Remodeling the Shape of the Skeleton in the Intact Red Cell

Jena K. Khodadad,* Richard E. Waugh,‡ Janice L. Podolski,§ Robert Josephs,¶ and Theodore L. Steck§
*Departments of Anatomy and Pathology, Rush Medical College, Chicago, Illinois 60612; ‡Department of Biophysics, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642; §Department of Biochemistry and Molecular Biology, The University of Chicago, Chicago, Illinois 60637; ¶Department of Molecular Genetics and Cell Biology, University of Chicago, Chicago, Illinois 60637 USA

ABSTRACT The role of the membrane skeleton in determining the shape of the human red cell was probed by weakening it in situ with urea, a membrane-permeable perturbant of spectrin. Urea by itself did not alter the biconcave disk shape of the red cells; however, above threshold conditions (1.5 M, 37°C, 10 min), it caused an 18% reduction in the membrane elastic shear modulus. It also potentiated the spiculation of cells by lysophosphatidylcholine. These findings suggest that the contour of the resting cell is not normally dependent on the elasticity of or tension in the membrane skeleton. Rather, the elasticity of the skeleton stabilizes membranes against deformation. Urea treatment also caused the projections induced both by micropipette aspiration and by lysophosphatidylcholine to become irreversible. Furthermore, urea converted the axisymmetric conical spicules induced by lysophosphatidylcholine into irregular, curved, and knobby spicules; i.e., echinocytosis became acanthocytosis. Unlike controls, the ghosts and membrane skeletons obtained from urea-generated acanthocytes were imprinted with spicules. These data suggest that perturbing interprotein associations with urea in situ allowed the skeleton to evolve plastically to accommodate the contours imposed upon it by the overlying membrane.

INTRODUCTION

In erythropoiesis, the biconcave disk shape of the human erythrocyte is established through the remodeling of its misshapen reticulocyte precursors over a period of several hours, during which time most biosynthetic processes have stopped (Palek and Lambert, 1990). This transformation of contour can be viewed as reflecting the reorganization of key elements in both the plasma membrane and the cell. Theoretical treatments postulate that these elements are the cell volume, the membrane surface area, the elastic moduli for the extension and bending of the membrane, and the natural curvature of the resting membrane (cf. Evans, 1974; Steck, 1989; Svetina and Zeks, 1989; Grebe and Schmid-Schonbein, 1990).

Shape maturation might then proceed as follows. Physiological modulation of the aforementioned determinants would drive contour change but would be opposed instantaneously by tension arising from the elasticity of the membrane. The molecules in the membrane would slowly rearrange to dissipate this tension, and the membrane would thereby evolve a new contour. Similar plastic deformations might also account for some of the permanent shape changes afflicting human red cells, of which irreversible sickling is the best-known example (Lux et al., 1976; Bennett, 1989; Palek and Lambert, 1990).

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Address reprint requests to Dr. Theodore L. Steck, Department of Biochemistry and Molecular Biology, University of Chicago, 920 E. 58th Street, Chicago, IL 60637. Tel.: 312-702-1329; Fax: 312-702-0439. E-mail: t-steck@uchicago.edu.

Dr. Podolski's present address is Department of Operating Room and Surgical Nursing, Rush College of Nursing, Chicago, IL 60612.

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In the bloodstream, normal erythrocytes withstand months of constant deformation without important changes in their rest shape. Micromechanical experiments indicate that, on the time scale of minutes, red cell contour is maintained passively by membrane elasticity; i.e., the membrane behaves as a solid. Over time, however, cell shape might be sensed and actively rectified by cellular mechanisms (i.e., contour homeostasis). Neither the notable durability nor the shape stability of the erythrocyte requires great membrane rigidity; indeed, normal mammalian erythrocytes are exceedingly deformable and compliant (Chien, 1987; Hochmuth and Waugh, 1987).

The elasticity of the human red cell derives from its two solid elements: the membrane lipid bilayer (with integral proteins dissolved therein) and the membrane protein skeleton (Steck, 1989; Mohandas and Evans, 1994). Although the glycoconjugates projecting from the extracellular surface of the membrane may contribute to the bending elasticity (Grebe and Schmid-Schonbein, 1990), this factor will be ignored here. As a planar fluid, the bilayer has negligible resistance to elongation (surface shear), moderate resistance to bending, and great resistance to area dilation.

The bending elasticity of the bilayer is ascribable to two principal mechanisms. First, each of the two leaflets is stiffened by lateral interactions among neighboring lipid molecules (Christiansson et al., 1985), leading to a preferred, intrinsic, local curvature. Second, because the transverse diffusion of lipids is limited (Steck, 1989), bending the membrane expands the surface area of one of its leaflets while compressing the other; the consequent tension/area work is manifested as stiffness (Evans, 1980).

The skeleton is a closed network of filamentous proteins affixed as a single layer to the cytoplasmic surface of the membrane (Yu et al., 1973; Hainfeld and Steck, 1977). The skeleton takes the form of an irregular lattice; the repeating

unit is built upon short, stiff actin protofilaments interconnected by multiple flexible spectrin filaments (Bennett, 1989, 1990). It is the skeleton that confers elastic resistance to shear deformation and reinforces the membrane against mechanical disruption (Chien, 1987). The bilayers of red cells with impaired skeletons are poorly supported and are notably fragile (Chasis and Shohet, 1987; Waugh and Agre, 1988; Palek and Lambert, 1990; Mohandas and Evans, 1994).

The bending stiffness of the membrane skeleton is apparently small compared to that of the bilayer. For one thing, the thermal fluctuations of skeletons, which are modulated by bending elasticity (Peterson et al., 1992), increase dramatically when they are freed of bilayer constraints by detergent extraction (Svoboda et al., 1992; Schmidt et al., 1993). Furthermore, bending stiffness scales with the modulus of surface area compression/expansion (Evans, 1974); this modulus is five orders of magnitude smaller for the red cell skeleton than for the bilayer (Discher et al., 1994).

The bilayer is intimately coupled to the underlying membrane skeleton (Hainfeld and Steck, 1977; Elgsaeter et al., 1986). As a result, the deformation of one should be resisted by the other (Liu et al., 1989; Discher et al., 1994). To explore this interplay, we treated intact erythrocytes with urea to weaken their skeletons in situ. We analyzed the consequent alterations of the contours and mechanical properties of these cells and their membranes. In a companion report (Waugh, 1996), model calculations were performed to assist in the analysis of our findings.

