The Molecular Basis of Erythrocyte Shape

ARNLJOT ELGSAETER, BJORN T. STOKKE, ARNE MIKKELSEN, DANIEL BRANTON*

Recent discoveries about the molecular organization and physical properties of the mammalian erythrocyte membrane and its associated structural proteins can now be used to explain, and may eventually be used to predict, the shape of the erythrocyte. Such explanations are possible because the relatively few structural proteins of the erythrocyte are regularly distributed over the entire cytoplasmic surface of the cell membrane and because the well-understood topological associations of these proteins seem to be stable in comparison with the time required for the cell to change shape. These simplifications make the erythrocyte the first nonmuscle cell for which it will be possible to extend our knowledge of molecular interactions to the next hierarchical level of organization that deals with shape and shape transformations.

ANYONE INTERESTED IN EXPLAINING HOW THE SHAPE OF A eukaryotic cell is maintained cannot help but be intrigued by the mammalian erythrocyte. This relatively simple cell pliantly conforms to the small passages and shear forces to which it is normally subjected in the circulatory system, returns to a biconcave shape when undisturbed, and transforms into cup-shaped and crenated forms in response to metabolic changes or chemical and environmental treatments.

The integrated system of molecules that determines a cell's shape, internal spatial organization, and motility is usually called the cytoskeleton (*1*). In most cells, this cytoskeleton is a complex transcellular network that includes microtubules, intermediate filaments, microfilaments, and associated proteins. Mammalian erythrocytes are simpler. They are filled with a dense, nonelastic solution of hemoglobin, do not contain a transcellular cytoskeleton, and have no microtubules or intermediate filaments. In the absence of internal organelles or other transcellular structures, the mammalian erythrocyte relies on its plasma membrane and a network of membrane-associated proteins, the so-called membrane skeleton, to serve as its cytoskeleton and to generate cell shape (*2*). Many aspects of the structure and molecular interactions within the membrane skeleton have been reviewed (*3*) and elucidated (Fig. 1). We believe this detailed knowledge can now be used to explain the shape and shape transformations of the erythrocyte.

Biologists and biochemists commonly explain the structure or function of a complex assembly by a building-block approach that assumes that the final assembly will be predicted by understanding the structure and mode of interaction of each subunit. This approach works well for relatively simple ordered assemblies, such as viruses or sarcomeres, where the assembly units can go together in only a limited number of ways and where thermal motion does not significantly affect the properties of the assembled structure. But it is hard to imagine how a building-block approach could be used to explain the overall shape and shape transformations of the erythrocyte; even though the components of the membrane skeleton are interconnected in a regular manner (Fig. 1a), the proteins in this skeleton and the lipids in the bilayer undergo vigorous thermally induced flexing. This marked thermal motion makes it impossible to gather detailed information about the conformation of each molecule and gives the assembled membrane emergent properties that would not exist in the absence of such motion.

Here we explain these properties and show that in this cell a two-step approach can be used to predict shape. The first step is to define the mechanochemical properties of the membrane according to principles that rely on our knowledge of the individual molecules and their interactions, but that do not require detailed information about the particular conformation of each molecule. Once the membrane's mechanochemical properties are defined, the second step is to explain shape by applying established techniques of continuum mechanics. Continuum mechanics is the mathematical formalism that allows one to determine shape by calculating stresses and strains in continuous bodies in which these stresses and strains may be anisotropic (*4*). It is the technique an engineer uses to calculate the contour a bridge will assume when spanning its supports. Although the mathematics may be complex, applying continuum mechanics to derive the shape of a structure with assumed mechanochemical properties is a standard engineering routine that has often been used to analyze the erythrocyte (*5*). But until recently, the molecular basis for important mechanochemical properties of the erythrocyte membrane has been unknown. Now our understanding of the molecular organization of the mammalian erythrocyte membrane and its associated proteins justifies a coherent theoretical analysis that can predict this cell's mechanochemical properties (*6, 7*). Although our analysis is limited to the erythrocyte, the insights it provides may be applicable to understanding aspects of cytoskeletal function in many eukaryotic cells.

Organization of the Membrane

A major component of the erythrocyte membrane skeleton is spectrin, a protein composed of two different polypeptides that are found as heterodimers (Fig. 2a), head-to-head tetramers (Fig. 2b), or higher order oligomers (*3*). Data from light-scattering (*8*), electrooptical measurements (*9*), viscometry (*10*), and electron microscopy (*11*) show that spectrin in solution is an elongated molecule that undergoes marked flexing at physiological tempera-

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tures. Thus, in electron micrographs spectrin appears wormlike (*11*), and calculations based on intrinsic viscosity data (*12*) indicate an equilibrium end-to-end distance (root-mean-square end-to-end length) of about 53 nm for the dimer and about 82 nm for the tetramer, even though the contour lengths of the heterodimer and tetramer are 97 nm and 194 nm, respectively. Together, the data imply that the spectrin repeat segments (Fig. 2c) are flexibly joined and that the molecules behave as chains whose statistical mechanical properties are mainly of entropic origin. To keep the end-to-end distance of such molecules at a value other than the equilibrium value requires a mechanical force whose magnitude increases with the distance from the equilibrium value. Such molecules are often referred to as entropy springs (*13*) because the restoring force is attributable to the tendency of the molecule to adopt a random conformation.

