VISCOELASTIC PROPERTIES OF THE HUMAN RED BLOOD CELL MEMBRANE

II. Area and Volume of Individual Red Cells Entering a Micropipette

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ABSTRACT Previous work demonstrated that human red cells can be drawn into cylindrical glass micropipettes of internal diameter approximately 2.0 μm without lysing. For pipettes of less than approximately 2.9 μm inside diameter, the red cell must become less spherical, that is, reduce its volume-to-area ratio. In this work measurements were made from 16-mm film records that allowed the determination of the cellular area and volume of individual erythrocytes as they were drawn into a 2.0-μm pipette with negative pressures. The results showed that the total surface area of the membrane remains constant and that the cell endures the passage into the pipette by losing volume. The volume loss was interpreted to be due to cell water and solute loss when the membrane is under stress. The loss of cell volume, rather than the stretching of the membrane, adds confirmation that although it is very deformable, the membrane is very resistant to two-dimensional strain.

INTRODUCTION

The toughness and flexibility of the membrane are important for red cell survival in the circulation. The red cell membrane is viscoelastic, resisting stretch as an elastic structure, and temporarily resisting deformation as a viscous structure. Rand and Burton (1964), and Rand (1964) studied the membrane viscoelasticity using negative pressures to draw single red cells into glass micropipettes, a procedure since used extensively by other investigators (Weed et al., 1969; LaCelle, 1972; Leblond, 1973; Evans, 1973; Evans and LaCelle, 1975; Evans and Hochmuth, 1976). Work done by Jay (1973) indicates that the behavior of a red cell entering a micropipette depends upon the size of the micropipette, the geometry of the pipette entrance, and the pretreatment of the cell (Beck, et al. 1972).

Investigators have used the magnitude of the negative pressure in the micropipette sufficient to draw a cell into the pipette as an index of the deformability. In this study we wish to draw attention to one further variable, the geometry of the individual red blood cell.

The area and volume of each cell entering a micropipette, and in particular the area-volume relation that leads to the derived parameter, the "minimum cylindrical diam-

eter," are very important (Canham and Burton, 1968; Jay, 1973, 1975). The minimum cylindrical diameter is the diameter of the narrowest cylindrical channel through which an individual red blood cell can pass without reducing its volume or increasing its area. Canham and Burton calculated the minimum cylindrical diameter to be 3.3 μm $\pm$ 0.17 SD for a normal population of red blood cells of variable shape in isotonic buffered Ringer solution. It was calculated that passage through channels significantly narrower than 3.3 μm requires that the cell reduce its volume, stretch its membrane, or both. There is experimental evidence that red cells will pass through narrower channels without hemolysis, for example the nucleopore studies by Gregerson et al. (1967) and Chien et al. (1971), and the micropipette studies of Weed et al. (1969). More recently Jay (1973) has observed that, although there may be no hemolysis, the cell is altered in other ways. Micropipettes in the range of 2.0–2.5 μm cause the indrawn cell to alter so that when the cell is released (ejected with positive pressure), the cell is intact but irreversibly crenated (echinocyte). Pipettes of inside diameter larger than 2.5 μm have no irreversible effect on the cell, in that the cell when released adopts the familiar biconcave shape. Micropipettes with a partly narrowed entrance owing to contamination, such as membrane fragments from lysed cells or platelets, cause the indrawn cell to divide into two hemoglobin-filled cells. Similarly, cells divide when the micropipette is very small, i.e. less than 0.9 μm in diameter. Our goal in this investigation was to measure the area and volume of individual cells during their passage into a small micropipette. In particular, we hoped to learn the timing of the adjustment in the volume-to-area ratio and to assess the transient stresses in the membrane.

The theoretical work of Burton (1970) regarding the distensibility of pores versus total surface of biological membranes and the experimental work of Canham and Parkinson (1970) suggest that the red cell membrane tolerates only the smallest degree of stretch before lysis. This limited stretch of the red cell membrane has also been considered by Katchalsky et al. (1960). That the volume might decrease for a cell entering a pipette under negative pressure seemed more probable to us than that the cell might stretch, and is our hypothesis in this study. Preliminary results of this study have been reported earlier (Canham and Jay, 1973).

