MECHANICAL PROPERTIES OF THE RED CELL MEMBRANE

I. Membrane Stiffness and Intracellular Pressure

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ABSTRACT The technique of Mitchison and Swann (1954) was modified for determining the resistance to deformation, or "stiffness," of the red cell membrane and the pressure gradient across the cell wall. It requires a measure of the pressure needed to suck a portion of the cell into a micropipette. Stiffness of hypertonically crenated cells was less than that of biconcave discs or hypotonically swollen cells. Crenated cells showed zero pressure gradient and a stiffness, probably due to pure bending, equivalent to 0.007 ± 0.001 (SE) dynes/cm. Normal and swollen cells showed a pressure gradient of 2.3 ± 0.8 (SE) mm H_2O and a stiffness, due to bending and tension in the membrane, equivalent to 0.019 ± 0.002 (SE) dynes/cm. No difference in stiffness was found between the rim and the biconcavity of the cell or between biconcave discs and hypotonically swollen cells. Micromanipulation showed that the membrane can withstand large bending strains but limited tangential strains (stretching). These results have significant implications in any theory explaining the cell shape. For example, the data give no indication that the physical properties of the membrane are different at the rim from those of the biconcavities, and the existence of a positive pressure in the normal cell is established.

INTRODUCTION

For years the problem of the shape of the red cell has stimulated the curiosity of many investigators. Theories to account for the biconcave shape, unique among cells, have ranged from those suggesting that internal structures, such as structural gels or sols or internal stroma, act to constrain the whole cell to be this shape, to those theories which place the constraining forces in the membrane itself. Ponder (1937, 1948) provides comprehensive reviews of the problem and suggests that the two theories are not mutually exclusive.

However, the nature of the constraint has not yet been clearly established. The contents of the cell have been considered to be in the state of either a gel or a fluid by various authors and under various conditions (see Ponder, 1948). We have chosen as a point of departure, to treat the cell as a fluid-filled membrane, and to

assume that the "shaping forces" lie in the membrane itself. One reason for this approach is the lack of evidence, particularly from studies with the electron microscope, that any internal structure exists. This suggests that the interior of the cell can be treated as a concentrated homogeneous fluid capable of exerting a hydrostatic pressure across a membrane that can develop both tension due to stretching and resistance to deformation by bending.

Two observations that support this approach are as follows. First, that tension and rigidity can be developed in the membrane, at least when the membrane is stretched; this has often been observed and is illustrated in Fig. (1*a*). Here a more

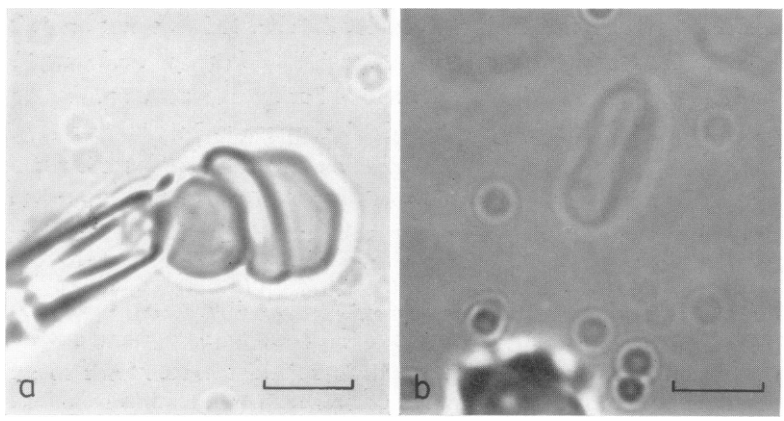

FIGURE 1 (*a*) A normal cell has been drawn into the pipette until the portion outside has become spherical. This portion has been pushed by micromanipulation against a normal cell outside. It is evidently rigid and deforms the second cell (a portion of this points up towards the reader) without suffering deformation itself. (*b*) Showing that a "ghost" (in the middle of the field) regains the biconcave shape. The portion of an unhemolysed cell, shown on the left, illustrates the difference in density when Hb is present. The scale indicates 5 μ.

flaccid cell of normal shape is distorted by a more rigid cell. The more rigid membrane was produced by pulling a portion of a red cell into a micropipette, as described subsequently, until the outer portion of the cell becomes the portion of a sphere and the cell as a whole cannot move further into the pipette without an increase in area of the membrane. The membrane then exhibits the rigidity illustrated. Secondly, that hemoglobin is not required in a highly concentrated form, as in the normal cell, to maintain the cell shape is documented (Teitel-Bernard, 1932; Hoffman, 1958; Weed *et al.*, 1963) and a hemolysed ghost will assume the biconcave shape (Fig. 1*b*). Further, changes in shape of the cell from biconcave disc to sphere, produced by osmotic swelling, are smooth and reversible, if made slowly, and do not show any evidence that at any stage internal constraints have to be overcome. From these observations one is led to the conclusion that the normal shape of the

cell is one of equilibrium, determined by the mechanical or elastic properties of the cell membrane, under the influence of "external" forces. It seems unlikely that internal structure can be one of these external forces, but hydrostatic pressure may well be. This report describes some experimental results on the mechanical properties of the red cell membrane. From these results the factors that appear important in an analytical investigation of the equilibrium shape are considered. A rigorous analysis of the equilibrium shapes of the cell must of course take into account all the external forces acting on the membrane. Only one of these possible forces, *i.e.* pressure within the cell, is investigated here.

Analytical Treatment of the Equilibrium Shape. On the assumption that the biconcave shape is determined by the action of external forces on an elastic membrane, regardless of the nature of these forces, it is necessary to know the components of stress and strain in the membrane, *i.e.* the reactions of the membrane to any external forces, and which of these components can be neglected. In very general terms, in order to characterize the deformation and equilibrium conditions of a deformable shell, it is necessary to determine the stresses tending first to increase the area of shell, *i.e.* to stretch the surface, and secondly to change the curvature of the shell; *i.e.*, to bend the membrane without stretching it. Fig. 2 illustrates these

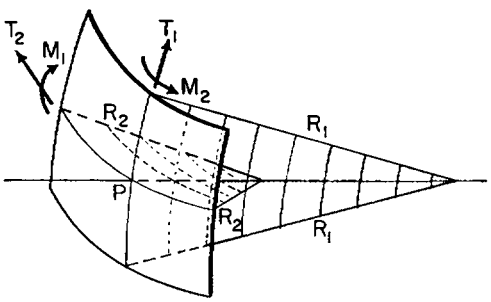

FIGURE 2 Illustrating the two principal radii of curvature of an element of the membrane, and some of the tensions and bending movements involved in its deformation.

two distinct types of deformation that can occur in an element of membrane. Not all components of stress are shown. Stresses T_1 and T_2 acting tangentially to the surface at point P and in the directions of the two "principal curvatures," tend to increase the membrane area. These stresses will be referred to as "tension" in the membrane. In addition and acting independently, M_1 and M_2 are moments acting to change the "curvatures," $1/R_1$ and $1/R_2$, of the membrane (to bend it). These components of stress will be referred to as "rigidity" of the membrane. Without going into the mathematical details of analysis, which in the general case is intractable without making some simplifying assumptions, four sets of basic assumptions about the elastic properties can be made.

