# VISCOELASTIC PROPERTIES OF THE HUMAN RED BLOOD CELL MEMBRANE

## I. Deformation, Volume Loss, and Rupture of Red Cells in Micropipettes

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ABSTRACT Single human red blood cells suspended in buffered Ringer's solution were rapidly drawn, at recorded pressures, into glass micropipettes of diameter 0.6–3.2 $\mu$m. Cells could enter micropipettes of diameter $\geq$ 2.9 $\mu$m with minimal pressure. In micropipettes of 0.9–2.9 $\mu$m, the pressure required increased linearly with decreasing diameter. For diameters 2.5–2.9 $\mu$m, pressures ranged up to 7 cm Hg, and the cells returned to normal biconcave shape on release. For diameters 1.9–2.5 $\mu$m, the required pressures ranged from 7 to 17 cm Hg. The released cells were crenated. In micropipettes 0.9–1.9 $\mu$m, the pressures required ranged from 17 to 34 cm Hg. The cells hemolyzed on entry. As diameter decreased from 0.9 to 0.6 $\mu$m, cells were drawn into dumbbell shapes and parts of the cells were pinched off without complete hemolysis of the cell. Using an accepted value of 138 $\mu m^2$ for the mean cell area, the mean volume of the human red cell was calculated to be 94 $\mu m^3$. Under mechanical stress, about 12% of this volume is rapidly exchangeable with the external medium. The cell volume may further decrease by 20% which is not reversible.

### INTRODUCTION

Since the original works of Rand and Burton (1964) and Rand (1964), the micropipette method of studying the mechanical properties of the red blood cells has provided a direct, quantitative method of recording the deformability of red blood cells. Deformability of individual red blood cells is important when one considers the flow of red cells through capillaries smaller than their own diameters. The ability of the biconcave red cell to deform easily and change shape, because its membrane area is larger than the minimum required to enclose its volume, makes it possible for the cell to traverse capillaries 4 $\mu$m in diameter with little resistance (Jay et al., 1972). A degradation of this deformability is likely to impede blood flow

and result in increased flow resistance in the microcirculation. Weed et al. (1969) have shown that deformability is dependent on the metabolism of the red cell, and LaCelle (1969) has reported decreased deformability of red cells in storage. Pathological conditions with decreased red cell deformability have also been reported. Among these were decreased deformability after severe burns (Braasch and Rogausch, 1971; Schachar et al., 1973) and in cholestatic jaundice (Rogausch, 1971).

Deformability of red cell depends on several factors. Firstly, the low resistance of the membrane to bending is a contributing factor (Rand and Burton, 1964). Any change, either in vivo or in vitro, which results in changes in the structure or chemistry of the membrane itself will alter the deformability (Rand, 1968; Beck et al., 1972). Secondly, deformability depends on the size and shape of the cell. In order for a cell to be deformable, it must have a large membrane area-to-volume ratio. Canham and Burton (1968) have measured the mean cell volume of the biconcave disk to be 107.5 $\pm$16.8 (SD) $\mu m^3$ and the mean membrane area to be 138.1 $\pm$17.4 (SD) $\mu m^2$. This area is about 25% greater than the minimum area required to enclose the volume. Assuming that the red cell membrane cannot tolerate any stretch without hemolysis, the authors calculated that the mean diameter of the smallest cylindrical channel which red cells can go through without damage is 3.33 $\pm$0.17 (SD) $\mu m$. Weed and LaCelle (1969), using glass micropipettes, estimated the minimum diameter for passage as 2.85 $\mu m$.

The red cell membrane is highly permeable to water (Sidel and Solomon, 1957; Paganelli and Solomon, 1957) and to small molecules. It may be expected that under mechanical stress, the cell may lose some of its water, resulting in a decreased volume. Such a reduction in volume may allow the cell to pass through even smaller channels, without destruction. Finally, the viscoelasticity of the membrane is also a determinant of cell deformability. If the membrane can stretch, then the cell would be able to traverse channels smaller than the calculated minimum. Rand and Burton (1963), in a study of osmotic hemolysis of red cells, estimated that the cell membrane increases in area by as much as 20% during hemolysis, depending on the original shape of the cell. On the contrary Canham and Parkinson (1970) refined the method of Rand and Burton (1963) and showed that the surface area of the human red blood cell remains constant throughout the process of osmotic hemolysis. It will be assumed therefore that the red cell membrane under stress will not increase in surface area, at least until hemolysis occurs.

In the present study, red cells are rapidly drawn into glass micropipettes of diameters ranging from 0.6 to 3.2 $\mu m$. The pressures required are recorded. Assuming that the area of the membrane is not increased up to the time of lysis, the minimum cylindrical diameter for free passage, the amount of reversible and irreversible volume losses under stress, as well as the tension in the membrane at the time of rupture are calculated.

## EXPERIMENTAL

### *Glass Micropipettes*

Glass micropipettes of internal diameter 0.6–3.2 $\mu$m were made from stock glass capillaries (15 cm long, 1 mm OD, 0.5 mm ID) supplied by Walter A. Carveth Limited (Vancouver, B. C., Canada). A deFonbrune microforge (A. S. Aloe Co., St. Louis, Mo.) was used to make micropipettes of uniform thickness with no taper at the tip section. The diameters of the micropipettes were measured from photomicrographs of the micropipettes on-end. A calibrated stage micrometer was also photographed using identical optics (40$\times$ objective, 25$\times$ eyepiece). This method of micropipette size has been described previously (Jay e tal., 1972). In the present study, however, the micropipettes were photographed before they were filled and used, and optics were much improved. Measurements of the diameters can be made to an accuracy of $\pm$ 0.1 $\mu$m. Typical photographs obtained from micropipettes photographed on-end are show in Fig. 1.

