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Role of the Bilayer in the Shape of the Isolated Erythrocyte Membrane

Yvonne Lange, Alice Gough, and Theodore L. Steck

Biophysics Section, Boston University School of Medicine, Boston, Massachusetts 02118, and
Department of Biochemistry, University of Chicago, Chicago, Illinois 60637

Summary. The determinants of cell shape were explored in a study of the crenation (spiculation) of the isolated erythrocyte membrane. Standard ghosts prepared in 5 mM NaP_i (pH 8) were plump, dimpled disks even when prepared from echinocytic (spiculated) red cells. These ghosts became crenated in the presence of isotonic saline, millimolar levels of divalent cations, 1 mM 2,4-dinitrophenol or 0.1 mM lysolecithin. Crenation was suppressed in ghosts generated under conditions of minimal osmotic stress, in ghosts from red cells partially depleted of cholesterol, and, paradoxically, in ghosts from red cells crenated by lysolecithin. The susceptibility of ghosts to crenation was lost with time; this process was potentiated by elevated temperature, low ionic strength, and traces of detergents or chlorpromazine.

In that ghost shape was influenced by a variety of amphipaths, our results favor the premise that the bilayer and not the subjacent protein reticulum drives ghost crenation. The data also suggest that vigorous osmotic hemolysis induces a redistribution of lipids between the two leaflets of the bilayer which affects membrane contour through a bilayer couple mechanism. Subsequent relaxation of that metastable distribution could account for the observed loss of crenatability.

Key words shape · bilayer · membrane · amphipaths · contour · erythrocyte

Introduction

A difference in the areas of the two leaflets of a bilayer can determine membrane curvature by what has been called a bilayer couple mechanism [13, 16, 36]. The contour of the human erythrocyte, a biconcave disk, corresponds to a difference of less than 0.5% between the areas of the two membrane surfaces; abnormal red cell shapes should arise if this small differential were either increased or decreased by an even smaller amount [3]. The shape of the human erythrocyte has been mathematically modeled, assuming small differences between the areas of the two membrane surfaces and the known surface-to-volume ratio of the cell [14].

The erythrocyte assumes a variety of abnormal shapes under pathological and experimental conditions [5]. Anionic amphipaths such as 2,4-dinitrophenylate induce evagination of the cell surface (echinocytosis or crenation), while cationic amphipaths such as chlorpromazine cause invagination and endocytosis [15]. These two classes of agents are antagonistic, so that cell shape seems to reflect a balance between them [15, 35]. Since the red cell bilayer is asymmetrical [8, 32], it has been suggested that the charged amphipaths partition electrostatically, so that anionic species preferentially enter and expand the outer (neutral) leaflet and cationic species, the inner (anionic) leaflet [36]. More generally, the differential accumulation of any charged or neutral amphipath in either leaflet in response to either a kinetic (trapping) or an equilibrium mechanism may alter cell contour [2, 29, 30, 35, 37].

Despite the cogency of this body of thought, there is currently a prevailing opinion that it is the submembrane reticulum of spectrin and actin that plays the dominant role in determining red cell membrane countour [4, 6, 7, 21, 28, 31]. Furthermore, Conrad and Singer [11, 12] found negligible uptake of dinitrophenol and chlopromazine in red cell membranes and have cast doubt on the bilayer couple hypothesis. The role of the lipid bilayer in the determination of red cell contour is thus presently uncertain.

The isolated erythrocyte ghost is a system of choice for the detailed exploration of the determinants of cell shape, since it is well defined in its composition, structure, and function and shows variations in contour which closely resemble those of the intact cell [6, 20, 21, 23, 28, 33–36]. Our studies of ghosts addressed several unanswered questions: Why do crenated red cells yield uncrenated ghosts? Why is it that ghosts but not red cells crenate in isotonic saline? Why do modest cholesterol depletion and gentle hemolysis suppress the crenation of ghosts? Why does lysolecithin induce eryth-

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rocyte and ghost crenation under some conditions and abolish it under others? Our results support a simple hypothesis which accounts for all ghost crenation data thus far reported: we propose that the balance of lipids in the bilayer can be driven into a new and metastable distribution by hemolysis which then slowly relaxes toward its original or some other low energy state. These small net shifts determine membrane contour by a bilayer couple mechanism.