1. The tension of the membrane can behave like surface tension, *e.g.* of a soap bubble, in which case the stresses in the membrane do not change with deformation. Also M_1 and M_2 are zero, $T_1 = T_2$, and this tension is not changed with strain or

deformation. The equations governing the analysis of this case are those of capillarity as applied to fluid interfaces (*i.e.* the general law of Laplace, $P = T(1/R_1 + 1/R_2)$). T would have to be the same over the surface of the red cell, and independent of stretch.

2. The membrane may be elastic, in which case the stresses vary with strain. If the stresses required to bend the membrane are insignificantly small compared with those required to stretch the membrane, the former can be neglected and the *"membrane theory"* of elastic shells can be used (Timoshenko, 1940).

3. If the membrane is rigid, *i.e.* if the stresses required to bend the membrane are significantly greater than those required to stretch the surface, or if the deformation is such that no tangential stresses develop, then the latter can be neglected and the theory of *"pure bending of shells"* (Novozhilov, 1959) can be used.

4. Finally if the stresses required to bend the membrane and to stretch the surface are of the same order of magnitude, both types of deformation must be considered and the general theory of deformation of shells must be used.

The attempt in this research was to determine which set of the above assumptions is applicable to the red cell, in considering the equilibrium discoid shape.

Technique of Measuring the Mechanical Properties of the Red Cell Membrane. Mitchison and Swann (1954) have described a technique for measuring the stiffness of the cell membrane of various marine eggs. In brief the method required a measure of the "deformation" x (Fig. 3) of a cell into a micropipette under a

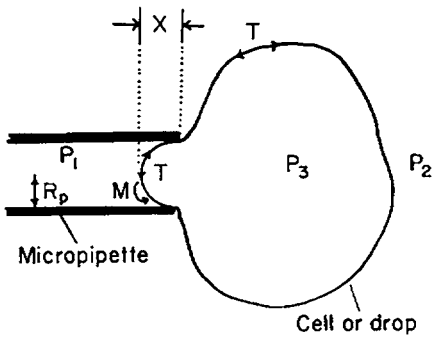

FIGURE 3 Illustrating the method used by Mitchison and Swann (1954) to measure the stiffness of the membrane of marine eggs, which in this research has been applied to the red cell. Symbols are explained in the text.

negative pressure difference $(P_2 - P_1)$. A plot of this pressure against the deformation x gave a linear relation, the slope of which was called the stiffness. The authors indicate that both the rigidity and tension of the membrane resisted the movement of the cell into the micropipette. We have used this general technique to investigate the properties of the red cell membrane. However, because of the differences between the red cell and marine eggs, listed below, it will be profitable to outline in detail the technique used here.

1. The small size of the red cell ($8\ \mu \times 2\ \mu$) precludes an accurate measurement of the distance x (Fig. 3) required to obtain the slope of the stiffness curve.

2. The marine eggs are very nearly spherical in shape and consequently when the egg was deformed by pulling a portion of it into the pipette, the area must have suffered an increase. The red cell, on the other hand, is not spherical and the cell could be drawn into the pipette without any increase in the total area of the cell, which is conserved by the residue of the cell becoming more spherical. The red cells behaved differently from the eggs in moving into the pipette. Because of these important differences the pressure required to pull the cell into the pipette will be called the "resistance to deformation" of the cell membrane. The relative contribution of rigidity and tension to this resistance will be discussed later.

Of the four basic sets of assumptions regarding the elastic stresses the first two lead to an analysis predicting that the curvature of the membrane will be inversely proportional to the pressure difference, P, across it; *i.e.*,

$$P = T(1/R_1 + 1/R_2) \tag{1}$$

where T is the tension in dynes/cm, P is in dynes/cm^2, and the radii in cm. If resistance to bending is also involved, there may be an approximate, similar relation; *i.e.*,

$$P = S(1/R_1 + 1/R_2) \tag{2}$$

where S is a parameter denoting the resistance to deformation, which may include both rigidity and tension. S will, like tension, be in dynes/cm. The sum of $1/R_1$ and $1/R_2$ will be called the "curvature" of the membrane. The general theory of shells, where solutions are in general intractable, might suggest that the portion of S that represents resistance to bending would not follow this simple law. The application of the law must then depend upon empirical demonstration that it is approximately true for the red cell, as it turned out to be in the work of Mitchison and Swann for the marine eggs. In the model experiments of Mitchison and Swann (1954*a*), using a system where the deformation was resisted by both rigidity and tension (that is the system represented by the third and fourth set of the above assumptions) the resulting deformation experimentally followed this relation. For this reason it will be assumed in the following that the laws which govern the relation between pressure, curvature, and resistance to deformation are the same as those of surface tension. This assumption must be validated by the results.

VALIDATION OF THE TECHNIQUE USED FOR MEASURING THE RESISTANCE TO DEFORMATION OF THE RED CELL MEMBRANE

When used to measure the interfacial tension between two liquids the technique requires a measurement of the pressure needed to draw a drop of liquid, suspended in water, into a pipette of known radius also containing water. The pressure is required to overcome the interfacial tension between the two liquids. In Fig. 3, con-

sider a drop of liquid being pulled into a pipette of radius R_p by a pressure difference $(P_3 - P_1)$. P_3 is the pressure inside the drop, P_2 the pressure of the liquid the drop is in, and P_1 the pressure inside the pipette. If the law of Laplace is applied to the spherical cap (meniscus) being pulled into the pipette, and when the cap is pulled into a hemispherical shape by decreasing pressure P_1, then $R_1 = R_2 = R_p$ and

$$(P_3 - P_1) = 2T/R_p \tag{3}$$

where T is the interfacial tension between the two liquids. When P_1 is decreased further the system becomes unstable and the drop flows into the pipette.

The difference in pressure between the water outside the drop and in the pipette can be measured experimentally. Let this be P. Then from (3)

$$P = (P_2 - P_1) = 2T/R_p + (P_2 - P_3) = 2T/R_p - P_d \tag{4}$$

where P_d is the excess pressure inside the drop over the outside pressure (*i.e.* $P_3 - P_2$). Two different tests of this equation are available.