### *Apparatus*

The experiments were carried out using a Leitz Laborlux II (E. Leitz, Inc., Rockleigh, N. J.) fixed stage microscope. A Leitz micromanipulator and micropipette holder provided support for the micropipettes. Two Statham pressure gauges (Statham Instruments, Inc., Oxnard,

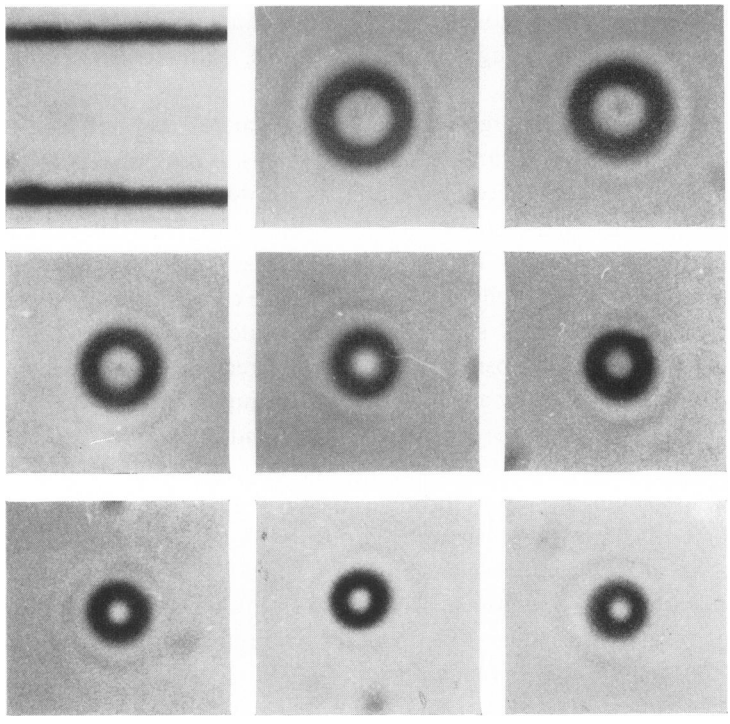

FIGURE 1 Photomicrographs of micropipette ends for measurements of internal diameters. The calibrated scale division shows 10.06 $\mu$m.

Calif.), one venous and one arterial type, were used to measure the pressures which were varied by a micrometer syringe. The venous pressure transducer was calibrated with a water manometer, and the arterial transducer was calibrated with a mercury manometer. Depending on the size of the micropipette used, the negative pressures required to draw the cells into the pipette ranged from less than 1 mm $H_2O$ to as high as 36 cm Hg. The venous pressure gauge was used to record pressures of up to 20 cm $H_2O$ and the arterial gauge was used to record the higher pressures. The pressures were recorded on a Hewlett-Packard chart recorder (Hewlett-Packard Co., Palo Alto, Calif.).

*Procedure*

Red blood cells were obtained from finger pricks and suspended in Tris-HCl buffered isotonic Ringer's solution (pH 7.40 ±0.02, 310 ±2 mosM), to make up a dilution of about 1:1,000. The cell suspension was placed in a Lucite chamber, open on one side where the micropipette was inserted. The pressure was adjusted to zero with the micrometer syringe so that there was no flow in or out of the micropipette. This was easily and accurately done by observing the movement of red cells near the micropipette tip. The recorder was adjusted for zero.

A slight negative pressure was introduced so that there was a very slow flow into the pipette. This enabled single red cells in suspension to be caught at the tip of the micropipette. The pressure difference was then rapidly increased until the cell suddenly flowed into the pipette. The pressure was immediately returned to zero and the procedure was repeated for 50 cells in each experiment.

The time required to raise the pressure to the final required value was 0.5–1.5 s in all measurements. The results appeared as spikes on the recording chart and the peaks represented the negative pressures required to suck the cells in. Provided that this time is kept within about 2 s, the measurements of the pressure are reproducible and independent of the speed of measurement. When the pressure was increased very gradually, the pressures required to suck the cells into the micropipettes were lower. Only very low pressures were used to catch the cells so that they would not be traumatized before entering the pipette. Examples of recordings are shown in Fig. 2.

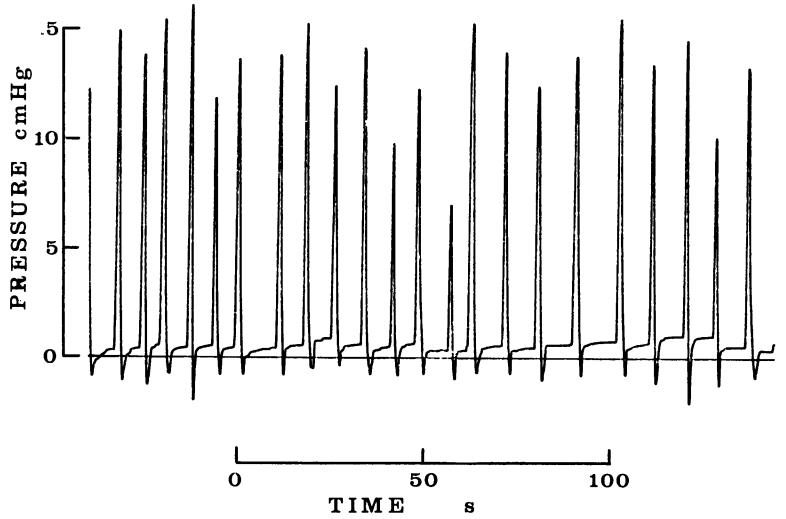


FIGURE 2 Example of pressures recorded in our experiments.

