

## **The Minimum Energy of Bending as a Possible Explanation of the Biconcave Shape of the Human Red Blood Cell**

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The membrane of the erythrocyte is assumed responsible for the cell's curious biconcave shape. Combined theoretical and experimental results explain the biconcave shape by the principle of least total curvature of the membrane. This has been interpreted to mean that the energy of bending is minimized. The red-cell membrane is considered uniform over its surface. To accommodate the known viscoelastic properties of the membrane the expression for the bending energy of an element of area,  $dA$ , was modified to:  $dU \propto (1/R_1^2 + 1/R_2^2) dA$ .  $R_1$  and  $R_2$  are the principal radii of curvature. The classical equation for the ovals of Cassini was altered to provide an expression for generating hypothetical red-cell profiles. Using the Cassini equation and the bending relation above (ignoring other membrane forces), the energy was determined for many surfaces of revolution having the same area and volume. The geometry and energy of the shape with least energy closely approximated the bending energy and geometry of the actual red-cell profile with the same area and volume. In addition to predicting the geometry of 23 cells in an isotonic medium and nine osmotically swollen cells, the bending hypothesis predicted a swelling sequence for a single cell which was similar to the observed series of Rand (1967). The existence of cup shapes frequently observed in hypotonic media fits within the framework of the bending hypothesis. The concept of bending energy adequately explains the shape of the erythrocyte.

### **1. Introduction**

Since the discovery of the red blood cell in the seventeenth century its curious biconcave shape has been an enigma. Several recent publications (Rand & Burton, 1964; Rand, 1964a; Murphy, 1965; Fung, 1966; Fung & Tong, 1968) deal specifically with the problem but the conflicting evidence in these papers suggests that there is need for further study. Brånemark & Lindström (1963) reported that cells resumed their equilibrium form within a fraction of a second after emerging from very small blood vessels. Rand (1964b) showed that a cell released from a micropipette returned to the biconcave shape within a few seconds. These observations imply that the

biconcave form requires the least energy to be maintained. We believe the energy minimized is the bending energy of the membrane, and that the membrane is solely responsible for the cell's shape. (Specifying the area and volume is insufficient to determine the shape of an object as shown in Fig. 1.)

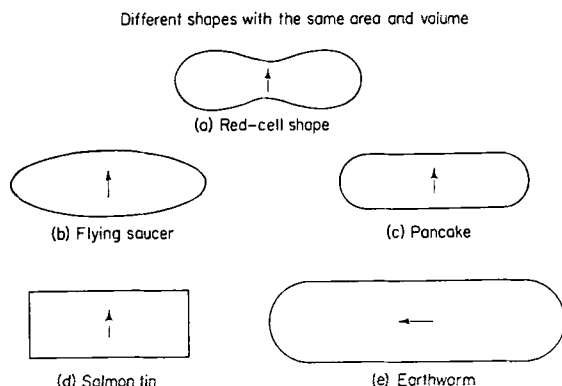


FIG. 1. Several shapes each having the same area and volume. The arrows indicate the axes of rotation. The earthworm shape appears too large because of an inability to project a plane view into three dimensions. Profiles (a) and (b) were generated by the modified equation for the ovals of Cassini.

Disregarding the influence of bending energy Rand & Burton (1964) and Rand (1964a) drew two conclusions from their investigation into the visco-elastic properties of the red-cell membrane. First, an internal positive pressure of 2 mm of water is indicated, and second, tensional forces alone are insufficient to account for the biconcavity. Rather than consider bending they discussed the necessity of an attractive force between the two opposing membranes in the central region of the cell. (This is inferred in their papers and clearly outlined in Rand's Ph.D. thesis, 1964, University of Western Ontario, London, Canada.) However, their experiments, repeated on crenated cells (cells subjected to a hypertonic environment), indicated that the bending of the cell membrane was not negligible but was as significant as tension. Lopez, Duck & Hunt (1968) discussed the possibility that the biconcave configuration represents an isopotential surface. They suggested that the membrane has a conducting charged layer and therefore adopts the biconcave shape in order to minimize its electrical potential energy. Fung & Tong (1968) took a theoretical approach to the problem and concluded that the bending energy is negligible and that the equatorial region of the membrane possesses a very specialized mosaic of tensional stiffness. However, Rand & Burton (1964) found no evidence of such regional variations in their experi-

ments. Shrivistav & Burton (1969) took another approach to the problem by considering that the contents of the cell are responsible for the biconcave shape.

Using the bending energy of the membrane as the basic consideration in our study, we sought a simple explanation for the shape of the red cell.

#### (A) PHYSICAL DESCRIPTION OF THE CELL

The mature erythrocyte is devoid of organelles and contains mainly hemoglobin, electrolytes and water. The membrane is a thin continuous viscoelastic envelope (Rand, 1964*a*). Many techniques have been used to measure the thickness of the membrane. Each method is based on a different property of the membrane and consequently yields a different value. This research is concerned with the mechanical thickness which is probably within the range of 100 to 300 Å. In isotonic buffered Ringer solution the average cellular dimensions are: diameter  $8.0\text{ }\mu$ , area  $136\text{ }\mu^2$  and volume  $107\text{ }\mu^3$ . These averages are from 1000 cells, measured individually (Canham & Burton, 1968).

During osmotic swelling the cellular area is considered to remain reasonably constant (Hoffman, Eden, Barr & Bedell, 1958; Ponder, 1948). The central region gets thicker and the biconcavity gradually disappears. At the final stage of swelling the cell becomes a perfect sphere and then hemolyzes (Rand, 1967). During osmotic shrinking the cylindrical symmetry persists until the tonicity is approximately 600 milliosmolar. Under these conditions the membrane goes into smooth bulges and depressions and is said to be crenated. These are reversible shape changes. The evidence that has been difficult for Fung & Tong (1968) to explain is that the red cell, before osmotic hemolysis, becomes a  $6.5\text{ }\mu$  sphere, a diameter significantly less than the original  $8.0\text{ }\mu$ .