1. *Very large drops.* On the same large drop of liquid, the critical pressure difference P may be measured using different sizes of pipettes. A plot of P *versus* $1/R_p$ should yield a straight line, the slope of which would be equal to $2T$, while the negative intercept should give P_d. In the case of a sufficiently large drop this intercept should be very small.

2. *Small drops of different radii.* The critical pressure difference P may be measured using the same pipette of radius R_p, but on drops of different radius R_d. By the law of Laplace

$$P_d = \frac{2T}{R_d} \tag{5}$$

Substituting in Equation (3):

$$P = \frac{2T}{R_p} - \frac{2T}{R_d} \tag{6}$$

Equation (6) predicts that a plot of P *versus* $1/R_d$ should give a straight line of negative slope equal to $2T$, and an intercept on the axis of $1/R_d$ equal to $1/R_p$. The value of this intercept can be checked with the measured value of the radius of the pipette.

RESULTS OF VALIDATION EXPERIMENTS

(*a*) *Measurement of Interfacial Tension Using Very Large Drops*

Procedure. Micropipettes were drawn using an apparatus (supplied by Leitz) capable of producing open pipettes with inside diameters down to less than 0.25 μ. The pipettes were usually shaped to enable work to be done in a hanging drop (Fig. 4). They were filled with distilled water using a technique described by Gesteland *et al.* (1959). A filled pipette was then connected by a water-filled tubing to an open reservoir which could

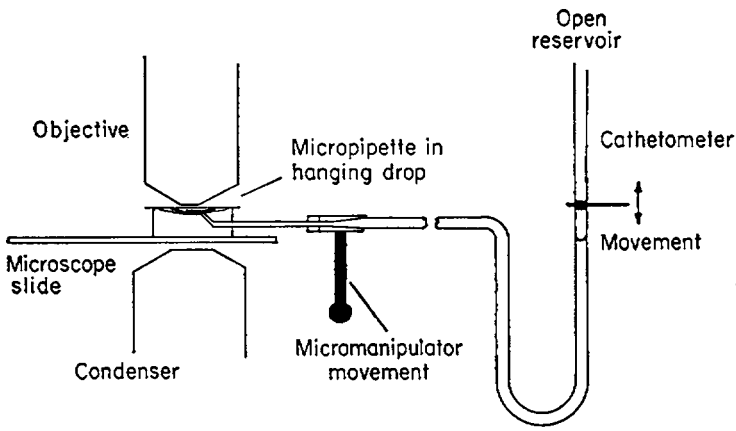

FIGURE 4 Apparatus used in technique of measuring resistance to deformation of the red cell membrane.

be raised and lowered and whose height could be measured to ±0.05 mm (a cathetometer movement was used). A schematic diagram of the apparatus is shown in Fig. 4. Movement of the pipette in the hanging drop was accomplished by using a micromanipulator, and the tip was observed with a high power microscope.

With the pipette filled with water and set into a hanging drop of isobutyl alcohol (of radius of curvature several centimeters), with this tip very near to the underside of the coverslip, the reservoir was progressively lowered and the height required to pull isobutyl alcohol into the pipette was observed. This was repeated many times to obtain a good mean value. The diameter of the pipette was then measured using a calibrated micrometer in the eyepiece of the microscope. Zero pressure ($P_2 - P_1 = 0$), or the height of the reservoir required to give zero flow, was obtained by pulling isobutyl alcohol far into the pipette and then raising the reservoir until small dirt particles remained stationary in the mouth of the pipette. This gives a very sensitive index indeed of the reference level of pressures.

Independent measures of the interfacial tension between isobutyl alcohol and water were made using a de Noüy balance, calibrated by an air-water interface.

Experiments using oleic acid and caprylic acid, each against water, were carried out using the micropipette technique described above.

Observations. The interface between isobutyl alcohol in the large hanging drop and the water in the pipette was observed to be very unstable, as a vibrating interface, until it moved into the pipette very suddenly at the critical pressure. This occurred consistently at the pressures reported for several trials with the same pipette (SEM $\approx \pm$ 0.2 mm H_2O). Fig. 5 is a plot of the critical pressure ($P_2 - P_1$) against the reciprocal of the radius of the pipette, $1/R_p$. The slope and intercept give a measure of interfacial tension of 2.42 ± 0.04 (SE) dynes/cm, and of internal pressure of the hanging drop + 0.202 ± 0.208 mm H_2O. The latter is not significantly different from zero. According to Equation (6) this intercept should be zero in

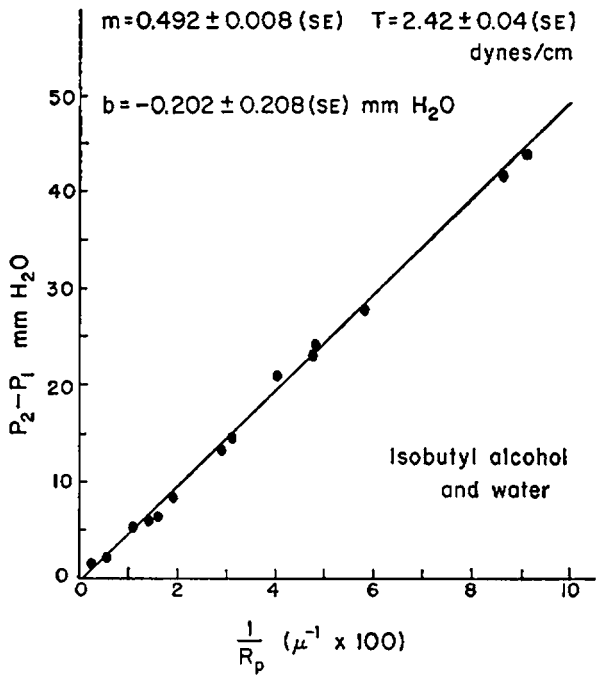

FIGURE 5 Results of measurement of critical pressure ($P_2 - P_1$) required to draw a meniscus of the interface of a large drop of isobutyl alcohol in water into pipettes of different radii R_p.

these experiments, since the radius of curvature of the hanging drop was extremely large, as indicated in Fig. 4. The interfacial tension between isobutyl alcohol and water, using the de Noüy balance, was 2.9 ± 0.1 (SE) dynes/cm. The value published by Antonow (1907) measured by the drop method is 2.1 dynes/cm. The reason for the discrepancy in these values is unknown, but is probably a result of variation in the technique of measurement.

Trials with the higher interfacial tensions of oleic acid and caprylic acid, each against water, resulted in movement of the meniscus so slight at the critical pressure that the latter was difficult to determine accurately. With the lower interfacial tension of isobutyl alcohol against water the critical pressure could be identified very accurately as this liquid flowed very quickly or not at all. The experiments with oleic acid and caprylic acid, each against water, gave tensions of 13.1 to 18.2 (mean = 15.7) dynes/cm and 7.7 to 8.9 (mean = 8.4) dynes/cm respectively compared to published values of 15.6 dynes/cm and 8.2 dynes/cm.