EXPERIMENTAL STRATEGY

We used 1.5 M urea to weaken the connections within the skeleton. This chaotropic solute equilibrates rapidly across the membrane (Sha'afi, 1977) and promotes the breakdown of red cells (Bessis, 1973, p. 157). The particular utility of urea lies in its ability to perturb the structure of spectrin at concentrations below those that denature most proteins (Calvert et al., 1980; Morrow et al., 1981; Yoshino and Marchesi, 1984; di Stasi et al., 1987). The action of urea on spectrin appears to be complex and at least partially reversible (Yoshino and Marchesi, 1984; Fischer, 1989). There is no evidence for an effect of even 6 M urea on the mechanical properties of lipid bilayers.

The disruption of either intramolecular structure or intermolecular connections should reduce the elasticity of the skeleton. For example, this was the interpretation placed on the apparent diminution of the stiffness of isolated skeletons caused by 10% ethanol (Vertessy and Steck, 1989). Furthermore, increasing the lability of connections within the skeleton could foster their rearrangement, hence leading to plastic shape change. Perturbation of skeleton structure might therefore be manifested in both reversible and irreversible changes in the shapes of intact cells. Further information can be obtained from ghosts and membrane skeletons derived from cells with weakened skeletons: will they be imprinted with the ambient contour of the parent cells (e.g., Lux et al., 1976)?

Two techniques were used to reversibly deform erythrocytes: palmitoyllysophosphatidylcholine (LPC) and micropipette aspiration. LPC partitions preferentially into the outer leaflet of the lipid bilayer and expands it differentially. The difference in area between the two lipid leaflets drives curvature through a bilayer couple effect (Sheetz and Singer, 1974; Farge and Devaux, 1992). In addition, the headgroup of LPC is large relative to its hydrocarbon tail; it may therefore alter the intrinsic natural curvature of the membrane in plane. The consequence of both these effects would be to promote outward curvature (projection).

Deformation by LPC typically creates spikes and is called crenation (Bessis, 1973, p. 205). There are two distinctive forms of such projections. We used the term *echinocytes* (meaning spiculated cells) when the projections took the form of axisymmetrical cones and were regularly spaced (e.g., Fig. 3 *C*). We applied the term *acanthocytes* (meaning spiny or thorny cells) when the projections were irregular and curved, with bulbous tips (e.g., Figs. 2 *D* and 3 *H*) (see Bessis, 1973, pp. 146–152 and 207–210.) The mechanistic relationship between these two forms is considered below.

We also used micropipette aspiration to modify cell shape. The membrane in the dimple region of biconcave disks was aspirated, and the length of the ensuing membrane projections was measured as a function of aspiration pressure, from which the membrane shear modulus was calculated (Waugh and Agre, 1988). In addition, cells held at a fixed pressure for a specified length of time were released from the micropipette, and the recovery of their rest shape was determined. The residual bump height and the time and pressure taken to form it reflect the rate of plastic remodeling of surface contour (Markle et al., 1983).

RESULTS

The effect of urea on red cell contour

Using fresh blood and benign conditions maintained the control red cells in their classical morphology: uniform biconcave disks free of spontaneous spicules (Fig. 1 *A*). Incubation at 37°C in the presence of 1.5 M urea did not substantially alter their contour beyond slightly reducing the depth of the concavities in the disks (Fig. 1 *B*). However, a slight increase in the concentration of urea (to 1.7 M) caused the fragmentation of cells into microcytes of varied contour, apparently by repeated membrane fission (Fig. 1 *C*; see also Bessis, 1973, p. 157).

It seems likely that the disruptive effect of urea was on spectrin, because the isolated protein is altered by urea in this concentration range (Calvert et al., 1980; Morrow et al., 1981; Yoshino and Marchesi, 1984; di Stasi et al., 1987). Furthermore, similar transformations abruptly occur when normal red cells are warmed beyond 48°C (Ham et al., 1948), the denaturation temperature of spectrin (Yoshino and Marchesi, 1984; di Stasi et al., 1987). Similar modes of

