THE STRESS-FREE SHAPE OF THE RED BLOOD CELL MEMBRANE

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ABSTRACT The two main proposals found in the literature for the stress-free shape of the red cell membrane are (*a*) the bioconcave shape and (*b*) the sphere of the same surface area. These possibilities are evaluated in this paper using theoretical modeling of equilibrium membrane shapes according to Zarda et al. (1977. *J. Biomech.* 10:211–221) and by comparison to experiments on red cells whose membrane shear modulus has been increased by treatment with diamide. Neither proposal is found to be compatible with all the experimental behavior of native red cells. To account for this discrepancy we propose that either the shear modulus of the native membrane is dependent on the membrane strain or that the bending stiffness is higher than estimated by Evans (1980. *Biophys. J.* 30:265–286). These studies suggest that the biconcave disk is the more likely possibility for the stress-free shape.

INTRODUCTION

In theoretical modeling of red blood cell behavior, several different assumptions have been made in the literature concerning the stress-free shape of the red cell membrane. Skalak et al. (1973) and Evans (1973*a*, *b*) assumed the biconcave shape to be stress-free. Brailsford et al. (1976) assumed that the membrane is free of bending moments when it is flat. It may be interpreted from their work that a red blood cell membrane is free of shear stresses in the spherical shape, but not free of bending moments. Zarda et al. (1977) considered both the biconcave shape and the sphere as stress-free shapes and showed that quite different membrane bending stiffnesses would have to be used to match the red cell shapes observed during the shape transformation from a disk to a sphere that can be induced by hypotonic swelling. Other authors have disregarded the shear stiffness of the membrane altogether and assumed the curvature at zero bending moment to be zero (Canham, 1970; Jenkins, 1977) or negative, ie., opposite to that of the sphere (Deuling and Helfrich, 1976). Recently Evans (1980) gave an upper bound for the membrane bending stiffness, making it possible to put the search for a stress-free shape of the red cell membrane on a more definite basis. This search is the subject of the present paper, where we carry out theoretical modeling using the approach of Zarda et al. (1977) and compare the results with experiments on red cells whose membrane shear modulus is increased by treatment with the SH-oxidizing reagent diamide (Fischer et al. 1978*a*).

METHODS

Methods of Theoretical Modeling

The theoretical computations are performed using the finite element program of Zarda et al. (1977). Given the stress-free shape of a shell, the program calculates the shape of the shell (red cell membrane) by minimizing the strain energy of the shell for specified increments of volume. The program maintains the constraint of constant surface area and assumes that the membrane is isotropic in the membrane plane. Two elastic constants enter the constitutive equations: the shear modulus (μ) and the bending stiffness parameter (B). μ is taken to be 0.005 dyn/cm according to Chien et al. (1978) and Waugh and Evans (1979). For B, a value of 0.67×10^{-12} dyn cm is adopted on the basis of Evans (1980). He gave 10^{-12} dyn cm as an upper bound for B. When his theoretical calculations (Evans, 1980) are compared with his experiments (Evans and La Celle, 1975), B is estimated as 10^{-13} dyn cm. We chose here a more conservative value of 0.67×10^{-12} dyn cm. One quarter of the cell contour is divided into six elements, each represented by five nodes. The program uses the stress-strain relation suggested by Skalak et al. (1973), in which the stretch ratio λ enters up to the fourth power. This is different from the stress-strain relation suggested by Evans (1973*a*), in which λ enters up to the second power. This latter stress-strain law was used by both groups cited above for the determination of the shear modulus μ. However, for the cases treated in this paper the stretch ratios λ are so close to one that the two stress-strain laws essentially coincide. Using $\lambda = 1 + \epsilon$ and expanding both stress-strain laws in terms of ϵ, they coincide up to the second power of ϵ.

Experimental Methods

The method of increasing the shear modulus of the membrane of red blood cells is described by Fischer et al. (1978*a*). Briefly, red cells are washed and suspended in isotonic phosphate-buffered saline plus 44 mM sucrose and the pH is adjusted to 8.0. The cells are then pretreated with 10 mM iodoacetate for 15 min at 37°C. This serves to oxidize intracellular GSH, which might reverse the effects of diamide. The iodoacetate treatment does not increase the membrane shear modulus appreciably. After three washings the cells are treated with diamide at concentrations between 0.05 and 1 mM. This leads to a concentration-dependent increase of the membrane shear modulus. Alternatively, after the second washing following the iodoacetate treatment, further washing and incubation procedures are carried out in a hypotonic medium prepared by diluting the normal medium (see above) with 1.3 vol water (i.e., 130 mosmol).

To study the resting shape, diamide-treated and control cells are suspended in isotonic phosphate-buffered saline (pH 7.4) or in plasma and placed in a rheoscope. The test chamber of the rheoscope consists of a cone and a plate, which are transparent and counterrotating. The chamber is viewed through an interference-contrast microscope (Leitz, Wetzlar, West Germany). By careful rotation of the cone and plate by hand it is possible to turn a red blood cell under microscopic control and to obtain a three-dimensional impression of its resting shape. The methods for studying the dynamic behavior of red blood cells in the shear field are described by Fischer et al. 1978*b*. Briefly, the red cells are suspended in isotonic solutions of dextran (DX 60; Knoll AG; Ludwigshafen; West Germany) in phosphate-buffered saline (pH 7.4). By driving the cone and the plate at constant speed, the suspension is subjected to preselected shear rates. The motion of the membrane is monitored by following the movement of membrane-bound latex spheres (0.8 μm Diam) in the microscope.