(*b*) *Measurement of Interfacial Tension Using Very Small Drops*

Procedure. Stable suspensions of small isobutyl alcohol drops in water could not be obtained. Hence a mixture of paraffin oil and detergent solution was used to obtain a

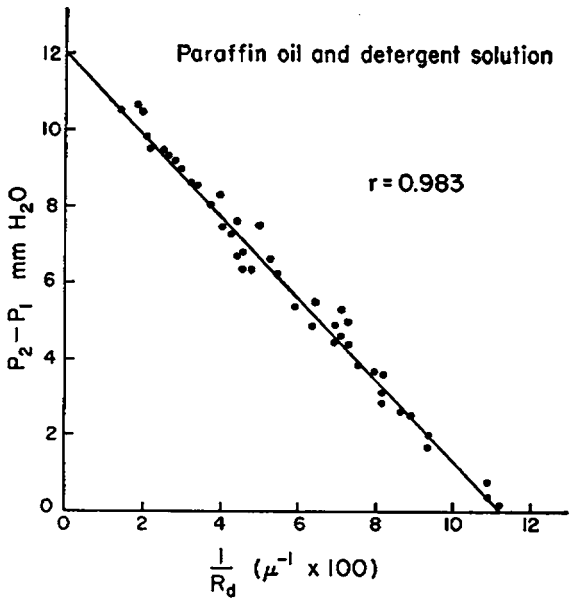

FIGURE 6 Results of measurement of critical pressure ($P_2 - P_1$) required to draw a meniscus of small drops of paraffin oil of different radii R_d into a pipette of fixed radius.

low interfacial tension and this mixture was shaken violently to produce very small suspended drops of the oil. Using the same pipette for all drops, the critical pressure required to pull drops of varying size into the pipette was determined as previously described. Measurements of the pipette diameter and of the diameter of each oil drop before it was pulled into the pipette were made with the eyepiece micrometer.

Observations. A plot of P against $1/R_d$ is given in Fig. 6. A linear relation ($r = 0.983 \pm 0.026$ SE) with negative correlation exists as predicted in Equation (6) proving that the critical pressure depends on the pressure P_2 inside the drop. The intercept on the $1/R_d$ axis, where P is zero, gave a radius of the pipette of 8.9 μ. By measurement with the eyepiece micrometer the radius was 8.3 μ, indicating that the diameter was underestimated by approximately 8 per cent. This could arise because of the poor optics in viewing through a hollow cylindrical glass pipette.

Discussion of the Technique

The combined results of these experiments indicated that the critical pressure required to pull a liquid drop into a pipette containing a second liquid depends on the size of the pipette, the interfacial tension between the two liquids, and the pressure inside the drop. Then knowing the size of the pipette, the interfacial tension and internal pressure can be determined as indicated by Equation (6).

The results of the second experiment also indicated that measurement of the pipette using the eyepiece micrometer can result in an error of approximately 10 per cent. This error may be higher for pipettes of smaller diameter where the optics of observing the internal diameter may be even poorer.

APPLICATION OF THE TECHNIQUE IN MEASURING THE DEFORMABILITY OF THE RED CELL MEMBRANE AND THE HYDROSTATIC PRESSURE ACROSS THE WALL

Introduction. The technique described above was used to measure the resistance to deformation of the red cell membrane and the pressure within the cell. In general, a part of the cell membrane was pulled into a small pipette with the application of negative pressures, as in the experiments with small drops of liquid. However, the red cell membrane is elastic, as are most cell membranes (*e.g.* Cole, 1932; Mitchison and Swann, 1954*b*) and the tension may increase with deformation. For this reason the cell does not flow abruptly and entirely into the pipette when the critical pressure is exceeded. At greater suction pressures, as the elastic membrane of the meniscus is stretched, the tension increases, and the cell then stops moving into the pipette unless the pressure is increased further. Three distinct stages are generally observed when a cell is pulled into a pipette. As the reservoir is lowered and the pressure difference $P_2 - P_1$ (Fig. 3) is gradually increased, the cell membrane interface (*a*) exhibits the same instability as do the liquid drop interfaces at pressures just below the critical pressure; (*b*) then becomes very stable and remains stationary, and finally (*c*) moves in very little further with increase in pressure difference. The first two stages are reversible and exhibit no "hysteresis" when the pressure is lowered again. If the critical pressure is exceeded so that the

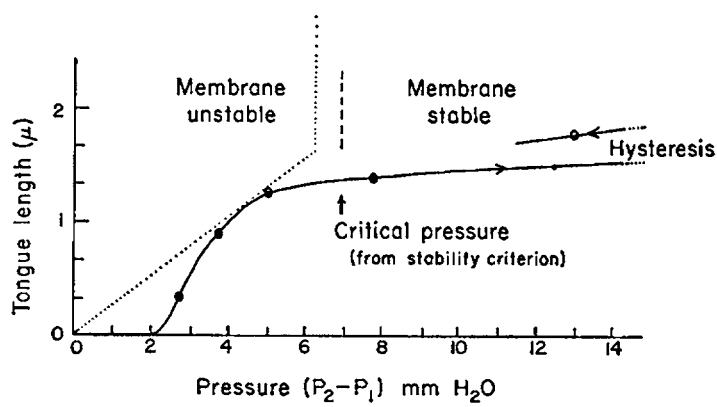

FIGURE 7 Illustrating the movement of the "tongue" into the pipette with increasing suction pressure ($P_2 - P_1$). Solid line, with red cells. Hysteresis is seen when the critical pressure, judged by observation of stability of the membrane, is exceeded. Broken line, schematic for the behaviour of liquid drops having a constant interfacial tension.

membrane moves in further, hysteresis is apparent. This process is represented graphically in Fig. 7, the values being obtained from measurements with the eyepiece micrometer for one of the largest pipettes. The distance the membrane moves into the pipette before it becomes stable is slightly more than the diameter of the pipette itself. The same dynamical behaviour is observed with the smaller pipettes but their small size precludes measuring the length of the tongue with any accuracy. The change in stability, however, could still be observed, quite accurately. The authors suggest that at stage (*b*) above, represented by the final increase in length of the tongue in Fig. 7, the membrane becomes stable and the critical pressure has been reached; at suction pressures above this the cell membrane in the meniscus tends to stretch, its resistance to deformation increases, and the cell will enter the pipette only a very little more. In these experiments the portion of the cell left outside the pipette was never in the form of a portion of a sphere, as shown in Fig. 1 (*a*), so that any stretching is probably confined to the meniscus, and the rest of the cell membrane is not stretched.