EXPERIMENTAL

Apparatus

A dilute suspension of human red blood cells (0.1% hematocrit approximately) was obtained by suspending cells from a finger prick of one of us (A.J.) in a Tris-buffered Ringer solution (310 mosM, pH 7.4), for which the composition is described elsewhere (Jay, 1973). The cell suspension was injected into an open-ended chamber with a 0.17-mm cover glass on the top (Fig. 1). A Leitz Ortholux II with bright field apochromatic 40× objective and 25× eyepiece was used in conjunction with a Leitz micromanipulator, 16 mm movie camera, and Leitz mercury arc lamp (E. Leitz, Inc., Rockleigh, N.J.). The micropipette was coupled to a Statham pressure transducer (Statham Instruments Div., Gould Inc., Oxnard, Calif.). Pressure was continuously recorded on a Hewlett-Packard chart recorder (Hewlett-Packard Co., Palo

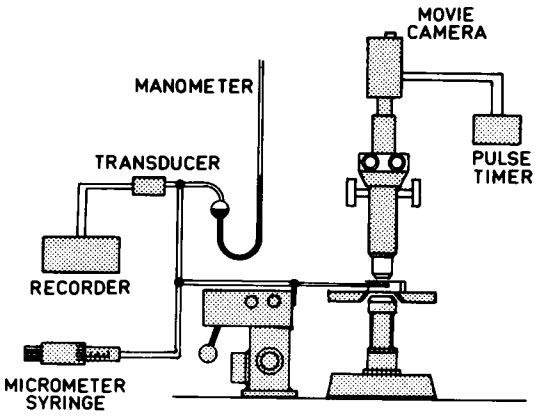

FIGURE 1 Schematic diagram of equipment used in the study.

Alto, Calif.) calibrated to 2 cm Hg pressure/cm and a paper speed of 15 cm/min. The micropipette was filled with Ringer solution. Pressures were adjusted by a micrometer syringe mounted on the microscope platform. The equipment has been described in detail elsewhere (Jay, 1973).

Procedure

The procedure was to capture a red cell in suspension with a small negative pressure that caused a slow flow of Ringer solution into the micropipette. The pressure in the micropipette was recorded continuously and the movie camera was started by a foot switch when the cell was first captured. The negative pressure was gradually increased at an average rate of approximately 0.5 cm Hg/s. Synchronization of the pressure tracing with the movie film was achieved by quickly reducing the negative pressure at the moment when the cell completely entered the pipette. The accuracy of timing was estimated to be better than 0.5 s. The camera speed, calibrated with a synchropulser, was 20.25 frames/s. The film used was Kodak Plus-X reversal (Eastman-Kodak Co., Rochester, Minn.), developed as a negative film.

Analysis

The micropipette used for this study was a long glass cylinder with a truncated entrance obtained by fracture. The internal diameter was calculated from the results of previous experiments (Jay, 1973). In that work it was shown that the pressure P required to draw an erythrocyte into a pipette of internal diameter D_p was given by the relationship $D_p = -0.0597\ P + 2.805$ (SEE = ± 0.10) μm. The micropipette in the present study was calibrated by drawing in quickly 50 cells from the standard subject A.J., which gave a mean sucking pressure of 14.3 cm Hg. This corresponds to a micropipette diameter of 2.05 μm as calculated from the above equation.

Measurements were made by projecting the film frame by frame onto a wall-mounted white card. The scale was adjusted to 0.50 inch/μm. The projector used was an LW motion analyzer (Photographic Analysis Ltd. Don Mills, Ontario, Canada). To determine the magnification, we used a portion of the roll of film to photograph a pair of calibrated etched lines on a Leitz stage micrometer.