#### (B) BASIC ASSUMPTIONS

(1) We assumed that the membrane consists mainly of two isotropic labile surfaces, an interpretation supported by the electron micrographs of membranes and the viscoelastic studies of Rand (1964*a*). It is acknowledged that micellar structures also exist in the membrane but since they are less stable (Stein, 1967, p. 22), it is assumed that the greater part of the membrane surface has the bimolecular leaflet arrangement proposed by Davson & Danielli (1952). The interpretation of the viscoelastic nature of the red-cell membrane is illustrated in Fig. 2. (One might consider the stress state in the membrane to be hydrostatic in two dimensions.) The flat membrane can only resist distortion temporarily, without area changes, in the form of a viscous

resistance, but can resist changes in area. Any attempt to change the area of either membrane surface leads to an elastic resistance to bending because the inner surface is compressed and the outer surface extended (Fig. 2). An essential assumption to this paper is that the membrane is not a slab of isotropic material. Otherwise material could change from one side of the membrane to the other because of the viscous property of the red-cell membrane. An elastic storage of bending energy at equilibrium would not be possible and one could then apply only "soap-bubble" physics to the isotropic

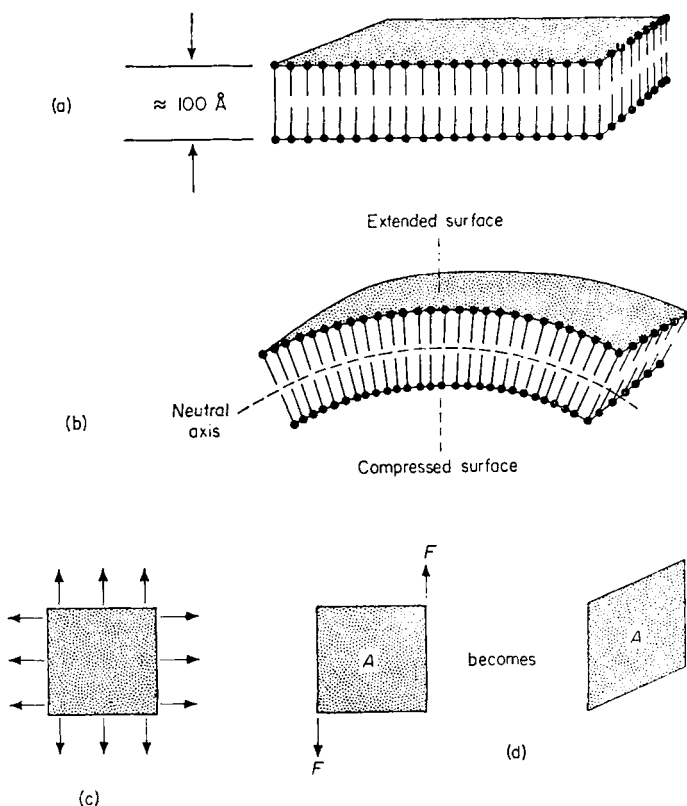


FIG. 2. A schematic interpretation of the red-cell membrane. (a) The edge of a flat portion of the membrane shows the leaflet model proposed by Davson & Danielli (1952). The many variations of this model which have been proposed are not relevant to this communication. (b) An element of membrane has been bent in one plane, showing the compression of the inner surface and the stretching of the outer surface. (c) An element of area which is stressed equally in all directions in the plane of the membrane (a two-dimensional hydrostatic stress) will resist the stress elastically. (d) The membrane will only offer viscous resistance to shear stresses, resulting in deformation with no storage of elastic energy.

membrane. The elastic property of films (Gibb's elasticity; see Adamson, 1967, p. 529) is supporting evidence for this elastic model of the red-cell membrane.

(2) The red cell is not assumed to be cast in the biconcave shape but rather that the biconcave shape minimizes the elastic energy stored in the membrane. We assumed that an element of membrane has no stored bending energy if it is flat, and that any curved element of membrane has stored elastic energy.

(3) Any shape changes owing to osmotic volume shifts are considered to take place without altering the total cellular area, that is the area of the neutral axis (Fig. 2). Local membrane shear stresses which must arise from shape changes could be accommodated by minor molecular re-arrangements in the labile surfaces. No molecular movement of structural material is assumed to take place between the inside and outside surfaces.

(4) The membrane has the same physical properties over the entire surface. This concurs with the results of Rand & Burton (1964) but contradicts those of Murphy (1965) and Fung & Tong (1968).

### (C) ENERGY OF BENDING

The stored energy from bending a simple beam in one plane is proportional to the integral of the square of the curvature over the length of the beam (Warburton, 1964, p. 87).

$$U = \int_0^L \frac{EI}{R^2} dx$$

$E$  is Young's modulus of elasticity,  $I$  the second moment of area about the neutral axis,  $R$  the radius of curvature at a distance  $x$  along the beam,  $U$  the total bending energy and  $L$  is the length of the beam.

Admittedly the red-cell membrane does not behave in a similar fashion to a homogeneous isotropic sheet. It is understood that the application of bulk constants to microscopic structures can lead to difficulties. For example  $E$ , Young's modulus of elasticity, and  $I$ , the second moment of area, are both physical bulk constants which are not applicable to membranes in the strictest sense but will be used.

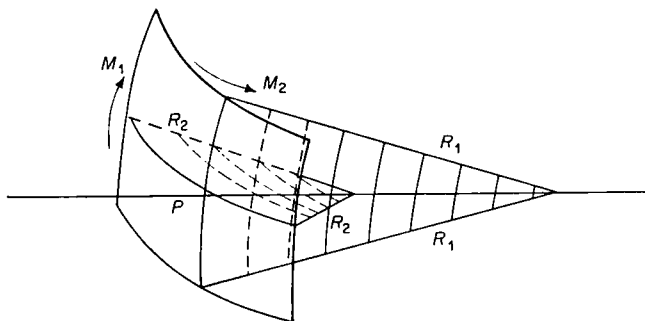
The calculation to obtain the stored elastic energy for bending a thin plate in two perpendicular planes is normally very complicated. However, if shear stresses must be zero, it would appear that the problem reduces to the integral of the addition of the squares of the two curvatures (Fig. 3).

It has been necessary to deviate considerably from the equations of structural engineering in order to accommodate the abundant biological observations. That red cells can adopt so many shapes without hemolysis

when the area must not be stretched is sufficient evidence to warrant this deviation from the standard approach. A red cell can change from the crenated form into a biconcave shape. Attempts to make similar transformations with models have been unsuccessful (Rand, 1967).

The author realizes that the general expression in Fig. 3 for the elastic energy of bending for a thin sheet is quite unsuitable for solid bulk structures

Two-dimensional bending



Without shear forces

$$U = \frac{D}{2} \int \left\{ \frac{1}{R_1^2} + \frac{1}{R_2^2} \right\} dA$$

area

$$D = \text{bending rigidity} = \frac{Eh^3}{12(1-\nu^2)}$$

$h$  = thickness of the membrane

$\nu$  = Poisson's ratio

FIG. 3. A section of membrane which is curved in two planes. Note that the two centers of curvature must lie on the normal to the surface. The equation shown is equation (1) and shows the relation between the bending energy,  $U$ , and the principal radii of curvature  $R_1$  and  $R_2$ . (The drawing was taken from Rand's Ph.D. Thesis, 1964, University of Western Ontario.)

made of steel. Even if the red cell were magnified by  $10^6$  we think that the membrane would be unlike any structural material analyzed by engineering technics.