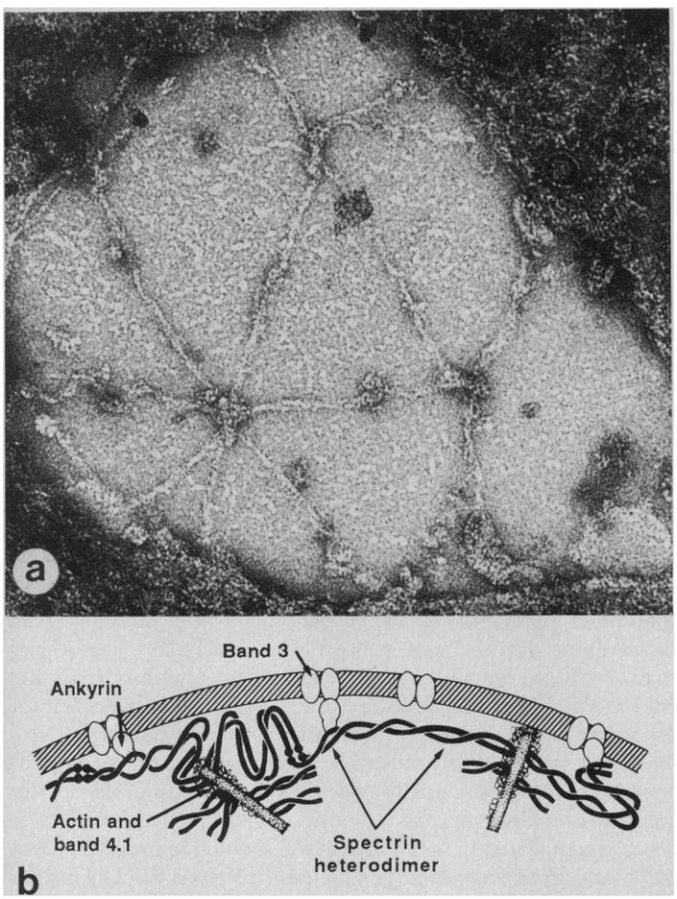

Fig. 1. The erythrocyte membrane skeleton. (a) Electron micrograph showing a region of the membrane skeleton (negatively stained, ×200,000) that has been artificially spread to a surface area nine to ten times as great as in the native membrane [reproduced from (*59*)]. Spreading makes it possible to obtain clear images of the skeleton whose protein components are so densely packed and so subject to thermal flexing on the native, unspread membrane that it is difficult to visualize the individual molecules and the remarkably regular way they are connected to each other. After spreading, views of the membrane skeleton show a predominantly hexagonal and pentagonal network composed of spectrin tetramers cross-linked by junctions containing actin oligomers and band 4.1 (*59, 60*). Band 4.9 (*61*) and tropomyosin (*62*) are probably also bound to the oligomers, whose length (13 actin monomers long) corresponds to the length of a tropomyosin molecule. (b) Diagram showing the unspread membrane in cross section. The network of skeletal proteins is localized exclusively on the cytoplasmic surface of the lipid bilayer to which it is anchored by ankyrin (*3, 63*), a molecule that links spectrin to band 3, the transbilayer anion channel. Band 4.1 can also connect the skeleton to the membrane by binding to integral sialoglycoproteins (*64*). Although the dimensions of the lipid bilayer and the proteins have been drawn to scale (1:500,000), for clarity the diagram underrepresents the density of spectrin and other network components.

The membrane skeleton is linked to the cytoplasmic face of the lipid bilayer by proteins that anchor spectrin to the major transbilayer proteins (Fig. 1b). While in part constrained by their linkage to the spectrin network, the transbilayer components are otherwise free to slide in the plane of the membrane (*14*), and the spectrin molecules in the network behave as polymers of nearly freely jointed segments (*15*).

The realization that the spectrin molecules in the network undergo marked thermal flexing is crucial because it allows one to predict the emergent properties of the assembled network. Networks of flexible, ionizable polymers are true ionic gels whose properties can be predicted by statistical thermodynamic analysis (*13, 16, 17*). Recent studies with macroscopic networks of artificially end-linked spectrin tetramers show that spectrin does indeed form structures whose viscoelastic properties can be accounted for by gel theory (*18*). To a first approximation, the spectrin network may be treated as a two-dimensional ionic gel and the membrane can be modeled as a protein gel connected to a fluid lipid bilayer. Because the lipid bilayer is fluid, the lipid monolayers can slip relative to each other and the gel can slip relative to the bilayer. Because there are multiple connections between the gel and the bilayer, the gel and the bilayer usually must follow each other's contours. Understanding the mechanochemical properties of the erythrocyte therefore requires us to consider the properties of both the spectrin network and the lipid bilayer with its integral membrane proteins.

Mechanochemical Properties

The spectrin network. Cell biologists sometimes attribute major changes in the viscoelastic properties of cells and cell extracts to gel-sol transformations. A gel-to-sol transformation involves the disassembly of the cross-links or bonds between gel polymers or polymer subunits. Although the integrity of the erythrocyte membrane skeleton depends in part on noncovalent bonds whose topology can change, under physiological conditions the rate constants for disassembling and reassembling the network's known molecular connections seem large in comparison with the time required for cells or cell ghosts to change shape in response to chemical and physical changes of environment (*19, 20*). Therefore we cannot invoke the breaking and making of these noncovalent bonds to explain rapid changes in cell shape, although such events must be considered in explaining shape changes over prolonged time periods.

Thus, we do not initially concern ourselves with gel-sol transitions, but with the changes in the properties of a continuous swollen network that retains its topology. The statistical thermodynamic theory of gels predicts that, in response to environmental change, networks constituted of ionic polymers may change their mechanical properties without disassembling and reassembling bonds between the polymers (*17*). Such changes in gel properties can be observed, for example by measuring the osmotic pressure in a gel (*17*). Osmotic pressure measures a gel's tendency to shrink (give up solvent) or expand (take up solvent). For the two-dimensional gel in an erythrocyte skeleton, the property equivalent to gel osmotic pressure is called gel tension, or Π_G.

Three components determine Π_G. The first is the elasticity of the gel. It derives from the springlike behavior of the flexible spectrin chains whose conformational free energy is mainly of entropic origin. The second component is spectrin-spectrin affinity, which is a measure of the attractive and repulsive forces between the polypeptide domains and between these domains and the solvent. Changes in the solvent, including changes in salt concentration and composi-

tion, can affect spectrin-spectrin affinity. The third component is the hydrogen ion tension attributable to the protons released into the gel fluid by dissociation of ionizable amino acids. Because of the electrostatic forces that arise when electroneutrality is not conserved, these protons are retained within the gel and maintain electrical neutrality. Their thermal motions generate tension just as moving molecules in a bottle of gas generate pressure. Increasing temperature will raise hydrogen ion tension, whereas decreasing the solvent *p*H toward the gel's isoelectric point will decrease hydrogen ion tension. Because the balance of the three components determines Π_G, very small changes in the environment can profoundly affect the spectrin network properties. For example, a single unit change in *p*H may produce a threefold change of Π_G (Fig. 3), and experiments with other ionic gels show that 100-fold changes in their osmotic pressure are not uncommon, even when no bonds or connections between the polymer chains are altered (*17*).

