

A POSSIBLE MECHANISM DETERMINING THE STABILITY OF SPICULATED RED BLOOD CELLS

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Abstract—In this work the stability of spiculated red blood cells, called echinocytes, is studied. It is assumed that the stable echinocyte shape corresponds to the minimum of its membrane elastic energy. It is shown that if the membrane skeleton shear elasticity is not taken into account the calculated stable echinocyte shapes always have only one spicule. However, by considering the skeleton shear elastic energy also, the calculated stable echinocyte shapes have many spicula in agreement with experimental observations. Copyright © 1996 Elsevier Science Ltd.

Keywords: Cell shape; Echinocyte; Shear elasticity; Bending energy; Membrane skeleton.

INTRODUCTION

The red blood cell (RBC) has no internal structure, therefore its shape is determined solely by the membrane properties (Canham, 1970; Evans, 1974; Evans and Hochmuth, 1978; Helfrich, 1973). The RBC membrane is essentially composed of two parts: the bilayer and the continuous network of proteins, the membrane skeleton (Steck, 1974). The bilayer which is composed of two layers of lipid molecules also contains some other molecules such as glycolipids and different membrane integral proteins. Under normal conditions the entire bilayer is underlaid with the skeleton (Iglič *et al.*, 1995; Liu *et al.*, 1989).

The normal resting shape of RBC is the biconcave discoid shape. Under different external conditions this normal red blood cell (discocyte) may be transformed into various other shapes such as spiculated RBC (echinocyte) or cup shaped RBC (stomatocyte) (Brecher and Besis, 1972). Echinocytes are spherical cells with 10–50 spicula uniformly distributed over the cell surface (Fig. 1). The most widely used method for changing the discocyte towards a stomatocyte or an echinocyte is incorporation of molecules into RBC membrane thereby changing the difference between the outer and the inner lipid layer areas (ΔA). It was shown that lowering of ΔA causes the discocyte RBC shape to change towards the stomatocyte shape, while increasing ΔA induces the transformation of the discocyte shape into the echinocyte shape (Evans, 1974; Helfrich, 1974; Sheetz and Singer, 1974).

It was shown that the stable axisymmetric discocyte and stomatocyte shapes correspond to the minimum of the membrane bending energy at the given external conditions (Deuling and Helfrich, 1976; Evans, 1974; Seifert *et al.*, 1991; Svetina and Žekš, 1989). The stability of echinocyte shapes has been also studied previously. However, these studies of echinocyte shapes have been limited to RBC shapes with a single spicule (Brailsford *et al.*, 1980; Stokke *et al.*, 1986) or to the RBC shapes with

short spicula approximated with spherical harmonics (Landman, 1984). In this work the stability of strongly nonaxisymmetric echinocyte shapes is analysed by taking into account all the arbitrary numbers and arbitrary lengths of echinocyte spicula.

A POSSIBLE PHYSICAL MECHANISM DETERMINING THE STABILITY OF ECHINOCYTE

Artificial phospholipid vesicles may attain discocyte and stomatocyte shapes or pear shapes with a single spicule (Berndl *et al.*, 1990; Käs and Sackmann, 1991; Sackmann *et al.*, 1986) but never echinocyte shapes with more than one spicule. Since the membrane of phospholipid vesicles consists only of the lipid bilayer, it can be assumed that the skeleton of the RBC membrane is responsible for the stability of the echinocyte shape with many spicula. Consequently, in the theoretical determination of the equilibrium echinocyte RBC shapes, the membrane shear energy should be taken into account (Brailsford *et al.*, 1980; Landman, 1984; Stokke *et al.*, 1986) which is contributed solely by the skeleton since the lipid bilayer has the properties of the two-dimensional liquid. In this work we shall determine the stable echinocyte RBC shapes by minimizing the membrane elastic energy (W), consisting of both bending (W_b) and shear (W_s) contributions:

$$W = W_b + W_s. \quad (1)$$

The bending energy of the bilayer can be decomposed into two contributions: local and nonlocal (Evans, 1974; Helfrich, 1974; Svetina and Žekš, 1989). The nonlocal bending resistance is due to net compression and expansion of both lipid layers resulting from the curvature change. Recently it has been experimentally determined that the nonlocal bending modulus B_n is approximately three times as large as the local bending modulus B (Vaugh *et al.*, 1992). However, it can be shown that for $B_n \simeq 3B$ the nonlocal bending energy of the pear-shaped RBC shape is approximately one order of magnitude smaller than the corresponding local bending energy (Iglič *et al.*, 1996). The nonlocal bending energy can be additionally decreased due to redistribution of molecules

