d). Points represent mean ± S.D. of two or more measurements on two or three donors. Where error bars are not shown, they fall within the limits of the symbols, except for the data (×) for standard ghosts which are shown in detail in Fig. 2. (A) Effect of resealing medium osmolality on mean ghost volume . Data are shown for ghosts resealed at four osmolalities (40 (○), 100 (□), 200 (△) and 300 (×) mosmol/kg), obtained by adding different quantities of 5×PBS to the lysate before the 37°C, 1 h incubation. (B) Effect of varying the red blood cell suspension quantity and concentration on mean ghost volume. Data are shown for ghosts prepared using different red blood cell suspension to lysing medium volume ratios (1:2.5 (○); 1:10 (□) and 1:30 (×)) and using different red blood cell suspension concentrations (12.5% open symbols, 100% closed symbols). The ghosts were all resealed (37°C, 1 h) at 300 mosmol/kg.

osmolality (Fig. 4A) affects ghost volume, indicating that the ratio of resealing medium to lysate osmolality is a determinant of the resealed mean ghost volume rather than the absolute osmolality value of either medium. For the lower red blood cell suspension to lysing medium volume ratios (i.e., 1:2.5 and 1:10), packed cell suspensions were also used (approx. 100% red blood cells) to prepare ghosts with high Hb content. The mean ghost volume values obtained using 100% red blood cells were elevated above those obtained using 12.5% red blood cells, especially for longer t_d and for the 1:2.5 ratio (Fig. 4B). For $t_d \geq 5$ minutes, the Hb contents of these ghosts averaged 37% and 9.6% of red blood cell levels for the ratios 1:2.5 (three measurements) and 1:10 (five measurements), respectively.

Ghost suspensions were centrifuged on dense sucrose cushions to test whether the incubated ghosts had indeed resealed and whether resealing depended on t_d. For t_d up to 1 min, 90% of standard ghosts remained at the top of the cushion; for $t_d \geq 2$ min more than 95% were at the top. Also, > 95% of ghosts incubated at 40 or 100 mosmol/kg remained at the top ($t_d = 2$ min). Incubated ghosts were also found to be osmotically active, shrinking or swelling in media which were hyper-osmotic or hypo-osmotic with respect to their resealing media. The ghosts acted as nearly 'perfect osmometers', with their volumes inversely proportional to osmolality (data not shown); this osmotic behavior was observed for ghosts resealed after various t_d and at various osmolalities. These findings indicate that, regardless of t_d and resealing medium osmolality, ghosts incubated at 37°C become resealed, i.e., they become impermeable to sucrose and regain selective permeability to buffer solutes. It was also noted that, when stored, the volume of incubated ghosts tended to increase slowly. For example, during 24 h at 4°C, the mean ghost volume rose by 10 ± 4% (data from 11 samples; $t_d \geq 2$ min), suggesting that there was slow accumulation of buffer ions during this period.

For comparison to ghosts prepared by the standard technique, 'white' ghosts were prepared, by the method of Dodge et al. [28]. Red blood cells were lysed then washed three times in hypotonic phosphate buffer at 0°C (20 ideal mosmol/kg (pH 7.4)) via centrifugation at 20000 × g, no salt was added nor was incubation carried out. In their final hypotonic medium these samples contained ghosts of widely varying shape, ranging from flat discs to spheroids. Their volume was intermediate (123 μm^3, mean of three samples) and their volume distribution was broad (mean coefficient of variation 41%, versus 26% for standard ghosts with

$t_d = 2$ min). Since ghosts lysed at 0°C otherwise remain nearly spherical (see above), centrifugation at high g probably collapsed these unsealed ghosts (i.e., forced water and solutes out). In fact, white ghosts resuspended from the lowest portion of the centrifuged pellet were mainly flattened in shape. Thus, using the method of Dodge et al. [28], it appears to be difficult to prepare unsealed, white ghosts with uniform shape and volume.

Discussion

The findings of the present study can be summarized as follows.

1. Ghost volume behavior at 0°C

(a) In 0°C lysate, before addition of concentrated buffer, ghosts had a nearly spherical volume for up to 120 min, though a slight increase in volume was apparent in the 5 minutes immediately following lysis.

(b) When concentrated buffer was added to the lysate, ghost volume rapidly fell, but then slowly increased toward an intermediate level.

(c) This fall in volume was greater, and the final volume achieved was smaller, if the delay time (t_d) between lysis and addition of buffer was made longer.

2. Volume and hemoglobin content of resealed ghosts

(a) Mean ghost volume (MGV) of incubated ghosts was smaller for longer delay times.

(b) Residual hemoglobin content was also lower for longer delay times.