Method. The method described previously for small liquid drops was applied to measurement of resistance to deformation of red cell membranes. Pipettes drawn and shaped were filled with hypotonic (0.6 per cent NaCl), isotonic (0.9 per cent NaCl), or hypertonic (1.2 per cent NaCl) solutions according to the concentration to be used in the suspension medium of the red cells. Red cells were obtained from a finger prick and were suspended in one of the above solutions in a small enough hematocrit so that individual cells could be studied in the hanging drop. The cells were mixed with the solution on a coverslip and small drops of the suspension were placed on other coverslips previously wiped with lens paper. These drops were immediately covered with paraffin oil to prevent evaporation. In approximately 30 minutes one of these coverslips was inverted to form the large hanging drop shown in Fig. 4. Many cells were found to be hanging from the underside of the coverslip. The pipette could then be placed against a cell and the height of the open reservoir required to pull the membrane into the pipette, according to the criteria described above, was determined visually. Reversibility of movement of the tongue, by decreasing the pressure slightly, was checked continuously. This was repeated 2 to 6 times on 10 to 15 cells of each group for each pipette. The pipette size was then determined with the eyepiece micrometer and then the height of the reservoir required for zero flow in the pipette was determined by observing cells or particles of debris flowing in and out of the pipette. (Sometimes it was necessary to break the very small pipette tips off against the coverslip to get a large enough orifice for dirt or cells to enter freely.) This reference height could be determined within ± 0.05 mm.

This procedure was performed on four groups of cells described below:

1. Cells suspended in 0.9 per cent NaCl (unbuffered pH $\approx$ 6): These cells were checked for good biconcave shape. Determinations were made on the rim of the cell, and in some cases on the rim and the biconcavity of the cell using the same pipette and, in many instances, on the same cell (Fig. 8*a*).
2. Cells suspended in 0.6 per cent NaCl (unbuffered pH $\approx$ 6): These cells were swollen into a spheroidal, but not spherical, shape (Fig. 8*b*).

3. Cells suspended in 1.2 NaCl (unbuffered pH $\approx$ 6): These cells were shrunken, very crenated spheres (Fig. 8*c*).
4. Attempts were made to apply the same method to cells that had become spherical, just short of the point of hemolysis. The results were very different.

Observations. Microphotographs of fields of the three types of cell are shown in Fig. 8 as well as a photograph of a tongue in a pipette.

Qualitative observations on the three groups of cell are as follows:

Group 1. Determination of the critical pressure on the rim of the cell followed the pattern previously described. The movement of the tongue pulled from the biconcavity appeared to be more of an "all or nothing" action than that of the rim. However, the poor optics in observing the pipette at the biconcavity precluded making a quantitative estimate of this difference. The critical point could, however, be measured accurately.

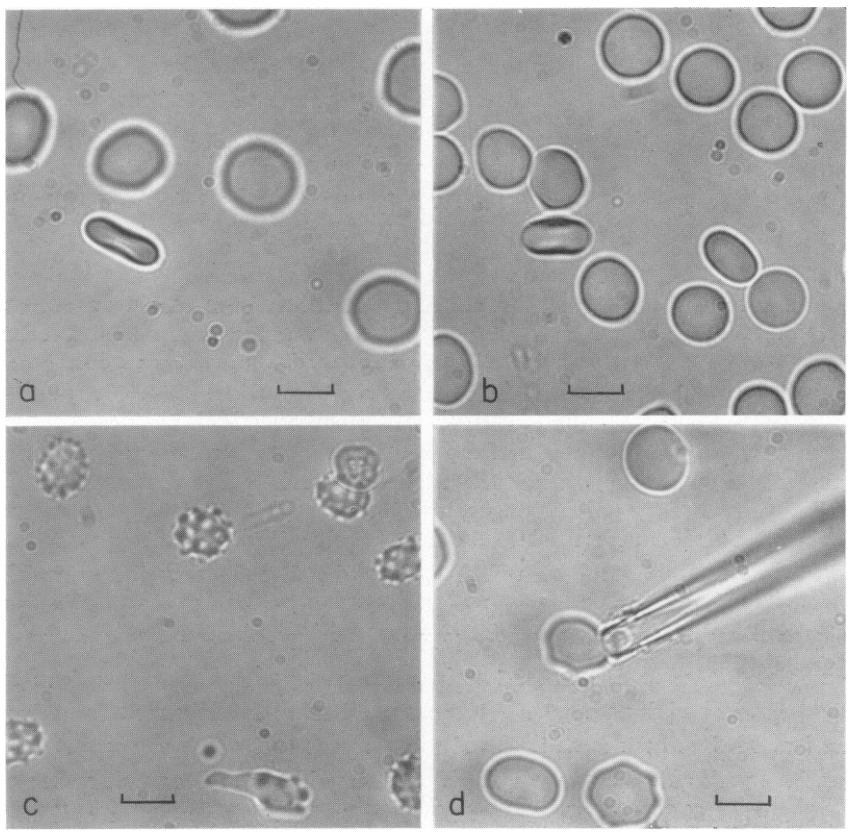

FIGURE 8 Microphotographs of red cells: (*a*) Group 1, of normal biconcave shape. (*b*) Group 2, swollen to ellipsoid. (*c*) Crenated after exposure to hypertonic saline. (*d*) A cell being drawn into the tip of the pipette, showing the meniscus in the pipette. The scale indicates 5 μ.

Group 2. The cells of this group were never swollen to the extent that when the tongue was pulled into the pipette, the part remaining outside the pipette was a portion of a sphere. If this did occur, the outside portion of the cell had an extremely high tension as described in Fig. 1 (*a*), and the behaviour was as in group 4, below.

Group 3. The tongues of the crenated spheres also had an all or nothing movement, and appeared to move into the pipette further at the critical pressure than did those of groups 1 and 2.

Group 4. Attempts to draw a tongue into the pipettes on cells that had become almost spherical and when the part remaining outside the pipette was a portion of a sphere as in Fig. 1, were unsuccessful. No matter how high a suction pressure was applied (even up to 100 mm Hg), no movement of the tongue could be produced without hemolysis. The time at which hemolysis occurred depended on the length of time the pressure was applied, and this has led, in further experiments, to information as to the "breaking stress" of the membrane when it is stressed appreciably. The tension in the membrane and probably the internal pressure in the cell increased to values many times those found in the non-spherical cells. Another important observation made with the swollen cells was as follows. When equilibrated with a sufficiently hypotonic medium, so that the dimple curvature has been reversed and the cell is a swollen ellipsoid, then the cells very abruptly "popped" from this swollen ellipsoid into a perfect, "glassy" sphere (Ponder, 1948). If the cells were returned to an isotonic medium immediately, no hemolysis took place, and the cell returned, not to the normal biconcave shape, but to a crenated sphere. If returned to an isotonic medium just before the point of "popping," the cells returned to the normal shape.

