Bending Stiffness of Lipid Bilayers: IV. Interpretation of Red Cell Shape Change

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ABSTRACT Two mechanisms are operative when the resting shape of human red cells is changed into an echinocyte or a stomatocyte. The first (bilayer couple) is a differential change in the surface area of the two monolayers. It rests on the two-dimensional isotropic elasticity of the two monolayers and their fixed distance. The second (single layer) is a change in the average cone angle of the molecules comprising a monolayer. It rests on the intrinsic bending elasticity of each single layer. With a few exceptions the first mechanism has been quoted to interpret experimentally observed shape changes. To reconsider this preference two types of spontaneous curvatures (in bilayer couple bending and in single-layer bending) are defined. It is shown that (*a*) disregarding the single-layer mechanism is not justified and (*b*) there is too little basic information for quantitative interpretations of shape change.

INTRODUCTION

The resting shape of the human red cell was and still is the subject of intensive research. The customary method of changing the biconcave disk toward an echinocyte or a stomatocyte is the incorporation of molecules into the membrane. In the first and, since then, dominating interpretation the shape changes were explained by a differential increase in surface area of the two leaflets of the membrane (1–3). This concept, termed *bilayer couple* (3), rests on the notion that the bending elasticity of the red cell membrane is caused by the area elasticity of the two lipid layers and their fixed distance.

The intrinsic bending stiffness of each monolayer is neglected in this view. In contrast, this intrinsic bending stiffness is exclusively considered by researchers studying the phase transitions between structures formed by lipids in water (4). The different liquid crystalline phases were explained by the packing behavior of different types of lipid molecules (5). This packing behavior was paraphrased by attributing intrinsic shapes (most of them axisymmetric) to the different lipid species (5). In this paper we use this notion and call a molecule conical when the cross section of its corresponding shape is larger at the hydrophilic than at the hydrophobic end. In this case the cone angle γ is defined as positive. Molecules with a corresponding shape that has a negative γ are called *inverted conical.* γ is defined as the maximum angle that can be enclosed by two surface generators of the cone. The concept of conical and inverted conical molecules was applied by Kuypers and co-workers (6) to red cell shape changes. It was, however, not picked up by others.

In this paper we will reevaluate the two concepts with respect to their relative contribution to understanding red cell shape change. Although we use information on the molecular level, our argumentation will be basically continuum mechanical. The definition of two kinds of spontaneous curvature given earlier (7) will be extended. Based on these definitions the interpretations of published experiments will be reconsidered. It will be shown that the neglect of single-layer bending is not warranted.

THEORETICAL CONSIDERATIONS

Bending elasticity

The bending elasticity of a lipid bilayer can be decomposed into two contributions (8, 9). Here these two contributions are called bilayer couple bending and single-layer bending (7). In bilayer couple bending the resistance against bending of a bilayer results from the resistance against a change in surface area of the monolayers and from their fixed distance. The elastic constant (bending stiffness) in bilayer couple bending is designated as B_c. The resistance of each monolayer alone against bending is taken care of by single-layer bending. This resistance results from the resistance of the molecules against a change in their average shape, say from cylindrical to conical. The values for the bending stiffness of the inner or the outer monolayer (B_i or B_o) are about 6 times smaller than B_c (7).

A deformation is called local when the contribution of a certain membrane element to the increase in elastic energy can be delineated from its deformation with respect to a reference state. As opposed to this, a balance between the deformation in different locations on the membrane may occur which makes the elastic energy depend on the deformation of the whole membrane envelope. A special case, called global bending by Evans (1), prevails when the elastic energy depends on the surface averaged value of the deformation parameter.

Bilayer couple bending is local when the two monolayers cannot slide relative to each other; it is global when slip is possible (1). In mechanical equilibrium there is enough time

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for slip (7). Consequently, bilayer couple bending is global in the resting shape of red cells.