RESULTS

Theoretical Results

The two stress-free shapes studied are (*a*) the biconcave shape determined experimentally by Evans and Fung (1972) and (*b*) the sphere of equal surface area. In both cases the membrane shear modulus $\mu = 0.005$ dyn/cm and the membrane bending stiffness parameter $B = 0.67 \times 10^{-12}$ dyn cm are the normal values assumed.

For case (*a*) a sequence of cell shapes with increasing volumes was calculated (Fig. 1). The interesting result is that a "pop-through" buckling of the dimples is predicted. For a certain range of volumes, there exist two stable shapes, one with inward- and the other with outward-buckled dimples. Fig. 2 shows the strain energy and the pressure difference (inside minus outside pressure) vs. the volume of the red cell. The curves end where the program ceases to give a stable solution. The volume at which the membrane pops through in an experiment would depend on the height of the energy barrier between the inward and outward buckled state and on the magnitude of the disturbance (e.g., Brownian motion) which is imposed on the membrane. However, such pop-through buckling has not been observed experimentally with normal cells by us or by others (Canham and Parkinson, 1970). It has been shown by Zarda et al. (1977) that the two stiffness parameters μ and B enter only through the dimensionless ratio $(\mu\ell^2)/B$ in determining the shape of the cell. Here ℓ is a characteristic radius of curvature, taken to be 1 μm. The values we used for the stiffness parameters of the normal red cell membrane give a ratio of 75. The shape at which buckling occurs is also determined by this ratio. At lower ratios, the tendency to buckle is reduced.

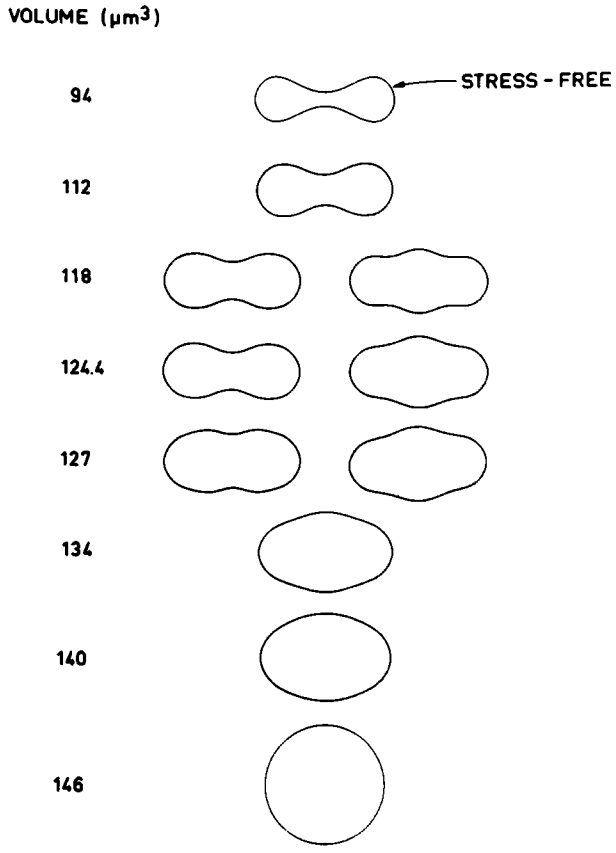

FIGURE 1 Pop-through buckling during the swelling of a (stress-free) biconcave disk, as predicted by the finite element program, $\mu = 0.005$ dyn/cm, $B = 0.67 \times 10^{-12}$ dyn cm. Where two shapes are shown, they correspond to increasing volume on the left and decreasing volume of the right.

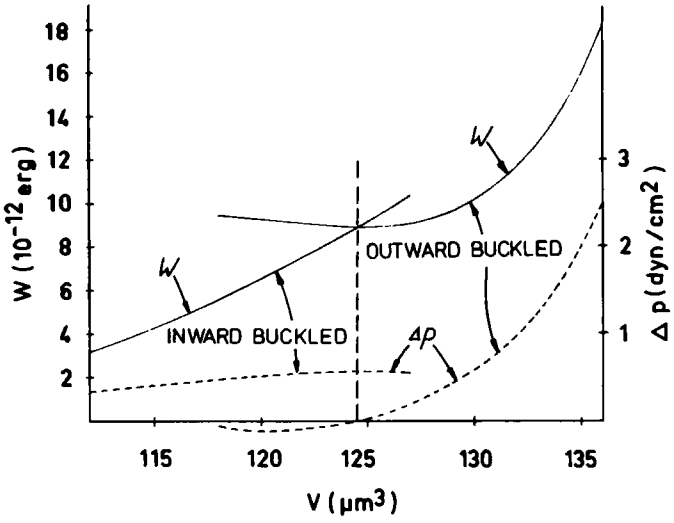

FIGURE 2 Strain energy (W; —) of the red cell membrane and pressure difference (Δp; inside minus outside; ----) versus volume (V) during the swelling of a biconcave disk, as predicted by the finite element program. $\mu = 0.005$ dyn/cm, $B = 0.67 \times 10^{-12}$ dyn cm.