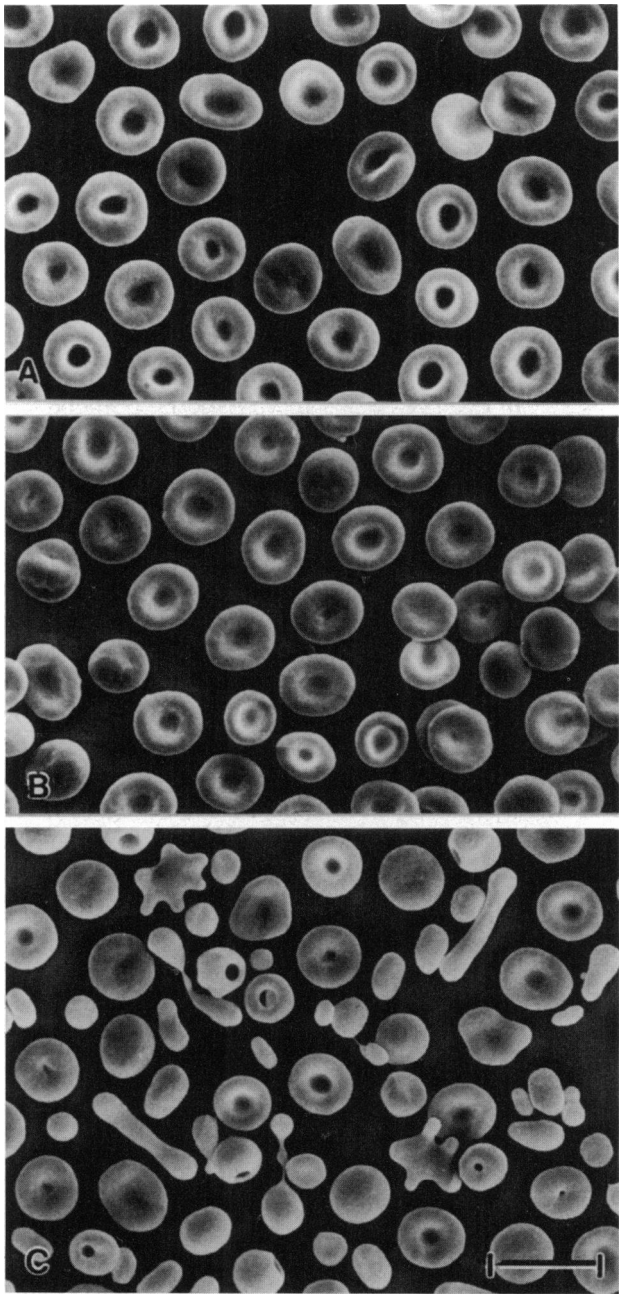

FIGURE 1 Effect of urea on erythrocyte morphology. All procedures were conducted at 0–5°C unless indicated otherwise. Blood was drawn from normal human volunteers into EDTA and used promptly (Fairbanks et al., 1971). The cells were washed three times in saline (0.15 M NaCl plus 5 mM NaP_i, pH 7.5), warmed to 37°C in saline containing 0.02% bovine serum albumin (defatted, from Sigma), and diluted in saline to give mixtures that were 5% (v/v) cells, 0.02% (w/v) bovine serum albumin, and urea at 0 (*A*), 1.5 M (*B*), or 1.7 M (*C*). After 10 min at 37°C, the mixtures were chilled on ice and fixed with 1% glutaraldehyde. The cells were washed twice with deionized water, spread on glass slides, air dried at room temperature, coated with gold-palladium (60:40), and photographed using a JEOL, JSM, 35C scanning electron microscope (Khodadad and Weinstein, 1983). Fixation and preparation for electron microscopy did not alter the morphology of the cells in this or the other figures compared to what was seen directly. Bar, 10 μm.

fragmentation are also characteristic of red cells and skeletons with defective spectrin (Palek and Lambert, 1990). Urea is not known to perturb lipid bilayers under these conditions.

The fragmentation seen in Fig. 1 *C* should not be considered a shape effect akin to those defining biconcave and spiculated contours. Rather, it is a manifestation of the mechanical instability of the expanses of bilayer that are left unsupported when the membrane skeleton fails (Lange et al., 1982b).

The effect of LPC on red cell contour

The concentration of LPC was adjusted in each experiment to achieve the desired degree of deformation. Four μM LPC usually evoked mild spiculation (i.e., a few bumps on a moderate fraction of the cells; Fig. 2 *B*), whereas 10 μM LPC caused strong spiculation (10–20 spikes visible on virtually every cell) (Fig. 4 *C*). In related studies, we determined that the heights of the projections in mild and extensive deformations were approximately 1.0 to 1.7 μm, respectively, and the corresponding diameters of the bases were approximately 2.5 and 1.0 μm, respectively.

Fig. 2 *B* also demonstrates that spicules arise in cells retaining their native concavities; thus, positive and negative curvatures can coexist (see also Bessis, 1973, pp. 148 and 209). The incidence of such compound curvature was variable; e.g., it was minimal in Fig. 3 *C*.

The effect of urea on spiculation by LPC

Fig. 2 *C* confirmed that 1.5 M urea had a minimal impact on the biconcave shape of erythrocytes. However, the urea significantly intensified the spiculation of erythrocytes by small amounts of LPC (compare Fig. 2, *B* and *D*). Furthermore, the form of many of the spicules was changed by the presence of urea from the type seen in echinocytes to the bulbous and irregular forms seen in acanthocytes. Some of the spicules in these cells still resembled those in echinocytes, as is also true in clinical conditions characterized by acanthocytosis (Lange and Steck, 1984; Khodadad et al., 1989).

Reversibility of spiculation

Erythrocytes were incubated for 10 min at 37°C with combinations of 9 μM LPC and 1.5 M urea. Then they were either fixed directly or diluted 1:20 to reverse the LPC effect while not changing the urea concentration. The undiluted preparations (Fig. 3, *left column of panels*) were comparable to those shown in Figs. 1 and 2. Dilution (*right column of panels* in Fig. 3) altered only slightly the contour of the control erythrocytes (compare panels *A* and *B*) and those incubated in urea alone (compare *E* and *F*). The dilution of cells that had been treated with LPC alone reversed their echinocytosis completely (compare *C* and *D*). However,

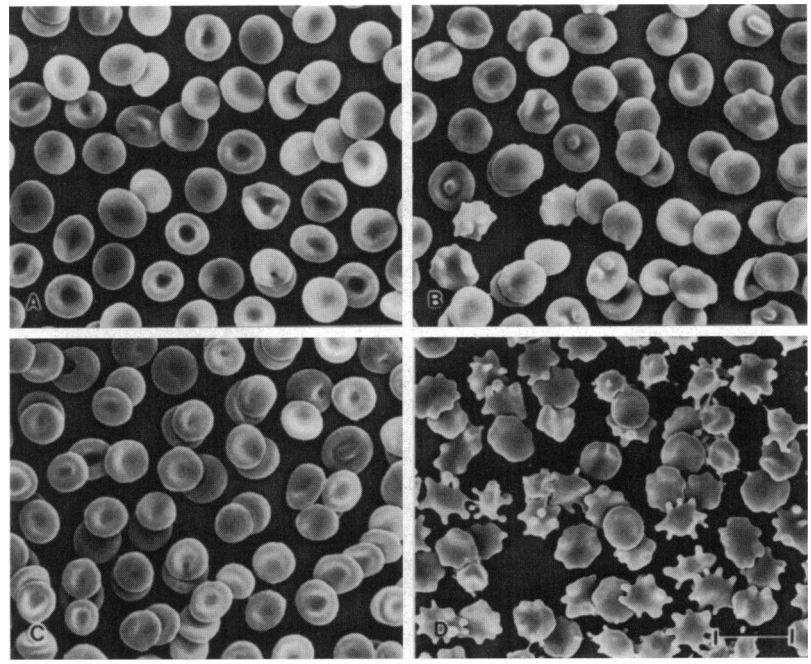

FIGURE 2 Effect of urea on red cell spiculation by LPC. Diluted red cells were warmed to 37°C for 10 min with or without a minimum concentration of egg yolk LPC (Sigma) and urea, fixed and processed as in Fig. 1. (*A*) No LPC; no urea. (*B*) 4 μM LPC; no urea. (*C*) No LPC; 1.5 M urea. (*D*) 4 μM LPC; 1.5 M urea. Bar, 10 μm.

dilution of the LPC from the acanthocytic cells induced by incubation with both LPC and urea reduced but did not eliminate their spiculation (compare *G* and *H*). We repeatedly noted that it was the axisymmetric cones (i.e., echinocytic spicules) that tended to disappear when LPC was diluted, whereas the bulbous acanthocytic spicules remained. Thus, as in naturally occurring acanthocytosis, the echinocytic spikes tended to be reversible, whereas the acanthocytic spikes tended to be irreversible (Lange and Steck, 1984).

