SPECTRIN, HUMAN ERYTHROCYTE SHAPES, AND MECHANOCHEMICAL PROPERTIES

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ABSTRACT Physical studies of human erythrocyte spectrin indicate that isolated spectrin dimers and tetramers in solution are worm-like coils with a persistence length of ~20 nm. This finding, the known polyelectrolytic nature of spectrin, and other structural information about spectrin and the membrane skeleton molecular organization have lead us to the hypothesis that the human erythrocyte membrane skeleton constitutes a two-dimensional ionic gel (swollen ionic elastomer). This concept is incorporated in what we refer to as the protein gel-lipid bilayer membrane model. The model accounts quantitatively for red elastic shear modulus and the maximum elastic extension ratio reported for the human erythrocytes membrane. Gel theory further predicts that depending on the environmental conditions, the membrane skeleton modulus of area compression may be small or large relative to the membrane elastic shear modulus. Our analyses show that the ratio between these two parameters affects both the geometry and the stability of the favored cell shapes and that the higher the membrane skeleton compressibility the smaller the values of the gel tension needed to induce cell shape transformations. The main virtue of the protein gel-lipid bilayer membrane model is that it offers a novel theoretical and molecular basis for the various mechanical properties of the membrane skeleton such as the membrane skeleton modulus of area compression and osmotic tension, and the effects of these properties on local membrane skeleton density, cell shape, and shape transformations.

INTRODUCTION

The beauty and simple geometry of human erythrocytes have long intrigued biophysicists, cell biologists, and others. Numerous studies suggest that spectrin plays a crucial role in determining the mechanical properties and stable shapes of erythrocytes by forming a macromolecular meshwork at the cytoplasmic side of the membrane lipid bilayer (Branton et al., 1981; Gratzer, 1983). The mechanical behavior of the erythrocyte membrane has been studied extensively (Evans, 1973; Evans and Skalak, 1979; Waugh and Evans, 1979), yielding membrane material parameters that are manifestations of its molecular composition and organization. The experimentally determined values of the elastic shear modulus, the modulus of area compression, and the maximum elastic extension ratio are all important quantities that can be used to test the validity of membrane models predicting these parameters. Despite the large amount of information available about the molecular organization of the erythrocyte membrane, no rigorous mathematical and molecular concept has yet been developed that relates the properties of the membrane components to the next membrane hierarchial level, membrane mechanical properties and cell shapes. Experimental data on spectrin and the molecular organization of the human erythrocyte membrane skeleton indicate that this membrane skeleton behaves like an ionic gel (swollen ionic elastomer). We have analyzed the implications of this concept with respect to the erythrocyte membrane material parameters and the observed erythrocyte shapes and shape transformations.

SPECTRIN AND THE HUMAN ERYTHROCYTE MEMBRANE SKELETON

It is well established that spectrin is the major component of the human erythrocyte membrane skeleton (Nicolson et al., 1971; Steck, 1974). Electron micrographs of isolated human erythrocyte spectrin dimers and tetramers vacuum-dried from glycerol-containing solutions reveal floppy, elongated macromolecules with a contour length of 100 and 200 nm (Shotton et al., 1979). Spectrin dimers consist of one α and one β chain associated side-by-side and connected at each end. Each α and β chain consists of 20 and 18 homologous segments connected by flexible regions (Speicher and Marchesi, 1984). The spectrin molecules are ionizable with an isoelectric point ~pH 4.8 and a net negative charge of ~200 on each spectrin dimer at physiological pH (Elgsaeter et al., 1976). The rotational relaxation time and intrinsic viscosity of spectrin dimers indicate that spectrin in solution is a worm-like molecule with a persistence length of ~20 nm (Mikkelsen et al., 1984; Stokke et al., 1985*a*). Studies of spectrin dimer intrinsic viscosity vs. temperature indicate that spectrin in solution behaves essentially as an entropy spring (Stokke et al., 1985*a*). Experimental studies of the elasticity of reconstituted spectrin networks has just been reported (Schanus et al., 1985; Stokke et al., 1985*b*). The elasticity of such

reconstituted macroscopic spectrin networks appears to be accounted for by simple elastomer theory.

Tyler (1980) and Liu and Palek (1980) obtained evidence suggesting that spectrin heterotetramers constitute the chains of the erythrocyte membrane skeleton network. Cohen et al. (1980) found that actin oligomers may be an integral part of the network junctions and that the maximum junction functionality may be determined by the length of the actin oligomers. The length of these oligomers may be controlled by protein 4.9, which has been identified as an actin bundling protein (Siegel and Branton, 1985). Recently Byers and Branton (1985) have been able to visualize the membrane skeleton directly using a negative staining electron microscopic technique. This study confirms many of the earlier findings and indicates that the membrane skeleton has a simple nearly replicating topology with an average junction functionality of 5–6. The membrane skeleton network, in contrast to most synthetic elastomers, depends in part on noncovalent bonds. The time-average equilibrium topology of the spectrin network may therefore depend on the environmental conditions, but the time constants involved in such topology changes (Ungewickell and Gratzer, 1978) appear to be large in comparison with the characteristic times involved in cell shape changes (Lange et al., 1982). Electron paramagnetic resonance measurements of spectrin in solution and bound to the membrane show no evidence of immobilization when bound to the membrane (Lemaigre-Dubreuil and Cassoly, 1983). This indicates that the thermal motion of the flexible spectrin molecules is not suppressed in vivo. One concludes that the erythrocyte membrane skeleton probably constitutes an ionic gel.