Besides Π_G, three other mechanical properties of the gel are important. The first is the elastic shear modulus, G, a scalar parameter that expresses the amount of tension required to reversibly deform the gel a given amount without changing its surface area. The statistical thermodynamic theory of gels predicts that G is proportional to both the number of polymer chains per unit area and the absolute temperature (*13*). If we assume the experimentally established number of spectrin molecules per unit membrane area, the value of G predicted by gel theory (*6*) is close to the observed value of 7×10^{-3} dyne/cm measured in an erythrocyte membrane (*21*). This observation provides further evidence that the erythrocyte membrane skeleton constitutes a true ionic gel whose properties are determined principally by the spectrin network. The second property with which we are concerned is the gel modulus of area compression, K_G, a scalar parameter that expresses the amount of tension required to expand or contract a gel to a given area without

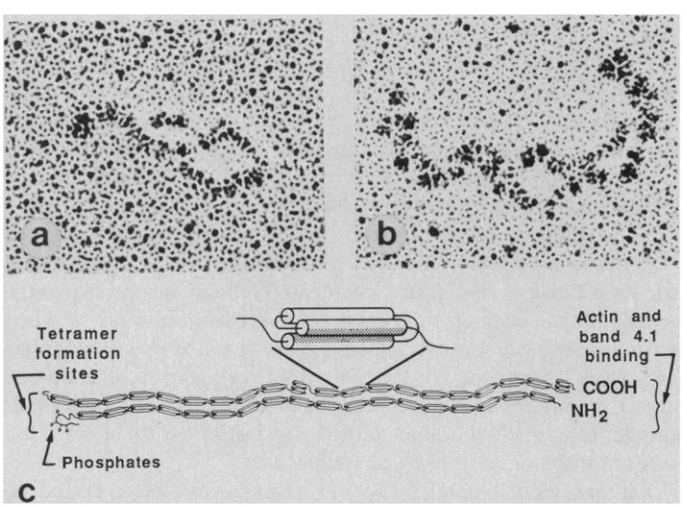

Fig. 2. The anatomy of spectrin. (a) The molecule of spectrin is a heterodimer composed of two different elongated chains, α and β, which, as seen in this electron micrograph of a rotary shadowed molecule, twine around each other or lie side-by-side. In solution, heterodimers can associate to form head-to-head tetramers (b) as well as higher order oligomers. The contour length of a tetramer is 200 nm, but, because the component chains are flexible, the end-to-end distance may be anywhere from 0 to 200 nm. For example, the end-to-end distance of the molecule in (b) is ≃ 95 nm (×250,000). (c) Peptide and nucleotide sequence data (*65, 66*) indicate that the predominant structural feature of both the α and the β chain is a 106–amino acid repeating segment. Each of the repeating segments (approximately 20 in α and 19 in β) has the capacity to fold into a helical triple-stranded structure (see expanded diagram). [Adapted from Speicher and Marchesi (*66*)]

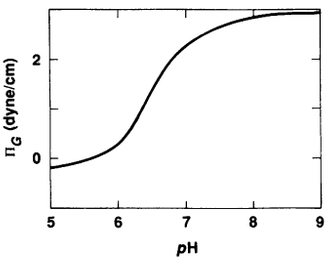

Fig. 3. Erythrocyte membrane skeleton tension, Π_G, as a function of *p*H. The values of Π_G were calculated from spectrin titration data (*37*) and according to the theory of ionic gels (*17*).

shear deformation. Theory predicts that K_G may vary over a wide range as environmental conditions are altered (*6, 17, 22*).

The final mechanical property of a gel with which we must be concerned is its stress-free shape (*23*), Ψ, a parameter that describes the shape the membrane skeleton assumes when the lipid bilayer is removed without perturbing the integrity of the network. To understand this notion, it may be helpful to imagine a gel that has been cast in a mold, such as a polyacrylamide gel cast in a tube. As cast, the gel would be stress-free (*24*). Experiments (*20*) suggest that Ψ of normal erythrocytes may be discoidal, but since this has not been firmly established, we have carried out our analysis with a number of different Ψ's ranging from spheres to thin flat disks.

The lipid bilayer. Because the erythrocyte lipid bilayer is fluid (*25*), it does not resist shear deformation. The shear modulus of each monolayer in the bilayer is therefore zero. But fluid lipid monolayers do exhibit tension, which changes as the surface area of the layer is varied. The relation between lipid tension and surface area has been determined for lipid monolayers with various lipid compositions and for varying environmental conditions (*26, 27*). When a fluid lipid monolayer is bent so that the center of curvature is on the same side of the monolayer as the lipid head groups, these will become more densely packed, whereas the region at the ends of the acyl chains will become less densely packed. Because of the interaction between the neighboring parts of the lipid molecules, the lipid monolayer will resist this kind of bending. The bending elastic modulus, B, is a parameter that expresses this resistance to bending or, more precisely, the amount of energy associated with a given curvature of the lipid monolayer. A rough estimate of B can be obtained from experimental values for the lipid modulus of area compressibility (*28*). Such estimates yield $B = 1 \times 10^{-13}$ to 10×10^{-13} dyne/cm and agree well with the experimental measurements (*29*) of 1×10^{-13} to 3×10^{-13} dyne/cm. Changes in a lipid monolayer—such as the introduction of modified lipids that alter the interaction between the acyl chains (*30*) or enzymatic or environmental changes that can alter the charge-charge interaction of the lipid head groups (*31*), the amount of bound water (*27*), or the sialic residues on glycolipids (*32*)—would affect the lipid properties in fairly predictable ways.