#### (D) THE MODEL EQUATION AND CALCULATIONS

(1) In order to predict the shape associated with minimum energy it is necessary to provide many alternative shapes and to find the one which possesses the least energy (i.e. the shape which has the least total square of

the curvature.) The most suitable equation was found to be a slight modification of the ovals of Cassini (Hodgman, 1959, p. 425). Fung & Tong (1968) supplied an equation which has the apparent advantage of using the radius and thickness as actual parameters, but it is less adaptable for this research than the ovals of Cassini. The following is the equation which generates the modified ovals of Cassini.

$$Y(x) = B. (\{C^4 + 4A^2x^2\}^{\frac{1}{2}} - A^2 - x^2)^{\frac{1}{2}} \quad (2)$$

$B$  is the modification parameter and is dimensionless.  $A$  and  $C$  have the units of length.

(2) The selecting of the Cassini equation was on the basis of its convenience. The modification parameter,  $B$ , is essential to provide the extra degree of freedom needed to vary the shape. There is no connection between this empirical equation and the physical behavior of the red-cell membrane.

(3) All numerical procedures for this communication were performed on the university IBM 7040 using FORTRAN IV. The closed formula for Simpson's rule was used for integration (Stanton, 1961, p. 116). Approximately 50 sub-intervals were used to minimize rounding-off errors. The standard approach for areas and volumes of revolution was used. The outer rim of the profile of the cell is a difficult portion to deal with when calculating the area because  $dy/dx$  approaches infinity. In this region equation (2) was modified to get  $x$  as a function of  $y$ . Where convenient a Calcomp plotter (model 563) was used to present the data.

## 2. Methods and Materials

### (A) CELLS IN ISOTONIC RINGER SOLUTION

(a) From two groups of photomicrographs, 23 red-cell profiles were selected on the basis of clarity. Moreover, they had to represent the full range of normal cellular size (Canham & Burton, 1968).

With an oil immersion objective, micrographs were obtained of single red cells on edge hanging from the underside of a coverglass. The cells were in a slightly modified Ringer solution which was Tris-buffered. The method has been described in detail previously (Canham & Burton, 1968; Rand & Burton, 1963). The two populations of cells came from two healthy males, the first aged 9 and the second aged 71 years. The details were already available regarding diameter, area and volume and other geometric parameters. For each cell the angle,  $\theta$ , subtended by the normal to the surface and the axis of rotation was measured at approximately 50 evenly spaced intervals around the profile of the membrane (Fig. 4). The two principal radii of curvature,  $R_1$  and  $R_2$  are respectively  $\Delta L/\Delta\theta$  and  $r/\cos\theta$ . The

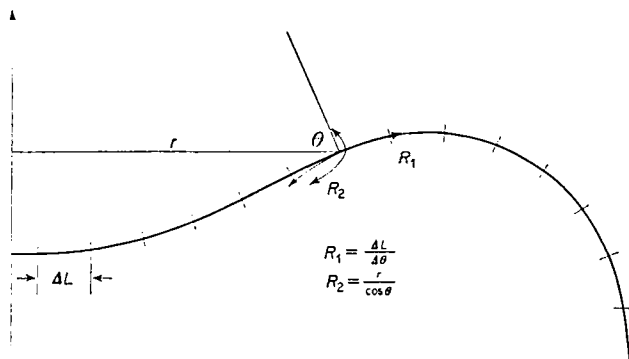


FIG. 4. A quadrant of the profile of the erythrocyte.  $R_1$  is in the plane of the paper and  $R_2$  perpendicular to it.

tangent angles were measured using a lucite prism mounted on the rotating arm of a drafting board. The bending energy of the complete surface was calculated using the equation (1) (Fig. 3). For a perfect sphere, the bending energy, as determined by equation (1), has the constant value of  $4\pi D$  and is not dependent on the size of the sphere. (As the area increases the curvature decreases.) Thus it is convenient to express all cellular bending energies as multiples of the bending energy of a sphere. (The spherical surface has the least energy of bending as calculated by equation (1), but the sphere has no flexibility with respect to shape.)

(b) For each cell, which had unique values for area and volume, a family of shapes was generated using equation (2). The criterion that area and volume must be constant made the generation of such a family of shapes rather complex. Because algebraic expressions could not be obtained for area and volume, many iterative technics were required. A brief formulation of the approach is illustrated in Fig. 5. The use of this cumbersome method is justified by its success in finding a family of shapes all having the same area and volume (to five-figure accuracy). The methods of central differences is described by Stanton (1961). Having obtained a set of  $A_j$ ,  $B_j$ ,  $C_j$ , it was possible to calculate the bending energy using equation (1) and the following standard relations for the two principal radii of curvature.

$$R_1 = \frac{(1 + \{y'\}^2)^{\frac{3}{2}}}{y''}$$

$$R_2 = \frac{x}{y'} (1 + \{y'\}^2)^{\frac{1}{2}}.$$

Equation (1) was integrated using Simpson's closed rule.



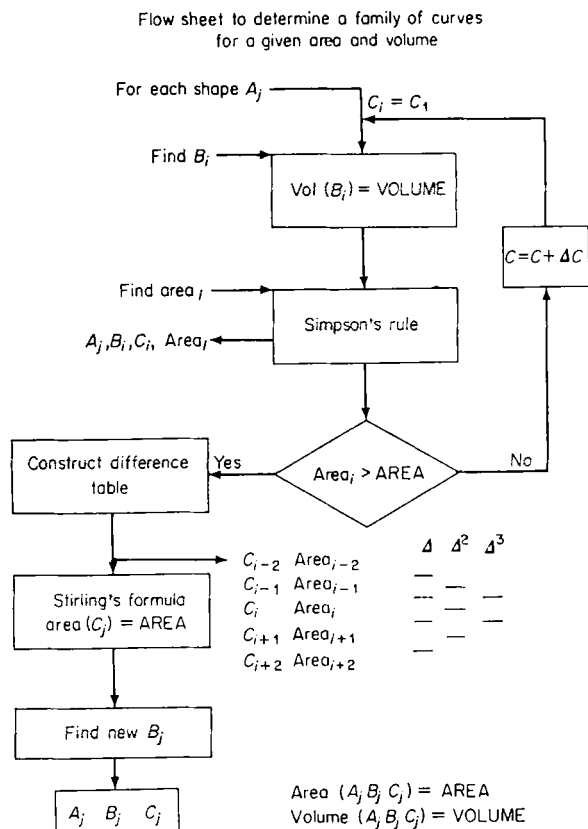


FIG. 5. Flow sheet for determining different shapes (a set of  $A, B, C_j$ ) for a given area and volume.

(c) Because of its flat sides the pancake form [Fig. 1(c)] appears to have less bending energy than the biconcave form with the same area and volume. It was of basic importance to this communication to determine the superiority of the biconcave shape for conserving bending energy. Once calculated, the energy of the pancake shape was used as a reference level for each area-volume set studied.

#### (B) CELLS IN HYPOTONIC RINGER SOLUTION

For the theory of bending energy to be at all acceptable it must correctly predict the different equilibrium shapes during osmotic swelling. For this test, similar studies to those outlined in section 2(A) (a), (b), (c), were made on 13 cells in a hypotonic environment (tonicity approximately 210 milliosmols) of buffered Ringer solution (pH 7.4).