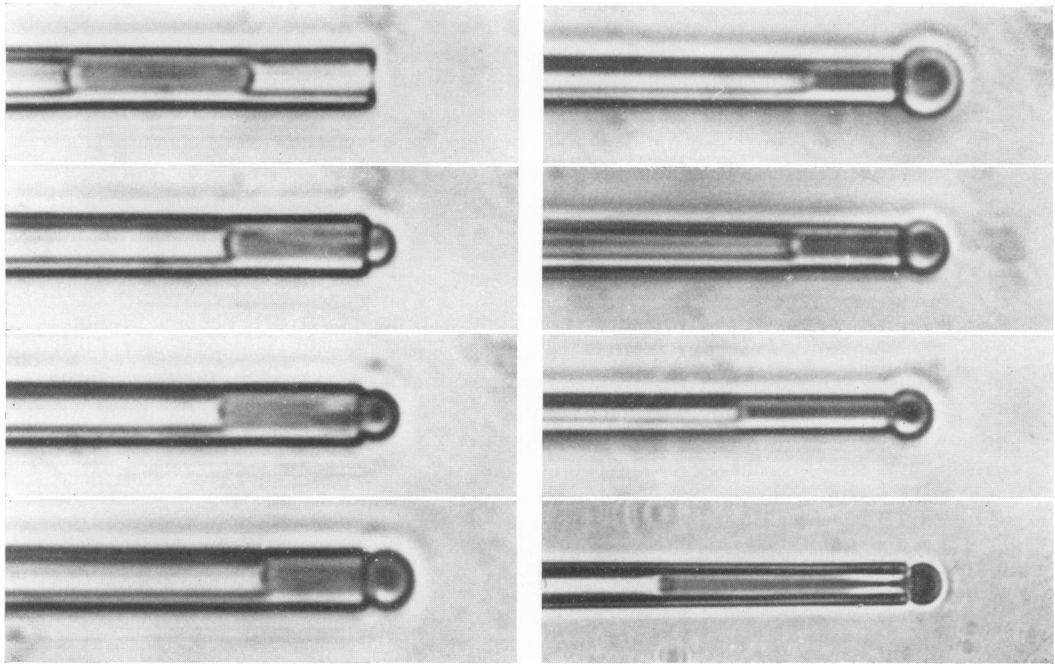

FIGURE 3 Photomicrographs of red cells in glass cylindrical micropipettes of various sizes. On the lower right, a cell is seen stretched in a very small micropipette. (Approx. 2,000×.)

The method of catching cells randomly in a suspension had two advantages over trying to pick either cells hanging on edge from a cover slip or cells which had settled to the bottom of the slide. Cells at either the top or bottom of the chamber are stuck to a solid surface, and significant force is required to detach them. This might result in errors in pressure measurements. In the case of cells hanging from the cover slip, the method itself might cause some selection, in favor of cells with stickier membranes. Since we were using glass micropipettes, any bias in selection of cells relating to their "stickiness" to glass would be undesirable. Fig. 3 shows photomicrographs of human red blood cells in micropipettes of various diameters.

The effect of initial cell volume has also been investigated. Cells were preswelled or shrunken by variation of the tonicity of the external medium. The pH and the strength of the buffer were maintained; the NaCl concentration alone was varied to make up solutions of final osmolarities ranging from 190 to 340 mosM.

## RESULTS

The results of a study of variation among 12 normal subjects, of mean pressures required to suck cells into 6 different micropipettes of various diameters are shown in Table I. In each experiment, the same micropipette was used to study cells from 5 to 7 subjects. The 12 subjects were all volunteers and were therefore not all available at any one time. Pressures were recorded for each of 50 cells from each subject, and the mean for each subject was calculated. The range of values obtained with

TABLE I

VARIATION AMONG SUBJECTS

Mean pressure required to draw cells into micropipette (cm Hg).

| Subject | Micropipette diameter ($\mu$m) | | | | | |
|---|---|---|---|---|---|---|
| | 1.5 | 1.7 | 1.9 | 2.1 | 2.2 | 2.4 |
| A.J. | 24.7 | 18.5 | 16.7 | 13.1 | 13.0 | 8.6 |
| B | 22.4 | 17.5 | 17.6 | 12.5 | 13.4 | 9.0 |
| C | 24.3 | 19.0 | — | — | 12.8 | — |
| D | 25.1 | — | — | — | — | — |
| E | 26.8 | — | — | — | — | — |
| F | 25.9 | 19.0 | 14.6 | 13.4 | 12.6 | 8.7 |
| G | 26.0 | — | — | — | — | — |
| H | — | 20.3 | — | — | 12.5 | — |
| I | — | 19.4 | — | — | 10.9 | — |
| J | — | — | — | — | 13.1 | — |
| K | — | — | 16.4 | 14.5 | — | 9.6 |
| L | — | — | 16.0 | 13.1 | — | 7.7 |
| Mean | 25.0 | 19.0 | 16.3 | 13.3 | 12.6 | 8.7 |
| ± SD | 1.4 | 0.9 | 1.1 | 0.7 | 0.8 | 0.7 |
| Maximum variation from mean | 10% | 8% | 10% | 9% | 13% | 11% |
| Variation of A.J. from mean | −1.2% | −2.6% | +2.5% | −1.5% | +3.2% | −1.1% |

each micropipette varied from 6 to 13% from the mean for all subjects, but in each case, the results from subject (A.J.) differed by no more than 3.2% from the mean. This established subject (A.J.) as an average normal subject, and all the blood samples used for the pressure vs. micropipette diameter study were taken from this one subject.

Fig. 4 shows the effect of initial cell volume on the mean pressure. Four micropipettes, ranging from 0.9 to 2.6 $\mu$m, were used in this study. Results showed that for larger micropipettes, the mean pressure increased with increasing cell volume in the lower range (i.e., decreasing osmotic pressure of the suspending medium) until some value was reached, and then it became insensitive to further increases in cell volume. The dependence of the pressure on cell volume decreased with micropipette diameter. In micropipettes 1 $\mu$m or less in diameter, the pressure was independent of the initial volume of the red cell.