PROTEIN GEL-LIPID BILAYER MEMBRANE MODEL

The current view of the human erythrocyte membrane can be summarized in what we have chosen to refer as the protein gel-lipid bilayer membrane model. This membrane model consists of a fluid lipid bilayer and an apposing membrane skeleton constituting an ionic gel. The two halves of the lipid bilayer and the ionic protein gel are all assumed free to slide relative to one another in the plane of the cell membrane. In this model, the membrane skeleton is responsible for the shear elasticity and part of the area compressibility, whereas the lipid bilayer resists both bending and area compression. The theory of ionic gels (Tanaka et al., 1980) predicts that for such a membrane model the membrane skeleton modulus of area compression, K_G, may be large as well as small relative to the membrane skeleton elastic shear modulus, G and that the ratio K_G/G can be strongly dependent on the environmental conditions. When this ratio is much larger than one, the membrane skeleton will behave as a nearly incompressible two-dimensional elastomer as discussed by Evans (1973), Evans and Skalak (1979), and others. However, under other environmental conditions, where K_G is of the same order of magnitude or smaller than G, one has to take into account the effects of a resulting nonuniform membrane skeleton density distribution.

PREDICTION OF HUMAN ERYTHROCYTE MEMBRANE MECHANICAL PARAMETERS USING THE PROTEIN GEL LIPID-BILAYER MEMBRANE MODEL

When there is no gel chain intra- or intermolecular interaction, the elastic free energy of the gel is of purely entropic origin and is sometimes attributed to the "rubber elasticity" of the gel (Flory, 1953; Treloar, 1975; Flory, 1976). For a gel area element that in the reference state is a square with sides l_r the elastic shear modulus G measured in a state where the gel area element has surface area l_u^2, equals (Treloar, 1975; Flory, 1976):

$$G = kT\,(N_c/l_u^2)\,\langle l_u^2\rangle/\langle l_r^2\rangle, \qquad (1)$$

where k is the Boltzmann constant and N_c/l_u^2 is the number of gel chains per unit area. Eq. 1 is valid for simple replicating gel topology as well as for randomly cross-linked two-dimensional gels. The shear modulus of the protein gel-lipid bilayer membrane model equals the one given by Evans (1973) and Evans and Skalak (1979).

Assuming that one spectrin heterotetramer constitutes one gel chain and using the number of spectrin heterotetramers per erythrocyte (Steck, 1974) and the erythrocyte surface area of 140 μm^2 (Canham, 1970), one obtains $N_c/l_u^2 \approx 780\ \mu m^{-2}$. The ratio $\langle l_u^2\rangle/\langle l_r^2\rangle$ is unknown, but because of the slow association equilibrium between the components of the spectrin network this ratio may normally be expected to be close to one. This yields $G \approx 3 \times 10^{-3}$ dyn/cm at room temperature for the human erythrocyte spectrin network. If each of the two subchains of the heterotetramers undergoes independent thermal motions, the predicted value of G equals 6×10^{-3} dyn/cm. Because the membrane lipid bilayer is assumed to be in the fluid state this also equals the predicted value of the elastic shear modulus for the whole erythrocyte membrane. Waugh and Evans (1979) reported $G = (6.6 \pm 1.2) \times 10^{-3}$ dyn/cm at 25°C and Chien et al. (1978) reported $G = 4.3 \times 10^{-3}$ dyn/cm in remarkable agreement with the predicted value of G. The agreement between the value of G obtained experimentally and the value of G predicted from gel theory suggests that spectrin molecules in situ also behave like entropy springs and that the membrane skeleton therefore does constitute an ionic gel.

Eq. 1 yields

$$T\,\partial \ln G/\partial T = 1 + T\,\partial \ln N_c/\partial T - T\,\partial \ln \langle l_r^2\rangle/\partial T \qquad (2)$$

The temperature dependence of the end-to-end distance of spectrin dimers estimated from intrinsic viscosity (Stokke et al., 1985*a*) indicates that $T\,\partial \ln \langle l_r^2\rangle/\partial T = -(1.2 \pm 0.6)$. Waugh and Evans (1979) found that the membrane elastic shear modulus decreased with increasing tempera-

ture. Their experimental data yield $T\,\partial \ln G/\partial T = -(3 \pm 1)$ in the temperature range 5–35°C. If the membrane skeleton behaves like a swollen elastomer this implies that the number of effective network strands, N_c, decreases with increasing temperature. An estimate of $T\,\partial \ln N_c/\partial T$ can be obtained assuming that the spectrin network consists of tetramers in equilibrium with dimers and each tetramer constitutes two gel chains. It can then be shown that

$$T\,\partial \ln N_c/\partial T = T\,(\partial \ln K_A/\partial T)/\sqrt{1 + 16\,K_A\,[S]}, \quad (3)$$

where K_A is the spectrin dimer-tetramer association constant and $[S]$ is the tetramer concentration when spectrin is tetramers only. The reported values of K_A in the absence of other molecules at 25–37°C (Ungewickell and Gratzer, 1978) yield $T\,\partial \ln N_c/\partial T = -(2 \pm 1)$ when the effective gel thickness is assumed to be 20 nm. However, Liu and Palek (1984) reported that hemoglobin enhances the self-association of spectrin dimers. In addition, the network junction integrity may also depend on temperature. Enough experimental data on the thermoelastic properties of the spectrin network to make a quantitative comparison between the experimental data of Waugh and Evans (1979) and the behavior predicted by the protein gel-lipid bilayer membrane model is therefore not yet available, but the observed temperature dependence of the erythrocyte elastic shear modulus is not incompatible with gel theory.