In an alternative form of this experiment, the urea was diluted 1:20 along with the LPC (Fig. 4). Because the cells were filled with hyperosmotic urea, the suspensions were diluted slowly to prevent osmotic lysis. The results were comparable to those in Fig. 3, except that the reversible echinocytosis (Fig. 4 *C*) and irreversible acanthocytosis (Fig. 4 *H*) were far more marked in Fig. 4. This incidental finding shows that severe echinocytosis (Fig. 4) was no less reversible than the mild form (Fig. 3).

The effects of urea showed critical behavior: reducing the period of incubation, the temperatures, or the concentration of urea acutely diminished the effects described above (not shown). Experiments were therefore conducted just beyond threshold conditions to achieve maximum perturbation with minimum cell disruption; namely, 1.5 M urea at 37°C for 10 min.

Ghosts from red cells treated with LPC and urea

Red cells were treated as in Fig. 4 except that LPC was held constant during urea dilution. Membranes were then isolated. The ghosts of control erythrocytes were generally smooth, round, dimpled disk-like sacs, with some ridges or folds (Fig. 5 *A*; see also Lange et al., 1982a). Prior incubation of the parent cells with LPC did not significantly alter ghost morphology (Fig. 5 *B*); the LPC effect was presumably reversed during ghost preparation (Lange et al., 1982a). Similarly, cells treated with urea yielded ghosts with an unchanged appearance (Fig. 5 *C*). Ghosts from cells that had been incubated in the presence of both LPC and urea remained spiculated (Fig. 5 *D*), although the projections were not as numerous as in intact cells. These data support the conclusion that incubation in the presence of urea beyond a critical point caused the contour change induced by LPC to become permanent.

Skeletons from red cells treated with LPC and urea

Red cells were treated essentially as in Fig. 4, ghosts prepared as in Fig. 5, and aliquots incubated in Triton X-100 to lay bare their membrane skeletons (Yu et al., 1973; Hainfeld and Steck, 1977; Johnson et al., 1980; Lange et al., 1982b). Skeletons from the control erythrocytes were round, somewhat crumpled sacs that resembled the parent membranes (Fig. 6 *A*). Prior incubation of the cells with either LPC alone (Fig. 6 *B*) or urea alone (Fig. 6 *C*) did not impart a significant lasting alteration to the skeleton morphology; in particular, these skeletons were not spiculated. In contrast, skeletons from cells that had been incubated in the presence of both LPC and urea (Fig. 6 *D*) bore projections similar to that seen in the parent ghosts (Fig. 5 *D*); again, these deformations were not as frequent as in intact cells.

Micropipette aspiration studies

Two series of experiments were performed. In the first, cells were incubated at 37°C for 10 min in the presence and

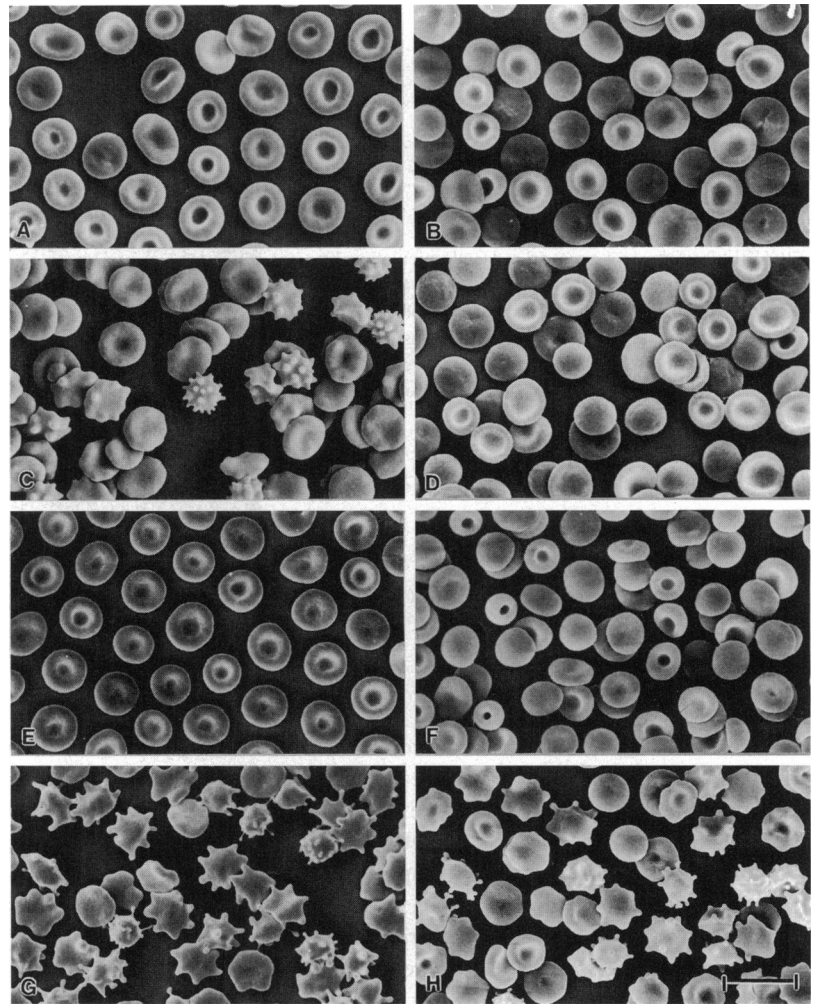

FIGURE 3 Reversal of spiculation by diluting LPC but not urea. Red cell suspensions were warmed to 37°C and incubated with LPC and urea as in Figs. 1 and 2. For the set of panels on the left (*A*, *C*, *E*, and *G*), aliquots were fixed directly for microscopy, just as in Figs. 1 and 2. For the set of panels on the right (*B*, *D*, *F*, and *H*), aliquots were diluted 20-fold with the corresponding solution lacking LPC (i.e., saline plus albumin ± 1.5 M urea) and then fixed. (*A* and *B*) No LPC; no urea. (*C* and *D*) 9 μM LPC; no urea. (*E* and *F*) No LPC; 1.5 M urea. (*G* and *H*) 9 μM LPC; 1.5 M urea. Bar, 10 μm.

absence of 1.5 M urea, then washed, resuspended in saline without urea, and probed at room temperature by micropipette aspiration. There was no difference in membrane shear modulus between these cells; these studies suggested that the effect of urea was either negligible or reversible.