Fig. 5 shows a plot of mean pressure against the diameter of micropipette. Each datum point represents the mean value of the pressures recorded for 50 individual cells. The standard deviations ranged from ±0.5 cm Hg for the lower mean values, to ±2.5 cm Hg for the higher mean values, and the standard error of the mean was at maximum ±0.4 cm Hg. In the range of diameter from 0.9 to 3.0 $\mu$m, the results show a straight line (coefficient of correlation: −0.985), which intersects the $x$ axis

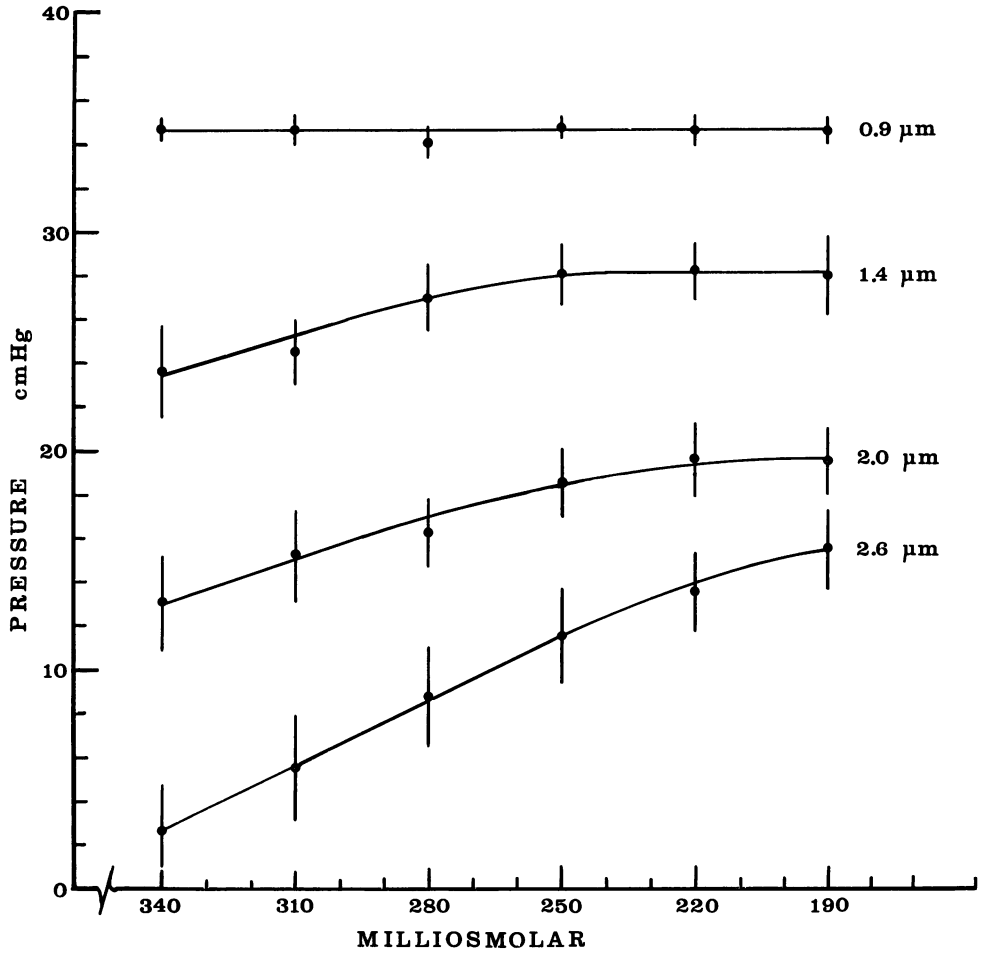


FIGURE 4 Plot of mean pressure (50 cells for each point) vs. osmotic pressure of the suspending medium for four micropipettes of different diameters.

at a diameter of 2.9 μm. The appearance of the released cells varied depending on the size of the micropipette used. In micropipettes larger than 2.5 μm in diameter, the cells were normal shaped on release from the micropipettes. In micropipettes 1.9–2.5 μm in diameter, the cells were crenated, and in micropipettes 0.9–1.9 μm in diameter, the cells hemolyzed. As micropipette diameter continued to decrease beyond 0.9 μm, the cells were drawn out into dumbbell shapes (Fig. 3), and parts of the cells were pinched off without complete hemolysis of the cell. The slope of the graph also reversed and the pressure decreased with reducing micropipette diameter. The point of discontinuity in the graph, which occurred at a diameter of about 0.9 μm and pressure of 34 cm Hg, is the transition point between two types of hemolysis which will be discussed later.

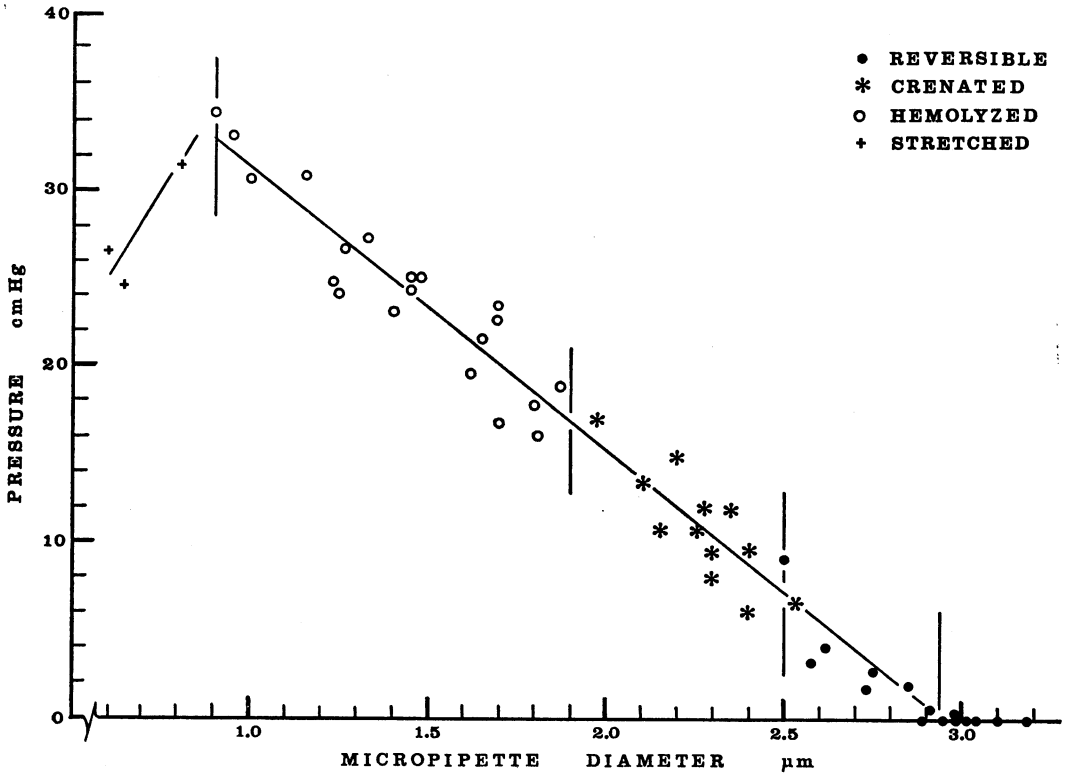


FIGURE 5 Plot of mean pressure (50 cells for each point) vs. micropipette diameter. Regression analysis of data points for micropipettes of diameter 0.9–3.0 $\mu$m gave coefficient of correlation = −0.985, standard error of estimate = ±1.7 cm Hg, $x$ intercept = 2.93 $\mu$m, and slope = −16.28 cm Hg/$\mu$m.