Materials and Methods

Materials

Human blood from normal donors was obtained either fresh or from outdated units generously provided by the University of Chicago Blood Bank. The age of the blood did not have a demonstrable effect on our results, although outdated cells were frequently mildly crenated.

Egg lecithin (grade I) was purchased from Lipid Products (Redhill, Surrey, U.K.). 2,4-Dinitrophenol, egg lysolecithin, chlorpromazine-HCl, and all other biochemicals were purchased from Sigma. (Chlorpromazine is of limited solubility in alkaline media, and 1–5 mM stock solutions were therefore prepared fresh in a weakly acidic solution.) L-1-[palmitoyl-1-^{14}C]-lysopalmitoylphosphatidylcholine was obtained from New England Nuclear. All chemicals were of reagent grade or better from Fisher, Mallinckrodt or Baker.

Methods

All procedures were conducted at 0 °C (in ice-water) at pH 8.0, and all centrifugations were for 15 min in a Sorvall SS-34 rotor at 15,000 rpm unless specified otherwise. Other procedures were as described previously [23].

Erythrocytes were prepared in isotonic saline (150 mM NaCl – 5 mM NaP_i) and ghosts in 5 mM NaP_i according to Fairbanks et al. [18]. The basic crenation experiment was as follows. Freshly prepared ghosts were suspended in 7 or 8 vol of buffer at 0–37 °C for a defined period. The ghosts then were fixed on ice for 15 min by the addition of 10% glutaraldehyde to 1% final. The ghosts were examined and photographed under dark-field illumination at 1000-fold magnification.

Three parameters were explored: (i) The crenatability of fresh, standard ghosts in various buffers. (ii) The effect of the preparative procedure on the crenatability of ghosts. Here red cells were lysed under various conditions, the ghosts treated to induce crenation and examined immediately. Further washing was avoided since it can affect crenation. Fixation was omitted in this set of experiments because crosslinking of the abundant hemoglobin present created intractable gels. (iii) The loss of crenatability was examined by preincubating fresh ghosts under various conditions before testing for crenation.

The extent of crenation was scored by analogy with the grading of red cell echinocytosis by Bessis [5].

Results and Discussion

This study examines a variety of factors affecting the curvature of the red cell membrane. In each case, we shall suggest a simple interpretation derived from theories linking differences in the surface areas of the two lipid leaflets in the bilayer to its contour.

Effect of Electrolytes on Membrane Contour

Ghosts, freshly prepared in a low ionic strength buffer like 5 mM NaP_i, were not biconcave disks