The extension ratio of a square gel area element with edge length l_r which is deformed to length l_x in the x-direction equals $\epsilon = l_x/l_r$. For all real gel chains there is a limit on ϵ. This is because the time-average end-to-end distance, $\langle l_{e-e} \rangle$, of the gel chains in the reference state cannot exceed the contour length L. Stretching beyond L is normally irreversible. For real gel chains the maximum extension ratio $\epsilon_{max} \approx L/\langle l_{e-e} \rangle$. L equals $N_s l_s$ where l_s and N_s is the length and number of identical segments making up a gel chain. For a freely jointed chain with many segments $\langle l_{e-e} \rangle \approx l_s\sqrt{N_s}$ and thus $\epsilon_{max} \approx \sqrt{N_s}$. For a gel not in its reference state, $\epsilon_{max} \approx L/\langle l_{n-n} \rangle$ where $\langle l_{n-n} \rangle$ is the time-average distance between nearest neighbor junctions. The calculated values of $\langle l_{n-n} \rangle$, ϵ_{max}, and N_s for some selected junction functionalities, Φ, for the human erythrocyte membrane skeleton, assuming that spectrin heterotetramers constitute the gel chains, are shown in Table I. If the topological first-neighbor junctions of the spectrin network are not assumed to be the nearest-neighbor junctions, the expected value of ϵ_{max} would be smaller than the values given in Table I by at least a factor of two.

Evans and LaCelle (1975) determined experimentally the maximum elastic extension ratio of erythrocyte membranes to be 3–4. Table I shows that the reported value of ϵ_{max} is compatible with a replicating network of spectrin tetramers with a junction functionality of 4 or 6. This is consistent with the findings of Byers and Branton (1985). The observed value of ϵ_{max} suggests that N_s equals 10–15, which implies that for extension ratios <1.5–2.0, the mechanical properties of the spectrin gel may be well accounted for using gaussian chain statistics. The observed value of ϵ_{max} shows that the erythrocyte membrane skeleton has a topology where the topological first neighbor junctions also are the nearest neighbor junctions.

TABLE I
NEAREST NEIGHBOR DISTANCE AND MAXIMUM EXTENSION RATIO OF THE HUMAN ERYTHROCYTE MEMBRANE SKELETON PREDICTED USING THE PROTEIN GEL–LIPID BILAYER MEMBRANE MODEL

Φ	$\langle l_{n-n} \rangle$/nm	ϵ_{max}	N_s
3	41	~5	~25
4	52	~4	~16
6	70	~3	~9

The tension needed to expand or compress isotropically an ionic gel depends not only on the entropy of the macromolecules constituting the network, but also on the gel chain intra- and intermolecular interactions and the degree of ionization of the gel chains. The osmotic surface tension, Π_G, of an ionic two-dimensional protein gel with negligible boundary effects is analogous to the osmotic pressure of three-dimensional gels (Treloar, 1975; Tanaka et al., 1980) and is given by:

$$\Pi_G = -(N_A, kT/V_1)\,(\phi + \ln(1-\phi) + (\Delta F_c/2\,kT)\,\phi^2) - \partial\,(\Delta F_r)/\partial l_r^2 + \nu_r\,kT\,g\phi/\phi_r, \quad (4)$$

where N_A is the Avogadro number, V_1 is the equivalent molar area of the gel solvent, ΔF_c is the chain-chain affinity and is a measure of the free energy decrease associated with the formation of contact between chain segments, ΔF_r is the elastic free energy of the rubber elasticity of the network, g is the number of effective dissociated hydrogen ions per chain, and ν_r is the number of chains per unit area in the gel reference state. ϕ and ϕ_r are the fractions of the surface area covered by the gel chains in the state under consideration and in the reference state. The osmotic pressure in the ionic gel is balanced by an equal pressure in the lipid bilayer in the stress-free shape. The modulus of area compression of the two-dimensional gel is given by

$$K_G(l^2) = -l^2\,\partial\Pi_G/\partial\,l^2. \quad (5)$$

Eqs. 4 and 5 predict that K_G may be negative for certain combinations of the molecular parameters, but thermodynamic analysis shows that K_G must be ≥ 0 to correspond to a stable equilibrium. Phase transitions and critical phenomena are therefore inherent properties of ionic gels. Tanaka et al. (1980) have demonstrated experimentally the presence of phase transitions and critical phenomena in ionic polyacrylamide gels.

The numerical values of V_1, ϕ, ΔF_c, and g for the erythrocyte membrane skeleton are not known. We have

calculated from Eq. 4 Π_G vs. l_G^2/l_r^2 using several values of the chain-chain affinity and values of other molecular parameters which may be reasonable for the erythrocyte membrane skeleton (Fig. 1). The expression of ΔF_r for nongaussian chain statistics (Treloar, 1975) was used. In the phase separation situation (the horizontal regions of Π_G) the two-dimensional gel maintains its topology, but is separated into one dense phase present as patches distributed more or less uniformly within the less dense phase, or vice versa. Such phase separation cannot take place without concominant shear deformation resulting from the topological restraints on the gel; the gel free energy will therefore depend on the geometry of the phase boundaries between the two phases. The macroscopic value of K_G for this situation will therefore be small, but not exactly zero. Because the erythrocyte intramembrane particles are partly bound to and partly trapped within the membrane skeleton (for review see Gratzer, 1983), such phase separation will be expected to lead to reversible freeze-etch particle aggregation. The existence of reversible freeze-etch particle aggregation in human erythrocyte membranes is well established (Pinto da Silva, 1973; Elgsaeter and Branton, 1974).