## DISCUSSION

In order to minimize scatter of experimental data, the major portion of the present study has been carried out on red cells from one subject. It has been established, however, that the selected subject does statistically represent the mean of the population. The results in Table I have shown that individuals may vary by as much as 14% from the mean even within the normal population, when the mean negative pressure required to draw red cells into glass micropipettes is measured. Moreover, samples taken from the same individual at different times may show variations. This is demonstrated by the data in Table I, which show that none of the subjects consistently records higher or lower pressures than another. The scatter is small enough that the present study is not significantly affected.

It has been shown, from our study of the dependence of mean pressure on initial cell volume, that higher pressures are required for cells with larger volumes. This is not surprising because it is the area-to-volume ratio of the cell which determines whether a specific passage is restrictive to a cell or not. Canham and Burton (1968)

have defined the term "sphericity index" (= 4.84 $V^{2/3}/A$) as a measure of the cell shape, relative to the sphere which has the maximum value of unity. The sphericity index is inversely proportional to the shape factor defined by Rand and Burton (1963). As the cell swells in an environment of low osmotic pressure (relative to isotonicity), its sphericity index increases and higher pressures are needed to force it into the same micropipettes. The study has also shown that the dependence of pressure on cell volume or shape is more evident in the larger micropipettes than in the smaller ones. In a micropipette 0.9 $\mu$m in diameter, the pressure was independent of cell volume. Such a micropipette would be useful to study the actual mechanical strength of the cell membrane itself, while the larger ones would be useful for studies of cell deformability, which is dependent on both the size and shape of the cell.

Within a normal population of red blood cells, the size as well as the shape varies from cell to cell, although within rather narrow limits. The sphericity index of individual cells ranges from about 0.7 to 0.9 (Canham and Burton, 1968). The variation in the mechanical strength of the membrane may be much less, i.e. cells of various sizes and shapes in the same population may have similar membranes, although membrane lipids (Westerman et al., 1963) have been reported to vary slightly with cell age, which is associated with alterations in cell geometry (Canham, 1969). These variations are demonstrated by the standard deviations in the data plotted in Fig. 4. The variation in cell geometry is related to the relatively large standard deviations in measurements obtained with the larger micropipettes ($\pm 2$ cm Hg, i.e., $\pm 50\%$ at isotonicity). The standard deviations appear to decrease slightly with decreasing micropipette diameter as the micropipettes become more sensitive to membrane strength and less sensitive to cell geometry. In the smallest micropipette, the uniformity in the mechanical strength of the cell membrane is demonstrated by the much decreased standard deviations ($\pm 0.5$ cm Hg, i.e., $\pm 1.5\%$).

The major cause of the scatter in the data presented in Fig. 5 is in the measurement of micropipette diameter. Repeated measurements showed reproducibility of $\pm 0.1$ $\mu$m, and depending on the quality of photographic negatives, the accuracy of measurement (possible error) varied from $\pm 0.1$ to $\pm 0.2$ $\mu$m. This accuracy is confirmed by the collected data which show that all points are within $\pm 0.2$ $\mu$m of the calculated regression line.

The changes sustained by the red cells, reversible and irreversible, vary, depending on the diameter of the micropipette which the cells have to go through. This statement is prompted by the observation that cells may be normal shaped, crenated, or hemolyzed after they have gone through the micropipettes, depending on the size of the micropipettes. For this reason, the data will be discussed in parts, according to the diameter of the micropipettes used.

### *Diameter > 2.9 $\mu$m: Reversible Deformation of the Red Blood Cell*

In the micropipettes of diameter greater than 2.9 $\mu$m, red blood cells can pass through with minimal driving pressure. In these experiments, the venous pressure gauge was

used. The precision in measuring the pressures and in setting the zero pressure (relative to atmospheric pressure) was $\pm 1$ mm $H_2O$. In these micropipettes, the pressures required were less than 1 cm $H_2O$. The cells go through the micropipettes with ease, deforming into sausage shapes during passage, and immediately returning to normal biconcave shapes when released or when they reached the larger sections of the micropipettes. The low pressures required to deform the red cells into these micropipettes are consistent with the reported high flexibility of the cell membrane (Rand and Burton, 1964). Passage of the cells through these micropipettes required only changes of shape and did not involve either area increase or volume decrease since such changes would have required much higher pressures.

### *Diameter 2.9 μm: the Minimum Cylindrical Diameter and the Mean Cell Volume*

The calculated regression line (Fig. 5) intersects the $x$ axis at a micropipette diameter of 2.9 μm. This represents the diameter of the smallest micropipette which red cells can traverse with ease and without undergoing area or volume changes. This corresponds to the "minimum cylindrical diameter" defined by Canham and Burton (1968), which has been calculated to be 3.33 μm from the areas and the volumes of individual cells. LaCelle (see Weed and LaCelle, 1969), using micropipettes, has estimated the minimum diameter to be 2.85 μm, a figure which is in agreement with our present result. Weed and LaCelle (1969) have explained the discrepancy between theory and experiment by "limited stretch" of the membrane. Calculations show, however, that the stretch required is in excess of 12%. This amount of stretch would result in hemolysis of the cell, and could not have occurred at such small driving pressures. One possible explanation may lie in the determination of the theoretical value itself, which has been calculated from the areas and volumes of individual cells, and which in turn have been calculated from measurements made from photographed cell profiles (Canham and Burton, 1968). Because the areas and volumes have both been calculated from measurements of linear dimensions, the results for the areas would necessarily be more accurate than those for the volumes. A value of 138.1 $\pm$17.4 (SD) $\mu m^2$ has been obtained by Canham and Burton (1968) for the mean cell area. This value agrees well with other figures in the literature, and has been confirmed in our laboratory using cells from subject (A. J.), by the same method. It is possible to calculate the mean volume from the area and the minimum cylindrical diameter. It is not implied that the figure for the volume of 107.5 $\pm$16.8 (SD) $\mu m^3$ (Canham and Burton, 1968) is in error; we have no evidence to suggest whether it is or not. We merely point out that the value of 138.1 $\mu m^3$ for the mean cell area is probably the more accurate, and has been confirmed by various investigators. It is this value for the area on which we would like to base our calculation of the equilibrium cell volume.