From a purely mechanistic point of view a balance appears to be possible in single-layer bending as well, provided molecules with different intrinsic shapes would distribute laterally according to their curvature preference. It can be shown, however, that the resulting gain in elastic energy per molecule is much less than the thermal energy tending to randomize the distribution of the molecules on the surface. On the other hand, some sort of attractive interaction could exist that is strong enough to induce a domain formation of certain molecular species. Such a process may be related to the observation that echinocytes after calcium influx look quite different from "ordinary" echinocytes (10). In our discussion we neglect this special case and assume the lateral distribution of the molecules to be uniform.

Definition of spontaneous curvatures

The spontaneous curvature is defined by the following *gedanken* experiment. We consider a circular piece in the membrane of a red cell (Fig. 1). The curvature of the neutral plane of this piece of bilayer is called the actual curvature in the following. Its mean value, c, is the arithmetic mean of the two principal values. The sign convention is such that c is positive at the tip of a membrane protrusion.

The size of the piece of membrane should be small enough so that within it the lateral distribution of the deformation is uniform. On the other hand, it should be large enough for continuum mechanics to apply. These requirements become irreconcilable when the length scale of curvature change becomes comparable to the mesh size of the spectrin-actin network. Such shapes are not covered by the continuum mechanical description suggested here.

We now imagine cutting this piece out of the membrane. We assume that the two lipid layers are kept flush at the rim (no slip) and that edge effects do not influence the shape of the piece. If the membrane is not free of shear stresses the circular piece will deform into an ellipse and release these stresses.

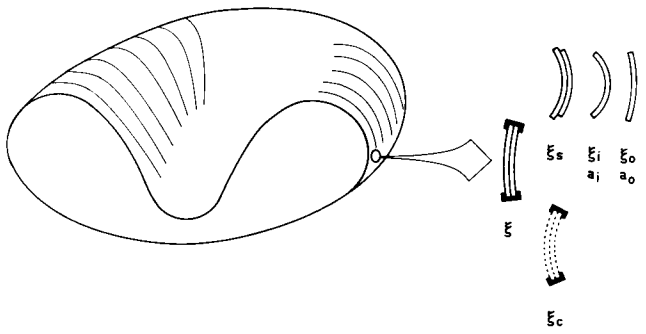

FIGURE 1 Spontaneous curvatures. Schematic drawing of an operational definition of the net spontaneous curvature of the bilayer (ξ), the spontaneous curvature in bilayer couple bending (ξ_c), and the spontaneous curvatures in single-layer bending: ξ_s for the bilayer, ξ_i for the inner leaflet, and ξ_o for the outer one. For details see text.

Furthermore, the piece will assume a uniform curvature characterized by a minimum in total bending energy. There is good evidence that the deviatoric contribution to this curvature vanishes (11). Its isotropic contribution (i.e., its mean curvature) will be termed the *net spontaneous curvature* (ξ) in the following.

If a slip between the two layers but not their separation were allowed, isotropic stresses in each layer would be released and the membrane would assume (Fig. 1) the spontaneous curvature ξ_s, which is characterized by a minimum in the elastic energy stored in single-layer bending.

We then assume that we are able to introduce a proper solvent between the two monolayers to allow their separation. If the solvent were able to mimic the natural hydrocarbon environment we could observe (Fig. 1) the spontaneous curvature (ξ_i and ξ_o) of the monolayers. Both values are characterized by zero elastic energy in single-layer bending. ξ_o and ξ_i can be expressed by the cone angles (γ) and the effective cross-sectional areas (α) of the shapes corresponding to the molecules in each layer:

$$\xi_o = (\Sigma\ \gamma)/(\Sigma\ \alpha)^{1/2}, \quad \text{and} \tag{1}$$

$$\xi_i = -(\Sigma\ \gamma)/(\Sigma\ \alpha)^{1/2} \tag{2}$$

where Σ indicates summation over the molecules. α is defined as the cross-sectional area at the position of the neutral plane of the monolayer. By definition α does not change during bending of a monolayer.

The spontaneous curvature due to bilayer couple bending (ξ_c) can be calculated from the unstrained surface area of the two monolayers (a_i and a_o, Fig. 1) and the distance (h) of their neutral surfaces.

$$\xi_c = \frac{a_o - a_i}{(a_o + a_i)h} \tag{3}$$

If we were able to abolish the elasticity in single-layer bending (Fig. 1, broken lines) and kept the two monolayers flush the membrane piece would assume the curvature ξ_c.