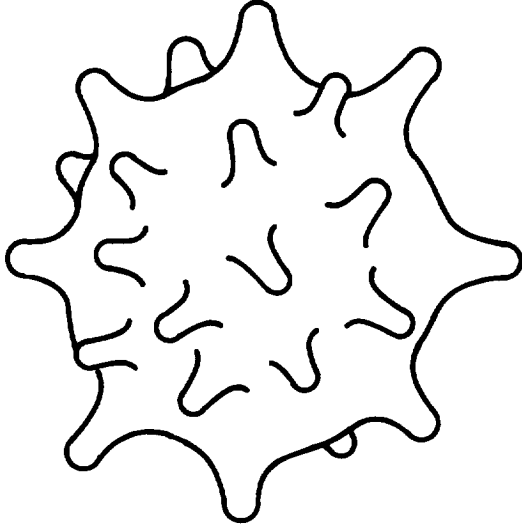


Fig. 1. Schematic representation of spiculated (echinocyte) red blood cell shape.

within each lipid layer in order to equalize the area per molecule and by exchange of lipid molecules between both lipid layers to alleviate curvature induced dilation or compression (Waugh and Hochmuth, 1995). In this work the nonlocal bending term is neglected, i.e. the bending energy of the bilayer is taken to consist only of the local contribution (Evans, 1974; Helfrich, 1974):

$$W_b = \frac{1}{2} B \int (C_1 + C_2)^2 dA, \quad (2)$$

where C_1 and C_2 are the two principal curvatures defined so that they are positive for a sphere and dA is the infinitesimal membrane area element.

The membrane bilayer greatly resists changes in surface area while the skeleton may dilate or condense relative to the constant density bilayer envelope; only the total area of the skeleton is conserved (Fischer, 1992; Mohandas and Evans, 1994; Svetina *et al.*, 1996). Namely, the very recent experiments have shown that the membrane skeleton is locally compressible which leads to a new constitutive model for the membrane skeleton behavior (Mohandas and Evans, 1994). For the sake of simplicity, in this work the RBC membrane is considered within the model of immobilized boundaries (Markin and Kozlov, 1986, 1988). Within this model the membrane skeleton cannot redistribute relative to the cytoplasmic surface of the bilayer, which prevents by lateral incompressibility of the bilayer, any local area changes both of bilayer as well as of the skeleton (Lerche *et al.*, 1991). Therefore the shear energy of the skeleton (Mohandas and Evans, 1994) is written using an approximate expression (Evans and Skalak, 1980):

$$W_s = \mu \int \left(\frac{1}{2\lambda_1\lambda_2} (\lambda_1^2 + \lambda_2^2) - 1 \right) dA, \quad (3)$$

where μ is the membrane area shear modulus, while λ_1 and λ_2 are the principal extension ratios of the membrane area element, which is chosen sufficiently small that we may consider it approximately flat. The local principal axes are chosen so that the shear resultants along the

local area element edges are zero. The assumption that the membrane skeleton is locally incompressible leads to the conclusion that $\lambda_1\lambda_2 = 1$. Since in this way λ_1 and λ_2 are not independent, we need only to consider a single principal extension ratio (Evans and Skalak, 1980). We choose the principal extension ratio along the meridional direction, λ_m . The other extension ratio is given by the reciprocal, λ_m^{-1} . Thus the membrane (skeleton) shear energy W_s can be written in the form (Evans and Skalak, 1980):

$$W_s = \mu \int \left(\frac{1}{2} (\lambda_m^2 + \lambda_m^{-2}) - 1 \right) dA. \quad (4)$$

GEOMETRICAL MODEL OF ECHINOCYTE SHAPE

The echinocyte shape is described by a geometrical model with five parameters (Iglič *et al.*, 1985). These parameters are the radius of the large sphere R , the number of axisymmetrical spicula distributed on the large sphere n , the length of the spiculum cylinder L , the radius ρ and the angle ϑ (see Fig. 2).