The quantitative results for groups 1 and 2 are given together in Fig. 9. Here a plot of $(P_2 - P_1)$, the critical pressure against $1/R_p$ for all cells of groups 1 and 2, indicates:

1. There is a linear correlation between these two variables as predicted by Equation (4). The coefficient of correlation was $r = 0.902$ (± 0.087 SE).
2. The measurements made on the rim of the cell, the biconcavity of the cell, and the membrane of the swollen cells all fit the same linear relation.
3. From the regression line calculated by grouping all these points the resistance to deformation, 2S, in the membrane is 0.037 ± 0.002 (SE) dynes/cm and an excess pressure of 2.32 ± 0.75 (SE) mm H_2O exists inside the cell, whether this is a biconcave disc or swollen spherical cell.

Fig. 10 represents the same plot of $(P_2 - P_1)$ *versus* $1/R_p$, for the crenated spheres of group 3. The regression lines indicates a membrane resistance to deformation 2S of 0.013 ± 0.003 (SE) dynes/cm, which is significantly less than for Fig. 9, and a pressure across the wall of -0.412 ± 0.63 (SE) mm H_2O; the latter is insignificantly different from zero.

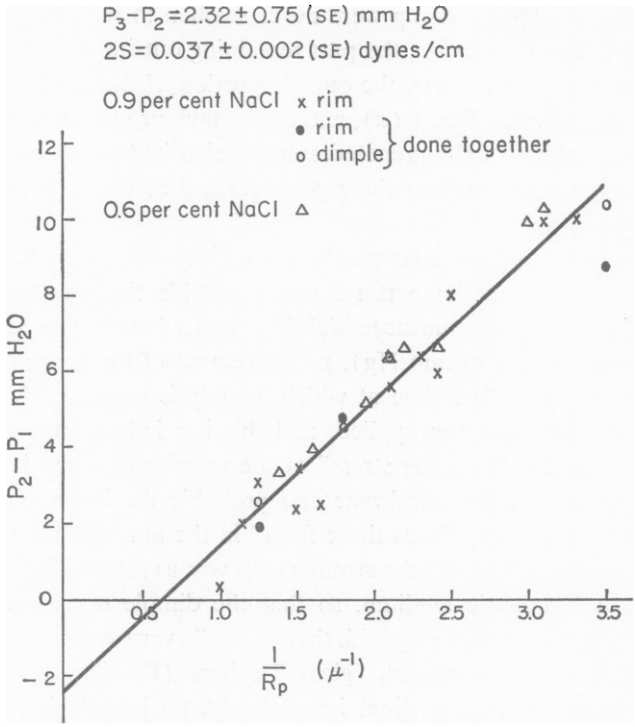

FIGURE 9 Results for cells of group 1 (normal biconcave cells) and group 2 (swollen to ellipsoids). The results of both types of cell fit the same straight line, which gives a value of the resistance to deformation S, and indicates an excess pressure within the cell.

DISCUSSION

(*a*) *Deformation of Crenated Cells*

Comparison with Results of Others. These results agree with those of Mitchison and Swann (1954*b*), both with their model experiments with thick rubber balloons and with the marine eggs. Experiments with rubber balloons indicated that although the pressure inside the balloon changed from nil to 15.3×10^4 dynes/cm^2, a plot of P, the pressure required to obtain a deformation x, equal to the radius of that pipette, (Fig. 3) against $1/R_p$ gave straight lines as in Figs. 9 and 10. It appears that when the pressure is raised from zero and the resistance to deformation changes from rigidity only to rigidity plus tension in the membrane, the straight line relation persists, but with changes in slope and intercept. Their experiments with the marine eggs indicated that when the eggs were placed in isotonic and hypertonic solutions, the stiffness or resistance to deformation of the membrane remained the same. Wrinkles occurred in the membrane in hypertonic solutions. When the eggs were placed in hypotonic solution, the stiffness increased. These re-

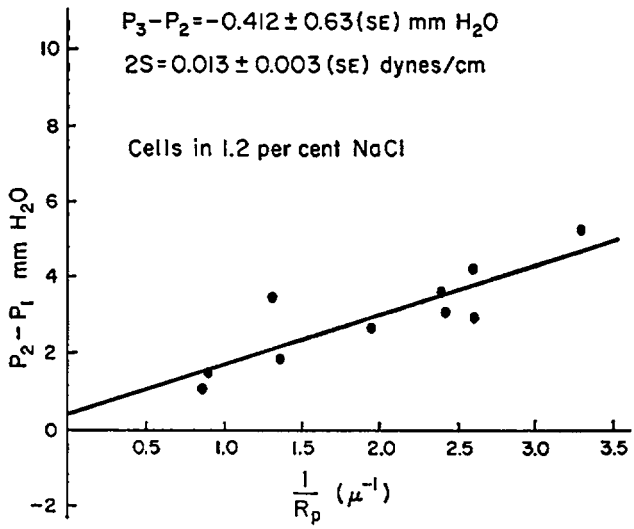

FIGURE 10 Results for cells of group 3 (shrunken and crenated). The line indicates a lower resistance to deformation, possibly resistance to bending only, and an insignificant internal pressure.

sults suggested that the pressure inside the marine eggs in isotonic and hypertonic solution was close to zero and stiffness was due merely to rigidity of the membrane. In hypotonic solution the cell took in water, the pressure increased, and increased stiffness was due to both rigidity and tension in the membrane. The membrane of the red cell, on the other hand, decreased in resistance to deformation and was deformed more easily when the cell was placed in hypertonic solution. These differences will be discussed subsequently.

Estimation of Internal Pressure. For two reasons it was possible to obtain indirectly an estimate of the internal pressure of the red cells. First, since the red cell is not in the form of a sphere, the cell can be distorted into the pipette with no increase in total surface area of the cellular membrane, and hence no change in tangential stress or tension in the membrane occurs. It can in fact be observed that the cell moves quite freely into the pipette; *i.e.*, that even the part of the membrane isolated by the pipette orifice is not stretched. No hysteresis, which would be expected if there were any adhesion or friction between the cell and the glass pipette, was ever observed. This difference in cell shape between the red cell and the marine egg leads to a difference in behaviour of the cells during deformation, such that the red cell exhibited a "critical pressure" at which the rigidity and tension of the membrane in the pipette appeared to be overcome. Secondly, by varying the pipette diameter it was possible to obtain the plots of Figs. 9 and 10, and the intercept gives at least a relative measure of the pressure difference across the cell wall.