For a cell to enter a micropipette of diameter equal to the minimum cylindrical diameter, it must take on a "sausage" shape. Fig. 6 shows a schematic diagram of a

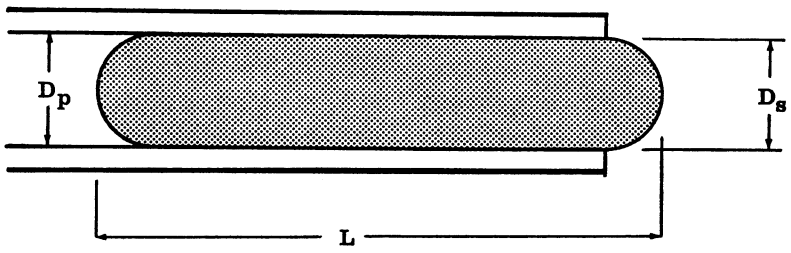


FIGURE 6 Schematic diagram (showing relative dimensions) of a cell, $A = 138\ \mu m^2$, $V = 94\ \mu m^3$ in a micropipette of diameter $D_p = 2.9\ \mu m$. $D_s = D_p$, and $L = 15\ \mu m$.

cell of area $A$, volume $V$, and length $L$, in a micropipette of diameter $D_p$. $A$, $V$, and $D$ are related by

$$V = A \cdot D_p/4 - \pi D_p^3/12.$$

$L$, which appears in expressions for both $A$ and $V$, has been eliminated. Taking $A = 138\ \mu m^2$ and $D_p = 2.9\ \mu m$, the volume is calculated to be $94\ \mu m^3$. This represents the mean volume for a normal cell population, and agrees with the generally accepted value of 90–95 $\mu m^3$ published in the literature.

*Diameter 2.5–2.9 μm: Rapidly Reversible Volume Losses from Cells under Stress*

When the diameter of the micropipette is smaller than the minimum cylindrical diameter, at the slightest negative pressure, the cell would take the geometrical shape as shown in Fig. 7. The spherical cap diameter $D_s$ is larger than the micropipette diameter $D_p$, and prevents the cell from entering the micropipette. If the applied pressure is increased, the cell will go in, but it requires a reduction of volume, since the membrane does not stretch at these pressures (see Introduction). Studies in our laboratory with a 2.1 μm micropipette (Canham and Jay, 1973), in which the area and volume of single erythrocytes were continuously monitored as they were drawn into the micropipette, have also shown that the area remained constant while the volume decreased. In micropipettes between 2.5 and 2.9 μm in diameter, cells which had gone through the micropipettes returned to normal shape. Moreover, repeated measurements, as many as three times, made on the same cell, gave reproducible pressure recordings. This indicates not only that there had been rapid volume loss which enabled the cell to enter the pipettes, but that the volume fluxes were just as rapidly reversed. The time taken for a cell to return to biconcave shape was about 1 s. When repeated measurements were continued using the same cell, the cell eventually crenated.

The amount of volume loss (maximum reversible loss) required to enable cells to enter a 2.5 μm micropipette has been calculated to be 11.5 $\mu m^3$, or 12% of the initial

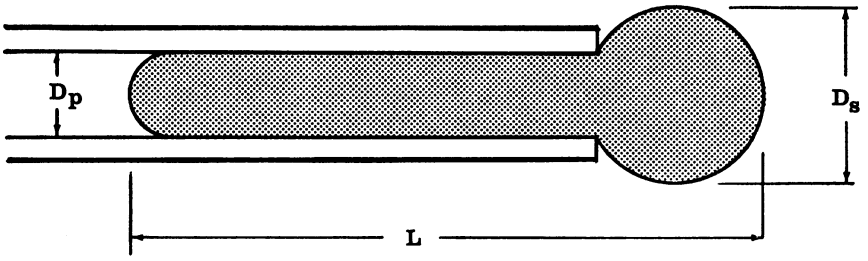


FIGURE 7 Schematic diagram (showing relative dimensions) of a cell $A = 138\ \mu m^2$, $V = 94\ \mu m^3$ in a micropipette of diameter $D_p = 2.2\ \mu m$. $D_s = 4.8\ \mu m$ and $L = 17\ \mu m$.

cell volume. It is possible that similar volume losses by red cells can take place in vivo in the microcirculation. Small volume fluxes can occur at very low pressures (several millimeters of mercury) which are available in the capillaries or when the cell is subjected to extreme deformations such as occur when cells reach the apices of capillary bifurcations. Sirs (1964) has shown that diffusion can account for only 10% of the gas exchange across the red cell membrane, and in fact has argued that the observed high rate of transport can be accounted for only if water is somehow pumped across the membrane carrying gas molecules with it. Our results appear to support this hypothesis.

*Diameter 1.9–2.5 μm: Irreversible Volume Losses and Crenation of the Red Blood Cells*

In micropipettes 1.9–2.5 μm in diameter, the released cells were crenated. Still, the cells were not hemolyzed. They must, however, in addition to losing the maximum reversibly exchangeable volume (12% of initial volume), also lose additional volume which is not reversible. Crenations as a result of mechanical stress on the membrane are probably different from osmotic crenations (hyperosmotic) and crenations due to high pH (Rand et al., 1965) which are both reversible, and take place without increase in membrane tension. Cells ejected from these micropipettes did not return to normal shape even after several minutes. Chien et al. (1971), in their study of mechanical hemolysis of red cells, have shown that crenations occur as a result of high perfusion pressures and small filter pore diameters. Their observations with a filter 2.2 μm in pore diameter, and at a perfusion pressure of 12 cm Hg, compare well with our results.