Volume and surface area of the model echinocyte are

$$\begin{aligned} V = & 4\pi R^3/3 + n(\pi\rho \cos \vartheta [(\rho + R)^2 \sin^2 \vartheta + \rho^2(1 - \cos^2 \vartheta/3)] \\ & - \pi\rho^2(\rho + R) \sin \vartheta (\sin \vartheta \cos \vartheta + \pi/2 - \vartheta) \\ & - \pi R^3(1 - \cos \vartheta)^2(2 + \cos \vartheta)/3 \\ & + \pi [(\rho + R) \sin \vartheta - \rho]^2 L \\ & + 2\pi [(\rho + R) \sin \vartheta - \rho]^3/3, \end{aligned} \quad (5)$$

$$\begin{aligned} A = & 4\pi R^2 + n(2\pi\rho [(\rho + R) \sin \vartheta (\pi/2 - \vartheta) - \rho \sin \vartheta] \\ & - 2\pi R^2(1 - \cos \vartheta) + 2\pi [(\rho + R) \sin \vartheta - \rho] L \\ & + 2\pi [(\rho + R) \sin \vartheta - \rho]^2). \end{aligned} \quad (6)$$

Since the distance between the neutral surfaces of the membrane lipid layers (h) is much smaller than the dimensions of the RBC the area difference between both the lipid layers (ΔA) can be approximately written as $\Delta A = h \int (C_1 + C_2) dA$ which gives us, in the case of presented model of echinocyte shape, the following expression for ΔA :

$$\begin{aligned} \Delta A = & 8\pi R h + n(-2\pi(\rho + R)h \sin \vartheta (\pi/2 - \vartheta) + 4\pi\rho h \cos \vartheta \\ & - 4\pi R h(1 - \cos \vartheta) + 4\pi R h(1 - \cos \vartheta) + 2\pi L h), \end{aligned} \quad (7)$$

where $8\pi R h$ is the area difference for the sphere with radius R .

ELASTIC ENERGY OF ECHINOCYTE MEMBRANE

The bending energy of the model echinocyte can be expressed by utilizing equation (2) as follows:

$$\begin{aligned} W_b = & 8\pi B + \frac{n\pi B L}{(\rho + R) \sin \vartheta - \rho} + \frac{2n\pi B (\rho + R)^2 \sin^2 \vartheta}{\rho((\rho + R)^2 \sin^2 \vartheta - \rho^2)^{1/2}} \\ & \times \operatorname{arctg} \left(\frac{((\rho + R) \sin \vartheta + \rho) \operatorname{tg}(\pi/4 - \vartheta/2)}{((\rho + R)^2 \sin^2 \vartheta - \rho^2)^{1/2}} \right). \end{aligned} \quad (8)$$

For the sake of simplicity we calculated the shear elastic energy W_s of the model echinocyte shape

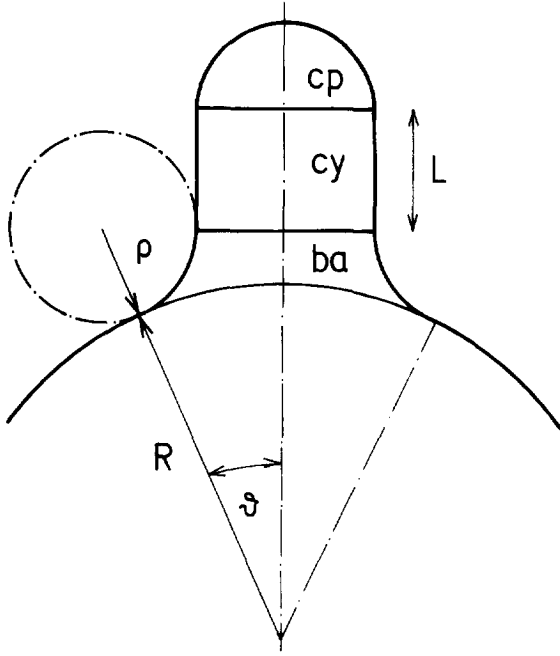


Fig. 2. The parameters characterizing the geometrical model of echinocyte shape. The bodies from which a single echinocyte spicule is composed are the base (ba), the cylinder (cy) and the cap of the spicule (cp).