In the second set of experiments, cells were suspended in buffer containing or lacking 1.5 M urea at 37°C. In the presence of urea, aspirations were begun within 3 to 5 min and repeated for up to 30 min. Membrane elasticity was essentially the same throughout this period. In urea, the mean modulus for 19 cells was 4.9 μN/m; the mean modulus of 11 cells analyzed in the absence of urea under the same conditions and with the same pipette was 6.0 μN/m. This reduction of ~18% was statistically significant at the 98% confidence level, as assessed by Student's *t*-test.

These studies were complicated by the spontaneous tendency of cells in 1.5 M urea to become progressively spiculated over prolonged periods in the micromanipulation chamber. Consequently, measurements were made only during an initial 30-min period on cells with normal shapes. A second complication was that urea increased the tendency of the aspiration deformations to persist after cells were released from the pipette (see Markle et al., 1983). To document this effect, a series of cells were each held in the pipette for a period of 90 s at constant aspiration pressure at 37°C, then released and observed for one additional minute. The deformations induced in 10 of 15 cells tested over 30 min of incubation in 1.5 M urea persisted after 1 min. Less than 10% of control cells lacking urea showed persistent deformations. It appears, therefore, that the urea treatment accelerated the rate of plastic shape transformation or creep.

In principle, this enhanced membrane remodeling in the presence of urea could mimic a reduction in the membrane elastic modulus. However, we observed a comparable reduction in the elastic modulus of urea-treated cells with and without the residual deformations induced by the pipette aspiration. We conclude that the presence of urea evoked both a reduction in membrane shear modulus and a greater propensity for residual deformations.

DISCUSSION

Reversible shape change

The red cell membrane is believed to resist deformation by storing elastic energy in at least four modes: a) the revers-

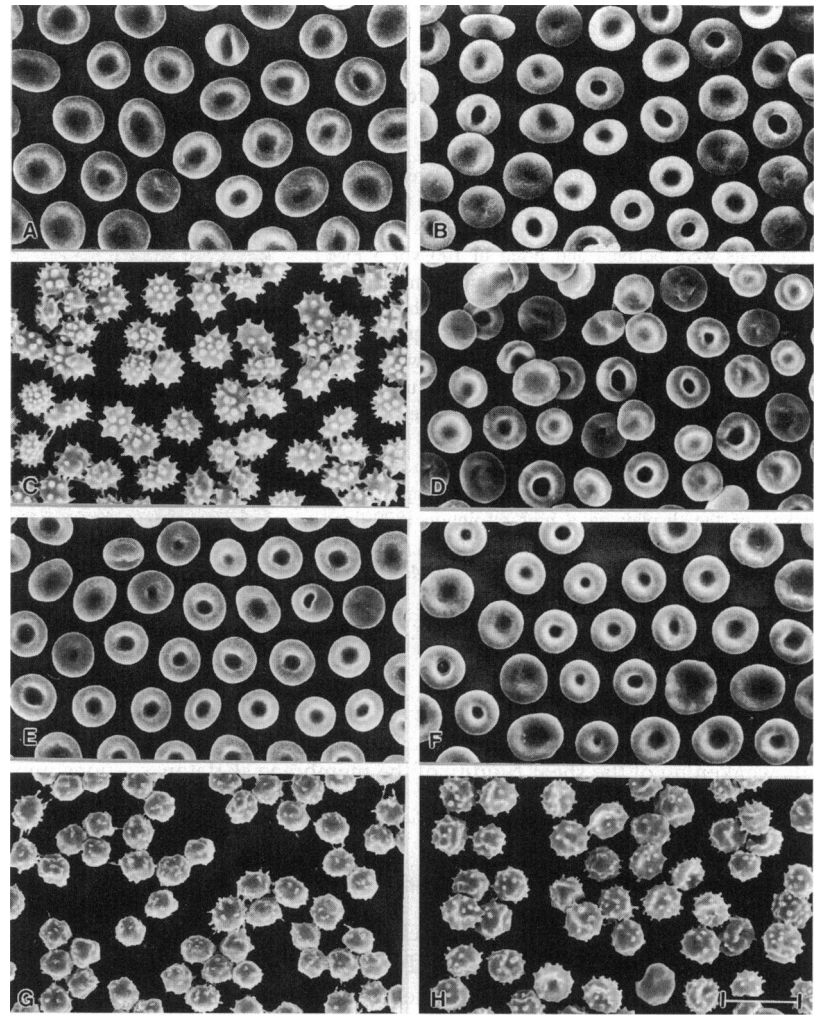

FIGURE 4 Reversal of spiculation by diluting LPC and urea. This experiment was identical to that shown in Fig. 3 except that dilution was performed stepwise on ice over ~5 min by adding small increments of the respective incubation solutions lacking urea (*right-hand panels*). This minimized the osmotic lysis of the cells containing urea. (*A* and *B*) No LPC; no urea. (*C* and *D*) 9 μM LPC; no urea. (*E* and *F*) No LPC; 1.5 M urea. (*G* and *H*) 9 μM LPC; 1.5 M urea. Bar, 10 μm.

ible extension and compression of the membrane skeleton; b) the reversible extension and compression of the membrane bilayer as a whole; c) the local curvature elasticity of each leaflet of the bilayer; and d) the nonlocal bending elasticity of the bilayer couple (Evans, 1980; Mohandas and Evans, 1994). For each of these, there is a resting or reference state with zero energy (i.e., zero net tension). During deformation, energy is stored in each mode, depending on its elastic modulus and on the magnitude of the geometric displacement from the reference state.

It is not known whether the skeleton in the resting red cell is free of tension or is under tension balanced against that in other solid elements (Stokke et al., 1986; Kozlov and Markin, 1987). In the latter case, decreasing the shear rigidity of the skeleton could alter the contour of the membrane. However, the results presented above showed that a reduction in membrane shear rigidity did not lead to such a shape change.

These data therefore suggest that the membrane skeleton is under no appreciable tension in situ. This conclusion is also supported by the weak elasticity of isolated membrane skeletons (Svoboda et al., 1992; Schmidt et al., 1993) and by fact that their contours often resemble those of native biconcave disks (e.g., Fig. 6; see also Johnson et al., 1980; Lange et al., 1982b). Presumably the long period of red cell maturation in vivo permits skeletons to dissipate shear tensions by remodeling to the shape of the cell (Steck, 1989).