3. Effect of lysate and resealing medium osmolality on resealed ghost volume

(a) Resealed ghosts could be prepared with internal osmolality from 40 to 300 mosmol/kg by varying the resealing medium osmolality. The mean ghost volume was inversely related to the resealing osmolality.

(b) Conversely, the mean ghost volume was greater if the lysate osmolality was raised (i.e., by increasing the proportion of red blood cell suspension added to the lysing medium).

(c) Thus, the mean ghost volume reflects the ratio of lysing/resealing medium osmolality.

4. Preparation of ghosts with high hemoglobin content

(a) Resealed ghosts with high residual Hb content could be prepared by using concentrated red blood cell suspensions.

(b) The mean ghost volume was also elevated for these high hemoglobin ghosts.

Finding 1a agrees qualitatively with previous studies [11–13]. The apparent rise in ghost volume immediately after lysis has previously been attributed either to a genuine increase caused by the colloid osmotic effect of residual internal hemoglobin [11] or to changes in membrane properties which could effect the electronic cell sizing procedure [12,13]. In one earlier report [20], ghost volume fell after lysis whilst the lysing medium was being continuously diluted with water; this is contrary to both our data and the above studies [11–13]. With regard to ghost volume after addition of salt, Palek et al. [19] indicated that the mean ghost volume falls to a new constant level; this observation is in contrast to finding 1b, where subsequent re-swelling was noted. In these previous studies [19,20], carried out at above 0°C, ghost resealing may have started during the course of volume measurements. Such resealing would affect the ghost volume response to alterations in the suspending medium, and cause disparities with our findings at 0°C. Note that in the present study, electronically sensed volumes were corrected for changes in shape factors. These corrections are particularly important for swollen ghosts.

The dependence of resealed, post-incubation mean ghost volume on t_d (finding 2a) is the direct result of finding 1c, i.e., the volume response of unsealed ghosts at 0°C is a function of delay time. The initial rapid ghost shrinkage, subsequent to addition of buffer but prior to incubation at 37°C (finding 1b), represents an efflux of water driven by osmotic forces; concurrently, salts will enter these still permeable ghosts. The fact that the degree of initial shrinkage is greater for longer t_d (finding 1c) suggests that ghost permeability to salts decreases with time at 0°C, i.e., the rate of salt influx decreases compared to the water efflux. However, complete resealing of the ghost membrane to salts does not occur at 0°C within the time period tested (i.e., up to about 120 min), since after shrinking, the ghosts slowly regain volume.

This swelling represents a slow influx of salts, driven by two possible mechanisms: (1) an imbalance in ionic content between the ghost interior and the exterior medium. Because of the compositions of the lysing medium and the added concentrated buffer, individual ion species (e.g, Cl^- and phosphate ions) will not be equally distributed inside and outside the ghosts, although the summed concentration of all ions will be equal in both regions. This imbalance can cause a net ghost swelling, if the different ions have different membrane permeabilites (e.g., Cl^- enters the ghost more rapidly than phosphate leaves); (2) colloid osmotic pressure exerted because Hb will be at a higher concentration within the ghosts than outside them after initial rapid shrinkage. Such a colloid osmotic effect will be greater when concentrated red blood cell suspensions are used to prepare ghosts with high residual Hb. This explains why high Hb ghosts have a greater final resealed mean ghost volume (finding 4b).

The permeability of ghosts to solutes other than salt ions is also of interest. In the present study, at 0°C, addition of sucrose to the lysate caused a greater degree of ghost shrinkage and, subsequently, a smaller degree of swelling than if concentrated buffer was added (Fig. 3). This suggests that sucrose can enter the 0°C ghosts, but at a rate slower than the ions in the buffered saline. With respect to hemoglobin, Anderson and Lovrien [15] have found that addition of salt to the lysate is sufficient to halt gradual Hb loss at 0°C. Thus, in the present study, the variation of final Hb content with t_d (finding 2b) reflects the rate of Hb loss at 0°C prior to addition of salt. Judging from Fig. 2, at 0°C most Hb (about 90%) is lost within 30 s after lysis; the remainder leaves more slowly, such that by 45 min the ghost Hb concentration essentially equals that of the lysate.

Although Hb loss is halted by addition of salt, return to isotonicity is neither necessary nor sufficient for resealing of ghosts to other solutes. In the present study, for example, ghosts could be fully resealed by incubation in the lysing medium (i.e., at about 40 mosmol/kg). It appears that incubation at 37°C, or at least above 0°C, is the most important step for inducing complete ghost resealing. Note also that ghosts resealed at 40 mosmol/kg retained nearly spherical volumes, indicating that resealing is not always accompanied by volume changes. This postulated temperature requirement is in agreement with the data of Minetti and Ceccarini [17]; they found that, even after addition of salt to the lysate, ghosts at 0°C did not become impermeable (i.e., resealed) to dextran. However, in the absence of added salt, ghosts did become impermeable to this polymer if they were incubated at a temperature of 12°C or above.