according to approximate relation:

$$W_s = nE_s, \quad (9)$$

where E_s is the shear elastic energy of a single spicule and its surrounding membrane. The energy E_s is calculated according to the method of Evans and Skalak (1980). While calculating the energy E_s the flat membrane is considered as an initial reference state (see Fig. 3), where it is assumed that in the reference state $E_s = 0$. Figure 3 schematically shows the membrane displacement and membrane constant area deformation which occurs at deformation of flat membrane into spicule. The undeformed reference coordinate system is characterized by the radius r_0 originating from the symmetry axis of the spicule and the polar angle φ in the initially flat membrane. The deformed coordinate system is defined by the curvilinear distance along the meridian (s) which begins at the pole of the spiculum cap and eventually becomes the radial coordinate (r) in the outer membrane surface. Because of the symmetry, the azimuthal angle is the same as the initial polar angle. The principal extension ratio along the meridian (λ_m) is then given by (Evans and Skalak, 1980)

$$\lambda_m = \frac{ds}{dr_0} = r_0/r, \quad (10)$$

where the fact that the area of the membrane (skeleton) is locally conserved is taken into account, i.e. $r_0 dr_0 d\varphi = r ds d\varphi$. The total area of the membrane segment must be conserved too (see Fig. 3):

$$\int_0^{r_0} r_0 dr_0 = \int_0^r r ds. \quad (11)$$

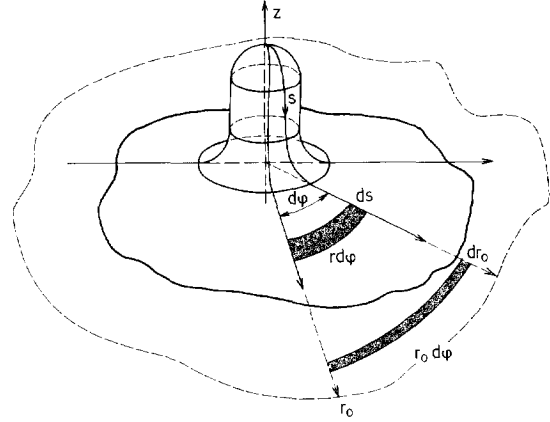


Fig. 3. Schematic diagram illustrating the mathematical procedure for calculating the membrane shear energy of a single echinocyte spicule. The flat membrane is considered as the reference state having shear energy equal to zero. The cylindrical coordinates (z, r, φ) are used to describe the surface geometry, while the curvilinear coordinate s is used to describe the distance along the meridian of the spiculum surface (Evans and Skalak, 1980). The constant area deformation of a membrane segment is shown schematically. The dotted line shows the borders of the chosen part of the membrane area before deformation in the reference state, i.e. before the formation of the spicule, while the bold line shows the corresponding borders after the formation of spicule.

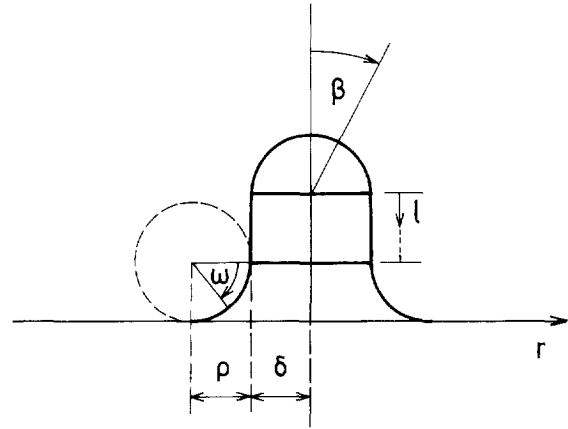


Fig. 4. Curvilinear distance along the meridian (s) from the pole of the spheroidal spiculum cap to the surface outside of the spiculum where $ds = dr$ (see Fig. 3). In the area of spiculum cap $ds = \delta d\beta$, where $\delta = (\rho + R)\sin\theta - \rho$ (see Fig. 2), in the area of spiculum cylinder $ds = dl$, while in the area of spiculum base $ds = \rho d\omega$.