Fig. 1 indicates that for some environmental conditions K_G of the erythrocyte membrane skeleton is small and probably of approximately the same order of magnitude as or smaller than G, whereas for other environmental conditions K_G is 2 to 4 orders of magnitude $>G$. These large values of K_G are approximately of the same order of magnitude as the modulus of area compression (450 dyn/cm at 25°C) for the whole erythrocyte membrane (Waugh and Evans, 1979). The modulus of area compression for synthetic bilayers is reported to be ~140 dyn/cm (Kwok and Evans, 1981). However, the modulus of area compression of intramembrane particle-containing lipid bilayers is not expected to equal that of synthetic bilayers. The contributions from the lipid bilayer and the membrane skeleton to the modulus of area compression of the entire membrane and in particular their temperature dependence, is therefore not known.

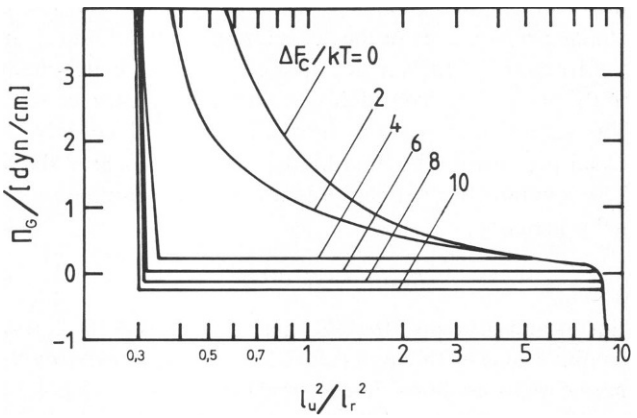

FIGURE 1 The osmotic surface tension, Π_G, of an ionic two-dimensional macromolecular gel vs. l_u^2/l_r^2, calculated using Eq. 8 and Maxwell's rule. The molecular parameters were chosen to correspond roughly to those of the human erythrocyte spectrin network: $\phi = 0.3$, $\nu_r = 1{,}600\ \mu m^{-2}$, $N_s = 9$, $g = 200$, $\Phi = 4$ and $V_1 = 0.5\ nm^2$. The osmotic surface tension is shown for several values of $\Delta F_c/kT$. The actual value of ΔF_c for a spectrin network is not known.

PREDICTION OF STABLE HUMAN ERYTHROCYTE SHAPES AND SHAPE TRANSFORMATIONS USING THE PROTEIN GEL-LIPID BILAYER MEMBRANE MODEL

The stable cell shape of a nonnucleated cell without a transcellular cytoskeleton will be the one associated with the lowest membrane free energy. To predict the stable cell shape, the free energy of any given cell shape, Ψ_d, has to be calculated relative to a stress-free shape. The stress-free shape of the membrane skeleton we refer to as Ψ_u. Lasting rapid random deformations would because of the so-called dynamic equilibrium of the membrane skeleton components lead to spherical Ψ_u. However, delipidated erythrocyte ghosts have been reported to be biconvex rather than spherical (Lange et al., 1982). At the molecular level, the spectrin network obviously does not constitute a continuum. However, limiting ourselves to situations where the time average gel density does not vary significantly within the time-average distance between gel nearest-neighbor junctions, one can use the standard methods of classical continuum mechanics including the formalism of infinitesimal area elements to analyze the gel free energy. This yields the following somewhat simplified expression for the total change in free energy for the cell shape change $\Psi_u \rightarrow \Psi_d$ (Stokke, 1985):

$$\Delta F_{tot} \approx \tfrac{1}{2}G \int\int (l_x/l_u - l_y/l_u)^2\, d_r^2 + \tfrac{1}{2}K_{Gu} \int\int (1 - l_x\, l_y/l_u^2)^2 d_r^2 - \Pi_{Gu} d\partial(A_d - A_u)/\partial z + \tfrac{1}{2}B_L \int\int ((1/R_1)^2 + (1/R_2)^2)\, d_r^2 \quad (6)$$

The first two terms constitute the total membrane gel shear deformation and nonuniform compression. the third term, $\Pi_{Gu}\, d\, \partial(A_d - A_u)/\partial z$, we refer to as the trilayer couple term because of its analogy to the bilayer couple hypothesis (Sheetz and Singer, 1974). d is the distance from the neutral plane to the gel, and A_d and A_u are the areas of the gel in the deformed and stress-free state. The last term is the energy associated with local bending of the lipid bilayer where B_L is the lipid bilayer elastic bending modulus, and R_1 and R_2 the principal radii of curvature.

Several different strategies can be followed to obtain the cell shape parameters needed to calculate the membrane free energy. We have used a geometric parameterization strategy because it facilitates separation of cell shapes into four classes with certain characteristic shape features in common: cell shape class I (oblate to biconcave discs); class II (cup-shaped cells); class III (cells with variable number of identical exocytic blebbs or spikes); and class IV (cells with a variable number of identical invaginations). All our numerical analyses were performed using Eq. 6 for ΔF_{tot} including some or all of the terms. The free energy terms

were calculated requiring that the relative gel surface area of shape Ψ_d, equals $1 + (A_d - A_u)/(4\pi R_0^2)$, and that the relative gel volume ξ_d had the preselected values. R_0 is estimated to 3.3 μm assuming that the area of the neutral plane equals 140 μm^2 (Canham, 1970). The details of our parameterization and the numerical calculations are given elsewhere (Stokke, 1985).