By analogy to the determination of the resting length of a parallel arrangement of springs ξ_s is obtained from ξ_o and ξ_i by the weighted sum

$$\xi_s = \frac{B_o\xi_o + B_i\xi_i}{B_o + B_i} \tag{4}$$

An analogous formula holds for ξ:

$$\xi = \frac{B_s\xi_s + B_c\xi_c}{B_s + B_c} \tag{5}$$

where $B_s = B_o + B_i$.

It follows from the definitions given above that in general ξ_c depends on location on the red cell surface and that its distribution varies with red cell shape. Its value averaged over the membrane surface, on the other hand, is a constant

which can be calculated according to

$$\langle \xi_c \rangle = \frac{A_o - A_i}{(A_o + A_i)h} \quad (6)$$

where A_o and A_i denote the unstrained surface area of the outer and inner monolayer, respectively, of the whole membrane envelope, and $\langle\ \rangle$ indicates the average over the red cell surface. It is clear that $\langle \xi_c \rangle$ is independent of red cell shape. For later use we note that $\langle c \rangle$ can be calculated from Eq. 6 when the actual areas instead of the unstrained ones are inserted.

ξ_i and ξ_o, in contrast to ξ_c, are uniform on the red cell surface. This is a consequence of the uniform distribution of the molecules comprising each monolayer (see above).

Red cell shape change

It was noted by Evans (1) that the static red cell shape is determined by a minimum in the elastic energy stored in bending and in (in-plane) shear of the membrane. A local formulation of the energy density due to shear deformation (of the membrane skeleton) has been given by Evans and Skalak (12). Recently it has been suggested that a balanced formulation is more appropriate to the real situation (8, 13).

As for bilayer couple bending (of the phospholipid moiety) it was argued above that in the resting shape only the global case prevails. The corresponding elastic energy (E_{gc}) can be written (7)

$$E_{gc} = 2AB_c(\langle c \rangle - \langle \xi_c \rangle)^2 \quad (7)$$

In single-layer bending we obtain for E_i, the elastic energy stored in the inner layer (7),

$$E_i = 2B_i \int (c - \xi_i)^2\, dA. \quad (8)$$

An analogous formula applies to the outer layer. In a strict sense Eq. 8 is approximate since the neutral surfaces of the monolayers are displaced from that of the bilayer. Consequently, the value of c in Eq. 8 should be different from that in Eq. 7. Taking this difference into account leads to a correction that increases with the difference between ξ_o and ξ_i (14). This correction is neglected in the following.

The two expressions in Eqs. 7 and 8 are formally different. It is therefore not possible to formulate the total bending energy with just a local or just a global term.

Relative impact of the spontaneous curvatures

In the following we account for four elastic contributions to the free energy of the membrane in static equilibrium: by shear deformation of the membrane skeleton, by bilayer couple bending according to Eq. 7, and for each monolayer by single-layer bending according to Eq. 8. The contribution from isotropic tension is not considered since we restrict ourselves to shapes in which the red cell volume is considerably smaller than the maximum volume that can be enclosed by the red cell surface area. In principle the shape can be changed by changing one or more of the following quantities: the elastic constants for shear or bending deformations, the reference configuration of the skeleton, or the spontaneous curvatures. With a few exceptions (15, 16) the spontaneous curvatures were manipulated in order to change red cell shape.

The relative impact (on red cell shape) of changes in the different kinds of spontaneous curvature is weighted by the corresponding elastic constants. A proof is given in the Appendix. Since B_s is about 3 times smaller than B_c (7) a change in ξ_s would therefore have to be 3 times larger than a change in $\langle \xi_c \rangle$ to elicit the same shape change.