When the cell entered the micropipette, it appeared to have three cylindrically symmetrical portions: a truncated sphere remaining outside the pipette, a straight cylindrical section as-

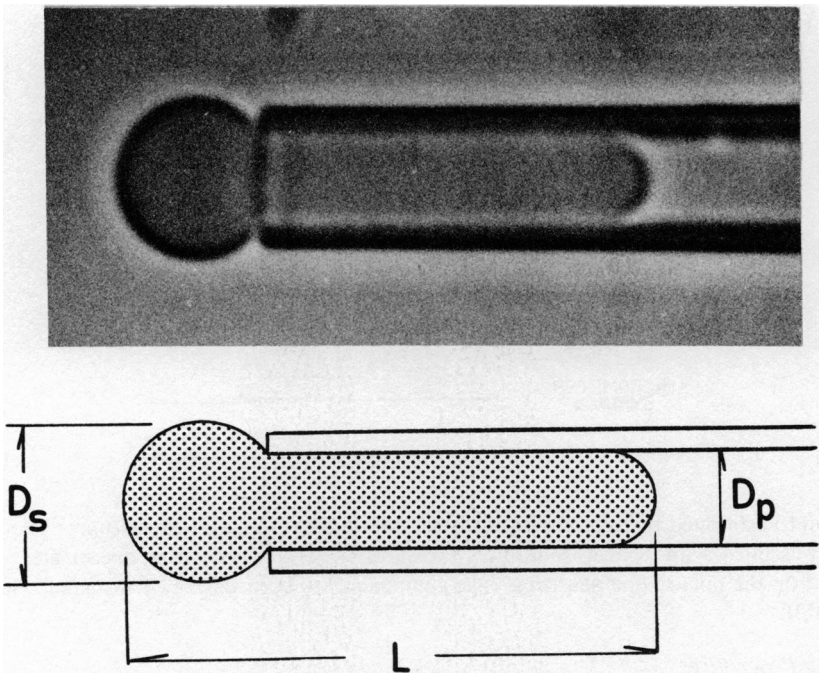

FIGURE 2 Entrance of cell into micropipette. Top, microphotograph from the exposed 16-mm film. Bottom, schematic representation showing the parameters measured.

sumed to have the diameter of the inside of the micropipette, and a hemispherical cap at the end of the cylindrical section. Fig. 2 shows our interpretation of the cell geometry. The camera was focussed to give the best-focussed image of the spherical cap of the cell outside the micropipette. The first movie frame from which measurements were taken was the first frame in which the cell appeared to have cylindrical symmetry, with a smooth spherical cap outside the micropipette. That is to say the previous frame, 0.05 s earlier, showed an image of the cell which was obviously not smooth or with cylindrical symmetry. It is possible that distortions were still present in a few subsequent frames but were not detectable optically.

The area and volume of the cell entering a micropipette can be calculated from two measurements, D_s and L, along with the measurement for the inside diameter of the pipette, D_p. The expressions for the area and volume of the red cell are: Area $= (\pi/2)\{2D_s^2 - D_s[D_s - (D_s^2 - D_p^2)^{1/2}] + D_p[2L - D_p - D_s - (D_s^2 - D_p^2)^{1/2}] + D_p^2\}$; Volume $= (\pi/12)\{2D_s^3 - [D_s + \frac{1}{2}(D_s^2 - D_p^2)^{1/2}][(D_s^2 - D_p^2)^{1/2}]^2 + D_p^3 + \frac{3}{2}D_p^2[2L - D_p - D_s - (D_s^2 - D_p^2)^{1/2}]\}$.

RESULTS AND DISCUSSION

Entry of the Cell with Increasing Negative Pressure

Of the cells drawn into the micropipette, eight were chosen to be analyzed in detail. The criterion for selection was clarity of focus as determined later with the processed film. Fig. 3 shows an example of the pressure tracing. This particular cell required 9.5 cm Hg to be pulled into the pipette. This pressure is much lower than the mean pressure for the 50 cells sucked in quickly, which was 14.8 cm Hg. This is because the