When the ratio is reduced by a factor three, no buckling is predicted. For the ratio 25,[1] Zarda et al. (1977) found good correspondence with the experimental results of Evans and Fung (1972).

In case (*b*) the membrane is taken to be stress-free in the spherical shape. Under this assumption, Fig. 3 shows red cell shapes for a range of volumes and two different dimensionless ratios $(\mu\ell^2)/B$. For a ratio of 75 (left column) the radii of curvature at the rim are much smaller than those of normal red cells. In order to make the shapes similar to the cross section published by Evans and Fung (1972), the ratio $(\mu\ell^2)/B$ has to be reduced by a factor of 30.

At first glance, the last two pictures in the left column of Fig. 3 might suggest that the membrane pops through when it reverses curvature. However, the program finds no instability in this volume range. The central deflection varies smoothly with volume. At a volume of 145.9 μm^3 the membrane in the center is almost flat and is in equilibrium. The absence of buckling predicted by the model sheds some doubt on the explanation given by Jay and Rowlands (1975) for the sudden displacement of cells before sphering. Instead of buckling, we suggest another possibility, ie., that the membrane is attached at more than one point to the underlying coverslip. During sphering, tensions build up in the attachments. The detachment of one of the points would release enough strain-energy to displace the cell several microns as observed.

Experimental Results

Various experimental results are shown in Figs. 4–7. Fig. 4 shows a photo-montage from a time-lapse sequence in which the swelling of a diamide-treated cell was recorded in 157

[1]Due to the redefinition of the shear modulus, this number is half that given by Zarda et al. (1977) for the same physical situation.

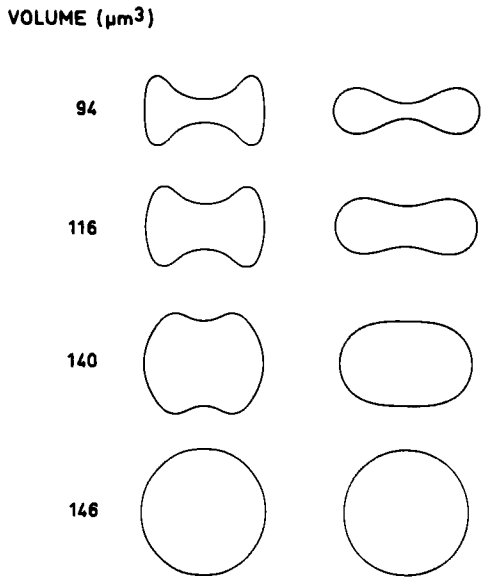

FIGURE 3 Swelling of biconcave cells whose membrane is stress-free in the spherical shape; $B = 0.67 \times 10^{-12}$ dyn cm. First column, $\mu = 0.005$ dyn/cm; second column, $\mu = 0.00015$ dyn/cm. The volumes below the spherical value of 146 μm^3 are accompanied by small negative pressure inside the cells.

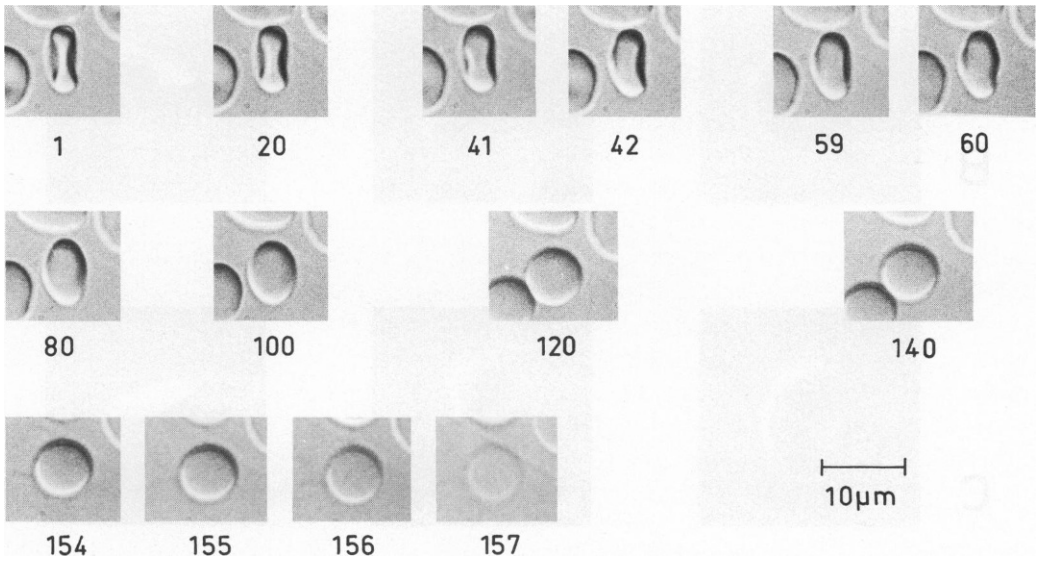

FIGURE 4 Swelling of an individual red cell treated with 1 mM diamide in the biconcave shape. Photomontage from a time-lapse movie (numbers of selected frames are indicated). Time interval between two successive frames is 15 s.