The actual change in the two kinds of spontaneous curvature depends on the experimental protocol. For an order of magnitude estimate we make a *gedanken* experiment that corresponds to a typical incorporation proctocol. We start with a closed membrane having an equal number of molecules of a long-chain phosphatidylcholine (PC) in each monolayer. According to Eqs. 4 and 6 we obtain $\xi_s = \langle \xi_c \rangle = 0$. We then imagine adding to the outer layer one LysoPC (LPC)/100 PC molecules. Using 0.65 nm^2 and 0.34 nm^2 (17) for the effective cross-sectional areas of PC and LPC, respectively, we obtain from Eq. 6 $\langle \xi_c \rangle = 1/(770\ \text{nm})$, where 2 nm, half the bilayer thickness (7), was taken for h.

For simplicity we assume LPC to have a truncated conical shape with cross-sectional areas of 0.65 nm^2 at the hydrophilic end and 0.34 nm^2 in the middle of the molecule. Assuming its length to be 2 nm we get $\gamma = 12.7°$. From Eqs. 1, 2, and 4 we obtain $\xi_s = 1/(73\ \text{nm})$, which is an order of magnitude above $\langle \xi_c \rangle$. This factor has to be compared with the factor of 3 originating from the ratio B_c/B_s. We conclude that in this example single-layer bending outweighs bilayer couple bending. With the incorporation of molecules with a smaller cone angle the relative influence of single-layer bending would decrease.

RECONSIDERATION OF PUBLISHED INTERPRETATIONS

Qualitative interpretations

There are many ways to change the spontaneous curvature of the bilayer of a red cell (the list given below may still be incomplete):

(a) asymmetric incorporation of exogenous molecules into the two leaflets
(b) interleaflet redistribution of endogenous molecules
(c) asymmetric removal of endogenous molecules from the two leaflets
(d) exchange of endogenous molecules against exogenous ones
(e) change in the solutions adjacent to the membrane

Along the same lines as in the estimate made above for case (a) it can be shown for cases (b) to (d) that single-layer bending does play a role besides bilayer couple bending, provided the involved molecules have a conical or inverted

conical shape. Before turning to case (*e*) I address the question of why single-layer bending was hardly ever accounted for in the interpretation of red cell shape changes.

In an elegant experiment Kuypers et al. (6) exchanged endogenous phospholipids from the outer layer in a one-to-one fashion, against exogenous ones that were either more or less inverted cone shaped. The inner layer was not changed. Based on the effective cross-sectional area of the exchanged molecules as obtained from monolayer studies the authors argued that the changes in $\langle \xi_c \rangle$ and ξ_o were antagonistic. Since the observed shape changes were in line with the change in ξ_o these experiments presented evidence for a dominance of single-layer bending over bilayer couple bending in the observed shape change.

The consideration of the spontaneous curvature in single-layer bending was not picked up by later authors to explain experimental data. Obviously arguments based just on bilayer couple bending were sufficient. As long as only qualitative statements are made, this may occur in two ways. First, the changes in the two types of spontaneous curvature are synergistic. Second, the changes are antagonistic but the change in $\langle \xi_c \rangle$ dominates the shape change. An example for the first case is a pure incorporation experiment (case (*a*)).

For an amphiphile to be incorporated from solution or from lipid vesicles into the membrane its hydrophobic part must be relatively small. This means after incorporation it acts as a conical molecule. A preponderance of such molecules, in either monolayer, changes both types of spontaneous curvatures in the same direction; that is, the action is synergistic.

For most experimental protocols it is impossible to distinguish between the two cases since there is very little information on the spontaneous curvature of monolayers, especially of mixed ones.

We now return to the question of the relative importance of single-layer bending in case (*e*). Shape changes according to this case require the interaction between the solution and the hydrophilic portion of the lipid molecules. It is obvious that such an interaction would if anything change the effective cone angle more than the effective cross section of the lipids.

As one alternative to an explanation via a bilayer couple mechanism of the two phospholipid leaflets, the influence of adjacent layers (on the outside the glyocalyx and on the inside the membrane skeleton) on red cell shape has been discussed (8, 18, 19) to explain shape changes according to case (*e*). In considering the molecular structure of these layers, a contribution to membrane bending stiffness appears possible only via bilayer couple bending. This means a change in spontaneous curvature requires a change in surface area of the layers. Such changes have been suggested to arise from a change in electrostatic interaction between the charges within the layers (8, 19).