Fig. 2 *A* shows the contributions from the membrane skeleton gel, the trilayer couple term and the lipid bilayer bending to the total membrane free energy for some selected class I shapes. The membrane skeleton gel free energy is calculated assuming that K_{Gu}/G is infinitely large and $K_{Gu}/G = 1$ for Ψ_u being spherical (Ψ_{us}). The lipid bilayer bending resistance is calculated assuming a planar unstrained bilayer shape. Note that the trilayer couple term alone does not predict any stable cell shape among the shown shapes, whereas the membrane skeleton gel free energy and the lipid bilayer bending energy both have minima for cell shape among the ones shown. The free energy associated with bending of the lipid bilayer and deformation of the membrane skeleton gel has a minimum for different cell shapes, indicating that the stable shape will change when the ratio between bending elastic modulus and the membrane skeleton shear modulus changes. Fig. 2 *A* also shows that both the free energy and the free energy change of the membrane skeleton associated with a given shape change, the cell shape stability, decreases when K_{Gu}/G decreases. It is shown that when $K_{Gu}/G = 0$, the free energy of the membrane skeleton gel does not depend on the cell shape, and the stable cell shape is determined by the other terms in Eq. 6 (Stokke, 1985). Fig. 2 *B* further shows how the gel density distribution along the surface of the human erythrocyte is predicted to change from being uniform for an incompressible gel to nonuniform when K_{Gu}/G is reduced to one. If all the erythrocyte intramembrane particles were attached to and distributed randomly over the spectrin network, then the intramembrane particle density would reflect the spectrin

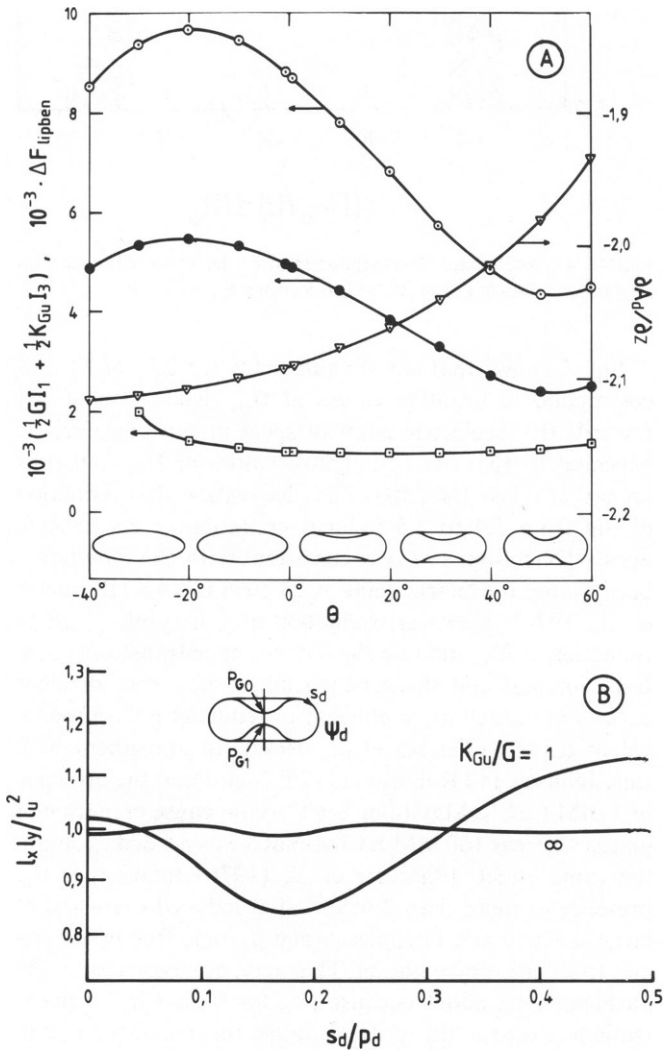

FIGURE 2 The elastic free energy $\frac{1}{2}(G I_1 + K_{Gu} I_3)$ of the membrane skeleton for some selected class I cell shapes with $\xi_d = 0.7$, and shape parameters $\phi = 90°$, and $\delta = 0$ for $\Psi_u = \Psi_{us}$ (*A*). The membrane skeleton elastic free energy were calculated for $K_{Gu}/G = 1$ (●) and ∞ (○). The lipid bilayer bending energy (⊡), and the derivative $\partial A_d/\partial_z$ (▽) are also given (*A*). The free energy is given in units of kT and $\partial A_d/\partial_z$ in units of $4\pi R_0$. The relative spectrin gel density distribution vs. distance s_d from the gel pole P_{G0} of the favored cell shape of Fig. 2 *A*, (*B*). The relative spectrin gel density distributions were calculated for spectrin gel with $K_{Gu}/G = 1$, and ∞. P_d is the pole-to-pole distance.