As far as we know, this is the first published experimental evidence that the hydrostatic pressure within the normal red cell is greater than outside the cell. It is close to the value (2.6 mm H_2O) found by Cole (1932) for the *Arbacia* egg. The excess pressure takes on a new interest in view of the theory of Teorell (1962), who has added the new parameter of the difference of hydrostatic pressure to the Hodgkin-Huxley model of bioelectric potentials. Since as yet there are no measured values for the potential difference across the membrane of red cells, the value found for the pressure has not yet been fitted into a "membrane pump" of this kind.

Rigidity of the Red Cell Membrane. In the deformation of the crenated cells, Fig. 10 indicates that the cell is more easily deformed than the discoid or swollen cells, and that the pressure across the cell wall is decreased to values insignificantly different from zero. The presence of crenations or wrinkles on the red cell membrane also suggests this. For zero pressure there can be no tension due to stretching in the membrane, and the significant slope of the line in Fig. 10 ($S = 0.0065$ dynes/cm from Equation (2)) must be the result of the rigidity or pure bending of the membrane rather than of an elastic tension.

The theory of pure bending involving large deformations in shells or membranes is mathematically intractable for the type of deformation used in this experimental study. However, from the straight line of Fig. 10, where it is assumed that pure bending of the membrane is occurring, an estimate of this rigidity can be obtained from the slope of this line. If stretching of the membrane in the pipette does occur, this estimate gives an upper limit for the rigidity. On this basis it can be said from the slope of the empirical curve, Fig. 10, that a pressure of 0.65 mm H_2O is required to produce a change in curvature, $(1/R_1 + 1/R_2)$, of the membrane of 1.0 μ^{-1}, and this is an upper limit for the rigidity.

(*b*) *Deformation of the Biconcave Disc and Swollen Cell*

Internal Pressure and Resistance to Deformation. The difference between Fig. 9 and Fig. 10 represents a change in resistance to deformation of the cell membrane (slope of the line) and a change in the internal pressure of the cell (intercept). In isotonic and hypotonic solution, the pressure inside the cell is approximately 2 mm H_2O, wrinkles or crenations are absent, and the cell is more difficult to deform as the resistance to deformation is here due to tension as well as to rigidity. The increased resistance to deformation is measured by the additional pressure required to produce an equivalent deformation for a given size of pipette.

Deformability in Relation to Cell Shape. Perhaps the most important deduction from Fig. 9 is that the resistance to deformation of the membrane of the discoid red cells appears to be identical at the rim of the cell and at the biconcavity ("dimple") region of the cell. This has important implications in considering the equilibrium shape of the red cell. The cell is deformed from its normal shape during our measurement, but because the resistance to deformation in these tests appears to be the

same all over the surface, it is unlikely, for instance, that a difference in thickness exists between rim and dimple regions. This makes an important discrimination between theories that might eventually be advanced to explain the normal discoid shape of the red cell.

A second important result from Fig. 9 is that as long as the cell in hypotonic solution is swollen to an elliptical shape (but not quite to a sphere) as indicated in Fig. 8*b*, no difference in resistance to deformation or in internal pressure is observed. Estimates of the surface area of several equilibrium shapes of the same cell in different tonicities (Fig. 11) (Rand and Burton, 1963) indicate that no change

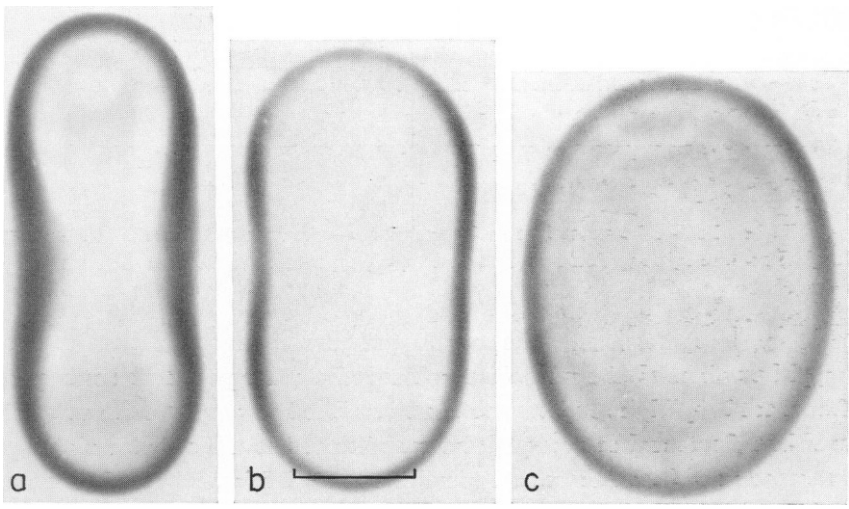

FIGURE 11 Examples of the equilibrium shapes of a single red cell in the process of osmotic swelling. The change from (*a*) to (*c*) is not associated with an abrupt reversal of curvature but the stage at which the sides are flat represents an equilibrium shape. The outline of the membrane is drawn according to rules explained by Ponder (1930). The scale is 2 μ.

in area can be detected as the cell passes from a biconcave shape to almost a spherical shape.

The results have an indirect bearing on the question of the causes of the change in shape, from discoid to the hemolysing sphere, which occurs in osmotic swelling. In this sequence, our method failed to indicate any significant change in the internal pressure. To recognize that if the cell is to have an increased volume without increase in area of the membrane, its shape must change towards the spherical, does not explain the nature of the forces that compel the membrane to change its shape. How much change in pressure would be required to effect these shape changes? The data provide an estimate of this.

No matter how we interpret the slope of the line in Fig. 9 (as a tension, "resist-

ance to bending," or anything else), this slope is an index of the pressure change that will produce a unit change in curvature (in μ^{-1}). This value is 1.85 mm H_2O pressure change per reciprocal micron change in curvature (this is $2/R_p$ in Fig. 9). An estimate of the change in curvature, at the rim region of the cell and at the dimple region, when the cell passes from shape (*a*) to shape (*c*) of Fig. 11, is given in Table I.

TABLE I

CHANGE IN CURVATURE OF THE RED CELL MEMBRANE FROM DISCOID TO ELLIPSOID SHAPE, IN μ^{-1}

	Discoid	Ellipsoid	Change in curvature
Region of rim	0.992	0.708	−0.284
Region of dimple	−0.441	0.454	+0.895

From the value 1.85 mm H_2O per μ^{-1}, these changes might be effected by a change of internal pressure of only about 0.8 mm H_2O. This is within the error of estimate for the intercepts on plots such as Fig. 9, particularly since we had not calculated regression lines for the points for the swollen cells, and for those of normal shape, separately. In other words, pressure changes sufficient to produce the changes of shape of the membrane in osmotic swelling would have been undetected in our experiments.