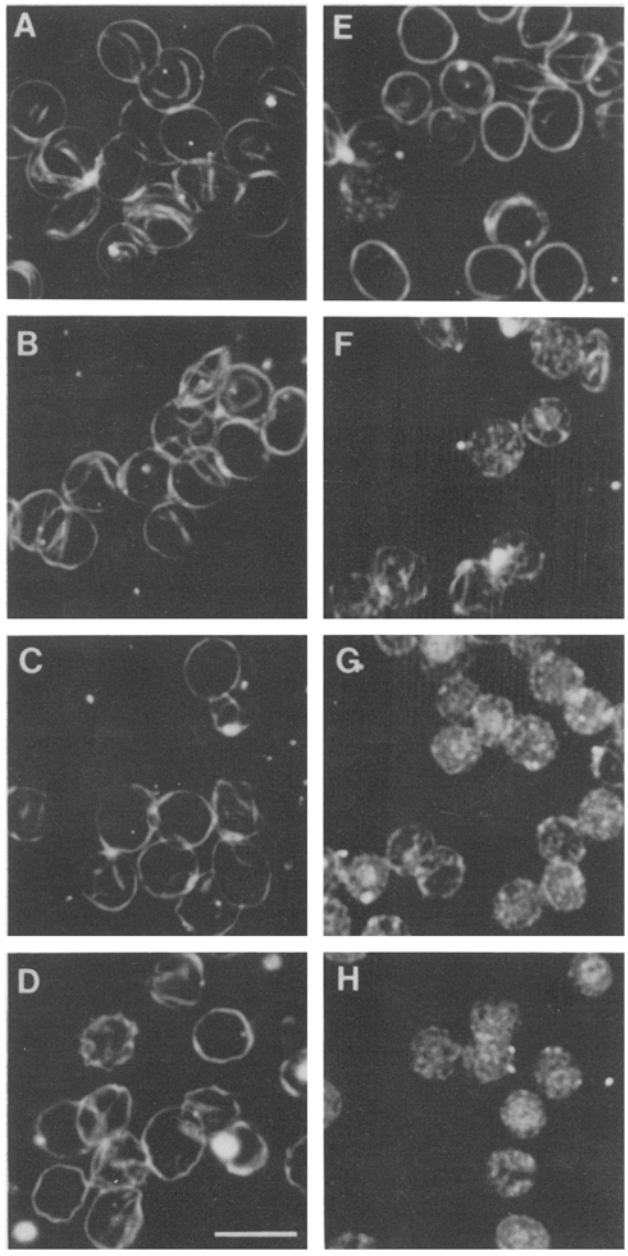

Fig. 1. Synergy between salt and lysolecithin. Packed ghosts, freshly prepared in 5 mM NaP_i (pH 8), were suspended at 0 °C in 7 vol of the phosphate buffers (pH 8.0) indicated below plus 1 vol water (*A–D*) or 1 mM lysolecithin (0.11 mM final; *E–H*). After a 5-min incubation on ice, 1 vol of 10% glutaraldehyde was added. After 15 min at 0 °C, the specimens were photographed by darkfield microscopy. (*A*) and (*E*), 5 mM; (*B*) and (*F*), 10 mM; (*C*) and (*G*), 15 mM; (*D*) and (*H*), 20 mM. Calibration bar = 10 μm

but rather plump bowls with a single shallow dimple (e.g., Fig. 1 *A*). From the point of view of the bilayer couple hypothesis, the change in contour from biconcave to monoconcave suggests that a relative increase in the surface area of the outer leaflet of the bilayer accompanies hemolysis.

As observed elsewhere [18, 20, 21, 34, 35], transfer to high ionic strength solutions converted the ghosts to shriveled spheres covered with spikes (e.g., Fig. 6 *A*). Uncharged molecules such as sucrose did not cause or alter crenation, although they can shrink (flatten) ghosts by a transient osmotic force. The first signs of crenation were the disappearance of the dimple and the conversion of the smooth surface of the ghost to an irregular, roughly-faceted contour (*see* Fig. 1 *A–D*). More intense crenation involved an increasing number of spicules and a corresponding decrease in ghost diameter (e.g., Fig. 1 *F–H*). This progression runs parallel to the echinocytosis of intact red cells [5]; we have no reason to doubt that the fundamental mechanisms underlying red cell echinocytosis and ghost crenation are the same.

A plausible interpretation of salt-dependent ghost crenation is that increasing the cation concentration in the medium causes a differential contraction of the more highly-charged cytoplasmic leaflet, thus bending the bilayer outward [20, 26, 34]. The fact that ghosts but not cells are crenated in physiologic saline supports the premise that hemolysis has caused a relative increase in the area of outer leaflet of the bilayer. At low ionic strength, this outer surface excess is compensated by the preferential electrostatic expansion of the inner leaflet (producing smooth ghosts), but at high ionic strength it drives crenation.

Ghost crenation increased with salt concentration in a sigmoid fashion. For example, in experiments such as that shown in Fig. 1, crenation was scant in 20 mM NaP_i (Fig. 1 *D*) but was maximal at 50 mM (not shown). Calcium and magnesium ions were far more potent than Na^+ or K^+, causing crenation at the millimolar level. The species of anion seemed indifferent, suggesting that crenation involved the neutralization of negatively-charged sites on the membrane by cations. Consistent with this hypothesis was the enhancement of crenation at pH 6 compared to pH 8.