A reduction in the charge of the glycocalyx by 50% or more has been reported to elicit a mild change in shape (20). In contrast, the same degree in shape change is observed after incorporation of one PC molecule per 100 native lipids of the outer monolayer (17). This comparison indicates that the influence of the glycocalyx on red cell shape is small. As for the membrane skeleton, its isotropic modulus is probably orders of magnitude smaller than that of a lipid monolayer (13). Therefore, despite its greater distance from the neutral surface of the membrane a direct influence on red cell shape is unlikely. The skeleton may, however, exert an indirect influence via electrostatic interactions with charged phospholipids of the bilayer and change their cone angle more than their effective cross-sectional area.

Taking together all cases enumerated above, we cannot neglect single-layer bending against bilayer couple bending in its effect on shape change.

Quantitative interpretations

Quantitative interpretations of red cell shape changes have been based to date solely on bilayer couple bending, where for the rest of the discussion we always mean bilayer couple bending of the phospholipid bilayer. In all studies a discrepancy was found in that the shape changes were smaller than expected on the grounds of estimated changes in surface area. In these expectations it was supposed that the change in $\langle \xi_c \rangle$ ($\Delta\langle \xi_c \rangle$ in the following) induced by the asymmetric incorporation of molecules would lead to an equal change in $\langle c \rangle$ ($\Delta\langle c \rangle$). Mechanically speaking, this expectation is equivalent to two assumptions: (*a*) Bilayer couple bending dominates over the other elastic contributions, that is, B_c is essentially infinitely large. As a consequence elastic energy would be stored in the other contributions but (due to the absence of strain) not in bilayer couple bending. (*b*) The curvature of the membrane is not limited by geometry (e.g., the ratio of surface area and volume). It may be noted marginally that such a cell would not assume typical red cell shapes at all but rather would shed an excess in $\langle \xi_c \rangle$ by vesiculation.

In contrast to the expected result, the experimental value for $\Delta\langle c \rangle / \Delta\langle \xi_c \rangle$ ranged from 0.65 (21) down to ~0.1 (22). In an attempt to explain this discrepancy Farge et al. (23) presented calculations in which the elastic energy stored in bilayer couple bending changed upon incorporation of molecules into the outer leaflet. Other contributions to the elastic energy were not considered by these authors. Since the shear elasticity is not global in nature the neglect of its contribution implies that the red cell shape is fixed. This can be achieved in two ways: (*a*) by a dominance of the shear elasticity (an example would be the increase of the shear modulus by treating red cells with wheat germ agglutinin) (24); or (*b*) by geometrical boundary conditions (an example would be swelling a red cell to a spherical shape).

Farge et al. (23) started from a shape with zero elastic energy and looked for the changes in area of the two leaflets after changing the unstrained area of the outer one. They calculated the ratio between the change in actual area of the outer layer and the change in its unstrained area. A value of 0.5 was obtained, which coincided with an experimentally determined value for $\Delta\langle c \rangle / \Delta\langle \xi_c \rangle$ (17). But note that values of

different quantities were compared. For the case of fixed shape $\Delta\langle c\rangle/\Delta\langle\xi_c\rangle = 0$.

The deviation of the experimental values for $\Delta\langle c\rangle/\Delta\langle\xi_c\rangle$ from unity as well as their variation can be interpreted when we assume that none of the three elastic contributions is dominant. $\Delta\langle c\rangle/\Delta\langle\xi_c\rangle$ must be between 0 and 1, the values for the two extreme cases considered above, when we (*a*) neglect single-layer bending and (*b*) assume the biconcave configuration to be free of shear stresses. If the shape change involves the formation of a bump or a strong invagination the same conclusion would be reached for a spherical stress-free shape. For moderate stomatocytes the situation may be less simple, but it appears that such shapes have not been used for quantitative interpretations. Upon inclusion of single-layer bending we reach an analogous conclusion: $0 > \Delta\langle c\rangle/\Delta\langle\xi\rangle > 1$, where $\langle\xi\rangle$ can be calculated from Eq. 5 when $\langle\xi_c\rangle$ instead of ξ_c is substituted.