frames, 15 s apart. The red cells were treated with diamide in their biconcave shape. A dilute suspension of these cells in isotonic saline was then put into the cone-plate chamber, which was held stationary. The suspension was surrounded by a large volume of water, thereby inducing a slow dilution of the original suspending medium. Between frames 41 and 42, one of the dimples bulges out; between frames 59 and 60, the other one follows. It seems likely that the bulging of the dimples in sequence is energetically more favorable than if both were to bulge at once. From theoretical and experimental work on pop-through buckling, it is found that non-axisymmetric shapes may occur during the bulging process (Gjelsvik and Bodner, 1962). This can be seen on frames 42 and 60 in Fig. 4. Shapes like those in frames 42, 59, 60, and 80 were not observed when untreated or iodoacetate-treated cells were subjected to the same swelling procedure. Occasionally we observed that iodoacetate-treated cells changed suddenly from a biconcave shape to a cup shape (stomatocyte I according to Bessis, 1977). The shapes of these cups were smoother overall and had little resemblance to the shapes

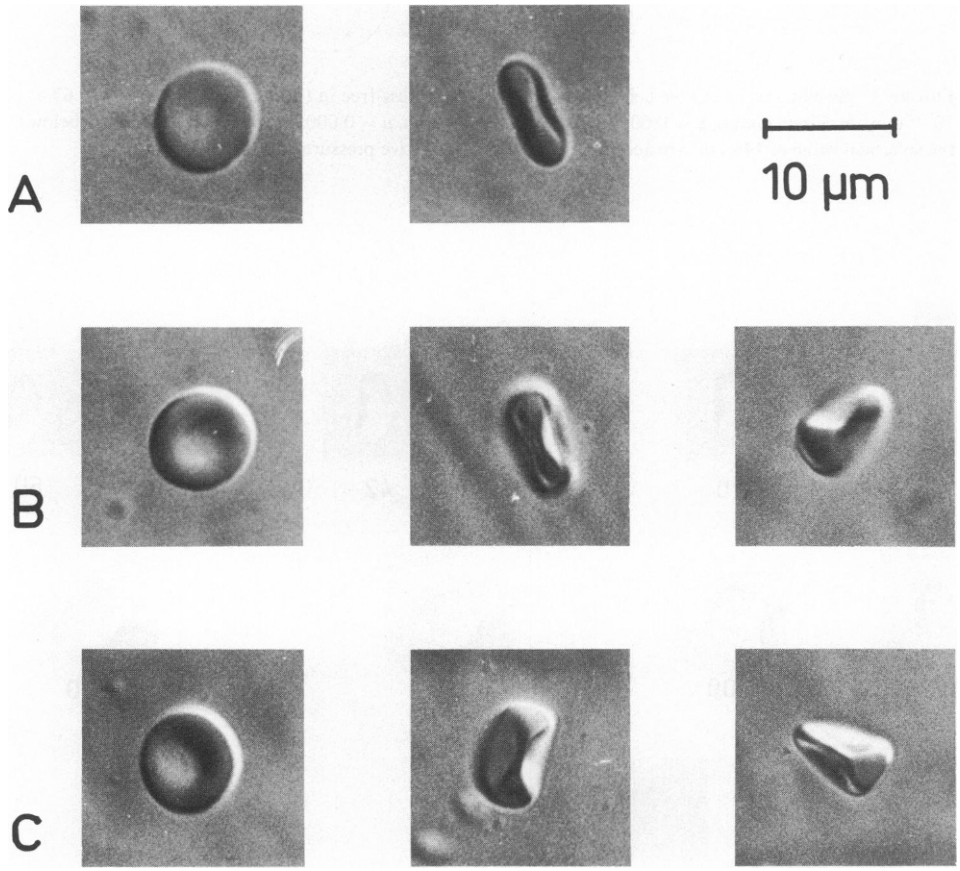

FIGURE 5 Red blood cells treated with diamide in the spherical shape and then shrunk back to normal volume. Each horizontal line represents the same cell in different views. Left column; face on; middle column; edge on; right column; edge on after 90° rotation (with respect to middle column). *A*, 10 mM iodoacetate; *B*, 10 mM iodoacetate + 0.05 mM diamide; *C*, 10 mM iodoacetate + 0.5 mM diamide.

shown in Fig. 4. The tendency to assume cup shapes may be related to the albumin added to the solution to prevent crenation (cf. Jay, 1975).

The sphere-to-disk transformation was studied by treating the red cells with diamide in a hypotonic medium in which most of the cells had already acquired a nearly spherical shape. The volume of these cells was subsequently reduced by resuspending them in their autologous plasma. Fig. 5 *B* and *C* shows the shapes most frequently observed after this procedure. Because the shapes were not axisymmetric, three views are shown for each cell. The most