In this work the shear energy of the single spicule E_s is approximately expressed as sum of some contributions:

$$E_s = E_{cp} + E_{cy} + E_{ba} + E_{pl}, \quad (12)$$

where E_{cp} is the shear energy of the cup of the spicule, E_{cy} is the shear energy of the spiculum cylinder, E_{ba} is the shear energy of the spiculum base, while E_{pl} is the shear energy of the plane around the spicule (see Fig. 4). In order to calculate these energies the corresponding values of the principal extension ratio λ_m in different points on the spicule and in its surrounding area are calculated by utilizing equations (10) and (11) (see Fig. 4):

$$\text{cap: } \lambda_m^2 = 1 + (1 - \cos\beta)^2 / \sin^2\beta, \quad (13)$$

$$\text{cylinder: } \lambda_m^2 = 2 + 2l/\delta, \quad (14)$$

$$\text{base: } \lambda_m^2 = \frac{2\pi\delta^2 + 2\pi\delta L - 2\pi\rho^2 \sin \omega + 2\pi(\rho + \delta)\rho\omega}{\pi[\delta + \rho(1 - \cos \omega)]^2}, \quad (15)$$

$$\text{plane: } \lambda_m^2 = 1 + [A_m - \pi(\rho + \delta)^2]/\pi r^2, \quad r > (\rho + \delta), \quad (16)$$

where

$$A_m = 2\pi\delta^2 + 2\pi\delta L + \pi^2(\rho + \delta)\rho - 2\pi\rho^2 \quad (17)$$

is the area of the spiculum which is placed in the plane (see Figs 2 and 4) while

$$\delta = (\rho + R)\sin \vartheta - \rho \quad (18)$$

is the radius of the spiculum cylinder.

Knowing the values of λ_m , the energies E_{cp} , E_{cy} , E_{ba} and E_{pl} can then be determined utilizing equation (4), where we integrate over the appropriate areas of the spicule (see Fig. 4). While we calculated the energy E_{pl} we extended the integration area to infinity. The corresponding error is small since the value of λ_m^2 in the surrounding area of the spicule strongly decreases as a function of the radius r as it can be seen in equation (16). It can be shown that the error involved in E_{pl} is around 20% if the distance between the two spicula is around few times of the length $(\rho + \delta)$. Consequently, the error in determination of the total shear energy of the single spicule E_s is much smaller (ca. 2%) because the shear energy of the plane around the spicule E_{pl} is around 10% of the energy E_s . In this way the energies E_{cp} , E_{cy} , E_{ba} and E_{pl} can be expressed as follows:

$$E_{cp} = 2\pi\mu\delta^2(\ln 2 - 5/8), \quad (19)$$

$$E_{cy} = \pi\mu L^2 + \frac{\pi}{2}\mu\delta^2 \ln(1 + L/\delta), \quad (20)$$

$$E_{pl} = \mu[A_m - \pi(\rho + \delta)^2] \ln\left(\frac{A_m}{\pi(\rho + \delta)^2}\right). \quad (21)$$

The shear energy of the spiculum base (E_{ba}) is obtained numerically by calculating the integral

$$E_{ba} = \mu \int_0^{\pi/2} \left(\frac{1}{2}(\lambda_m^2 + \lambda_m^{-2}) - 1 \right) \times 2\pi[\delta + \rho(1 - \cos \omega)]\rho d\omega, \quad (22)$$

using the trapezoidal formula, where λ_m is defined by equation (15).

RESULTS AND DISCUSSION

As we mentioned before, the aim of this work is to determine the stable echinocyte RBC shapes by minimization of the membrane elastic energy. At given external conditions the cell volume and the areas of the outer and the inner membrane lipid layers are considered to be constant and independent of the cell shape (Svetina and Zekš, 1989). So the five parameters of the described geometrical model of the echinocyte ρ , n , ϑ , L and R cannot assume arbitrary values. Therefore we introduce three constraints for the cell volume (V), the cell area (A) and the area difference between outer and inner membrane lipid layers (ΔA). These three constraints can be

written in our case as follows:

$$V(\rho, n, \vartheta, L, R) = V_0, \quad (23)$$

$$A(\rho, n, \vartheta, L, R) = A_0, \quad (24)$$

$$\Delta A(\rho, n, \vartheta, L, R) = \Delta A_0, \quad (25)$$

where V_0 , A_0 and ΔA_0 denote the values of V , A and ΔA for a given cell shape. The expressions for V , A and ΔA are given by equations (5)–(7). At given V_0 , A_0 and ΔA_0 , the parameters ϑ , L and R can be determined numerically as functions of ρ and n by solving equations (23)–(25). Thus the parameter R can be expressed analytically as a function of ρ , n , ϑ and L from equation (25) and inserted in equation (24). Then the parameter L is determined as a function of ρ , n and ϑ by solving analytically equation (24). In this way the functions $R(\rho, n, \vartheta)$ and $L(\rho, n, \vartheta)$ are determined. The parameter ϑ is then calculated numerically at given ρ and n from equation (23) using the tangential method, where the functions $R(\rho, n, \vartheta)$ and $L(\rho, n, \vartheta)$ are taken into account.

Since, in this way, the values of ϑ , L and R are known at given ρ and n , the elastic energy of model echinocyte W can be determined as a function of parameters ρ and n . By minimizing numerically the energy W with respect to parameter ρ at given n , the elastic energy of the echinocyte W as a function of number of spicula n can be determined as presented in Fig. 5(a). In addition, Fig. 5(b) shows the corresponding dependencies of the parameters ρ , ϑ , L and R on the number of spicula n . The chosen values $V_0 = 90 \mu\text{m}^3$ and $A_0 = 138 \mu\text{m}^2$ in Figs 5 and 6 are the normal values for the RBCs, while $\Delta A_0 = 1.0 \mu\text{m}^2$ is in the range of those ΔA_0 where the model axisymmetrical stomatocyte and discocyte shapes cannot exist due to geometrical restrictions (Beck, 1978; Svetina *et al.*, 1994). It can be seen in Fig. 5(a) that the portion of the elastic energy due to shear deformation (W_s) increases with decreasing number of spicula, i.e. with increasing length of the spicula.

Figure 6 shows the dependence of the number of spicula (n_{\min}) corresponding to the minimal membrane cell elastic energy (W_{\min}) (see Fig. 5a) as a function of the ratio μ/B . It can be seen in Fig. 6 that for $\mu/B = 0$ the stable echinocyte shape has only one spicule ($n_{\min} = 1$) which is not in accordance with experimental observations (Brecher and Besis, 1972). However, for $\mu/B \neq 0$, the stable echinocyte shapes corresponding to the minimal elastic energy W_{\min} have always more than one spicule ($n_{\min} > 1$) in agreement with the observed echinocyte shapes.

It is shown in Fig. 6 that n_{\min} is an increasing function of the ratio μ/B . Since the length of the spiculum cylinder decreases with increasing number of spicula (Fig. 5b) this also means that larger values of the ratio μ/B imply smaller spicula in accordance with experimental observations (Haest *et al.*, 1980).

The measured value of RBC membrane area shear modulus μ is about $6.6 \times 10^{-6} \text{ N m}^{-1}$ (Waugh and Evans, 1979), while the measured value of RBC membrane bending modulus B is about $1.8 \times 10^{-19} \text{ N m}$ (Evans, 1974), which gives us the value about $35 \times 10^{-12} \text{ m}^{-2}$ for the ratio μ/B .

In this work the spontaneous curvature of the membrane bilayer (Helfrich, 1973), introduced in order to