These data lead to the prediction that membrane evagination would occur through an electrostatic mechanism if the ionic strength at the outer surface were made much lower than that at the

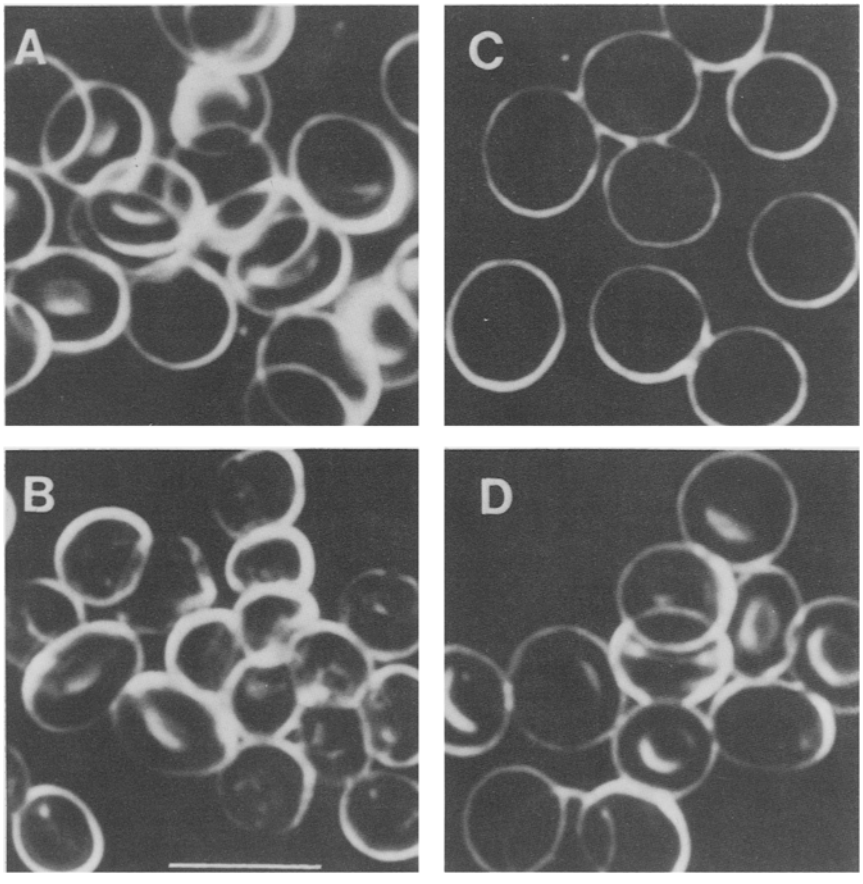

Fig. 2. Effect of ionic strength on erythrocyte shape. Freshly-drawn red cells were washed thrice in 0.15 M NaCl–5 mM NaP_i (pH 8), then once more in the same buffer or in 310 mM sucrose. The cells were pelleted and resuspended in 8 vol of the same media containing or lacking 0.1 mM chlorpromazine. After 15 min at 0 °C, 1 vol of 10% glutaraldehyde was added and the fixed specimens photographed by dark-field microscopy. (*A*): Saline alone. (*B*): Saline plus chlorpromazine. (*C*): Sucrose alone. (*D*): Sucrose plus chlorpromazine. Calibration bar = 10 μm

cytoplasmic surface, since the extracellular face also bears a substantial net negative charge. Such an experiment is straightforward in red cells, since they are highly impermeable to salts. As shown in Fig. 2 (*A* and *C*) substituting 300 mM sucrose for saline caused red cells to lose their dimples. This increase in outward curvature is not the result of cell swelling, since no increase in volume was detected in fixed cells by the Coulter Channelyzer. More dramatically, isosmotic sucrose solutions suppressed the pocking of the membrane by chlorpromazine (Fig. 2, *compare B* and *D*). These results are an illustration of the prediction of Deuling and Helfrich [14] that an asymmetry in the *environment* of a membrane can determine curvature.

Ghost crenation initially was reversible: spiculated shapes alternated with smooth as saline was added and removed in successive wash cycles. However, the capacity of ghosts to crenate was gradually lost, as detailed below. Crenation was not instantaneous, but emerged without a lag over a period of several seconds following the addition of salt. The rate of crenation increased with both salt concentration and temperature. For example, in one experiment at 0 °C, the half-time of crenation in 20 mM NaCl − 5 mM NaP_i was approximately 45 sec, while increasing the NaCl to 50 mM reduced the half-time to about 5 sec.