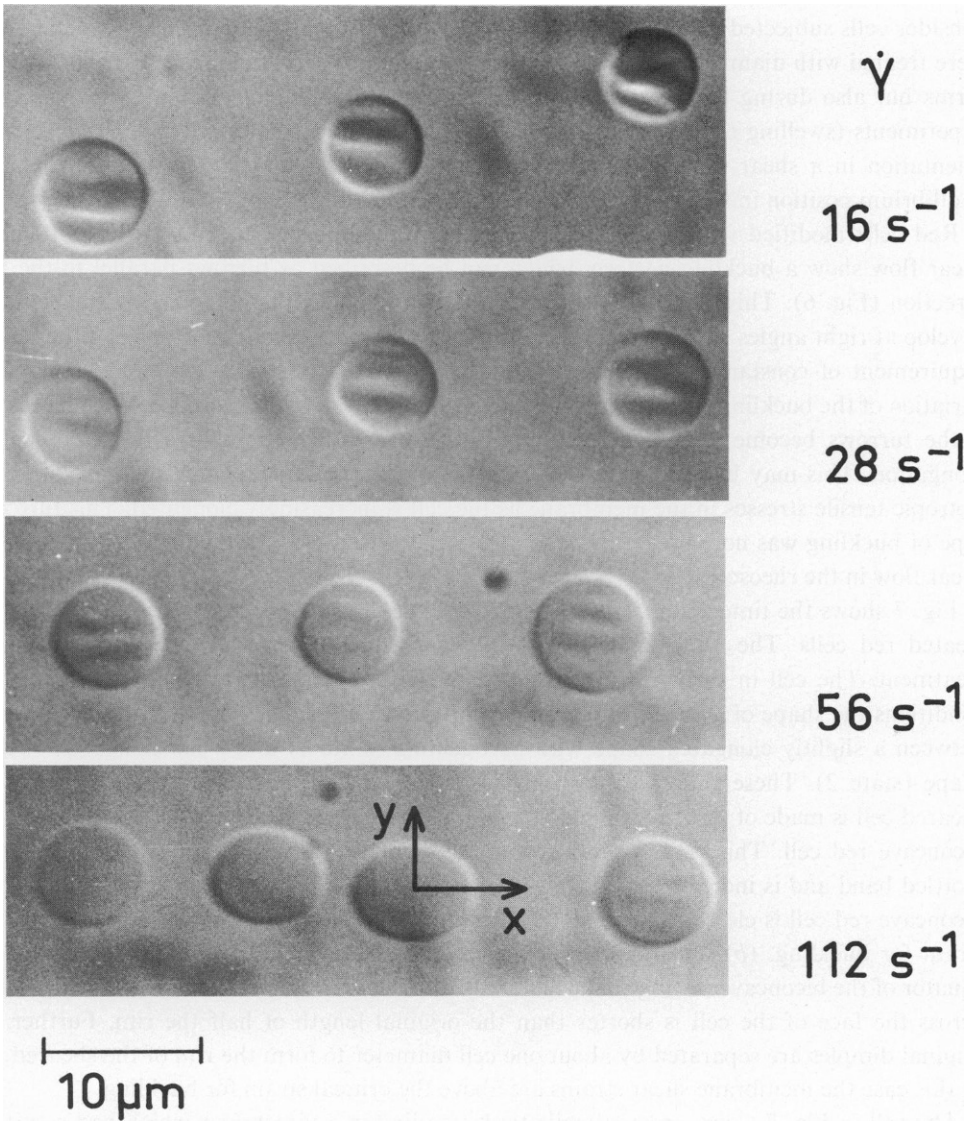

FIGURE 6 Membrane buckling of red cells treated with 0.5 mM diamide in the spherical shape, suspended in a dextran solution (34 cP) and subjected to different mean shear rates ($\dot{\gamma}$) in the rheoscope (x, flow direction; y, radial direction in the cone-plate chamber).

striking difference between the shape of normal red cells and diamide-treated cells can be seen at the rim. The thickness of the rim varies around the equator; the extremes of this thickness are shown in the views given in the right column of Fig. 5 *B* and *C*. In the view after 90° rotation of the disk, shown in the middle picture, the rim appears approximately uniform in thickness. The rim, however, is much thicker than in the iodoacetate-treated cells (Fig. 5 *A*). Furthermore, considering the sequence from the iodoacetate-treated cells (Fig. 5 *A*) to cells treated with 0.05 and with 0.5 mM diamide (Fig. 5 *B* and *C*) there is a definite increase in curvature of the membrane at the rim.

The above discussion pertains primarily to static axisymmetric deformations. We now consider cells subjected to periodic non-axisymmetric deformations in a shear field. The cells were treated with diamide as in the previous experiments, namely in biconcave and spherical forms but also during the deformations. An important distinction from the previous static experiments (swelling or shrinking series) is that the membrane of a red cell with stationary orientation in a shear field undergoes a periodic state of motion, i.e., there is a dynamic equilibrium position in which the cell has a tank-tread motion.

Red cells modified with diamide ($\geq$ 0.05 mM) and subjected to tank-tread motion in a shear flow show a buckling pattern, which can be described as furrows parallel to the flow direction (Fig. 6). This can be interpreted as buckling under the compression that tends to develop at right angles to the direction of stretch. This compression (shortening) is due to the requirement of constant surface area as the length of the cell increases. Fig. 6 shows the variation of the buckling pattern with the mean shear rate $\dot{\gamma}$ in the rheoscope. With increasing $\dot{\gamma}$ the furrows become shallower and finally disappear when the cells show a perceptible elongation. This may be due to the development of an internal pressure and concommitant isotropic tensile stresses in the membrane as the cell is increasingly elongated. This furrowed type of buckling was not observed when native or iodoacetate treated cells were subjected to shear flow in the rheoscope.