Findings 2 to 4 show how resealed ghosts can be prepared with desired volume and ionic and Hb content. In general, the mean ghost volume can be controlled by varying the delay time t_d (finding 2a), though the ghost volume also depends on the ratio of the resealing medium to lysate osmolality (finding 3c). This ratio presumably affects the degree of shrinkage first experienced by ghosts at 0°C when concentrated buffer is added. Ghosts can be prepared with internal osmolalities in the range from the lysate level up to 300 mosmol/kg by varying the quantity of $5 \times$ PBS added; the lower the internal osmolality, the larger the resulting mean ghost volume will be (finding 3a). The residual ghost Hb content can be changed by varying t_d (finding 2b). For standard ghosts, Hb falls from about 10% of the intact cell level down to less than 1% when t_d is increased from 30 s to 45 min (Fig. 2). Higher residual Hb levels can be obtained by increasing the red blood cell suspension concentration, but this also affects the final ghost volume (finding 4). In general, the final mean ghost volume can easily be adjusted since resealed ghosts behave like perfect osmometers (i.e., the mean ghost volume is inversely proportional to the osmolality). However, it should be noted that this relationship will not be exact when ghosts have high residual Hb content, since a significant proportion of the ghost volume is then occupied by the Hb molecules.

Acknowledgements

We gratefully acknowledge the technical assistance of Ms. Victoria Lovelace and Ms. Rosalinda Wenby, and the helpful discussion of Dr. Robert Farley. This work was supported in part by National Institute of Health grants HL15722 and HL15162, and by American Heart Foundation -

Greater Los Angeles Affiliate Investigative Award 537IG5.

References

1 Schwoch, G. and Passow, H. (1973) Mol. Cell. Biochem. 2, 197–218
2 Bodeman, H.H. and Karsch, B. (1984) Biochim. Biophys. Acta 772, 77–83
3 Wood, P.G. (1984) Biochim. Biophys. Acta 774, 103–110
4 Goldsmith, H.L (1967) Fed. Proc. 26, 1813–1820
5 Usami, S. and Chien, S. (1973) Biorheology 10, 425–430
6 Tillman, W., Levin, C., Prindull, G. and Schroter, W. (1980) Klin. Wochenschr. 58, 569–574
7 Heath, B.P., Mohandas, N., Wyatt, J.L. and Shohet, S.B. (1982) Biochim. Biophys. Acta 691, 211–219
8 Heusinkveld, R., Goldstein, D.A., Weed, R.I. and LaCelle, P.L. (1977) Blood Cells 3, 175–182
9 Nash, G.B. and Meiselman, H.J. (1983) Biophys. J. 43, 63–73
10 Hoffman, J.F. (1958) J. Gen. Physiol. 42, 9–28
11 Weed, R.I. and Bowdler, A.J. (1966) J. Clin. Invest. 45, 1137–1150
12 Seaman, P., Sauks, T., Argent, W. and Kwant, W.O. (1969) Biochim. Biophys. Acta 183, 476–489
13 Akeson, S.P. and Mel, H.C. (1982) Biochim. Biophys. Acta 718, 201–211
14 Jay, A.W.L. and Rowlands, S. (1975) J. Physiol. 252, 817–832
15 Anderson, P.C. and Lovrien, R.E. (1977) Biophys. J. 20, 181–191
16 Kirkwood, D.H., Hudson, R.A. and Brown, R.K. (1972) Fed. Proc. 31, 412a
17 Minetti, M. and Ceccarini, M. (1982) J. Cell Biochem. 19, 59–75
18 Teorell, T. (1952) J. Gen. Physiol. 35, 669–701
19 Palek, J., Curby, W.A. and Lionetti, F.J. (1971) Am. J. Physiol. 220, 19–26
20 Tatsumi, T. (1981) Biochim. Biophys. Acta 641, 276–280
21 Ponder, E. and Barreto, D. (1957) Blood 12, 1016–1027
22 Shank, B.B., Adams, R.B., Steidley, K.D. and Murphy, J.R. (1969) J. Lab. Clin. Med. 74, 630–641
23 Grover, N.B., Naaman, J., Ben-Sasson, S. and Doljanski, F. (1969) Biophys. J. 9, 1398–1414
24 Richieri, G.V., Akeson, S.P. and Mel, H.C. (1983) Biophys. J. 41, 218a
25 Kachel, V. (1974) Microsc. Acta 75, 419–484
26 Nash, G.B. and Wyard, S.J. (1981) Biorheology 17, 479–484
27 Linderkamp, O.L. and Meiselman, H.J. (1982) Blood 59, 1121–1127
28 Dodge, J.T., Mitchell, C. and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119–130