Effect of Amphipaths on Ghost Crenation

As previously observed [36], crenation of ghosts was evoked by amphipaths which induce echinocytosis in intact erythrocytes; e.g., 1 mM 2,4-dinitrophenol and 0.1 mM lysolecithin (Fig. 1). Dinitrophenolate presumably equilibrates across the bilayer in favor of the outer surface, while lysolecithin has been shown to be kinetically trapped at the surface of insertion because of its slow rate of transmembrane movement [30]. It is a good hypothesis that both act through a bilayer couple mechanism.

A novel finding was that crenation induced by both amphipaths was highly salt-dependent. For example, crenation by 0.1 mM lysolecithin was undetectable in 5 mM NaP_i but was maximal in 15 mM NaP_i(Fig. 1). A similar potentiation of the action of amphipaths was observed by adding 0.1–1.0 mM $MgSO_4$ to the 5 mM NaP_i. Cations and amphipaths potentiated each other and were synergistic; i.e., crenation was maximal in mixtures of the two where the omission of either abolished the effect entirely (Fig. 1). These results support the premise that the amphipaths serve to expand the outer leaflet while the elevation of the cation concentration contracts the cytoplasmic leaflet.

Capacitation of Ghosts for Crenation

The capacity of ghosts to crenate depended on how they were prepared. In particular, the acquisition of crenatability was blocked by minimizing the osmotic stress attending hemolysis, as follows. Ghosts prepared in water or 5 mM NaP_i were strongly crenated when suspended in 150 mM NH_4HCO_3, but ghosts formed by hemolysis in 150 mM NH_4HCO_3 were globoid rather than discoid and were scarcely more scalloped than the control at low ionic strength (Fig. 3 *A–C*). (Cells lyse osmotically in 150 mM NH_4HCO_3 because this electrolyte is in equilibrium with the uncharged species, NH_3 plus CO_2, which freely equilibrate across the membrane to reform NH_4HCO_3 inside the cell [19]). The more dilute the NH_4HCO_3 hemolysis medium, the stronger the ghost crenation when the NH_4HCO_3 was subsequently restored to 150 mM. Ghosts lysed in 150 mM NH_4HCO_3 did not crenate when 1 mM 2,4-dinitrophenol was added, although this medium caused strong crenation of standard ghosts prepared in 5 mM NaP_i.

We also have shown that the addition of 150 mM NH_4HCO_3 or 150 mM NaCl immediately after hemolysis in 5 mM NaP_i elicited only weak crenation; the longer the freshly-lysed membranes were incubated at low ionic strength over a period of a few minutes at 0 °C, the more they crenated when isotonicity was restored. Hemolysis in 20–50 mM $MgSO_4$ generated uncrenated ghosts even though 1 mM $MgSO_4$ or more caused strong crenation of ghosts prepared in 5 mM NaP_i (not shown). Thus, conditions which themselves induce crenation of standard ghosts suppress crenation if present at or shortly after the time of hemolysis, presumably because they suppress a time-dependent process of capacitation following hemolysis.

A similar phenomenon was observed in ghosts prepared by hemolysis in 300 mM glycerol. Glycerol was chosen because it penetrates the membrane slowly, leading to gradual swelling and gentle osmotic lysis. Ghosts prepared in this medium were globoid and shrunken but were not crenated in the presence of 150 mM NaCl (Fig. 3 *D*). In control experiments, ghosts prepared in 5 mM NaP_i and then suspended in 150 mM NaCl plus 300 mM glycerol (i.e., the same final medium as above) crenated strongly. When ghosts generated by hemolysis in 150 mM NH_4HCO_3 or 300 mM glycerol were washed twice in 5 mM NaP_i, they regained sensitivity to crenating agents.

We take these data to suggest that lipids may shift between the two leaflets in a bilayer during or shortly after hemolysis so as to affect the response of ghosts to standard crenating treatments.