## (C) SWELLING SERIES

The profile of an osmotically swelling cell has been studied by Rand (1967). By using the modified equation of Cassini it was possible to generate a swelling series based on the criterion of minimum bending energy. For this series the cellular area was held constant at  $135 \mu^2$ , the mean for most cell populations. At each volume, ranging from  $80$  to  $147 \mu^3$ , in steps of  $5 \mu^3$ , a configuration was determined which had the minimum total bending energy.

## 3. Results and Discussion

## (A) CELLS IN ISOTONIC RINGER SOLUTION

A consideration of Fig. 1 is helpful in providing a qualitative impression of the bending energy. Profiles (a) and (b) have the same area and volume of revolution. For the oblate spheroid (flying saucer) the bending energy is concentrated at the rim. The stored energy at the rim could be reduced by allowing the curvature at the rim to decrease and the central curvature to increase (because volume must be conserved). This would relieve the bending strain at the rim by redistributing the curvature towards the center. Intuitively a net decrease in energy is realized because the total surface at the center is much less than the total surface at the rim. The implication that the red-cell shape resulted from minimizing the total curvature of the membrane initiated a thorough theoretical and experimental study.

The general features of the curve of bending energy vs. the parameter  $B$  are shown in Fig. 6, which is an energy plot for a cell of average area and volume. Several different shapes were generated, each represented by a point on the plot. These shapes are geometrically possible in that they all have the same area and volume, but they are *not observed* equilibrium shapes. To illustrate the variety of profiles a few have been traced by the computer. The choice of abscissa is arbitrary and the parameter  $B$  was chosen for its relation to the shape. The following features are important: (i) there is a minimum for the curve at 2.45 and it occurs at a point when the contour is biconcave; (ii) if the profile is thinner than the optimum profile (right end of the curve) then the energy begins to rise; (iii) the energy calculated numerically from the actual cell profile is 2.37 and is slightly less than the minimum; (iv) the energy of the flat-sided profile (2.61) is considerably higher than the minimum.

A great variety of cellular geometries exists within a normal blood sample. Figure 7 shows the bending energy curves for the same cell as in Fig. 6 along with two other cells. Large cells are much thinner than smaller cells (Canham & Burton, 1968). It is evident from Fig. 6 that the very thin profile represents the minimum energy for large cells but the smaller more spherocytic cell is only moderately thin for an energy minimum. This is borne out by

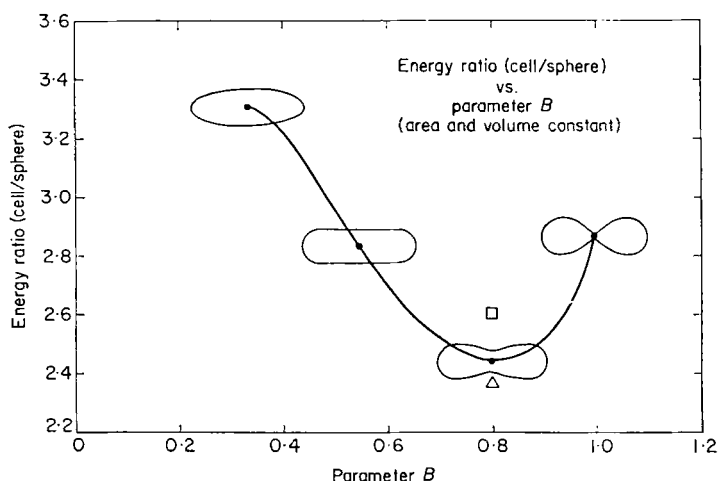


FIG. 6. Bending energy ratio (cell/sphere) plotted against  $B$ , a parameter in the ova. equation (2). The ordinates for the energy levels for the pancake shape and the observed shape are indicated. The area and volume for this particular cell were  $137 \mu^2$  and  $107 \mu^3$ . (□) Pancake energy level; (△) observed profile energy, measured from actual cell profile.

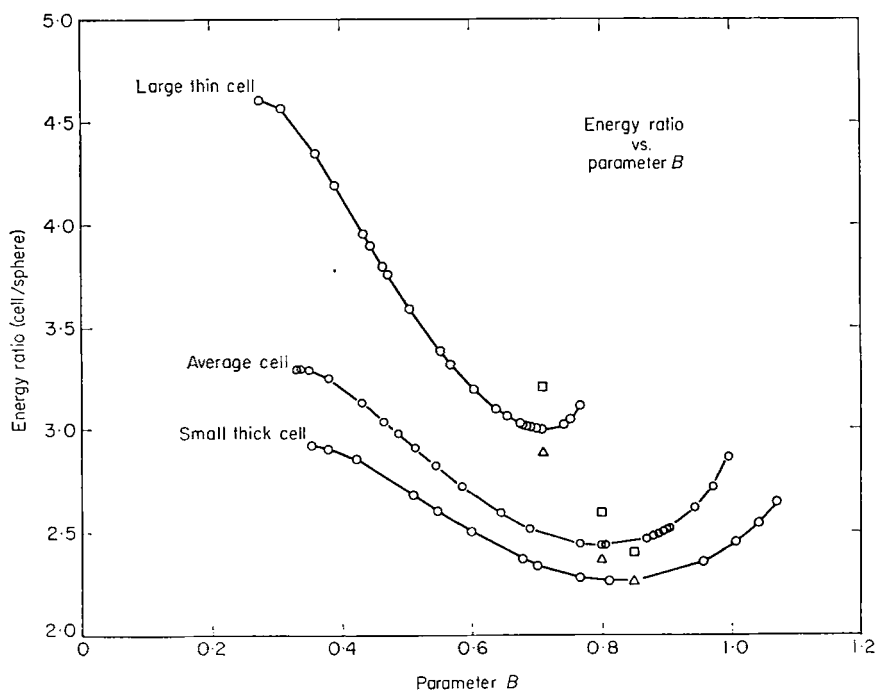


FIG. 7. The bending energy ratio plotted against  $B$  for three different cells. The areas and volumes for the three cells are: thin cell  $168 \mu^2$ ,  $128 \mu^3$ ; average cell  $137 \mu^2$ ,  $107 \mu^3$ ; thick cell  $110 \mu^2$ ,  $80 \mu^3$ . (□) Pancake energy level; (△) Energy level measured on photographed red-cell profile.

observation; indeed, the smaller, more spherocytic cells are not as thin as permitted by geometry.