The maximum volume loss without hemolysis occurs in micropipettes 1.9 μm in diameter. Calculations show that the total volume loss is as much as 30 $\mu m^3$, or 32% of the initial cell volume. This figure includes the 12% loss which is reversible. The maximum irreversible volume loss then amounts to 20% of the initial cell volume. The chemical compositions of the volume fluxes have not been studied, but it would be expected that the reversible and irreversible fluxes would differ. Chien et al. (1971) have demonstrated that in passing through a polycarbonate sieve with 2.2-μm

pores, at a driving pressure of 12 cm Hg, red cells lose about 15% of their hemoglobin, but about 35% of their intracellular potassium.

The apparent three volume compartment system in the red cell may represent (*a*) a volume fraction of 12% consisting mainly of water freely and rapidly exchangeable with the extracellular environment, (*b*) a volume fraction of 20% consisting of a solution of electrolyte, mainly $K^+$ and $Cl^-$, which can be forced out of the cell but is not readily replaced, and (*c*) the remainder 68% consisting of hemoglobin etc. which cannot be released without hemolysis of the cell. The expulsion of these volumes must take place in sequence because of the relative pressures at which each occurred and because of the relative molecular sizes involved.

The permeability of the red blood cell membrane to water is extremely high, and water fluxes can occur reversibly and at low driving pressures (Sidel and Solomon, 1957; Paganelli and Solomon, 1957). The rapid exit of solutes may occur only after some limited stretch of the membrane has taken place such that the "pores" become sufficiently enlarged. This seems to contradict our earlier assumption that the membrane area cannot be increased without cell lysis. However, Burton (1970) has shown that the "pores" in the red blood cell membrane may actually enlarge by many times their own diameters with very little membrane stretch. The preexisting pores in the red cell membrane are functionally of diameter about 7 Å. While a membrane stretch of 6% is enough to enlarge the pores sufficiently for the exit of hemoglobin molecules (Seeman et al., 1969), which have a diameter of at least 60 Å, the amount of membrane stretch required to open up the pores enough for forced passage of the hydrated $K^+$ ions is probably no more than 1%. Such a small increase in membrane area would not significantly affect our calculations based on the assumption that the area remained constant.

The opening of pores in the cell membrane as a result of mechanical stretch is likely to be similar to that which occurs in osmotic hemolysis of the red cell. Seeman (1967) has shown that the opening of pores is transient during hemolysis. In our experiments, after the cell had gone through the pipette, the stress (tension) in the membrane no longer existed. The pores would reseal immediately. The $K^+$ lost would not be replaced, the cell could not return to its original volume and therefore would become crenated. The maximum amount of $K^+$ released, calculated from the reversible and irreversible volume losses, is about 35% of the total intracellular $K^+$, ignoring $K^+$ bound to protein, assuming that $K^+$ is not lost in the reversible portion and that the initial cell volume consists of 70% water (LeFevre, 1964; Savitz et al., 1964; Gary-Bobo and Solomon, 1968).

### *Diameter 0.9–1.9 μm: Rupture of the Red Blood Cell Membrane*

If the diameter of the micropipette is small enough so that even after the volume has been reduced by the maximum of 32% of the initial volume, the cell is still unable to go through the micropipette, and if the pressure is maintained or further increased,

the cell membrane must rupture. This occurred in micropipettes of 0.9–1.9 $\mu$m in diameter. The hemolyzed cell ghosts were irregular in shape, and often appeared as membrane fragments. When a cell loses 32 % of its volume or almost half of its water, the consistency of its contents may well approach that of a "gel," or the contents are at least far from being the "hemoglobin solution" as found in the normal erythrocyte. If membrane stress is increased, the result is gross rupture of the membrane. This is quite different from osmotic hemolysis which leaves an intact cell ghost. The breaking tension ($T$), i.e. the tension in the cell membrane at the time of rupture, in dynes per centimeter, can be calculated from LaPlace's law, assuming uniform membrane tension in the cell (Rand, 1964):

$$T = \Delta P \cdot \rho \cdot g/4(1/D_p - 1/D_s) \cdot 10{,}000$$

where $\Delta P$ is the final pressure in centimeters of mercury; $\rho$, the specific gravity of mercury; $g$, the acceleration due to gravity; $D_p$, the diameter of micropipette; and $D_s$, the diameter of the spherical cap (see Fig. 7). $D_s$ can be calculated from the unchanged area 138 $\mu m^2$, the final volume 64 $\mu m^3$ and the diameter of the micropipette $D_p$. Results are plotted in Fig. 8, for micropipettes used in the experiment. The breaking tension increased from 13 to 27 dyn/cm with micropipette diameter

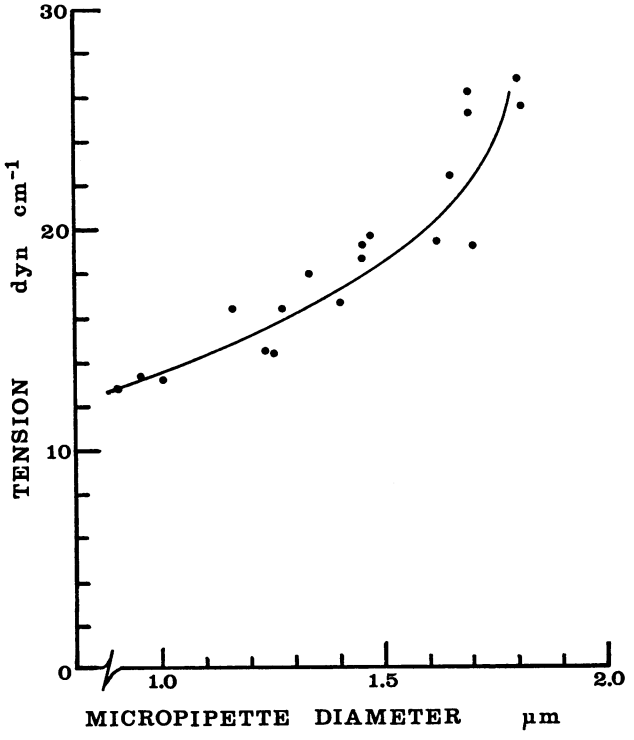


FIGURE 8 Plot of membrane tension at the time of hemolysis vs. micropipette diameter.