Fig. 7 shows the time history of the membrane buckling during tank-treading of diamide-treated red cells. The buckling pattern varies according to the shape at the time of the treatment. The cell in Fig. 7 *A* was modified in the biconcave shape. Under tank-treading conditions the shape of this cell fluctuates with twice the frequency of the tank-tread motion between a slightly elongated shape with no buckling (state 1) and a nonelongated buckled shape (state 2). These shapes can be interpreted as follows: (*a*) In state 1 the rim of the sheared cell is made of parts of the membrane which correspond to the equator of the resting biconcave red cell. This ring is marked in the schematic drawings in Fig. 7 *B* by a broad mottled band and is indicated in Fig. 7 *A* by two membrane-bound latex spheres. When the biconcave red cell is elongated into state 1 the membrane shear strains are below the critical strain for buckling. (*b*) On the other hand, in state 2 the membrane band that forms the equator of the biconcave resting shape lies across the buckled face of the red cell. The distance across the face of the cell is shorter than the original length of half the rim. Further, the original dimples are separated by about one cell diameter to form the rim of the sheared cell. In this case the membrane shear strains are above the critical strain for buckling.

The cell in Fig. 7 *C* was treated while tank-treading in a viscometer which had a suitable total volume (3 cm^3). The sample was continuously sheared so that during the treatment the cell was always deformed and tank-treaded. When the cell, after the treatment, was observed

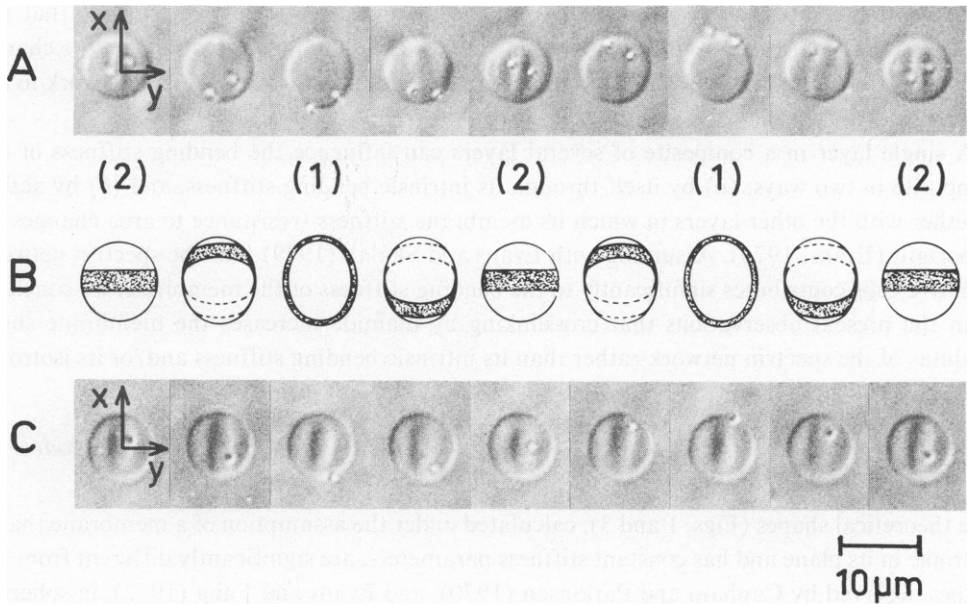

FIGURE 7 Membrane buckling and tanktreading of red cells treated with diamide, suspended in dextran solutions of viscosity η_0 and subjected to mean shear rates ($\dot{\gamma}$) in the rheoscope (x, flow direction; y, radial direction in the cone-plate chamber). Photomontages from motion pictures (Δt, time interval between two successive frames). Tanktread motion of the membrane is visualized by membrane-attached latex spheres. *A*, cells treated with 0.1 mM diamide in the biconcave shape; $\dot{\gamma} = 72\ s^{-1}$; $\eta_0 = 27$ cP; $\Delta t = 33$ ms; states 1 and 2 are discussed in the text. *B*, schematic drawing of the cell in *A*. The location of the membrane band that forms the equator of the biconcave shape is represented by the mottled area. *C*, cells first treated with 0.05 mM diamide in a viscometer while being sheared; $\dot{\gamma} = 37\ s^{-1}$; $\eta_0 = 22$ cP; $\Delta t = 80$ ms. The views shown are after the treatment had taken effect.

in the rheoscope it showed stationary buckling patterns. This is interpreted as follows. When any cell is tank-treading in the shear field with a stationary shape, the deformation of individual membrane parts changes periodically. Therefore during the treatment with diamide, the S–S crosslinks introduced are such that there are no differences between any two points of the membrane and no memory of the disk shape.

When the cell is treated in the spherical shape (Fig. 6) one might expect stationary furrows when the cell buckles in a shear field because of symmetry of all locations on the sphere. We observed, however, intermediate behavior of such cells under shear between that shown in Fig. 7 *A* and *C*. This might result from a slightly nonspherical shape during the treatment with diamide which may be due to the statistical distribution in the response of red cells to osmotic swelling and/or to the memory of some properties of the initial biconcave shape not erased by sphering.

DISCUSSION

Implications of Buckling in Diamide-treated Cells

The fact that diamide-treated cells show membrane buckling during sphering experiments for the same cell geometry for which normal cells do not show buckling can be explained by an

increase in the ratio $(\mu \ell^2)/B$ resulting from the diamide treatment. This means that the change in the shear modulus of the membrane induced by diamide is greater than the change in bending stiffness. This has implications as to the contribution of the spectrin network to the bending stiffness of the native membrane.