Despite the absence of an effect on resting cell shape, urea nevertheless potentiated shape deformation by chemical and physical agents (e.g., Fig. 2 *D*). That a mere 18% decrease in membrane shear elasticity might underly such effects can be understood as follows. The dependence of red cell crenation upon LPC concentration is sigmoidal (i.e., shows a threshold); we routinely chose LPC concentrations at (e.g., Fig. 2) or just above (e.g., Fig. 4) this critical point. Model calculations predict that bilayer couple (area differential) effects drive spicule formation in a highly nonlinear fashion (Waugh, 1995). Specifically, these calculations predict that the areas of the two leaflets can differ by 1–2% without a visible change in membrane geometry, but that a small further increment in this differential can provoke a large shape change. The model also predicts that a small reduction in the shear modulus of the membrane (skeleton) can promote marked shape transformations in cells near their threshold. It is therefore plausible that the observed potentiation of induced shape change by urea resulted from

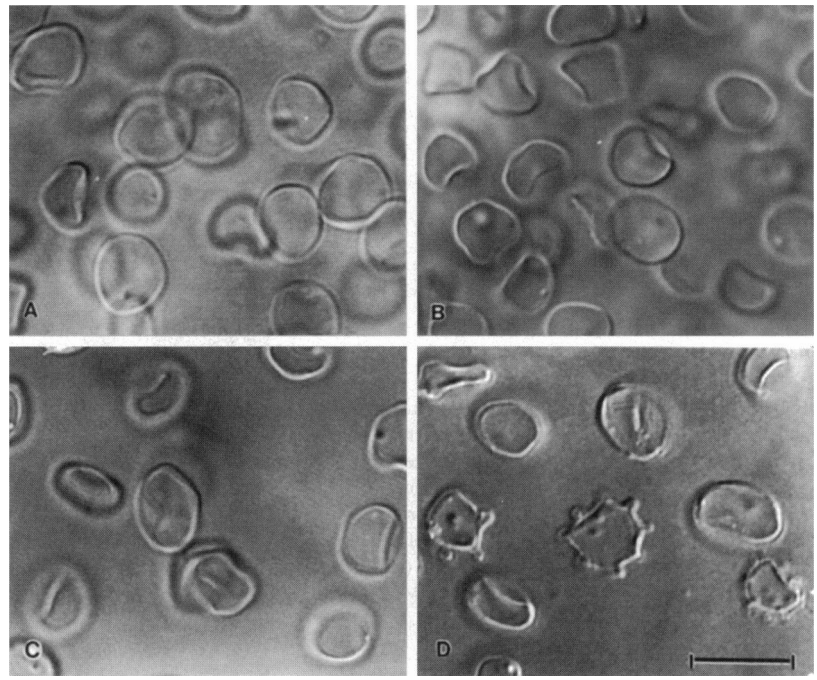

FIGURE 5 Spiculation of ghosts from red cells incubated with LPC and urea. Red cells (10% v/v) were suspended in 0.02% bovine serum albumin ± 10 μM LPC ± 1.5 M urea at 37°C, as in Fig. 1. After 10 min, each sample was slowly diluted tenfold with the corresponding incubation solution lacking urea, as in Fig. 4. The cells were pelleted and lysed in 100 vol 5 mM sodium phosphate (pH 8.0), and the ghost membranes were pelleted (Fairbanks et al., 1971). The ghosts were resuspended in the lysis buffer and the aliquots were saved for Fig. 6. The remaining portions were fixed with 1% glutaraldehyde and visualized by using Nomarski interference contrast microscopy (Zeiss Photo Microscope II). Images were acquired with a Hamamatsu Newvicon camera C2400 and an Argus 10 image processing computer, and photographs were taken of the display on a high-resolution (800-line) video monitor. (*A*) No LPC; no urea. (*B*) 10 μM LPC; no urea. (*C*) No LPC; 1.5 M urea. (*D*) 10 μM LPC; 1.5 M urea. Bar, 10 μm.

its weakening of the shear rigidity of the membrane skeleton near a threshold point.

Irreversible shape change in urea

The reversible spiculation of red cells by LPC was made irreversible by the presence of urea (Fig. 3 and 4). Similarly, urea treatment promoted the persistence of the projections imposed by micropipette aspiration. The most likely explanation of these effects is that urea enhanced the irreversible remodeling of the membrane skeletons to match the deformations imposed on the contour of the overlying membranes. In particular, the weakening of the linkages in the skeleton network by urea could accelerate its stress relaxation (Markle et al., 1983). The 10-min time interval and elevated temperature critical to making deformations irreversible would then reflect the kinetic requirements for the intermolecular rearrangements underlying stress relaxation. This hypothesis is supported by the persistence of residual deformations in ghosts and membrane skeletons isolated from cells treated with a combination of urea and LPC (Figs. 5 and 6). Similarly, the urea-dependent transformation of the contour of spicules from the echinocytic

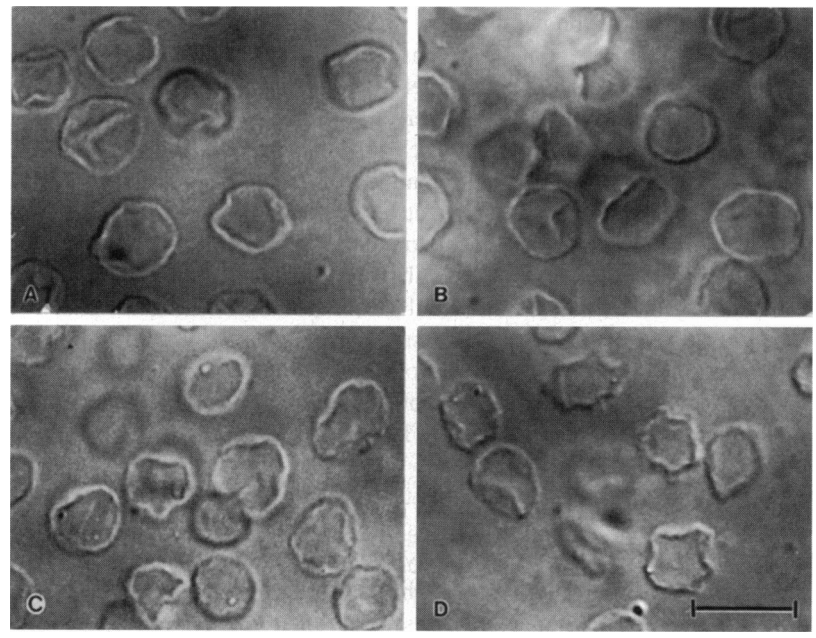

FIGURE 6 Spiculation of skeletons from red cells incubated with LPC and urea. Aliquots of the unfixed ghosts from the experiment shown in Fig. 5 were incubated with 2% Triton X-100 for 1 min on ice, then fixed as above, and photographed as in Fig. 5. (*A*) No LPC; no urea. (*B*) 10 μM LPC; no urea. (*C*) No LPC; 1.5 M urea. (*D*) 10 μM LPC; 1.5 M urea. Bar, 10 μm.

to the acanthocytic form (compare Fig. 3, *C* and *H*) might represent plastic remodeling of the membrane skeleton.