Free Energy and Cell Shape

In the absence of internal cytoskeletal elements, only the lipid bilayer with its integral proteins and associated membrane skeleton determines the shape of the red cell (*2*). We can therefore assume that the stable cell shape is the one for which the entire membrane, including the bilayer and the skeleton, has the lowest free energy. Predicting cell shape requires that we derive the mathematical expression for the total elastic free energy associated with shape changes in terms of the membrane's mechanochemical properties and then use this expression to find the cell shape with the lowest free energy (*33*). Such analyses are well-established exercises in continuum mechanics, and several studies that have assumed various

membrane material properties have been published (*5, 28, 34*). The change in free energy for a cell with a protein gel-lipid bilayer membrane that changes from Ψ to a deformed shape is given in detail by Stokke *et al.* (*6*). To show which parameters enter into the calculations, the main terms of their equation are presented here:

$$\Delta F_{total} = G \cdot f_1 + K_G \cdot f_2 + \Pi_G d \cdot f_3 + B \cdot f_4$$

The equation includes functions, f_1 to f_4, that involve integrations for which the integrand depends on the changes from Ψ to some deformed shape whose contours or volume differ from Ψ. To calculate ΔF_{total} requires mathematical descriptions of the contour and volume and the values for Ψ and the following parameters: G, elastic shear modulus; K_G, gel modulus of compression; Π_G, gel tension; d, a length dependent on the distance from the membrane skeleton to the lipid bilayer; and B, the energy associated with local bending of the lipid bilayer.

From a continuum mechanical viewpoint, the only major difference between Stokke *et al.*'s (*6, 7*) analysis and previous analyses is that Stokke *et al.* take into account Tanaka *et al.*'s (*17*) results which show that in ionic gels the modulus of compression can change significantly in response to environmental changes.

The first term of the equation represents the change in energy due to shear deformation as the cell shape changes from Ψ to the deformed shape, while the second term represents the change due to nonuniform compression. The third term depends on both the lipids and the gel and represents a mathematical expression of the trilayer analogue (two lipid monolayers plus one gel layer) of the bilayer couple hypothesis (*35*). The last term represents free energy associated with bending of the lipid bilayer. When the exact values for the various parameters are inserted into the equation, this mathematical expression can be used to make accurate predictions of cell shape. At this stage, our main objective has been to learn how the various parameters influence shape as they are varied within experimentally observed and theoretically estimated values.

Analysis of Erythrocyte Shapes

Three classes of erythrocyte shape are commonly observed (Fig. 4): discocytes, among which are found the normal biconcave disk; stomatocytes, which are cup-shaped; and echinocytes, which bear nearly uniformly spaced projections. As the values for the parameters of the free-energy equation are systematically varied, all three shape classes arise as free-energy minima, or favored shapes. It is striking that the parameter values that favor these shapes make physical sense in terms of the known or predicted responses of a spectrin gel and a lipid bilayer to reasonable changes of *p*H, temperature, and ionic environment. For example, the parameter values that favor discocytes, stomatocytes, or echinocytes—the three most commonly observed erythrocyte forms—exist within a relatively narrow range of gel tensions and lipid tensions (*36*) that have been observed in erythrocyte membranes.

Within each shape class, a broad continuum of shapes has been studied (*7*), and analysis (Fig. 5) shows which parameters are important and how each can influence erythrocyte shape.

1) The value assumed for K_G determines the depth of the free-energy minimum, but has little influence on which specific shape is favored for discocytes and stomatocytes (Fig. 5, a to c). The value of K_G does influence the shape of echinocytes (Fig. 5d).

2) Assumptions regarding Ψ affect both the depth of the free-energy minimum and the specific shape that will be favored (Fig. 5, a and b). Experiments with delipidated cells (*20*) suggest that Ψ has a discoidal contour.

3) For reasonable values of the parameters, the energy minimum in each class corresponds to a commonly observed erythrocyte shape. For example, among the discoid shapes, the biconcave disk with intermediate concavities is favored by a lower energy than for the disks with deep or no concavities (Fig. 5, a and b).

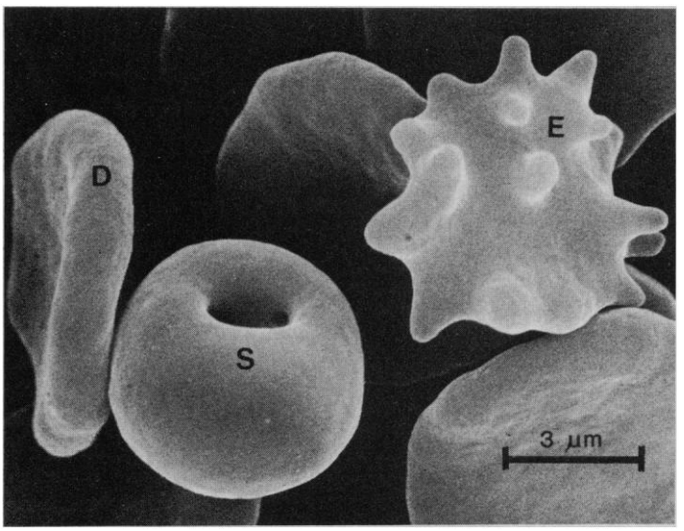

Fig. 4. Common erythrocyte shapes. Scanning electron micrograph illustrating discocytic (D), stomatocytic (S), and echinocytic (E) shapes. ×10,000.

4) For the same given set of conditions and parameter assumptions (Fig. 5, b and c), both the discocyte and the stomatocyte classes can exhibit energy minima, and the difference between these minima is often small. This suggests that these two shape classes may coexist, one shape stable and the other metastable.

5) Changes in cell volume, such as those brought about by osmotic factors, will affect the specific shape that is favored within a shape class and can also play an important role in determining which shape class will be favored (Fig. 5, e and f).

6) When the protein gel is very compressible (low values of K_G), the gel free-energy minima are very shallow. This implies that as K_G becomes smaller, less pressure is required to deform an erythrocyte or force it through a narrow capillary. Providing the erythrocyte with a very compressible protein gel may be nature's way of producing a pliant but robust cell membrane.

7) Changes in the gel tension and the related lipid tension (*36*) play particularly important roles in selecting which shape class has the lowest energy. For example, crenated cells are favored when the tension in the external lipid monolayer becomes positive or when tension in the gel becomes negative. Cell shapes with invaginations are favored when the converse becomes true (Fig. 5, e and f). A crucial feature of our analysis is that it predicts that changes in either the gel tension or the lipid tension can transform the cell from a discocyte or stomatocyte to an echinocyte.