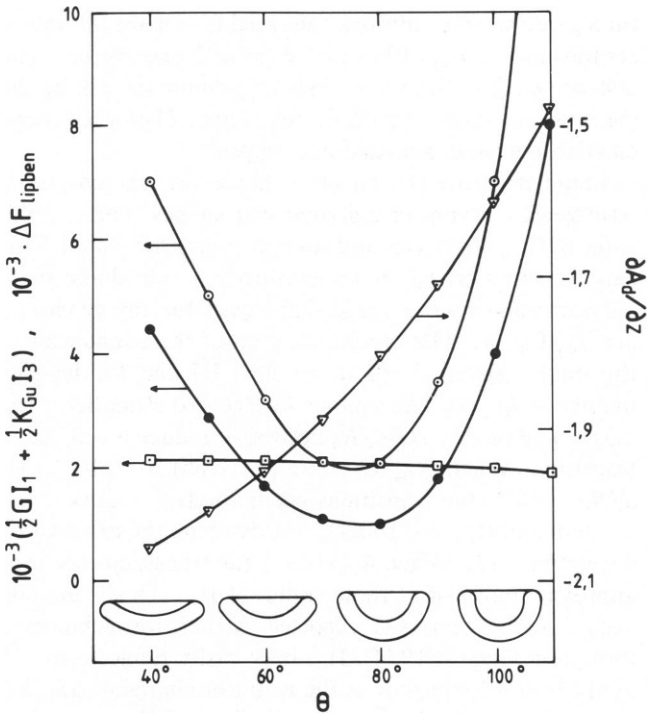

FIGURE 3 The elastic free energy $\frac{1}{2}(G I_1 + K_{Gu} I_3)$ of the membrane skeleton for some selected class II cell shapes with $\xi_d = 0.5$ and shape parameter $\gamma = 0$ for $\Psi_u = \Psi_{us}$. The membrane skeleton elastic free energy were calculated for $K_{Gu}/G = 1$ (●) and ∞ (○). The lipid bilayer bending energy (□), and the derivative $\partial A_d/\partial_z$ (▽) are also given. Dimensions as in Fig. 2.

gel density. If so, a careful analysis of the intramembrane particle density distribution for known ψ_u could be used to obtain information about K_{Gu}/G, and vice versa.

Fig. 3 shows the contributions from the membrane skeleton gel, the trilayer couple term, and the lipid bilayer bending to the total free energy for some selected class II shapes. It can be shown that cell shapes with exocytotic protrusions generally have negative $\partial(A_d)/\partial z$ whereas the opposite is the case for cell shapes with invaginations (Stokke, 1985). Environmental conditions leading to a negative osmotic gel tension therefore favor crenated cell shapes (Eq. 6). The protein gel-lipid bilayer membrane model thus provides a firm molecular and theoretical basis for the concepts of the bilayer couple hypothesis. Although the bilayer couple hypothesis incorporates an important mechanism for cell shape transformations, our analysis shows that this hypothesis alone is completely unable to predict the observed stable shape within each erythrocyte shape class. All the terms of Eq. 6 generally have to be incorporated in the analysis to predict the stable and metastable cell shapes.

HUMAN ERYTHROCYTE CELL SHAPE CLASS DIAGRAM

For given shape Ψ_u and values for ξ_d, K_{Gu}/G, $B_L/(GR_0^2)$, Π_{Gu}/G, and d, the favored cell shape within a shape class is the one that corresponds to the lowest value of the elastic free energy ΔF_{tot}. To determine the favored cell shape class for a given set of conditions one should compare the values for the lowest ΔF_{tot} within each class with one another. The cell shape class with the lowest ΔF_{tot} minimum will be the class that contains the stable cell shape. The other shape classes may contain metastable shapes.

Large negative Π_{Gu} favors echinocytes whereas large positive Π_{Gu} favors invaginated cell shapes. For a small value of Π_{Gu}, discocytes and stomatocytes are favored. This can be summarized in an erythrocyte cell shape class diagram showing the stable shape class for any given Π_{Gu} and ξ_d (Fig. 4). The precise location of the boundaries of the stable region of cell shape class III and IV depends mainly on K_{Gu}/G. The smaller K_{Gu}/G is, the smaller is the magnitude of $(\Pi_{Gu}/G)d/R_0$ needed to induce a cell shape transition. When $K_{Gu}/G = \infty$, $B_L/(GR_0^2) = -10^{-4}$ and $d/R_0 = 10^{-3}$, the transitions occur for $\Pi_{Gu} \approx 20G \approx 0.2$ dyn/cm and $\Pi_{Gu} \approx -100G \approx -1$ dyn/cm, the exact value depending on ξ_d. When $K_{Gu}/G = 1$ the transitions occur at approximately half of these values of Π_{Gu}. This is in good accord with what was reported earlier for echinocyte formation (Evans, 1974). Π_{Gu} may easily range from -1 dyn/cm to $+2$ dyn/cm as the result of changing $\Delta F_c/kT$ and g (Fig. 1). These calculated values of Π_{Gu} needed to induce erythrocyte shape transformations are consistent with the observation that lipid bilayers can generally sustain tension down to about -100 dyn/cm (Papahadjopoulos, 1968) and up to about $+3$–4 dyn/cm (Kwok and Evans, 1981) and still maintain their normal structure.

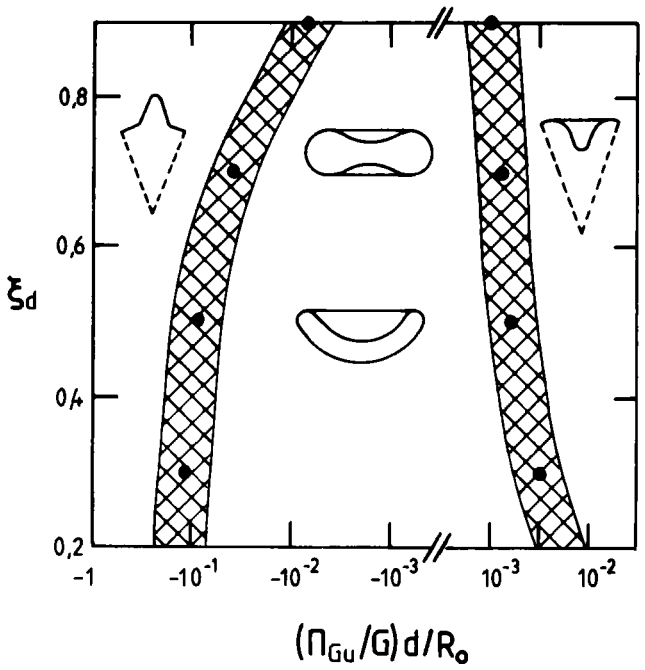

FIGURE 4 A cell shape class diagram showing the stable cell shape class for various values of ξ_d and $(\Pi_{Gu}/G)d/R_0$ when $K_{Gu}/G = 1.0$.