However, when the theory of shells is applied to the sequence of changes of shape that occur, which are certainly equilibrium configurations (there is no sign of instability of shape at any stage of osmotic swelling except at the final abrupt change from the swollen ellipsoid, Fig. 11*c*, to a rigid sphere), grave doubts are raised whether changes in pressure could explain the changes of shape. The considerations that should be introduced, such as possible anisotropic stresses in the membrane (different in the longitudinal and latitudinal directions at each point), bending moments, and so on, are so numerous and difficult that further discussion of this point would be inappropriate, and would tend to detract from the positive empirical results of this research. It may be concluded, however, that since no changes in deformability can be detected at different regions on the cell, or for the swollen cell, it is impossible to say that these shape changes result from differences in rigidity *or* tension. It is entirely likely that the contribution of these two components of stress in resisting deformation changes from pure rigidity for the crenated cell to nearly wholly tension in the spherical cell (shown in Fig. 1(*a*)) with significant contributions from each for the stages in between these two extremes. This makes it impossible on the basis of the evidence presented to say that the rigidity and tension are significantly different and to use any of the three sets of simplifying assumptions given previously. Hence analysis of the equilibrium shapes of the cell,

from disc to ellipsoid, becomes mathematically intractable, even assuming that the only external force acting is the pressure difference across the cell wall. It is still possible that there are other external forces acting and contributing to the cell shape, which have not yet been taken into account.

Stability of the Red Cell Membrane to Bending versus Stretch. There is much evidence that the membrane of the red cell can suffer great degrees of bending without damage. Examples are given in Fig. 12. Prothero and Burton (1962)

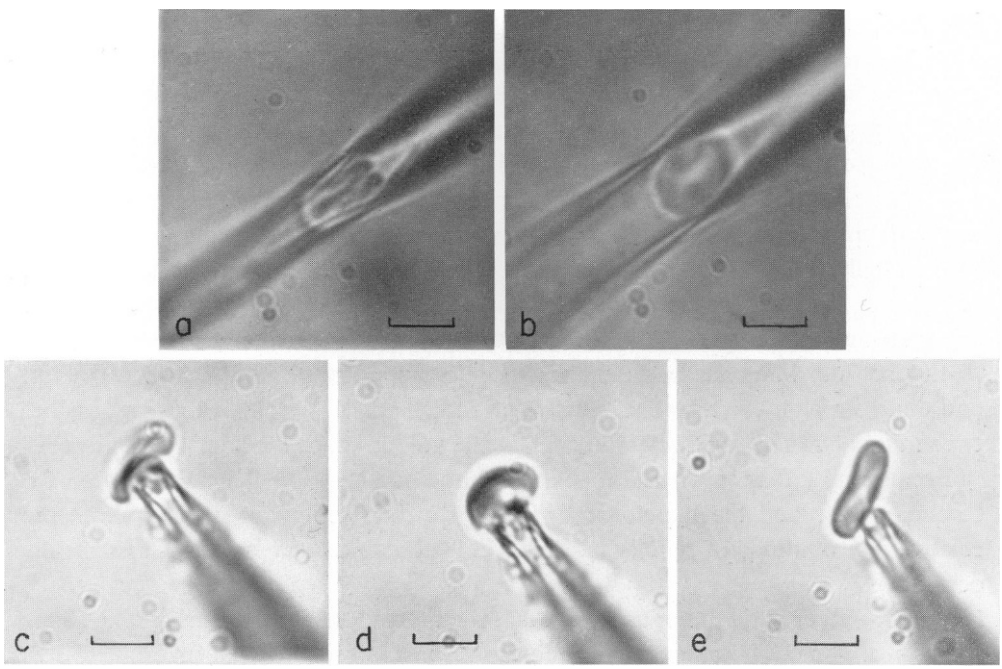

FIGURE 12 Illustrating how a normal red cell can be submitted to very great bending strain and yet regain its normal shape. (*a*) Tightly folded cell in a micropipette. (*b*) The same cell farther in the pipette, where it regains its normal shape. (*c*) A cell drawn into a pipette, broadside. It entered the pipette completely. (*d*) Leaving the pipette. (*e*) Restored to normal shape outside. The scale is 5 μ.

showed that red cells will pass through 3.0 μ pores under a pressure of less than 1 cm H_2O without hemolysis. Fig. 12*c* shows a red cell entering broadside a pipette about 2 μ in diameter under a pressure of 3 mm H_2O. In contrast, a very slight degree of tensional strain resulting in a small increase in area of the membrane (probably less than 10 per cent (Rand and Burton, 1963)), results in a drastic change in the membrane (the popping already described) and eventually to hemolysis. The necessary deformability of the red cell in the circulation is possible only because the normal shape is far from spherical, so that the changes in shape do not necessi-

tate stretching of the membrane, and because the resistance to bending of the membrane is so slight.

SUMMARY

1. A modification of a technique developed by Mitchison and Swann (1954*a*, *b*) is described for determining the resistance to deformation of the red cell membrane, at various regions over the cell, and for determining the pressure gradient across the cell wall. It requires a measure of the pressure needed to suck a portion of the cell into a micropipette.

2. The resistance to deformation of crenated cells was less than that of biconcave discs and of hypotonically swollen red cells, and in crenated cells there was no significant pressure gradient across the cell wall. The resistance to deformation of the crenated cells was therefore attributed to pure bending of the membrane.

3. The resistance to deformation of the biconcave disc was the same at the rim as at the dimple region of the cell, and this was the same as that of the membrane of swollen cells. It was higher than that of the crenated cells and an internal pressure of 2.3 mm H_2O, higher inside, was the same for discoid and for swollen cells. This increased resistance to deformation was attributed to a tension in the membrane.

4. Since no difference in resistance to deformation was found between the rim and dimple region of the cell, there was no indication of mechanical differences between these two regions that could explain the unique shape of the normal red cell.

Because no difference in resistance to deformation could be detected between the biconcave disc and the hypotonically swollen cell, nothing can be said about the relative contributions of rigidity and tension in the membrane as this shape change occurs.

5. The changes in curvature of the membrane that occur during the shape changes of osmotic swelling would require an increase of internal pressure of less than 1 mm H_2O, which might not have been detected by the method used. However, there are many difficulties in explaining the sequence of shapes as equilibrium configurations in terms of internal pressure alone, and other forces on the membrane, not yet recognized, are probably acting.

6. When the membrane is stretched, as in cells reaching the spherical shape by osmotic swelling, an abrupt increase in rigidity or tension of the membrane occurs as the resistance to deformation increases very many times and the internal pressure of the cell probably rises to high values. Hemolysis follows this abrupt change in the membrane, though it is not coincidental.

7. The membrane of the red cell can suffer very great bending strains without irreversible changes but is drastically changed by any tangential stress that results in increase in area.

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