These general observations are tabulated for these three representative cells in Table 1. The area and volume were the two criteria which determined the parameters of the ovals. The sphericity index is a dimensionless number

TABLE 1  
*Measured and predicted dimensions for three representative cells*

		M or P†	Cell 1	Cell 2	Cell 3
Area	$\mu^2$	M	110.4	137.3	167.6
Volume	$\mu^3$	M	80.4	107.1	128.3
Sphericity index		M	0.8162	0.7940	0.7339
Diameter	$\mu$	M	7.143	8.036	8.893
		P	7.300	8.190	9.236
Thinness	$\mu$	M	1.68	1.75	0.79
		P	1.73	1.58	1.00
Thickness	$\mu$	M	2.54	2.68	2.71
		P	2.38	2.52	2.40
Bending energy ratio		M	2.270	2.366	2.893
		P	2.263	2.448	3.105
Pancake energy ratio		P	2.405	2.605	3.217

† M = measured on actual cell profiles. P = predicted from bending energy theory.

ranging from zero to unity and is defined as  $4.83 \times \text{vol.}^{2/3}/\text{area}$  (Canham & Burton, 1968). This term is useful in that it provides a quantitative way of saying "thick" or "thin".

For each cell presented in Table 1 the following are apparent: (i) the predicted diameter is greater than the measured; (ii) the predicted thickness is less; (iii) the predicted thinness at the center is usually greater; (iv) the predicted bending energy is greater than the measured energy and this difference is greater for larger cells. For the small thick cells the predicted bending energy is very close to the measured energy and is sometimes even less; (v) the energy of the pancake configuration [Fig. 1(c)] is always considerably higher. This is summed up for the 23 cells in the following table:

Diameter ( $\mu$ ) : average difference =  $0.28 \pm 0.11$  P > M†  
 Thickness ( $\mu$ ) : average difference =  $0.28 \pm 0.14$  P < M  
 Thinness ( $\mu$ ) : average difference =  $0.25 \pm 0.29$  P > M  
 Bending energy : average difference =  $0.084 \pm 0.126$  P > M

† P = predicted; M = measured. Means are given with standard deviations.

Figure 8(a) shows the superposition of the observed and predicted profiles.

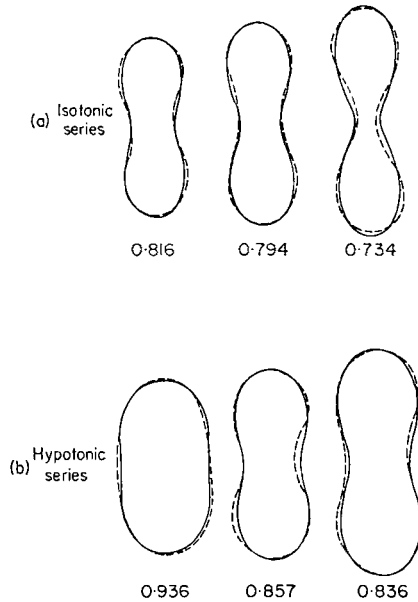


FIG. 8. Observed (— — —) and predicted (——) profiles for six cells. (a) Three representative cells observed in isotonic Ringer solution. (b) Three representative cells in hypotonic Ringer solution. The sphericity index is given below each profile.

The differences are small but significant and point to the disadvantage of using an empirical equation rather than an equation which actually satisfies the proposed bending-moment relation [equation (1)]. The oval equation is less suitable for thin cells (lower sphericity index). It makes too sharp an indentation at the center and a non-circular cross-section at the rim. (Most of the energy of bending is due to  $R_1$  and therefore the ideal equation would keep the profile at the rim as circular as possible. This is observed in actual red-cell profiles.)

The basic assumptions leading to the hypothesis of bending energy are not exempt from criticism. No attempt has been made to investigate the effect of changing some of the original assumptions. For example, one might alter the relation between the bending energy and the curvature of the membrane; however, further experimental evidence would be required to justify any alterations.

## (B) CELLS IN HYPOTONIC RINGER SOLUTION

(i) *Biconcave cells*

When subjected to a reduced tonicity the erythrocyte swells quickly and adopts a new volume to preserve osmotic equilibrium. Table 2 shows the geometric quantities for 24 swollen cells which were photographed singly, and on edge, in hypotonic Ringer solution (tonicity approximately 0.21

TABLE 2  
*Dimensions of the hypotonic cell series*

	M or P†	Cell B1	Cell B2	Cell B3
Area ( $\mu^2$ )	M	114.0	126.3	174.9
Volume ( $\mu^3$ )	M	103.6	106.0	166.3
Sphericity index	M	0.9358	0.8574	0.8362
Diameter ( $\mu$ )	M	7.011	7.509	9.004
	P	6.955	7.636	9.093
Thinness ( $\mu$ )	M	3.70	2.03	2.46
	P	3.50	2.27	2.39
Thickness ( $\mu$ )	M	3.70‡	3.04	3.33
	P	3.54	2.86	3.17
Bending energy ratio	M	1.442	2.050	2.135
	P	1.417	1.961	2.110
Pancake energy ratio	P	1.484	2.061	2.233

† M = Measured; P = predicted.

‡ Biconvex.

osmolar). (Only 13 of these cells were analyzed for bending energy). The 24 cells are compared to 103 cells taken from the same person one year previously (Canham & Burton, 1968). There was a significant shift of all the parameters except area, which provides additional evidence for the belief that the cellular area is unchanging during osmotic volume changes.

The nine biconcave cells of the hypotonic series had profiles which were closely predicted by the modified Cassini equation. The results are presented in Table 3 and Fig. 8(b) for three representative cells. The comparison between the measured (M) and predicted (P) is presented in the following table for the nine swollen cells:

Diameter ( $\mu$ )	: difference = $0.09 \pm 0.14$ S.D.	P > M
Thickness ( $\mu$ )	: difference = $0.09 \pm 0.13$	P < M
Thinness ( $\mu$ )	: difference = $0.12 \pm 0.31$	P > M
Bending energy	: difference = $0.14 \pm 0.18$	P > M
(compared to the sphere)		

TABLE 3

*Geometric quantities for 24 swollen cells which were photographed in hypotonic Ringer solution. (Not all of these were analyzed for bending energy)*

Sample size	Hypotonic environment 210 milliosmols	Isotonic environment 310 milliosmols	P value
	24	103	
Diameter ( $\mu$ )	$7.790 \pm 0.647^\dagger$	$8.116 \pm 0.631$	$<0.05$
Area ( $\mu^2$ )	$139.3 \pm 19.3$	$136.9 \pm 19.4$	$>0.5$
Volume ( $\mu^3$ )	$125.1 \pm 24.1$	$104.8 \pm 18.5$	$<0.001$
Sphericity Index	$0.867 \pm 0.038$	$0.786 \pm 0.032$	$<0.001$

† Standard deviations.

One significant difference from the isotonic-cell series was that the predicted bending energy was usually less than the measured energy. It would seem that the oval equation can suggest a more optimal shape than is actually observed. If the bending energy theory is valid then it would be contradictory to be able to suggest a profile which has an associated lower energy.