The fact that spiculated forms persist after the LPC or the micropipette is removed presumably signifies that the stiffness of the skeletons, although softened by urea, is sufficient to oppose the recovery of native cell contour driven by bilayer elasticity. However, other factors might also contribute to the promotion of irreversible projections by urea, as follows:

a) Urea could stiffen the membrane skeleton. Indeed, it has been observed that urea (Fischer, 1989) or warming above the denaturation temperature of spectrin (Rakow and Hochmuth, 1975) increases the shear rigidity of red cells. However, under our conditions, the membrane shear modulus was actually reduced by urea. Furthermore, membrane stiffness was not significantly increased in spiculated cells which arose spontaneously in preliminary micropipette aspiration experiments.

b) Urea might exert a bias toward outward membrane curvature and thereby potentiate deformations by LPC and micropipette aspiration. This influence would have to be subthreshold, because no shape change is observed with urea alone (Fig. 2). Furthermore, the induced deformations persisted in red cells, ghosts, and membrane skeletons after the urea was gone (Figs. 4, 5, and 6).

c) Urea might affect the transport or distribution of lipid molecules across the membrane, thereby altering bilayer couple tension. The site of action could be membrane lipid flipases (Devaux and Zachowski, 1994). Such perturbations might also account for the spiculation of cells exposed to urea at 37°C for an extended time. Again, such an effect would have to be subthreshold because urea alone did not affect red cell shape.

d) Persistent projections might also be seen if the urea treatment prevented complete washout of the LPC. While the driving force for spiculation might be reduced to a subthreshold level in such a case, the net elastic energy of the bilayer might be insufficient to overcome the shear elasticity of the skeleton, which presumably would have relaxed to assume the spiculated shape.

We cannot be certain about the site of action of either LPC or urea. Nevertheless, there is no evidence that LPC acts on spectrin or another membrane protein as opposed to bilayers. Similarly, 1.5 M urea is not known to affect lipid bilayers but does perturb spectrin. Urea might also act on other membrane proteins or on the linkage of the skeleton to the membrane proper. However, modulation of the strength of the interaction of the skeleton with the membrane should not influence the generation of echinocytes, because the bending resistance of and tension in the skeleton are negligible. In contrast, alteration of skeleton-to-membrane linkages could contribute to irreversible creep and the formation of acanthocytic forms.

CONCLUSIONS

Our findings document that urea alters the mechanical properties of the red cell membrane, reducing its shear rigidity and accelerating the rate at which the membrane and its skeleton adapt irreversibly to an imposed deformation. That such urea treatment does not affect the resting geometry of the cell supports the view that skeleton elasticity is not dominant in defining resting cell shape; rather, it helps to maintain the resting shape against deformation (Steck, 1989). Finally, our results concur with model calculations which predict that small changes in membrane shear elasticity can lead to dramatic changes in the geometry of cells near a shape change threshold (Waugh, 1995).

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REFERENCES

Bennett, V. 1989. The spectrin-actin junction of erythrocyte membrane skeletons. *Biochim. Biophys. Acta.* 988:107–121.

Bennett, V. 1990. Spectrin-based membrane skeleton: a multipotential adaptor between plasma membrane and cytoplasm. *Physiol. Rev.* 70: 1029–1065.

Bessis, M. 1973. Living Blood Cells and Their Ultrastructure. Springer-Verlag, New York.

Calvert, R., E. Ungewickell, and W. Gratzer. 1980. A conformational study of human spectrin. *Eur. J. Biochem.* 107:363–367.

Canham, P. B. 1970. The minimum energy of bending as a possible explanation of the biconcave shape of the human red blood cell. *J. Theor. Biol.* 26:61–81.

Chasis, J. A., and S. B. Shohet. 1987. Red cell biochemical anatomy and membrane properties. *Annu. Rev. Physiol.* 49:237–248.

Chien, S. 1987. Red cell deformability and its relevance to blood flow. *Annu. Rev. Physiol.* 49:177–192.

Christiansson, A., F. A. Kuypers, B. Roelofsen, J. A. F. Op Den Kamp, and L. L. M. Van Deenen. 1985. Lipid molecular shape affects erythrocyte morphology: a study involving replacement of native phosphatidylcholine with different species followed by treatment of cells with sphingomyelinase C or phospholipase A_2. *J. Cell Biol.* 101:1455–1462.

Devaux, P. F., and A. Zachowski. 1994. Maintenance and consequences of membrane phospholipid asymmetry. *Chem. Phys. Lipids.* 73:107–120.

di Stasi, A. M. M., T. C. Petrucci, and M. Minetti. 1987. Comparison of thermal properties of bovine spectrin and fodrin. *Arch. Biochem. Biophys.* 256:144–149.

Discher, D. E., N. Mohandas, and E. A. Evans. 1994. Molecular maps of red cell deformation: hidden elasticity and in situ connectivity. *Science.* 266:1032–1035.

Elgsaeter, A., B. T. Stokke, A. Mikkelsen, and D. Branton. 1986. The molecular basis of erythrocyte shape. *Science.* 234:1217–1223.

Evans, E. A. 1974. Bending resistance and chemically induced moments in membrane bilayers. *Biophys. J.* 14:923–931.

Evans, E. A. 1980. Minimum energy analysis of membrane deformation applied to pipet aspiration and surface adhesion of red blood cells. *Biophys. J.* 30:265–284.

Fairbanks, G., T. L. Steck, and D. F. H. Wallach. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry.* 10:2606–2617.

Farge, E., and P. F. Devaux. 1992. Shape changes of giant liposomes induced by an asymmetric transmembrane distribution of phospholipids. *Biophys. J.* 61:347–357.