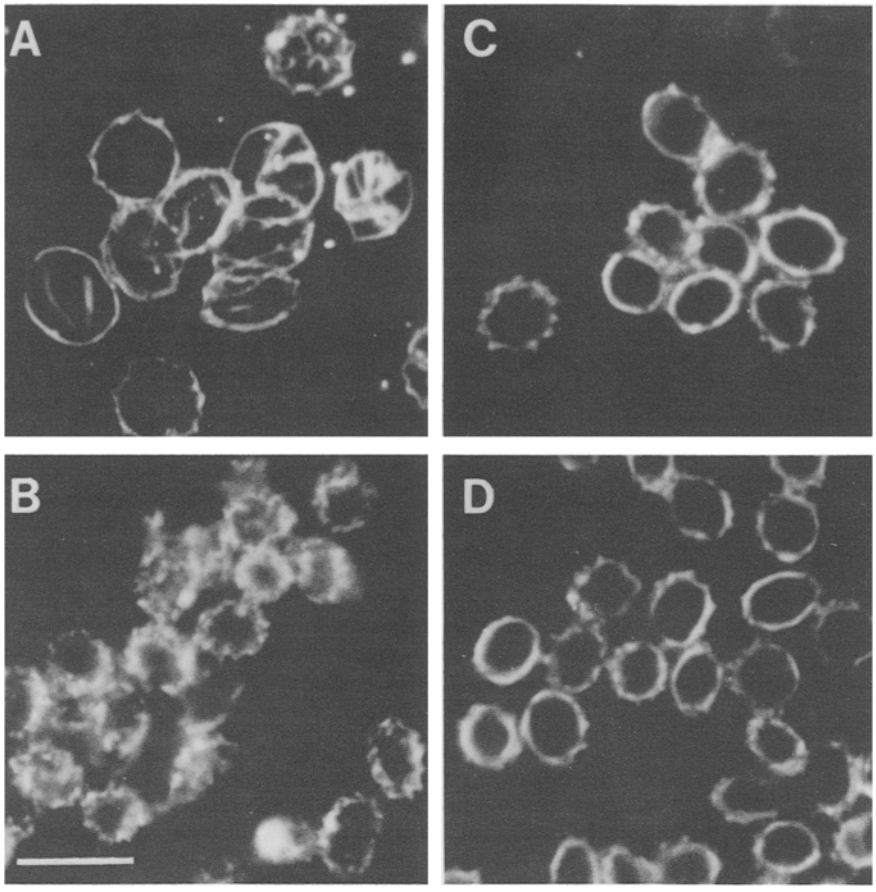

Fig. 3. The effect of minimal osmotic hemolysis on crenatability. Red cells were washed in isotonic saline and the pelleted cells hemolyzed in 9 vol water (*A* and *B*), 150 mM NH_4HCO_3 (*C*) or 300 mM glycerol (*D*) at 0 °C. After 5 min on ice, 1 vol of 1.5 M NH_4HCO_3 was added to restore isotonicity to the water and glycerol-lysed samples (*B* and *D*) while *A* received 1 vol water and *C* received 1 vol 150 mM NH_4HCO_3. After 5 min on ice, each sample was promptly photographed (unfixed) by dark field microscopy. (*A*): Lysis in water without restoration of isotonicity. (*B*): Lysis in water followed by introduction of 150 mM NH_4HCO_3. (*C*): Lysis in 150 mM NH_4HCO_3. (*D*): Lysis in 300 mM glycerol followed by introduction of 150 mM NH_4HCO_3. Calibration bar = 10 μm