Because ionic strength and *p*H changes can be expected to substantially change Π_G, Π_G and its derivative K_G, together with the tension in the two lipid layers (*36*), are the primary parameters through which ordinary changes in the environment or the presence of pharmacological agents (*35*) would be expected to influence cell shape. But at the low *p*H and high ionic strengths that are predicted to produce large negative values of Π_G, a collapse of the membrane bilayer with a concomitant protrusion of lipid vesicles should, and in fact does, occur (*37*). In contrast, at the high *p*H and low ionic strengths predicted to produce high positive Π_G values, spectrin dissociates from the membrane and endocytic breakdown of erythrocyte ghosts should, and in fact does, occur, as when inside-out spectrin-depleted vesicles are produced (*38*).

Applications of the Analysis

Can cell shapes be predicted? We have suggested a mechanism to account for how shape can be maintained and influenced by environmental change, but to test our suggestion we should be able to predict shape. Thus far, we have shown that the property changes needed to effect shape transformations make sense in terms of the network and the lipid bilayer structure, but we have not actually predicted specific cell shapes. To do so, it will be necessary to predict or measure the actual values for the various parameters in the equation that describes the total elastic free energy of the membrane. These parameters are Ψ, G, K_G, Π_G, d, and B. Many of these can be measured with structural or biophysical techniques. We already have some information about Ψ (*20*), G (*21, 39*), Π_G (*40*), and B (*29, 41*), although additional measurements are needed. Parameter d can be determined from the shape and location of binding sites on ankyrin and other molecules that connect the membrane skeleton to the lipid bilayer. The theory needed to calculate G for a network consisting of elongated, highly flexible macromolecules is well established (*16*), and, as noted above, the calculated value and the experimentally determined value are in close agreement. Accurate measurement of K_G and Π_G is difficult: estimates of K_G from measurements on the intact membrane are inaccurate because of yet unmeasured integral membrane protein effects on the lipid bilayer. The theory for predicting K_G and Π_G from three-dimensional ionic gels is fairly well established, but has not yet been extended to the topologically two-dimensional membrane skeleton. Improvement in theory can be expected, but the needed biochemical data for the total charge on the protein, the spectrin-spectrin segment affinities, and the spectrin-lipid affinities will be difficult to obtain.

After the membrane mechanochemical properties are known, the second step in predicting cell shape is to calculate the most favored shape, that is, the shape with the minimum total free energy. Because these calculations involve well-established principles of classical continuum mechanics, they can be expected to yield quantitative predictions whose accuracy is limited only by the accuracy with which the mechanochemical properties of the membrane have been measured or calculated.

Stability and change in network topology can affect shape. Because the assemblies that sustain the normal erythrocyte membrane skeleton seem to dissociate slowly in comparison with the rate at which shape changes occur, the topology of the gel, and hence Ψ, will remain constant when the cell changes shape. This unique feature of the erythrocyte membrane skeleton permits an analysis that deals only with equilibrium states, but the limits on the applicability of the analysis must be appreciated; temporally and spatially controlled reactions that lead to, or metabolically alter, the mature Ψ have not been considered. For example, if a cell is constrained to a particular shape for a time that is longer than the time for disassembly and reassembly of the gel junctions [for example, a sickled cell in a prolonged sickling crisis or a cell in a micropipette aspiration experiment (*42*)], the topology of the gel, and hence the contour of Ψ, may change. Because Ψ has an important effect on the favored cell shape, such long-term constraints on cell shape should have enduring effects on how cell shape will respond to a given set of environmental conditions. For example, a cell that is sickled long enough to allow Ψ to change may retain an abnormal appearance long after the sickling crisis has passed (*43*).

Data that explain erythrocyte shape in terms of lipid need not be discordant with data that explain shape in terms of protein. Lange *et al.* (*20*) observed that erythrocyte ghosts crenated by saline or dinitrophenol yielded smooth discoid skeletons when the lipid bilayer was extracted with non-ionic detergent. They concluded that the skeleton, because it assumed an independent contour when delipidated, "... was not the cause of the ghost shape change but accommodated it" (*20*, p. 719). Such conclusions overlook evidence that shape change depends on the proteins that link spectrin to the lipid bilayer (*44*). Thus, neither the protein gel nor the lipid bilayer alone, but the two together, are responsible for erythrocyte shape.

Some aspects of erythrocyte shape are clearly under metabolic controls that involve proteins. Upon adenosine triphosphate (ATP)