increasing from 0.9 to 1.8 $\mu$m. When the micropipette diameter approaches 1.9 $\mu$m, $D_s$ approaches $D_p$, the system is unstable, with the cell flowing into the pipette. The tension calculated from the equation approaches infinity. Theoretically, the breaking tension should be independent of the diameter of the micropipette used. Rand (1964) has demonstrated that the red cell membrane ruptures at a critical strain (stretch) and not critical stress. The hemolysis of red cells given different constant stresses is time dependent. This is because of the time dependent stretch of the viscous component of the membrane. Given enough time the membrane will rupture at lower stress (tension). In our experiments (see Methods), because the pressures were applied by turning a screw gauge micrometer piston, the higher the pressure, the longer the time required to reach it. In micropipettes about 1.8 $\mu$m in diameter, the time required was less than 1 s, to reach pressures of about 18 cm Hg. In micropipettes about 0.9 $\mu$m in diameter, the pressures required were about 35 cm Hg, and the times required to produce such pressures were approximately 1.5 s. The difference in time would have allowed more time for the viscous component of the membrane to stretch in the case of smaller micropipettes and therefore rupture took place at lower stress. In experiments with micropipettes in this diameter range, in which the pressures were preset at constant values, the times required for cells to hemolyze after they were caught were found to be no less than 0.4 s. This is to say, the time required to reach the critical strain, even at very high pressures, was at least 0.4 s. Although our calculated tensions do not give very accurate results due to the time dependence of measurements, the higher figures which were obtained in the faster measurements undoubtedly would give at least an order-of-magnitude estimate of 50 dyn/cm for the breaking tension, for the "fastest" rupture of the membrane.

*Diameter 0.6–0.9 $\mu$m: Incomplete Lysis of the Red Cell*

Although only three experiments were carried out in this range of micropipette diameters, it is clear that cells behave very differently from those in experiments with larger micropipettes. The slope of the line is reversed, and the pressure decreases with decreasing diameter. The cell appeared drawn out into a long, dumbbell shape, with a thin section connecting two portions of the cell (see Fig. 3). When the pressure was increased, the thin section was pulled into a long thread which eventually broke. This procedure was repeated as many as three times on the same cell with a part of the cell being pinched off each time, until a sphere remained at the end of the pipette. One interesting observation is that if part of the micropipette tip was broken off, resulting in a slightly larger pipette (but still less than 0.9 $\mu$m in diameter) the pressures required to break the cells became higher. The nonuniform lysis of the red cell suggests that the stress was not uniform in all parts of the cell membrane. Perhaps the micropipettes were small enough that the cell membrane at the tip of the micropipette had reached a critical curvature and could no longer slide around the glass edge of the pipette tip, resulting in discontinuity in membrane tension at

the pipette tip. One observation which is consistent with this idea is that in the larger micropipettes where the cells were uniformly hemolyzed, as soon as debris (usually membrane fragments from hemolyzed cells) became stuck at the pipette tip, the subsequent cells would be drawn out into dumbbell shapes such as seen in these very small pipettes. Another point to remember is that after the cell has lost half of its water, its contents can no longer be considered as "without internal structure." Perhaps cell lysis in such small pipettes resembles the breaking of a membrane-enclosed "gel" or "pseudosolid." Further research is being carried out concerning this aspect of the problem.

## SUMMARY

(*a*) The pressures required to draw human red blood cells rapidly into cylindrical glass micropipettes have been studied in the range of pipette diameter 0.6–3.2 $\mu$m. The results are reproducible provided that the time taken to reach the required pressure is kept within 2 sec.

(*b*) The relation between micropipette diameter and pressure is linear in the range of diameters 0.9–3.0 $\mu$m, the pressure increasing with decreasing pipette diameter. Correlation coefficient for the calculated regression line was $-0.985$, and the standard error of estimate was $\pm 1.7$ cm Hg.

(*c*) The "minimum cylindrical diameter," i.e. the smallest cylindrical diameter through which red cells can pass without area or volume changes, has been determined to be 2.9 $\pm 0.1$ $\mu$m. From this, and an accepted value of 138 $\mu m^2$ for the area, the mean equilibrium volume of the human red blood cell has been calculated to be 94 $\mu m^3$.

(*d*) Red cells can traverse cylindrical channels as small as 2.5 $\mu$m in diameter under driving pressures of up to 7 cm Hg, without irreversible shape changes. In doing so, the cells lose up to 12% of their volumes. The volume is regained rapidly upon release of the cells from the micropipettes.

(*e*) Red cells can be forced through cylindrical channels as small as 1.9 $\mu$m without hemolysis. The released cells are crenated. In going through these small channels, the cells lose as much as 32% of their volume, 12% of the loss has been shown to be reversible. The irreversible volume loss is estimated to be 20% of the initial cell volume. The irreversibility suggests loss of intracellular potassium, which has been calculated to be maximally 35% of the total intracellular potassium.

(*f*) Red cells cannot go through cylindrical channels smaller than 1.9 $\mu$m without hemolysis. Results show that membrane tensions of about 50 dyn/cm are required for "fast" rupture of the red cell membrane, but even at such high stresses, the membrane takes about 0.5 s, to reach the critical strain for hemolysis of the cell.

(*g*) When the micropipettes have diameters smaller than 0.9 $\mu$m, or have pinched or partially blocked tips, the cells are stretched such that lysis is incomplete and parts of the cells are removed with the membrane resealing at the point of rupture,

preventing gross release of hemoglobin. Destruction of cells in this manner occurs at a lower pressure than uniform mechanical hemolysis.

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