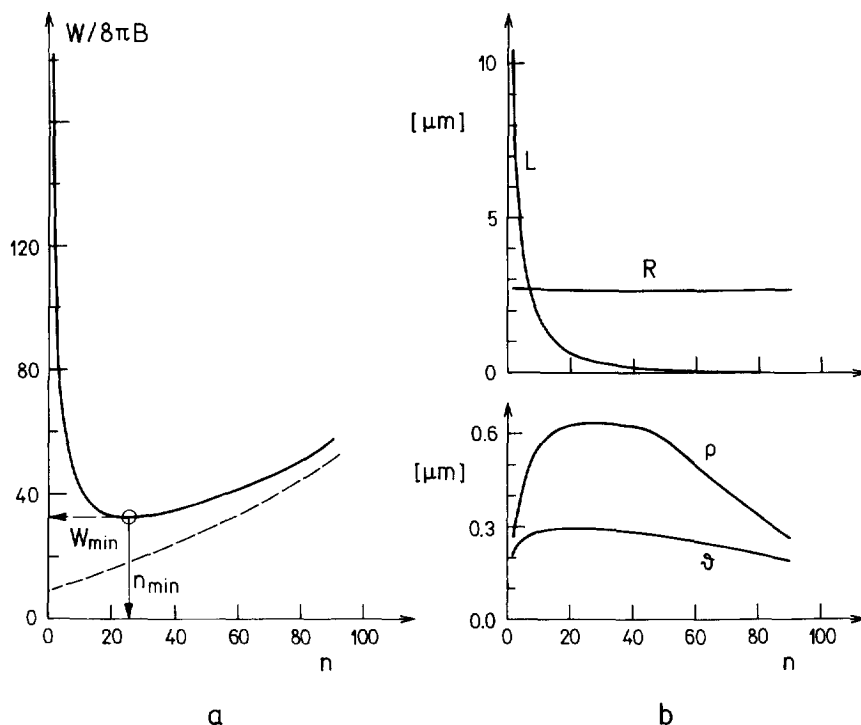


Fig. 5. The calculated total echinocyte membrane elastic energy W as a function of the number of spicula n (full line) for ratio $\mu/B = 3.8 \times 10^{12} \text{ m}^{-2}$ where $n_{\min} = 25$ (a). Figure also shows the corresponding dependencies of the parameters ρ , ϑ , L and R on n for the same value μ/B (b). The values of parameters V_0 , A_0 and ΔA_0 are: 90, 138 and $1.0 \mu\text{m}^2$ (Beck, 1978), respectively. The energy W is normalized relative to the bending energy of the sphere $8\pi B$. The dashed line shows the portion of the elastic energy due to bending (W_0).

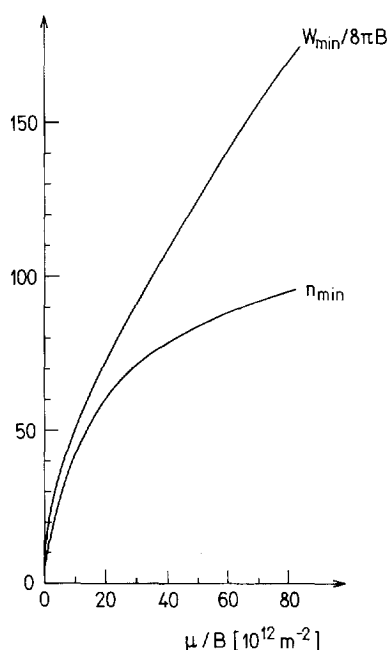


Fig. 6. The calculated equilibrium number of echinocyte spicula n_{\min} and the corresponding minimal elastic energy W_{\min} , see Fig. 5(a) as functions of the ratio μ/B . The values of V_0 , A_0 and ΔA_0 are the same as in Fig. 5.

describe the asymmetry of both lipid layers (Svetina *et al.*, 1994), was not taken into account. However, the inclusion of the spontaneous curvature would not alter the

basic conclusion of this work. Namely, for $\mu/B \neq 0$ the nonzero spontaneous curvature would only change the value of n_{\min} while it cannot explain the stability of echinocyte shapes with more than one spicule for $\mu/B = 0$.

CONCLUSIONS

The requirement of the minimal RBC membrane bending energy can explain the stability of numerous observed RBC shapes (Deulin and Helfrich, 1976; Evans, 1974; Kralj-Iglič *et al.*, 1993; Seifert *et al.*, 1991; Svetina and Žekš, 1989). However, the previous studies of RBC shapes were limited to the cell shapes which cannot describe the arbitrary shape of strongly nonaxisymmetric spiculated RBC cells. Therefore in this work the analysis of the RBC shape stability was extended to the range of strongly nonaxisymmetric echinocyte shapes having arbitrary number and arbitrary length of spicula.

It was shown that within the limitation of the presented geometrical model of echinocyte shape, the requirement of the minimum bending energy cannot explain the stability of echinocyte RBC shapes with more than one spicule. However, by the inclusion of the membrane shear energy in the minimization procedure the existence of such stable echinocyte shapes can be explained.

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