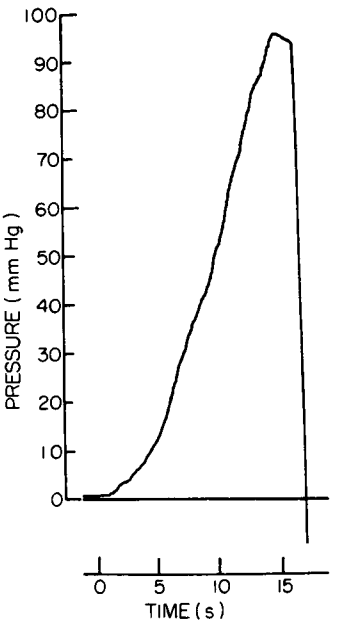

FIGURE 3 Pressure tracing from chart recorder. Note that although the control of pressure was manual, the pressure was varied smoothly.

resistance to deformation is time-dependent, owing to the viscoelastic nature of the cell membrane. As reported earlier (Jay, 1973), the cells drawn in rapidly require approximately twice the negative pressure as cells drawn in gradually. The mean sucking pressure for the eight cells in this study was 9.6 cm Hg.

In response to increasing negative pressure, the cell gradually moved into the micropipette. During the first stage of the process, for about 2 s, as the pressure increased by several millimeters of mercury, the diameter of the spherical cap D_s external to the micropipette decreased rapidly while the length of the cell L increased and moved further into the pipette. Then, for a period of 5–10 s as the pressure increased to about 5 cm Hg, the changes were much slower. As the pressure increased further, the rates of decrease of D_s and increase of L again became greater until the cell abruptly entered the micropipette completely. All eight cells analyzed in this study entered the micropipette without hemolysis, i.e. rupture of the cell membrane.

Area Versus Time

Fig. 4 shows a plot of the calculated area against time. It is typical of all the cells analyzed. There is considerable scatter in the data due to optical limitation and accuracy of measurements, but it is clear that the cell area remains constant to the time when the cell completely enters the micropipette. The last three points shown in the data are measurements made while the cell was moving along inside the pipette. These points are less accurate than the previous points.

The plot of area as a function of the time adds confirmation to the gathering evi-

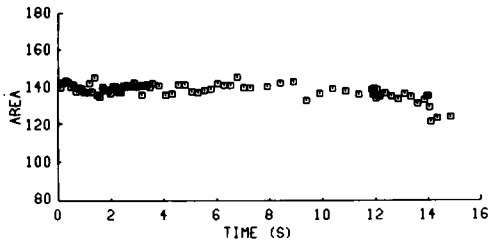

FIGURE 4 Plot of cell area in square micrometers vs. time for one cell.

dence that the membrane of the human red cell, although highly flexible, will withstand very little stretch without hemolysis. The current view of some authors is that the membrane is intolerant of any detectable stretch during gradual osmotic lysis (Canham and Parkinson, 1970).

Volume Versus Time

We have concluded that the membrane does not stretch during entrance into the micropipette and we must accept therefore, that the volume decreases to facilitate entry. The volume versus time plot for the cell is shown in Fig. 5. There appears to be a substantial total volume loss of approximately 30% before the cell entered the micropipette. This is consistent with the work of Jay (1973). The reduction in volume occurs in three different stages during the 15-s procedure. The stages of volume decreases are typical behavior of all the eight cells.

The first phase of volume change shows a rapid reduction of about 18% occurring in the first 2 s. This figure ranges from 10 to 20% for the eight cells. The second phase, lasting to about 9 s, is a plateau phase in which the volume either remained constant or showed a slight decrease as the pressure increased. The red blood cell membrane is viscoelastic and even the slight structural change in response to applied tension may depend on time and stress. This phase might be considered to involve gradual, progressive opening of membrane pores for the exit of cell content. The duration of this phase varies for the different cells and is continuous with the third phase, in which there was a more rapid volume loss until the cell was completely drawn into the glass tube.

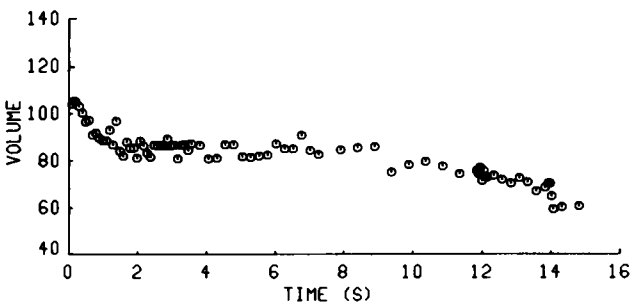

FIGURE 5 Plot of cell volume in cubic micrometers vs. time for the same cell shown in Fig. 4.