The main consideration focuses on the error in obtaining the measured bending energy of the actual cell profile. In tracing the membrane from photographs of red-cell profiles we have followed the rules established by Ponder (1930). One traces to the inside of the gray area in the central region of the cell and more toward the outer edge at the rim. An example is given by Rand & Burton (1963). In my work medium contrast film and printing paper were used which made the profiles less contrasted in appearance but easier to trace. Although there seems to be room for considerable interpretation in following Ponder's guidelines it is possible to test the method by tracing the membranes of two touching cells. From the electron microscopy of red cells (Seeman, 1966) it is known that two adjacent cells will have membranes which are touching. Therefore, when a photomicrograph is obtained on a light microscope of two cells touching which have deformed each other and have a common flat side, it is possible to verify that Ponder's guidelines for tracing the membrane are correct. The central area of the cell is still the most difficult area in which to be confident when outlining the membrane. The configuration of least energy is perfectly symmetrical and perfectly smooth with no sudden changes in curvature. Therefore, measurement inaccuracies, irregularities in tracing the membrane (shaky-hand

syndrome) and quadrant assymetries† all bias the measured energy towards a higher value. If these effects are independent of the sphericity of the cell (which is reasonable) then the effect would be more evident on a percentage basis for the thicker, more spherical cell. (The more spherical cell has a lower bending energy.)

(ii) *Cup-shaped cells*

Many cup-shaped cells were observed when erythrocytes were suspended in the Tris-buffered Ringer solution which had a tonicity of 0.21 osmolar (Plate I). Is it possible that the cup-shaped profile represents a metastable configuration?, i.e. does the cup shape represent a local minimum for the bending energy? Although only four such cells were photographed and analyzed in detail this seems to be consistent with our bending energy theory. In 7 of the 13 cells from the hypotonic series the pancake energy was between the measured bending energy and the predicted minimum for their respective areas and volumes, whereas in the isotonic series the pancake energy was consistently higher than both the measured bending energy and the predicted

TABLE 4  
*Energy levels of the four cup-shaped cells observed in  
hypotonic solution*

	Cell 1	Cell 2	Cell 3	Cell 4
Area ( $\mu^2$ )	111.2	137.8	144.5	147.5
Volume ( $\mu^3$ )	91.0	112.6	103.8	134.1
Sphericity index	0.880	0.818	0.739	0.859
Pancake energy	1.89	2.39	3.16	2.05
Measured energy	2.01	2.58	3.04	2.15
Predicted energy for the biconcave shape	1.80	2.25	2.97	1.94

minimum. The flat-sided profile (pancake) represents an uncommitted shape because it is neither biconcave nor monoconcave (cup-shaped). Since both configurational energies are close to the pancake energy it is conceivable that the disturbances of preparing the dilute suspension of cells could easily disturb the cell sufficiently to make a cup shape from a biconcave shape or vice versa. In fact, the probability of observing a particular configuration should be in proportion to the energy gap between the energy of the flat-sided profile and the respective mono or biconcave energy. (This has not

† All four quadrants are never exactly the same owing to the quivering of the cell when on edge.



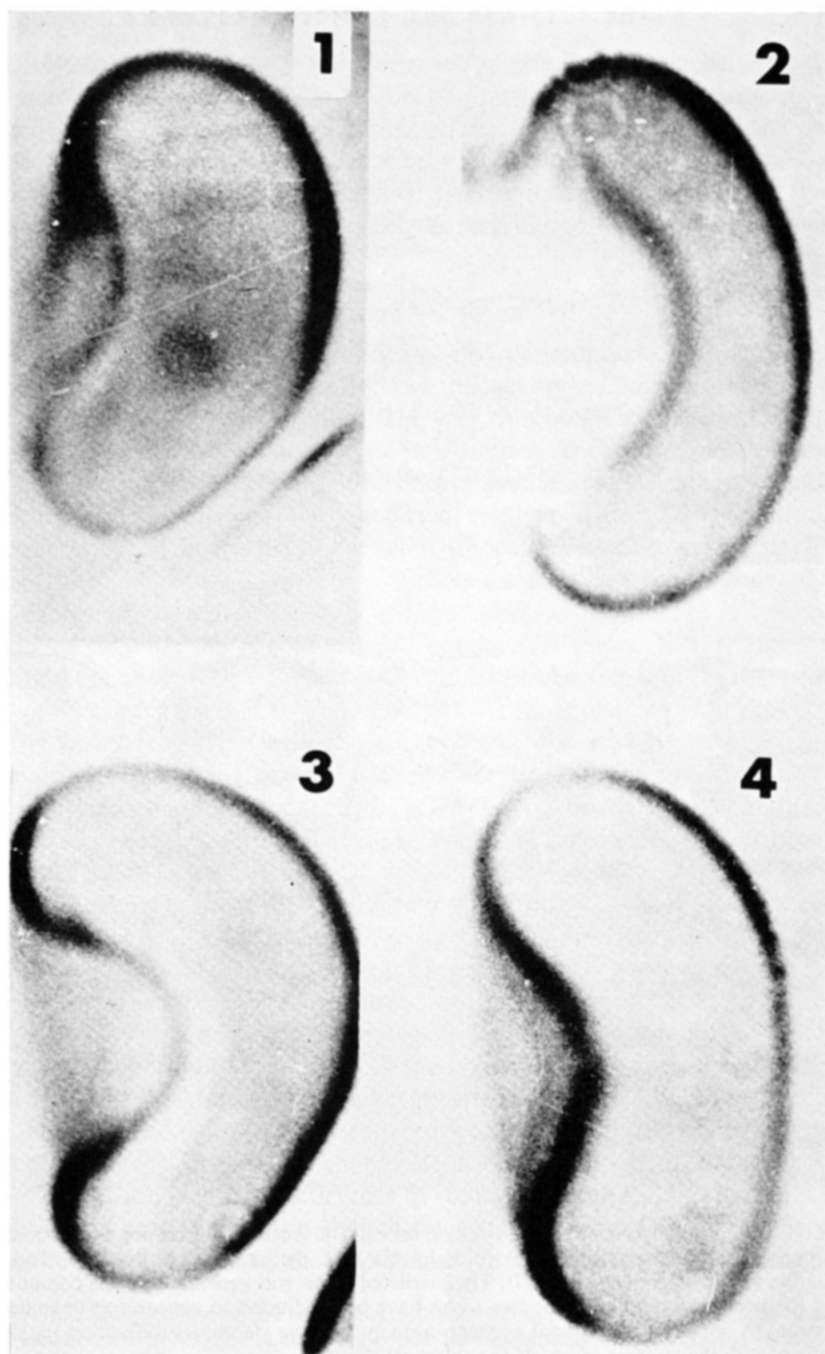


PLATE I. Cross-section photomicrographs of cup-shaped erythrocytes seen on edge. The numbers correspond to those in Table 4. The membrane outline was traced using the rules described by Ponder (1930).