A single layer in a composite of several layers can influence the bending stiffness of the composite in two ways: (*a*) by itself through its intrinsic bending stiffness, and (*b*) by acting together with the other layers in which its membrane stiffness (resistance to area changes) is important (Evans, 1974). Assuming with Evans and Skalak (1979) that the spectrin network in native cells contributes significantly to the bending stiffness of the membrane, we conclude from the present observations that crosslinking by diamide increases the membrane shear modulus of the spectrin network rather than its intrinsic bending stiffness and/or its isotropic modulus.

Implications of the Comparison Between Experiments and Theoretical Models of Axisymmetric Deformations

The theoretical shapes (Figs. 1 and 3), calculated under the assumption of a membrane that is isotropic in its plane and has constant stiffness parameters, are significantly different from the shapes observed by Canham and Parkinson (1970), and Evans and Fung (1972), in sphering experiments. To explain the discrepancy, there are at least four possibilities, which are based on the properties of the native membrane: (*a*) The stress-free shape of the membrane is neither biconcave nor spherical. We tried for example a biconvex shape (oblate spheroid), which was fairly flat near the axis of symmetry. This shape was generated by deflating a stress-free sphere with $\mu = 5 \times 10^{-5}$ dyn/cm and $B = 0.67 \times 10^{-12}$ dyn cm to a volume of 127 μm^3. If we use this as the stress-free shape ($\mu = 0.005$ dyn/cm, $B = 0.67 \times 10^{-12}$ dyn cm) no pop-through buckling is predicted. However, the shapes are significantly thicker at the dimple than the shapes measured by Evans and Fung (1972). (*b*) The membrane is anisotropic in the membrane plane in that its shear modulus depends on the direction in the membrane. For example an orthotropic membrane whose shear modulus is larger when stretched in meridional direction than when stretched in latitudinal direction would deviate from the theoretical predictions under the assumption of an isotropic membrane and behave more like the cells in the experiments. The extent of this deviation would depend on the extent of the anisotropy. (*c*) The shear modulus μ at the small membrane deformations involved in the disk-to-sphere transformation of normal cells (maximum stretch ratio $\lambda_{max} = 1.3$) is <0.005 dyn/cm, which has been measured in micropipette experiments employing λ_{max} in the range 2–4. As shown above, μ would have to be reduced by a factor 3 or 30 depending on whether the biconcave disk or the sphere is assumed to be the stress-free shape of the membrane. Instead of proposing a strain-dependent shear modulus we could alternatively change the stress-strain law appropriately. (*d*) Since the shape of the red cell is only determined by the ratio $(\mu \ell^2)/B$ the discrepancies could be explained as well by a stiffness parameter B which is higher by a factor 3 or 30.

On the other hand, when the red cells are treated with ~0.5 mM diamide, the cells behave during sphering and shrinking essentially as the theoretical model predicts, if we assume that the shape in which the diamide treatment is applied corresponds to the stress-free shape in the model calculations. For reasons of simplicity in the computations, we assumed symmetry with

respect to the plane normal to the axis of rotational symmetry for the theoretical modeling of pop-through buckling. Therefore the dimples are allowed only to pop simultaneously. We believe that this constitutes only a minor deviation from the more realistic asymmetric case. The coincidence of model and diamide-treated cells suggests that the additional shear stiffness produced by the diamide treatment is stress-free in the shape in which the treatment is applied. There are also good biochemical reasons for this explanation. It was shown by Fischer et al. (1978*a*) that the treatment with diamide induces inter- and intramolecular disulphide bonds in membrane proteins, and especially in spectrin, which is believed to be responsible for the membrane shear modulus. The oxidation of two adjacent SH-groups to one disulphide bond cannot introduce a tension between the two amino acids to which the SH-groups belong. Therefore we assume that the effect produced by the diamide treatment is equivalent to adding material that is stress-free in the shape in which the treatment is applied. If the diamide-induced shear stiffness is much greater than the native one, these treated cells should be expected to behave essentially as if the shape in which they were treated was their stress-free shape. We conclude that we may use the diamide-treated cells to illustrate the behavior of a given stress-free shape (in place of our theoretical model) for cases in which no theoretical solutions are available, e.g., non-axisymmetric cases discussed below.

Implications of the Comparison between Normal and Diamide-treated Cells with respect to their Behavior in a Shear Field

The elongation of red cells in a shear field of a viscous dextran-saline solution (~20 cP) allows the study of non-axisymmetric deformations. Three different diamide treatments were used: (*a*) treatment in the biconcave shape, (*b*) treatment in the spherical shape, and (*c*) treatment while the cell is elongated by the shear field and the membrane is continuously tank-treading. In all three cases we observed buckling of the membrane in contrast to native cells where buckling is not observed.

We will consider below whether the four properties of the native membrane suggested in the previous section dealing with axisymmetric deformations can be used to explain the observed behavior in the non-axisymmetric case.

STRESS-FREE SHAPE Because the cells treated with diamide in either the biconcave or the spherical shape showed membrane buckling in the shear field, we assume that any intermediate stress-free shape may do so too. Therefore we believe that a particular stress-free shape cannot serve as an explanation for the absence of buckling in native tank-treading red cells.