Jay (1973) has shown that the first phase of the volume loss, amounting to an average of about 12%, is reversible. It is not possible that such a volume change would involve outflux of cell water alone because of the very large osmotic imbalance that would result. More probably, some solute had accompanied the water flow. The red cell membrane is highly permeable to H^+ and Cl^- ions. It is conceivable that an intracellular pH change occurred with the volume loss. This could occur even at very low pipette pressures. Since the external medium contains a buffer system, this pH change would reverse just as readily when the cell was released. Work is in progress in our laboratory to study intracellular pH in deformed red blood cells. Possibly too, there may be a localized environmental change for the cell associated with its nearness to the glass of the pipette. Ponder (1948) discussed the shape transformations of erythrocytes in the wedge region of two converging cover glasses, and those transformations might possibly have had concurrent, small, reversible changes in volume.

The second and third phases of volume loss were continuous, with the second phase occurring more gradually and involving only slight reduction. During this time, the pipette pressure and therefore the stress in the membrane was continuously increasing. Stressing the red cell membrane results in the progressive opening of pores that allow exit of intracellular material (Seeman, 1967; Burton, 1970). The membrane behaves as a molecular sieve (MacGregor and Tobias, 1972), such that material with increasing molecular size leaves the cell in sequence. Intracellular potassium can leave the cell in the prelytic stage of hemolysis (Seeman et al., 1969; Canham and Parkinson, 1970; Jay and Rowlands, 1975; Jay, 1976). In the final phase of cell entry into the pipette, we suggest that potassium and chloride ions leave the stressed cell. This electrolyte loss would be accompanied by water outflow so that osmotic balance is maintained. If the solute and volume loss is sufficient to allow the cell to enter the pipette, the stress on the membrane is relieved. In the absence of stress, the membrane pores would reseal. The released red cell membrane is relatively impermeable to K^+, hence the K^+ loss was irreversible, and the released cell became crenated due to loss of intracellular solute (Jay, 1973). This crenation is consistent with observations made by Chien et al. (1971) on cells which passed through a 2.2-μm nucleopore filter. In these experiments, the authors have confirmed the loss of intracellular potassium from cells without lysis.

Volume Versus Tension

The tension in the membrane can be calculated from the cell geometry, the pipette pressure, and the law of Laplace (Rand, 1964; Jay, 1973). One makes the assumption that the tension in the membrane is isotropic and that the resistance to bending is negligible. The second assumption is probably not valid for very low pressures but probably true for most of the period of cell entry. The tension T in the membrane is given by $T = D_s(P_c - P_0)/4 = D_p(P_c - P_p)/4$, where P_c is the pressure inside the cell, P_p is the pressure inside pipette, and P_0, that of the suspending medium, i.e. atmospheric pressure. Other parameters are as shown in Fig. 2. The pressure difference between the inside and outside of the pipette, which is the negative pipette pressure as measured in the experiment was $(P_p - P_0)$.

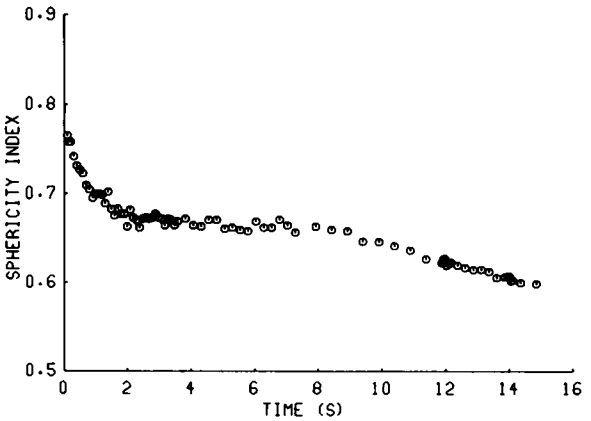

FIGURE 6 Plot of sphericity index vs. time. The sphericity index is a dimensionless geometric parameter relatively insensitive to the measurement errors.