Fig. 1 shows that small values of g for $\Delta F_c/(kT) \geq 6$ correspond to negative values of Π_{Gu}. Reduction of pH towards the isoelectric point of spectrin can therefore be expected to give rise to negative values of Π_{Gu} and thus crenation (class III cells). The observation that reduction of pH from 7.4 to 5.5 induces crenation of erythrocyte ghosts (Nicolson, 1973) is consistent with this prediction because the isoelectric point of spectrin is ~4.8 (Elgsaeter et al., 1976). However, reduction of g may also lead to reduction of K_{Gu} and thereby induce shape transformation. Experimental and theoretical studies show that divalent cations are much more efficient in reducing g than monovalent cations (Tanaka et al., 1980). In agreement with this, Johnson and Robinson (1976) found that the presence of 1 mM $CaCl_2$, $MgCl_2$, or $SrCl_2$ would cause crenation of ghosts whereas 100 mM KCl or NaCl was needed to obtain the same effect. Elgsaeter et al. (1976) found that the presence of more than 2 mM $CaCl_2$ induced extrusion of large spectrin and intramembrane particle free lipid vesicles from erytrocyte ghosts. This may, as suggested by the authors, come about because Π_{Gu} for these Ca^{2+}-concentrations exceeds the critical value for membrane lipid bilayer collapse. Elgsaeter and Branton (1974) found that increase of the NaCl concentration to $\gtrsim$100 mM gave rise to intramembrane particle aggregation in ghosts where the spectrin network was partly broken down. This may be interpreted as a manifestation of increased negative osmotic tension in the gel as a result of the increase in the NaCl concentration. Large values of g can give rise to the high positive values of Π_{Gu} (Eq. 4) which favor membrane invaginations (Fig. 4). For a spectrin network, high pH and

low ionic strength (Elgsaeter et al., 1976) yield a large value of g. But, because high pH and low ionic strength are also the conditions that lead to spectrin release from the membrane (Elgsaeter and Branton, 1974), these conditions might be expected to cause membrane breakdown at the same time as they cause membrane invagination. High pH and low ionic strength are indeed the conditions used to form inside-out membrane vesicles (Steck et al., 1970).

Any asymmetric changes in the lipid composition of the two halves of the membrane lipid bilayer can be expected to give rise to a change in parameter d in Eq. 6. Asymmetric incorporation of drugs can be expected to have the same effect. The membrane model therefore also offers a molecular basis for the shape transformations observed if amphiphatic drugs or phosphorylated lipids are asymmetrically incorporated into the membrane or if there is an asymmetric enzymatic breakdown of the membrane lipid bilayer (Sheetz and Singer, 1974; Tamura and Fujii, 1981).

Lange et al. (1982) observed that the isolated spectrin shells of crenated ghosts were smooth and concluded from this that the lipid bilayer and not the spectrin network is responsible for crenation. The protein gel-lipid bilayer membrane model shows how erythrocytes with a smooth spectrin shell can become crenated and predicts that neither the lipid nor the spectrin network alone, but the lipid bilayer and the spectrin network together are responsible for crenation. This model offers a theoretical basis for how changes in the osmotic surface tension occur as a result of changing spectrin-solvent interactions, and in turn, how the changes in the osmotic surface tension affect the stable cell shape of human erythrocytes. The main feature of the new model is that it relates knowledge at the molecular level to cell membrane mechanical properties and cell shape using the theory of ionic gels.

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DISCUSSION

Discussion Chairman: Donald L. D. Caspar
Scribes: Lucia García-Iniguez and Joe O'Neil

BLOOMFIELD: I have several questions. One relates to your model for the spectrin tetramer itself. I guess I'm so used to seeing it drawn out as a fairly rigid although worm-like model that the more or less random coil behavior you are now attributing to it is somewhat surprising. I was wondering if, for example, the hydrodynamic properties, the sedimentation coefficients, and so on of the isolated spectrin tetramer are consistent with that.

STOKKE: Yes. The plot of the radius of gyration of spectrin dimers vs. the ionic strength shows a radius of gyration at ~22 nm at high salt concentrations, while after removal of bound charges, spectrin expands. The large increase in the radius of gyration is compatible with a flexible chain. One can also deduce this from intrinsic viscosity data.

BLOOMFIELD: In your paper you say that the two halves of the lipid bilayer and the protein matrix are assumed to be free to slide past each other. How is that consistent with the linking of the internal protein matrix through transmembrane proteins?

STOKKE: The major anchoring point between the membrane skeleton and the lipid bilayer is through the association between ankyrin and band 3. Band 3 diffuses freely in the fluid lipid bilayer.

BLOOMFIELD: Your model implies that the inner protein matrix could move independently, and I don't see how it could be totally independent. If it were purely shearing motion, they could move independent of one another, but in a bending motion the two would have to be tied together. Does that influence your calculation? Is it taken into account?

STOKKE: We are taking that into account. We have incorporated the distribution of the spectrin molecules along the surface and their ability to slide relative to the lipid bilayer both for shearing and bending deformations relative to a stress-free shape.