For constant $\Delta\langle\xi\rangle$ we exspect $\Delta\langle c\rangle$ to be the smaller the larger the shear modulus, in accordance with experimental observation (16). In typical shape change assays the shear modulus and the stress-free shape of the membrane remain unaltered. Since the percentage of molecules causing the shape change is relatively small, B_c and B_s remain essentially constant; $\Delta\langle c\rangle/\Delta\langle\xi\rangle$ should therefore be constant. $\Delta\langle c\rangle/\Delta\langle\xi_c\rangle$, on the other hand, is expected to show a variation depending on the ratio of the cone angle of the molecules causing the shape change and their effective cross-sectional area. $\Delta\langle c\rangle/\Delta\langle\xi_c\rangle$ is greater than $\Delta\langle c\rangle/\Delta\langle\xi\rangle$ when the changes of spontaneous curvature in single-layer bending are synergistic to those in bilayer couple bending and is, respectively, smaller for antagonistic changes. Another part of the observed variation may be due to a transbilayer reorientation of endogenous molecules (25, 26) not accounted for in the determination of $\Delta\langle\xi_c\rangle$.

CONCLUSIONS

From the arguments raised it can be concluded that neglect of single-layer bending is not warranted in a discussion of red cell shape changes. A differentiation or a quantitative evaluation of the contributions of bilayer couple bending and single-layer bending, however, is difficult since there is very little information on the spontaneous curvature of pure monolayers, let alone of mixed ones. Inclusion of the single-layer mechanism into the argumentation, although correct, will therefore increase the existing ambiguity in the interpretation of red cell shape changes.

APPENDIX

We start with a resting shape of a red cell determined by its volume, surface area, and the balance between shear and bending elasticity. Bending comprises (local) single-layer bending and global bilayer couple bending, which are characterized by the spontaneous curvatures $\xi_o + \chi$, ξ_i, and $\langle\xi_c\rangle$ and the respective elastic constants B_o, B_i, and B_c. For all locations on the surface the balance of bending moments reads

$$B_o[c - (\xi_o + \chi)] + B_c(c - \xi_c) = M, \tag{9}$$

where M is the moment that balances the combined moments from single-layer bending of the outer layer and bilayer couple bending. Please note that global bending means that $(c - \xi_c)$ is constant on the surface. By a simple rearrangement we obtain

$$B_o(c - \xi_o) + B_c[c - (\xi_c + b\chi)] = M, \tag{10}$$

where $b = B_o/B_c$. Obviously, replacing a part χ of the spontaneous curvature of the outer layer by a part $b\chi$ of the spontaneous curvature in bilayer couple bending does not change the moment balance. Since χ is a constant the condition for global (bilayer couple) bending is conserved as well. It remains to be shown that the shape characterized by the distribution of c still corresponds to a minimum in total elastic energy. To this end we assume that another shape exists characterized by a distribution c' so that the total elastic energy is smaller. This assumption reads

$$2B_o \int (c' - \xi_o)^2\, dA + 2B_c A(\langle c'\rangle - \langle\xi_c\rangle - b\chi)^2 + E' < 2B_o \int (c - \xi_o)^2\, dA + 2B_c A(\langle c\rangle - \langle\xi_c\rangle - b\chi)^2 + E, \tag{11}$$

where E and E' denote additional energy terms from single-layer bending of the inner monolayer and from shear. With the original spontaneous curvatures, on the other hand, c corresponded to the shape with minimal energy. This condition reads:

$$2B_o \int (c' - \xi_o - \chi)^2\, dA + 2B_c A(\langle c'\rangle - \langle\xi_c\rangle)^2 + E' > 2B_o \int (c - \xi_o - \chi)^2\, dA + 2B_c A(\langle c\rangle - \langle\xi_c\rangle)^2 + E. \tag{12}$$

From the inqualities 11 and 12 we obtain

$$\chi(\langle c'\rangle - \langle c\rangle) > \chi(\langle c'\rangle - \langle c\rangle), \tag{13}$$

which disproves the assumption. This means there is no other shape with a smaller total elastic energy. The proof would run in an analogous fashion if we were to replace in addition part of the spontaneous curvature of the inner layer with a spontaneous curvature in bilayer couple bending.

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