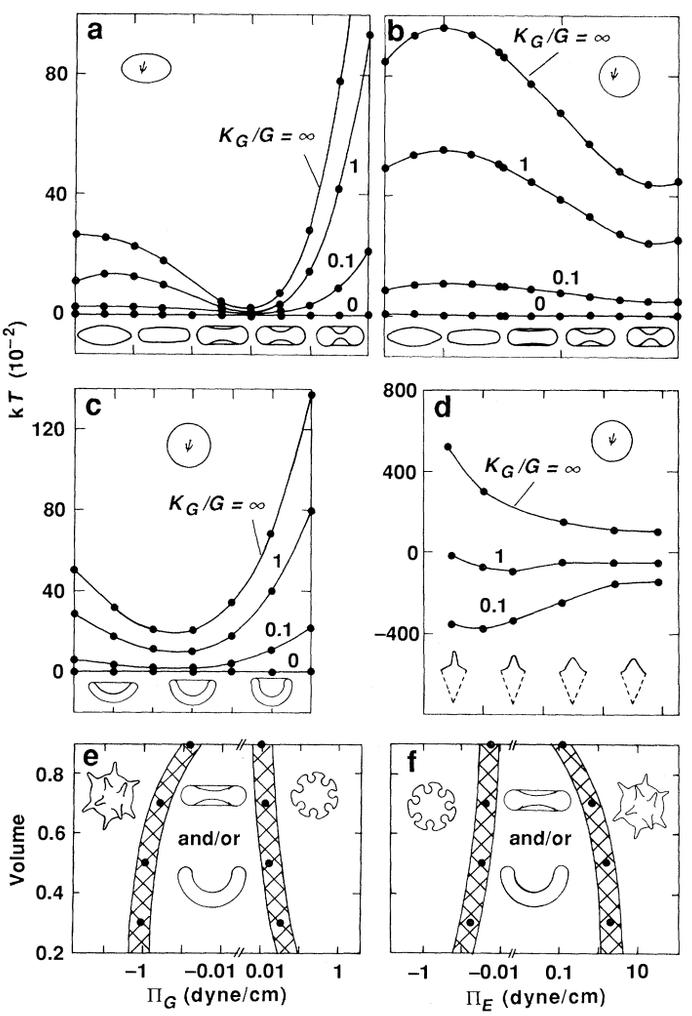

Fig. 5. Erythrocyte shape and membrane free energy. (a to d) The elastic free energy of an erythrocyte membrane for some selected shapes. (a and b) Discocyte, (c) stomatocyte, and (d) echinocyte. In each case, the elastic free energies are plotted in units of thermal energy and were calculated for membrane skeletons with K_G/G ranging from 0 (infinitely compressible gel) to ∞ (incompressible gel). In (a), the stress-free gel shape, Ψ, is assumed to be a discocyte; in (b) to (d), Ψ is assumed to be spherical. (e and f) Phase diagrams that indicate the favored cell shape as a function of (e) cell volume and tension in the gel or (f) cell volume and tension in the external half of the lipid bilayer, Π_E. Cross-hatching indicates the regions of uncertainty between the phases; within each phase, the favored shapes are indicated by diagram. The graphs illustrate how changes in some of the parameters of the energy equation influence the favored shape. For example, (a) and (b) assume identical values for all parameters except Ψ. If Ψ is discoidal (a), shallow concavities are energetically favored for all values of gel compressibility; if Ψ is spherical (b), fairly deep concavities are favored for all gel compressibility values. The calculations in (b) to (d) assume identical parameters, but comparing (b) with (c) shows that for any value of gel compressibility, the most favored biconcave discocyte has nearly the same energy as the most favored cup shape; in contrast, the free energies associated with many crenated shapes (d) are greater than for the cup or biconcave shapes, and gel compressibility can determine which crenated shape is most favored.

depletion, biconcave disks transform into echinocytes. If the cells resynthesize ATP, the effect is reversed. While some ATP effects may be mediated by consequent changes of cellular ionic composition, analogous effects can be seen in ghosts, where shape change can be critically correlated with the activity of a vanadate-sensitive magnesium-activated ATPase (*45*). But if we accept that the tension in any of the three membrane layers (membrane skeleton or either of the two lipid layers) can affect shape, lipids may also play a role in ATP-dependent shape change. The state of phosphoinositide and phosphatidic acid phosphorylation (*46*) or the movement of lipid from one layer to the other (*47*), both of which depend on ATP, can also be correlated with the discocyte-echinocyte transformations.

Conclusions and Prospects

We have shown that erythrocyte shape can be understood in terms of its molecular organization, but a critical test of this understanding will be possible only when we can quantitatively predict cell shape. Although we do not have numerical values for some parameters that would allow quantitative predictions, our analysis provides a coherent theory that explains the general features of erythrocyte shapes, the sequence in which the various shape classes appear, and the trend of shape changes within a class. This explanation does not require knowledge of the exact conformation and location of the constituent molecules, which, in the case of the erythrocyte, would be impossible. Rather, it requires that we know something about the collective properties of the protein gel and the lipid bilayer, data that we can hope to obtain from studies of the membrane itself or from studies of purified membrane components and their synthetic assemblies.

An important conclusion that emerges from our analysis is that major changes in shape can be explained without invoking the making and breaking of either covalent or noncovalent bonds. The success of the sliding filament model in explaining muscle action and concern with the polymerization dynamics of actin, tubulin, and other proteins in nonmuscle cells have justifiably led many workers to focus their attention on assembly-disassembly and association-dissociation reactions. While we do not deny the importance of assembly-disassembly phenomena or of temporal and spatial regulation of polymerization events, other factors that can affect cell shape and motility should be considered. Many nucleated cells have membrane skeletons (*48, 49*) and extensive transcellular networks of interconnected polymers (*50*) that may behave in part like ionic gels. Many nucleated cells contain spectrin (*3, 49, 51*) and other elongated, flexible actin-binding proteins (*50*). Thus, the interaction of ionic gels with lipid bilayers or of ionic gels with microfilaments, microtubules, or other relatively inflexible components may be a more frequent determinant of cell, nuclear, and vesicular shape than is commonly recognized.

Although it is widely held that microtubules are the overall organizers of cell morphology, polarity, and motile activity (*52*), the underlying endogenous determinants of shape and motile activity can persist in the absence of microtubules (*53, 54, 55*). For example, neuroblastoma cells regain their original morphology after treatments that temporarily cause the cells to round up, retract their neurites, depolymerize their microtubules, and undergo complete rearrangement of their microfilaments and intermediate filaments (*56*). If the endogenous determinants of shape depend on a physically interconnected network to coordinate recapitulation of original morphology, this network must be a persistent but pliant structure that endures marked alteration in the overall pattern of the cytoskeleton and persists through interruptions in its expression. Because networks of entropic springs are among the few structures whose elastic extension can exceed more than a few percent (*16*), it is possible to have gels that are extraordinarily compressible (values of K_G approaching zero) and yet resistant to rupture. Just as a fishing net stored on dry land has a memory of the very different shape it will assume when pulled through water, gels react to environmental factors by exhibiting large changes in volume (or surface area in the case of a two-dimensional gel) without breaking any connections between the polymers in the network. For gels that are constrained by attachment to another structure, such as a lipid bilayer, changes in gel volume or surface area can manifest themselves as a predictable change in contour. Because they have memory, gels may be important in persistent, repetitive, or cyclic shape and motility processes such as those observed in neuroblastoma cells (*53*), epidermal keratocytes or chromatophores (*55, 57*), fibroblast fragments (*58*), and other cell types (*1*).