BLOOMFIELD: A lot of the shape transitions of red cells from biconcave disks to crenated or spiky echynocytic forms are provoked by drugs. Did you see any connection there between the drugs that cause those very pronounced reversible shape changes and the predominantly ionic mechanism that you are proposing here?

STOKKE: Incorporation of drugs into lipid bilayers is part of our model, but we have focused on the role of the membrane skeleton in shape transformations. If you incorporate drugs into the outer membrane, it is expected to change the resistance against area deformations of the lipid bilayers. It may also change the surface tension in the lipids. In our paper we show various shapes predicted by the variations in the outer tension of the extra-cellular lipid monolayer. The surface tension in the membrane skeleton can remain constant. As the surface tension in the outer half increases and becomes larger, the shape changes from the biconcave, or cup-shape, to the crenated shape, and vice versa. Drugs may also affect the membrane skeleton because the membrane skeleton is in close proximity to the lipid bilayer.

BLANK: You have pictured band-3 protein to be just within the bilayer portion and attached by ankyrin there to the spectrin-actin network. What is the evidence that the band 3 does not go through the spectrin-actin layer? Some years ago, we measured the ion transport through spectrin-actin films adsorbed at mercury-water interfaces. We calculated from these measurements that there would be a significant resistance to ion movement if an anion had to move through the spectrin-actin layer. We then reasoned that band 3 protein must extend through the spectrin-actin network. Anions that move very rapidly through the band-3 protein in red blood cell membranes would encounter a resistance on the basis of our ion transport measurements unless the band 3 extended through the spectrin-actin layer.

STOKKE: Band 3 might well go through the spectrin network rather than in the way we have depicted it schematically. The spectrin network is not so homogeneously packed near the lipid bilayer; it is distributed in a more flexible way.

POLLARD: But you know that there is a large cytoplasmic domain of band 3 that one can cleave off proteolytically. It seems difficult, I believe, for many people in the audience to accept your assumption that these two parts of the system are independent of each other. Is the reason you can make that assumption that you are dealing only with equilibrium states?

STOKKE: Yes.

POLLARD: What is missing from your model, which may be interesting and important, is time. If you were looking at dynamics, then it would be unreasonable to assume free movement of these two components relative to each other. There would be viscous resistance to the movement of the band 3 through the lipid bilayer. Isn't that correct?

STOKKE: Yes.

POLLARD: Have you attempted to include any temporal factors in your analysis? The question is what is happening dynamically to the system . Are these bonds at all stable? Are any of them being made and broken in a time-frame that is relevent to the motion of the mechanical properties of the system?

STOKKE: If you are talking about the elastic properties of the membrane on a short time scale, below 10 seconds, the association between spectrin dimers is stable and it turns out that the cell goes back readily to the undeformed state. If you deform a cell and keep it for a long time, say 2-3 minutes, as in a micropipette aspiration experiment, the deformation doesn't go back. We believe that reflects the association equilibrium in the topology between spectrin dimers and tetramers.

POLLARD: So on the time scale you are considering, which I guess is seconds, you can consider the membrane skeleton to be similar to an organic polymer gel where there are covalent bonds between all the elements of the system. Is that the sort of prototype you are using for this analysis?

STOKKE: Yes.

POLLARD: I think it is worthwhile to consider the possibility that in other sorts of biological systems which have mechanical properties one might need a different model. We have studied a network of actin filaments that can be cross-linked by α actinin. We find in that system, which is different from yours, that the cross-linker binds to and dissociates from the actin filaments at fairly high frequencies. In consequence, the physical properties of the system vary considerably with the rate of deformation of the system. Time is an important factor. If you use a relatively high frequency of deformation, such as 1/s, the material looks like an elastic solid. If you look at very low frequencies, the bonds that join the polymers can rearrange at a frequency that is higher than the deformation of the network, and you can't even see the presence of the bonds between the polymers. This is a sort of "silly putty" model for how this particular biological structure works. I think it may not be relevant to the red cell but I think it is worth considering that temporal factors might be important in understanding these mechanical properties.

EISENBERG: Let's come back to the flexibility. Do you find the same flexibility for the dimer as for the tetramer or is it not convenient for such a study?

STOKKE: From the relaxation spectra of the spectrin dimers after the electric field across the solution is suddenly shut off, one finds a relaxation time ~2 ms at 20°C; the same has been observed for the tetramers. If spectrin were a stiff rod you would expect that the rotational time for spectrin would increase with a factor of about six. If the dimer were stiff and the tetramer were connected by flexible hinges, you would expect it to be about a factor of three increase in the rotation time.

POTSCHKA: I wonder whether one might be able to form gels without having definitive attachment sites cross-linked. Couldn't one imagine some higher-ordered structures (gel-types) which have these sorts of properties without having particular crosslinking and attachment in all these problems?

STOKKE: It is well known that polysaccharides can associate side-by-side and form gels by nonspecific aggregation for instance in the presence of ions. But for spectrin I don't know of any kind of associations that are side-by-side.

CASPAR: What is the experimental evidence that spectrin forms a net with five or six strands connected at nodes?

STOKKE: The electron microscopic work of Branton (1985. *Proc. Natl. Acad. Sci. USA* 82:6153), clearly points this out. In our reconstitution approach we take colloid gold beads with a diameter of 5 nm and attach protein 4.1 to them. Spectrin dimers bind only at one end, whereas with tetramers we get large aggregates. This is the network we use to reconstitute three-dimensional gels in solution.