Fischer, T. M. 1989. Cross bonding and stiffening of the red cell membrane. *Biochim. Biophys. Acta.* 985:218–228.

Grebe, R., and H. Schmid-Schonbein. 1990. Closed fluid quadrilamina model of the erythrocyte membrane. *In* Biomechanical Transport Processes. F. Mosora, D. G. Caro, E. Krause, H. Schmid-Schonbein, C. Baquey, and R. Pelissier, editors. Plenum Press, New York. 223–233.

Hainfeld, J. F., and T. L. Steck. 1977. The sub-membrane reticulum of the human erythrocyte: a scanning electron microscope study. *J. Supramol. Struct.* 6:301–311.

Ham, T. H., S. C. Shen, E. M. Fleming, and W. B. Castle. 1948. Studies on the destruction of red blood cells. IV. Thermal injury. *Blood.* 3:373–403.

Hochmuth, R. M., and R. E. Waugh. 1987. Erythrocyte membrane elasticity and viscosity. *Annu. Rev. Physiol.* 49:209–219.

Johnson, R. M., G. Taylor, and D. B. Meyer. 1980. Shape and volume changes in erythrocyte ghosts and spectrin-actin networks. *J. Cell Biol.* 86:371–376.

Khodadad, J. K., and R. S. Weinstein. 1983. The band 3-rich membrane of llama erythrocytes: studies on cell shape and the organization of membrane proteins. *J. Membr. Biol.* 72:161–171.

Khodadad, J. K., R. S. Weinstein, L. W. Marsh, and T. L. Steck. 1989. Shape determinants of McLeod acanthocytes. *J. Membr. Biol.* 107: 213–218.

Kozlov, M. M., and Markin, V. S. 1987. Membrane skeleton model of a red blood cell: electrical and mechanical properties. *J. Theor. Biol.* 129: 439–452.

Lange, Y., A. Gough, and T. L. Steck. 1982a. Role of the bilayer in the shape of the isolated erythrocyte membrane. *J. Membr. Biol.* 69: 113–123.

Lange, Y., R. A. Hadesman, and T. L. Steck. 1982b. Role of the reticulum in the stability and shape of the isolated human erythrocyte membrane. *J. Cell Biol.* 92:714–721.

Lange, Y., and T. L. Steck. 1984. Mechanism of red blood cell acanthocytosis and echinocytosis in vivo. *J. Membr. Biol.* 77:153–159.

Liu, S-C., L. H. Derick, M. A. Duquette, and J. Palek. 1989. Separation of the lipid bilayer from the membrane skeleton during discocyte-echinocyte transformation of human erythrocyte ghosts. *Eur. J. Cell Biol.* 49:358–365.

Lux, S. E., K. M. John, and M. J. Karnovsky. 1976. Irreversible deformation of the spectrin-actin lattice in irreversibly sickled cells. *J. Clin. Invest.* 58:955–963.

Markle, D. R., E. A. Evans, and R. M. Hochmuth. 1983. Force relaxation and permanent deformation of erythrocyte membrane. *Biophys. J.* 42: 91–98.

Mohandas, N., and E. Evans. 1994. Mechanical properties of the red cell membrane in relation to molecular structure and genetic defects. *Annu. Rev. Biophys. Biomol. Struct.* 23:787–818.

Morrow, J. S., W. B. Haigh, and V. T. Marchesi. 1981. Spectrin oligomers: a structural feature of the erythrocyte cytoskeleton. *J. Supramol. Struct. Cell. Biochem.* 17:275–287.

Palek, J., and S. Lambert. 1990. Genetics of the red cell membrane skeleton. *Semin. Hematol.* 27:290–332.

Peterson, M. A., H. Strey, and E. Sackmann. 1992. Theoretical and phase contrast microscope eigenmode analysis of erythrocyte flicker: amplitudes. *J. Physique II France.* 2:1273–1285.

Rakow, A. L., and Hochmuth, R. M. 1975. Effect of heat treatment on the elasticity of human erythrocyte membrane. *Biophys. J.* 15:1095–1100.

Schmidt, C. F., K. Svoboda, N. Lei, I. B. Petsche, L. E. Berman, C. R. Safinya, and G. S. Grest. 1993. Existence of a flat phase in red cell membrane skeletons. *Science.* 259:952–955.

Sha'afi, R. I. 1977. Water and small nonelectrolyte permeation in red cells. *In* Membrane Transport in Red Cells. J. C. Ellory and V. L. Lew, editors. Academic Press, New York. 221–256.

Sheetz, M. P., and S. J. Singer. 1974. Biological membranes as bilayer couples. A molecular mechanism of drug-erythrocyte interactions. *Proc. Natl. Acad. Sci. USA.* 71:4457–4461.

Steck, T. L. 1989. Red cell shape. *In* Cell Shape: Determinants, Regulation, and Regulatory Role. W. D. Stein and F. Bronner, editors. Academic Press, New York. 205–246.

Stokke, B. T., A. Mikkelsen, and A. Elgsaeter. 1986. The human erythrocyte membrane skeleton may be an ionic gel. II. Numerical analysis of cell shapes and shape transformations. *Eur. Biophys. J.* 13:219–233.

Svetina, S., and B. Zeks. 1989. Membrane bending energy and shape determination of phospholipid vesicles and red blood cells. *Eur. Biophys. J.* 17:101–111.

Svoboda, K., C. F. Schmidt, D. Branton, and S. M. Block. 1992. Conformation and elasticity of the isolated red cell membrane skeleton. *Biophys. J.* 63:784–793.

Vertessy, B. G., and T. L. Steck. 1989. Elasticity of the human red cell membrane skeleton: effects of temperature and denaturants. *Biophys. J.* 55:255–262.

Waugh, R. E. 1996. Elastic energy of curvature-driven bump formation on red blood cell membrane. *Biophys. J.* 70. In press.

Waugh, R., and P. Agre. 1988. Reductions of erythrocyte membrane viscoelastic coefficients reflect spectrin deficiencies in hereditary spherocytosis. *J. Clin. Invest.* 81:133–141.

Yoshino, H., and V. T. Marchesi. 1984. Isolation of spectrin subunits and reassociation in vitro. Analysis by fluorescence polarization. *J. Biol. Chem.* 259:4496–4500.

Yu, J., D. A. Fischman, and T. L. Steck. 1973. Selective solubilization of proteins and phospholipids from red blood cell membranes by nonionic detergents. *J. Supramol. Struct.* 1:233–248.