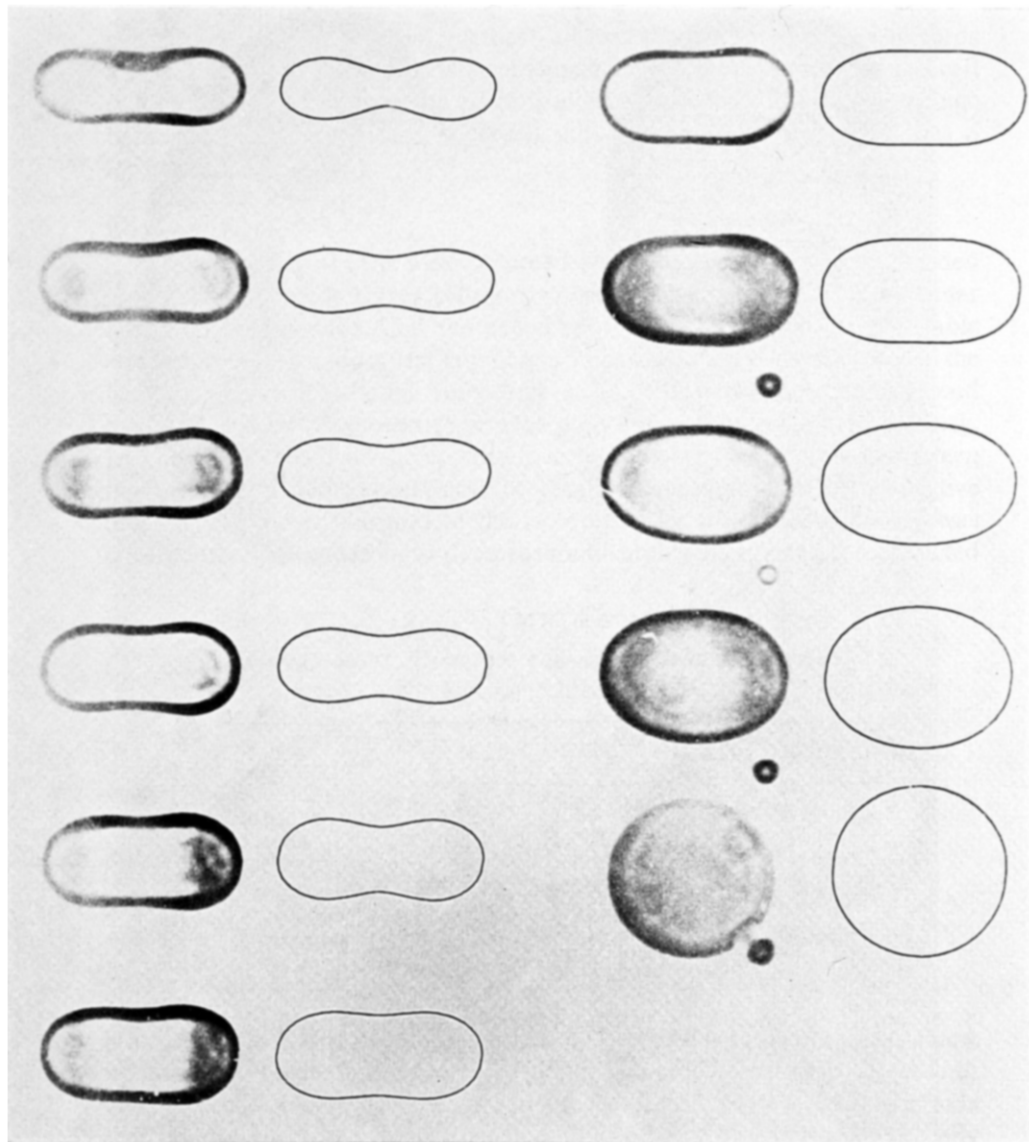


PLATE II. Observed and predicted swelling series. The observed series are from Rand's results and were reproduced in this form by his kind permission. He has published a similar series elsewhere (Rand, 1967). The predicted series was generated by the computer using the bending hypothesis. The two scales have been adjusted to appear approximately the same. (A small bit of material appears in many swelling stages. Its diffraction pattern interferes with the circular image of the spherical shape.)

been tested experimentally.) The difference in bending energy between the flat-sided configuration and the biconcave configuration could be called the stability of that biconcave configuration. Similarly the stability of the cup shape would be the energy difference between the cup configuration and the flat-sided pancake shape.† The fact that the cup-shape's stability is less than that of the biconcave makes possible the use of the term metastable with reference to the cup shape.

### (C) SWELLING SERIES

Rand (1967) has published a series of photomicrographs of a single cell during osmotic swelling. Figure 9 shows the bending energy curves for a cell at four different stages of swelling. The very thin initial step shows the high degree of stability for the biconcave configuration. At near spherical volumes the minimum does not occur at a biconcave profile. Plate II shows an actual swelling series compared to a predicted swelling series. The incremental volume is  $5.0 \mu^3$  in the predicted series (except for the last stage) starting from  $100 \mu^3$ ; however, the volume increments have not been measured in the experimental series. The cell thickens considerably before it becomes biconvex in both the predicted and experimental series. Eleven stages of swelling are shown for both the predicted swelling series and the observed; the cell becomes flat and then biconvex at the same place in both series.

The comparison between the predicted and observed is good but only qualitative. We felt that it would be necessary to improve the optics before obtaining cross-section micrographs which would be suitable for outlining the membrane in the central region. The rules for tracing the membrane (Ponder, 1930) describe how one should trace to the inside of the central region in the cross-sectional photograph and to the middle of the dark zone elsewhere. Therefore, in a swelling series where the cell passes continuously from the biconcave to the biconvex, one must decide when to stop tracing on the inside and begin tracing through the center.

Rand (1967) has described a "popping" phenomenon when the cell changes from a flat ellipsoid to a perfect sphere. A simple explanation seems possible. The theoretical series in Plate II has equal incremental volumes except for the last stage. (At this stage the volume goes from  $145$  to  $147 \mu^3$ .) There is a much greater visible profile change for the last few stages than for the first few. The reason is that the difference in volume between spherical and almost spherical is very slight, even though the two-dimensional image of the cell is quite different between the two shapes. In the last stages of sphering, in

† A discussion of the errors which overestimate the bending energy were presented at the end of section 3(B) (i). A lowering of the bending energy of the cup-shaped cells by approximately 10% would be consistent with that section.