REFERENCES AND NOTES

1. M. Schliwa, *The Cytoskeleton: An Introductory Survey* (Springer-Verlag, Vienna, 1986).
2. Although the interactions of the internal hemoglobin solution with the membrane may affect cell shape, the effects of hemoglobin on cell shape must be through its effects on the properties of the membrane skeleton or the lipid bilayer and its integral membrane proteins.
3. D. Branton, C. M. Cohen, J. Tyler, *Cell* **24**, 24 (1981); C. M. Cohen, *Semin. Hematol.* **20**, 141 (1983); S. R. Goodman and K. Shiffer, *Am. J. Physiol.* **13**, C121 (1983); W. Gratzer, in *Muscle and Nonmuscle Motility*, A. Stracher, Ed. (Academic Press, New York, 1983), pp. 37–124; J. Palek and S. E. Lux, *Semin. Hematol.* **20**, 189 (1983); D. M. Shotton, in *Electron Microscopy of Proteins*, J. R. Harris, Ed. (Academic Press, London, 1983), pp. 205–330; V. Bennett, *Annu. Rev. Biochem.* **54**, 273 (1985).
4. Y. C. Fung, *Biomechanics* (Springer-Verlag, New York, 1981).
5. P. R. Zarda, S. Chien, R. Skalak, *J. Biomech.* **10**, 211 (1977); E. A. Evans and R. Skalak, *Crit. Rev. Bioeng.* **3**, 181 (1979); J. D. Brailsford, R. A. Korpman, B. S. Bull, *J. Theor. Biol.* **86**, 513 (1980).
6. B. Stokke *et al.*, *Eur. J. Biophys.* **13**, 203 (1986).
7. B. Stokke *et al.*, *ibid.*, p. 219.
8. A. Elgsaeter, *Biochim. Biophys. Acta* **536**, 235 (1978); A. Boe, A. Elgsaeter, G. Oftedal, K. A. Strand, *Acta Chem. Scand. A* **33**, 245 (1979); M. H. Reich *et al.*, *Biophys. Chem.* **16**, 307 (1982).
9. A. Mikkelsen and A. Elgsaeter, *Biochim. Biophys. Acta* **536**, 245 (1978); *ibid.* **668**, 74 (1981); B. Roux and R. Cassoly, *Biophys. Chem.* **16**, 193 (1982).
10. G. B. Ralston, *Biochim. Biophys. Acta* **455**, 163 (1976); J. C. Dunbar and G. B. Ralston, *ibid.* **667**, 177 (1981); B. T. Stokke and A. Elgsaeter, *ibid.* **640**, 640 (1981).
11. D. M. Shotton, B. Burke, D. Branton, *J. Mol. Biol.* **131**, 303 (1979).
12. B. T. Stokke, A. Mikkelsen, A. Elgsaeter, *Biochim. Biophys. Acta* **816**, 102 (1985).
13. L. R. G. Treloar, *The Physics of Rubber Elasticity* (Clarendon, Oxford, 1975).
14. D. E. Golan and W. Veatch, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2537 (1980); C. H. Chang, H. Takeuchi, T. Ito, *J. Biochem. (Tokyo)* **90**, 977 (1981); D. W. Koppel, M. P. Sheetz, M. Schindler, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 3576 (1981); M. P. Sheetz, *Semin. Hematol.* **20**, 175 (1983).
15. Y. L. Dubreuil and R. Cassoly, *Arch. Biochem. Biophys.* **223**, 495 (1983).
16. P. J. Flory, *Principles of Polymer Chemistry* (Cornell Univ. Press, New York, 1953); *Proc. R. Soc. London Ser. A* **351**, 351 (1976).
17. T. Tanaka *et al.*, *Phys. Rev. Lett.* **45**, 1636 (1980).
18. B. T. Stokke, A. Mikkelsen, A. Elgsaeter, *Biochim. Biophys. Acta* **816**, 111 (1985).
19. V. Bennett and D. Branton, *J. Biol. Chem.* **252**, 2753 (1977); E. Ungewickell and W. Gratzer, *Eur. J. Biochem.* **88**, 379 (1978); E. Ungewickell, P. Bennett, R. Calvert, V. Ohanian, W. Gratzer, *Nature (London)* **280**, 811 (1979); V. Bennett and P. J. Stenbuck, *J. Biol. Chem.* **255**, 6424 (1980); C. M. Cohen and C. Korsgren, *Biochem. Biophys. Res. Commun.* **97**, 1429 (1980); S. C. Liu and J. Palek, *Nature (London)* **285**, 586 (1980); J. C. Pinder and W. B. Gratzer, *J. Cell Biol.* **96**, 768, 775 (1983); M. P. Sheetz and J. Casaly, *J. Biol. Chem.* **255**, 9955 (1980); V. Ohanian *et al.*, *Biochemistry* **23**, 4416 (1984).
20. Y. Lange, R. A. Hadesman, T. L. Steck, *J. Cell Biol.* **92**, 714 (1982).
21. R. Waugh and E. A. Evans, *Biophys. J.* **26**, 115 (1979).
22. K_G is the first derivative of Π_G with respect to gel area. Direct measurements of K_G or of K_G/G for the membrane skeleton are not currently available, and it is not yet known whether the large variations in K_G/G reported for other ionic gels will occur in the erythrocyte membrane skeleton within a physiological range of conditions.
23. We use the word "shape" to designate a specific cell volume and contour. An isotropic contraction of a cell changes its volume (and hence its shape) but not its contour.
24. We simplify the concept of Ψ for this presentation. In formulating the free-energy equation we actually defined Ψ as the shape of the gel that has a homogeneous chain density and no shear deformation, but which may be strained as a result of isotropic compression or expansion and for which the Π_G equals the countertension exerted by the lipids.
25. R. J. Cherry, *Biochim. Biophys. Acta* **559**, 289 (1979); R. Peters, *Cell Biol. Int. Rep.* **5**, 733 (1981); M. Seigneuret, A. Zachowski, A. Hermann, P. F. Devaux, *Biochemistry* **23**, 4271 (1984).
26. D. Papahadjopoulos, *Biochim. Biophys. Acta* **163**, 240 (1968); L. Lis, J. McAlister, M. Fuller, R. P. Rand, V. A. Parsegian, *Biophys. J.* **37**, 667 (1982).
27. S. H. White and G. I. King, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 6532 (1985).
28. P. B. Canham, *J. Theor. Biol.* **26**, 61 (1970).