Fig. 6 shows a plot of volume versus tension. The first phase of volume reduction occurred rapidly at membrane tensions of less than 2 dyn/cm. The volume then remained stable until a tension of 7 dyn/cm was reached. The final phase occurred up to a tension of over 30 dyn/cm without lysis, when the cell flowed into the pipette. The pressure difference between the cell interior and the inside of the micropipette ($P_c - P_p$) is greater than that between the cell interior and the suspending medium ($P_c - P_0$), and was calculated from the above equations to be 7×10^5 dyn/cm^2 just before entry into the pipette. Because of this high pressure difference across the cell membrane, we suggest that for the final stage of entry, the cell lost water hydraulically, that is, water was squeezed out of the cell resulting in a small gradient of osmotic pressure. This volume loss is in addition to the volume loss associated with the exit of intracellular solutes.

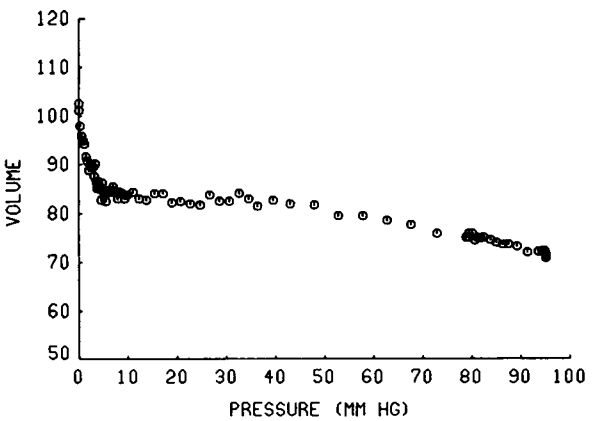

FIGURE 7 Plot of recalculated volume in cubic micrometers vs. pressure.

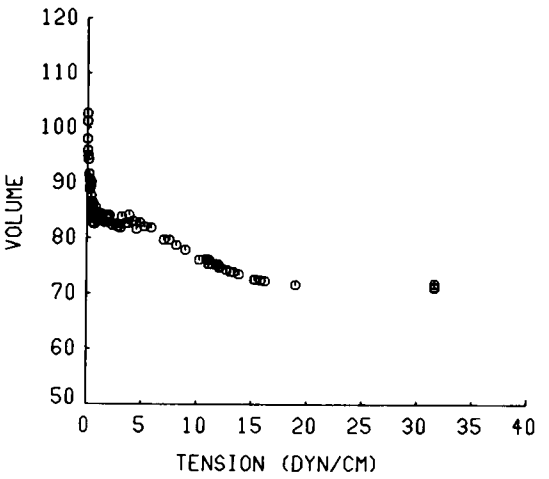

FIGURE 8 Plot of cell volume in cubic micrometers vs. membrane tension. The tension in the membrane is very sensitive to the area-volume relation of the cell and the size of the pipette.

SUMMARY

In summary, we have done a frame-by-frame film analysis of cells entering a restricting glass tube. The eight cells analyzed entered the 2.0-μm pipette without lysis by losing volume and not by stretching the membrane. The volume loss appeared to be in three phases, the first possibly involving a change in intracellular pH, which is reversible when the cell is released; the second, a plateau phase, involving progressive increase in membrane tension and opening of the membrane pores; and the terminal phase, thought to be due to loss of intracellular K^+ and cell water.

We have calculated membrane tensions at each level of entry into the pipette to emphasize the known interrelation between pipette size and cell size. The recording of pressure alone is not adequate to assess the applied stress on the cell membrane.

Dr. Jay is a Research Scholar of the Canadian Heart Foundation. Send reprint requests to Dr. Jay.

Dr. Canham was a Medical Research Council of Canada Visiting Professor at Calgary in the Summer of 1972 when this work was begun. His present address is: Department of Biophysics, Faculty of Medicine, The University of Western Ontario, London, Ontario, Canada N6A 5C1.

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