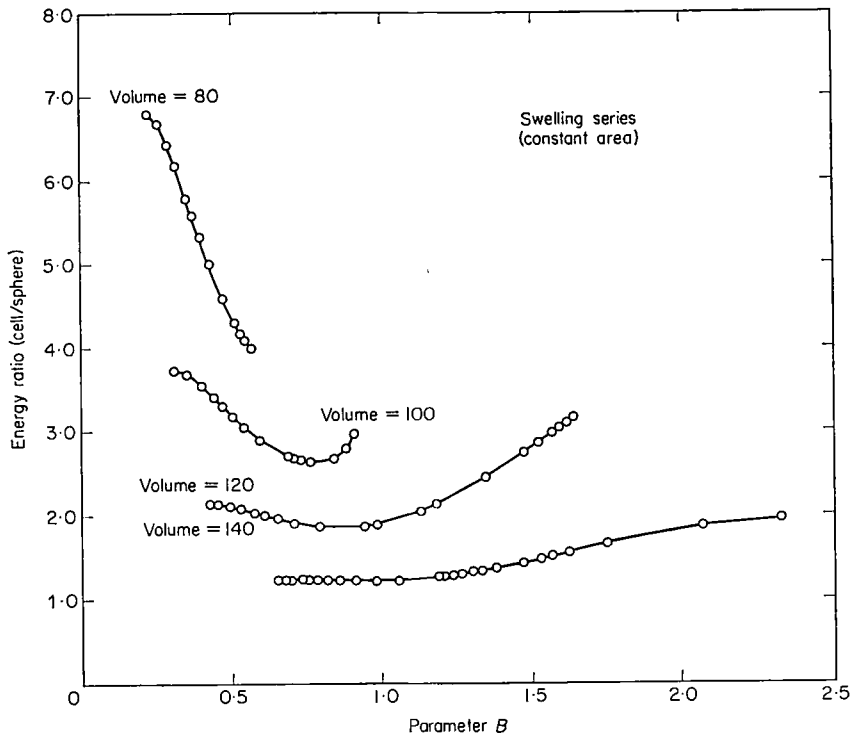


FIG. 9. Bending energy ratio for a hypothetical swelling sequence assuming a constant area of  $135 \mu^2$ .

which the rate of change of the volume is constant, the cell would appear to be "popping" into a sphere because of the more accelerated adjusting of the profile.

#### (D) CRENATED CELLS

Red cells which are suspended in hypertonic saline will adopt a shape which reminds one of a flattened mine. The surface goes into many smooth bumps and depressions which number about 15 (Ponder, 1948, Chap. 2). The crenated shape which is characteristic of cells in Ringer solution or saline at osmotic pressures of about 500 to 600 milliosmols has been attributed to the properties of the very concentrated hemoglobin solution within the cell. Perhaps the resistance of the cell's membrane to bending can provide an alternate explanation.

The minimum energy for shrunken cells increases markedly for model cells (generated by the Cassini equation) which have a volume much less

than isotonic. For example cells with volumes 90, 80, 70 and  $60 \mu^3$  have energies of 3.3, 4.0, 5.3 and 7.4 (compared to a sphere) all having the average area of  $135 \mu^2$ . One might assume that the single crenations or bumps are smooth spherical caps and that the depressions are also spherical caps. The depressions and bumps would be approximately equal in number. If the spherical caps and depressions represented approximately one-third of a sphere each (based on a preliminary microscopic investigation), the total energy would be 10, i.e.  $2/3 \times 15$ . With 60% of the cell's volume osmotically active (Gary-Bobo & Solomon, 1968) the volume of the average cell is about  $70 \mu^3$  in Ringer solution with a tonicity of 600 milliosmols. At this volume the average cell has a bending energy of approximately 5.0. Therefore, within the framework of the bending energy hypothesis one can suggest, partly quantitatively, why a cell crenates at higher osmotic pressures, rather than remain a very thin biconcave disc.

#### (E) MEANING OF THE BENDING ENERGY

A biconcave shape which is determined by bending rigidity alone requires an internal negative pressure. What order of magnitude of pressure would be in accordance with the known physical properties of the red cell membrane?

The bending energy of an average cell in isotonic solution is approximately  $35D$  where  $D$  is the bending rigidity [equation (1)]. This must be balanced by the work done,  $V\Delta P$ , in producing a negative pressure  $\Delta P$  with a volume,  $V$ , of  $100 \mu^3$ . Initially one can crudely approximate  $D$  by  $h^3 E / (12\{1 - \nu^2\})$  which is true for an isotropic plate. Using a value of  $10^8$  dynes/cm<sup>2</sup> for Young's modulus,  $E$  (Rand, 1964a), and ignoring Poisson's ratio,  $\nu$ , one gets a  $D$  of  $10^8 h^3 / 12$ . Assuming a membrane thickness,  $h$ , of 200 Å one gets a negative pressure of less than 1/3 mm of water. From this it is apparent that a very small negative pressure can account for the red-cell shape.

### 4. Summary and Conclusions

(1) The explanation for the shape of the red cell is based on minimizing the energy of bending the membrane. The engineer's approach for determining the bending energy of a homogeneous plate was modified. The modification was devised to incorporate both the mechanics of structural materials and the viscoelastic property of red-cell membranes. The assumption that the membrane is homogeneous across its thickness was only used to obtain an approximate value for the rigidity coefficient of bending,  $D$ . The need for such a coefficient was circumvented by expressing all bending energies in terms of the sphere's bending energy. The energy stored in an

element of area with radius of curvature  $R$  is assumed to be proportional to  $1/R^2$ , the same as for a bending beam. One would be unjustified to assume otherwise until more information about the physical behavior of membranes is available.

(2) Of fundamental importance to the research was the modified classical equation which describes the ovals of Cassini. This equation is empirical in nature and is in no way associated with the physics of bending. It is capable of generating a set of variations on the red-cell profile, all with the same area and volume. The profile in such a set which minimized the bending energy coincided very well with the actual cell of the same area and volume. This modified equation might prove useful to persons interested in studying the red-cell and who wish to have a mathematical expression to generate the shape.

(3) The energy of bending was calculated for 23 normal cells and 13 swollen cells (four of which were cup-shaped). All of these cells (other than the cup-shaped ones) had energies and profiles closely approximated by their counterpart produced by the altered Cassini equation.

(4) The bending energy hypothesis predicts that the cup shape should appear much more frequently in hypotonic media than in isotonic media. This has been supported qualitatively.

(5) A theoretical swelling series was produced, each profile of which represented an energy minimum for its particular area and volume. The area was constant at  $135 \mu^2$  for the series and the volume was increased in even steps from the isotonic value to the spherical value.

(6) The crenated erythrocyte has a shape which might also fit within the framework of the bending hypothesis.

(7) Rand & Burton (1964) obtained indirect evidence for a small positive internal pressure of 2.3 mm of water inside the erythrocyte. They assumed bending forces were negligible compared to tension. However, if bending forces are the dominant shaping forces there must be a small internal negative pressure of the order of 1.0 mm of water. To our knowledge no one has measured the internal pressure directly.

(8) The shape of the red-cell minimizes the surface integral of the square of the curvatures. It has been shown how this integral might represent the total bending energy of the membrane. The term bending energy has been borrowed from engineering, and we acknowledge that it is not strictly applicable to membranes which are only continuous in two dimensions. However, the viscoelastic property of red-cell membranes makes the concept plausible. Probably other workers interested in the shape of the erythrocyte should take into account the evidence that the biconcave shape of the cell minimizes the total square of the curvature of the membrane.

(9) It is unlikely that this bending energy hypothesis provides the complete answer to the shape of the red cell. However, it does provide another approach to this puzzling problem of the biconcavity.

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