ANISOTROPY An anisotropy built into the cell membrane would be expected to have axial symmetry and could therefore not explain the absence of buckling for the non-axisymmetric deformations of the native cells in the shear field. To explain the observations in terms of anisotropy, one would have to assume that anisotropy is not preformed but is induced by the deformation itself, e.g., the modulus at right angles of the direction of stretch decreases as a function of stretch. Such an effect, however, would be expected to be zero for a strain equal to zero. When a cell is stationary in the shear field and shows no perceptible elongation, the strains in the membrane are small. We therefore expect the effects of a hypothetical strain-induced anisotropy to be small and neglect them in the further discussion.

VARIABLE SHEAR MODULUS A strain-dependent shear modulus can explain the

absence of buckling in non-axisymmetric, as well as in axisymmetric deformations and is taken as the basis of the discussion below.

BENDING STIFFNESS PARAMETER HIGHER THAN ASSUMED An increase in B can explain the absence of buckling also in non-axisymmetric deformations. However, B would have to be more than an order of magnitude higher than the value estimated from the comparison of experiment and theory (see above). Therefore a variable shear modulus appears more likely.

Discussion of Stress-free Shapes

We showed above that a reconciliation between theory and experiment can be achieved for both stress-free shapes tested (biconcave disk and sphere) if the ratio $(\mu\ell^2)/B$ is lowered appropriately. Since it seems very unlikely that B is responsible, we propose that μ is strain dependent starting from a small value μ_0 at small membrane strains and ending up at the value of 0.005 dyn/cm at the high strains employed in a micropipette experiment. If we take the biconcave disk as the stress-free shape we have to assume μ_0 to be about 0.002 dyn/cm. If we take the sphere to be unstressed shape, μ_0 would become 0.0002 dyn/cm. Unfortunately there is no method available to measure μ at small strains. Following are some of the obstacles to such a measurement: (*a*) It is difficult to resolve optically small deformations of red cells. (*b*) Separation of shear and bending effects is very difficult as long as the bending stiffness parameter B and the relative contributions of local and global bending stiffness (cf. Evans, 1974) are not known. (*c*) The stress-free shape of the membrane is unknown. The determination of the stress-free shape is the object of the present study, but would be required to be known in advance for measurements of μ_0. The stress-free shape of the red blood cell membrane remains, therefore, the subject of speculation. However, since the μ_0 predicted for the biconcave stress-free shape is much closer to the large strain value of 0.005 dyn/cm than the μ_0 for the spherical stress-free shape, it appears that the biconcave shape is the more natural choice for the stress-free shape of the red blood cell membrane.

If the biconcave shape is taken to be the stress-free shape ($\mu_0 = 0.002$ dyn/cm) it remains to be explained why experimentally observed red cell shapes can be matched theoretically by deflating a membrane that is free of shear stresses in the spherical shape and has zero bending moments when it has zero curvature (Brailsford et al., 1976; Korpman et al., 1977) or negative curvature (Deuling and Helfrich, 1976) or positive curvature (Zarda et al., 1977). The shear modulus that was used by these investigators lies between 0 and 0.0002 dyn/cm. This shear modulus, however, does not have to be the actual shear modulus of the membrane. The actual shear modulus can have any greater value without changing the calculated shape, provided that the additional shear stiffness is stress-free in exactly this (biconcave) shape. To be in keeping with the above suggestion of a biconcave stress-free shape, the actual shear modulus should have a value of 0.002 dyn/cm.

Besides the theoretical reasons for such a model, there is also a physical interpretation for why most or all of the elements contributing to the shear stiffness can be stress-free in the biconcave shape. Evans and La Celle (1975) observed stress-relaxation phenomena at large membrane deformations. The membranes proposed above could be interpreted as a membrane with $\mu_0 = 0.002$ dyn/cm which is at some time free of shear stresses in the spherical shape. When the sphere is deflated quickly, there would be shear stresses in the

membrane. These, however, although being much smaller than those applied by Evans and La Celle (1975), in pipette experiments, could relax over a long period of time. A complete relaxation of shear stresses would correspond with shapes that could be matched by deflating a sphere without any shear stiffness. An incomplete relaxation of shear stresses would correspond to shapes that could be matched by introducing a small shear modulus ($\mu < 0.002$ dyn/cm). In this case there would be no stress-free shape but a mixture of the memory of a spherical and a biconcave stress-free shape.

Finally, the question arises as to whether the bending moments in the membrane might be relaxed as well. To throw some light on this question, we calculated the shapes during deflation of a stress-free sphere whose shear modulus was so small ($\mu = 0.00005$ dyn/cm) that the calculated shapes were essentially dictated by the bending stiffness alone. The shape that was obtained by deflation of the sphere to the normal red cell volume was then taken as a new stress-free shape and was reinflated to the sphere. The shapes that were calculated for intermediate volumes were almost indistinguishable for the two different stress-free shapes (data not shown). This shows that the experimental observations of the disk-to-sphere transformation cannot be used to determine in which shape of the membrane the bending moments are zero.

SUMMARY

In summary, observations and calculations on normal and diamide-treated red cells suggest that the membrane shear modulus may be quite strongly dependent on the strain, i.e., the shear modulus being smaller at small strains. Another possibility would be that the bending stiffness is higher than it was estimated by Evans (1980). A mixture of both possibilities would of course also explain the results. It is not possible at present to decide definitively the question of whether the red cell membrane is free of shear stresses in the biconcave shape but it appears to be the most likely possibility. The estimation of the membrane shear modulus at small strains could be an important step in resolving the question.

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