29. R. M. Servuss, W. Harbich, W. Helfrich, *Biochim. Biophys. Acta* **436**, 900 (1976).
30. A. Christiansson, F. A. Kuypers, B. Roelofsen, J. A. F. Op den Kamp, L. L. M. Van Deenen, *J. Cell Biol.* **101**, 1455 (1985).
31. A. Tamura and T. Fuji, *J. Biochem.* **90**, 629 (1981).
32. E. A. Doulak, W. T. Coakley, D. Tilley, *J. Biol. Phys.* **12**, 44 (1984).
33. Deriving this mathematical expression and expressing erythrocyte shape numerically is simplified because the normal biconcave discocyte is rotationally symmetrical around an axis through the centers of the two convexities. The convexities and equator of biconcave cells can be relocated [B. Bull, *Nouv. Rev. Fr. Hematol.* **12**, 835 (1972)], and the tank tread motion of the cell membrane in response to shear flow has shown that any point on the surface of the cell can be the center of a convexity [H. Schmid-Schonbein and P. Gaehtgens, *Scand J. Clin. Lab. Invest.* **41** (suppl. 156), 13 (1981)].
34. Y. C. Fung and P. Tong, *Biophys. J.* **8**, 175 (1968); E. A. Evans, *ibid.* **30**, 265 (1980).
35. M. P. Sheetz and S. J. Singer, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 4457 (1974); M. P. Sheetz, R. G. Painter, S. J. Singer, *J. Cell Biol.* **70**, 193 (1976).
36. Because the sum of the tensions in the three layers (membrane skeleton plus two lipid bilayers) must always be zero, the tension in any two layers completely determines the tension in the third.
37. A. Elgsaeter, D. Shotton, D. Branton, *Biochim. Biophys. Acta* **426**, 101 (1976).
38. T. Steck, *Methods Membrane Biol.* **2**, 245 (1974).
39. S. Chien, K.-L. P. Sung, R. Skalak, R. Usami, A. Tozeren, *Biophys. J.* **24**, 463 (1978).
40. M. J. Conrad and S. J. Singer, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 5202 (1979); *Biochemistry* **20**, 808 (1981).
41. E. A. Evans, *Biophys. J.* **43**, 227 (1983).
42. D. R. Markle, E. A. Evans, R. M. Hochmuth, *ibid.* **42**, 91 (1983).
43. S. E. Lux, K. M. John, M. J. Karnovsky, *J. Clin. Invest.* **58**, 955 (1976).
44. Y. Jinbu *et al.*, *Biochim. Biophys. Acta* **773**, 237 (1984); D. P. Carter and G. Fairbanks, *J. Cell Biochem.* **24**, 385 (1984).
45. V. P. Patel and G. Fairbanks, *J. Biol. Chem.* **261**, 3170 (1986).
46. J. E. Ferrell, Jr., and W. H. Huestis, *J. Cell Biol.* **98**, 1992 (1984).
47. M. Seigneuret and P. F. Devaux, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 3751 (1984).
48. A. Ben-Ze'ev, A. Duerr, F. Solomon, S. Penman, *Cell* **17**, 859 (1979); E. J. Luna, V. M. Fowler, J. Swanson, D. Branton, D. L. Taylor, *J. Cell Biol.* **88**, 396 (1981); M. F. Mescher, M. J. L. Jose, S. P. Balk, *Nature (London)* **289**, 139 (1981); J. R. Apgar, S. H. Herrmann, J. M. Robinson, M. F. Mescher, *J. Cell Biol.* **100**, 1369 (1985); F. D. McKeon, M. W. Kirschner, D. Caput, *Nature (London)* **319**, 463 (1986).
49. V. T. Marchesi, *Annu. Rev. Cell Biol.* **1**, 531 (1985).
50. R. E. Kane, *J. Cell Biol.* **71**, 704 (1976); K. R. Porter, *Proc. Int. Cong. Elect. Microsc.* **3**, 627 (1978); S. Penman *et al.*, in *The Modern Cell Biology Series: Spatial Organization of Eukaryotic Cells*, J. R. McIntosh, Ed. (Liss, New York, 1983), pp. 385–415; T. P. Stossel *et al.*, *Annu. Rev. Cell Biol.* **1**, 353 (1985).
51. W. J. Nelson and E. Lazarides, in *Cell Membranes: Methods and Reviews*, L. Glaser, W. A. Frazer, E. L. Elson, Eds. (Plenum, New York, 1984), vol. 2, pp. 219–246.
52. R. Goldman, T. Pollard, J. Rosenbaum, Eds., *Cell Motility* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1976); *Cold Spring Harbor Symp. Quant. Biol.* **46** (1982).
53. F. Solomon, *J. Cell Biol.* **90**, 547 (1981).
54. M. C. Beckerle and K. R. Porter, *ibid.* **96**, 354 (1983).
55. U. Euteneuer and M. Schliwa, *Nature (London)* **310**, 58 (1984).
56. F. Solomon, *Cell* **21**, 333 (1980).
57. K. R. Porter and M. A. McNiven, *ibid.* **29**, 23 (1982).
58. G. Albrecht-Buehler, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 6639 (1980).
59. T. J. Byers and D. Branton, *ibid.* **82**, 6153 (1985).
60. B. W. Shen *et al.*, *J. Cell Biol.* **102**, 997 (1986).
61. D. L. Siegel and D. Branton, *ibid.* **100**, 775 (1985).
62. V. M. Fowler and V. Bennett, *J. Biol. Chem.* **259**, 5978 (1984).
63. V. Bennett, *J. Cell Biochem.* **18**, 49 (1982).
64. T. J. Mueller and M. Morrison, in *Erythrocyte Membranes: Recent Clinical and Experimental Advances*, W. Kruchberg, J. Eaton, G. Brewer, Eds. (Liss, New York, 1981), vol. 2, pp. 95–112; R. A. Anderson and R. E. Lovrien, *Nature (London)* **307**, 655 (1984); R. A. Anderson and V. T. Marchesi, *ibid.* **318**, 295 (1985).
65. D. W. Speicher, *J. Cell Biochem.* **30**, 245 (1986).
66. ——— and V. T. Marchesi, *Nature (London)* **311**, 177 (1984).
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"I now pronounce you prey and predator."