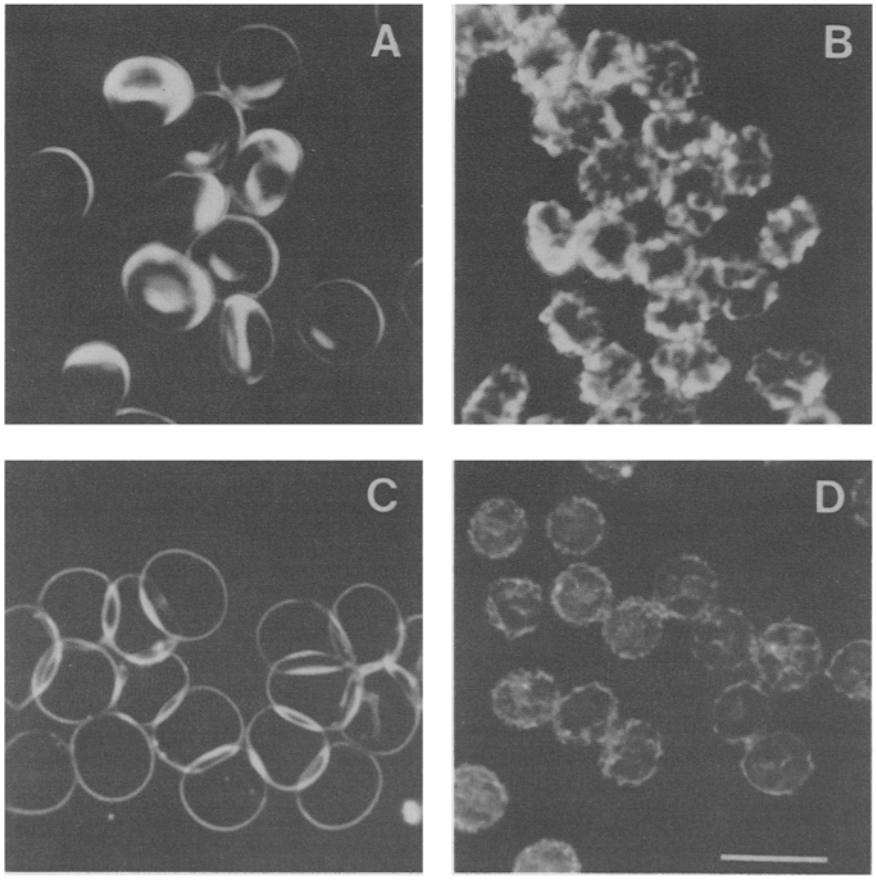

Fig. 4. Lysolecithin-crenated erythrocytes yield uncrenated ghosts. Washed red cells were preincubated in 7 vol saline lacking or containing 0.1 mM lysolecithin. Aliquots were fixed for microscopy. Ghosts in 5 mM NaP_i (pH 8) were prepared from the remaining cells exposed to lysolecithin and either not treated or incubated with 0.11 mM lysolecithin as described in Fig. 1. (*A*): Untreated red cells. (*B*): Lysolecithin-treated red cells. (*C*): Ghosts from lysolecithin-treated red cells. (*D*): Ghosts from lysolecithin treated red cells themselves treated with lysolecithin. Calibration bar = 10 μm

Evidence for Membrane Derangement During Hemolysis

Echinocytic red cells were generated by metabolic depletion (overnight incubation at 37 °C in buffered saline) as described [39]. The ghosts from those cells were indistinguishable from unincubated controls; i.e., smooth, plump bowls (not shown). This result speaks against the hypothesis that an irreversible shape-determining alteration in the sub-membrane reticulum occurs during metabolic depletion, perhaps as a result of an increase in intracellular calcium [27, 31]. Instead, the result suggests that the red cell echinocytosis resulted from the inhibition of an energy-dependent process which maintains the relative surface areas of the two sides of the membrane, perhaps involving the synthesis of lipids at the cytoplasmic surface [2]. We suggest that the imbalance causing echinocytosis may have been dissipated during hemolysis to yield smooth ghosts.

Red cells crenated by lysolecithin yielded smooth ghosts in 5 mM NaP_i (Fig. 4). The smooth contour of the ghosts did not reflect a loss of the ability to crenate in saline, as shown in Fig. 4*D*. The smooth contour of the ghosts did not result from the loss of the lysolecithin since, in control experiments, greater than 90% of the ^{14}C-lysolecithin added to the cells was retained by the hemoglobin-free ghosts. Instead, we suggest that a redistribution of lipid occurred during hemolysis, in this case expanding the cytoplasmic leaflet and therefore opposing ghost crenation.

Direct evidence for a membrane derangement during hemolysis comes from further study of lysolecithin-induced crenation. First, we measured the redistribution of ^{14}C-lysolecithin upon hemolysis, exploiting the observation [30] that only lysolecithin in the outer leaflet is extracted from red cell membranes by serum albumin (i.e., lysolecithin extraction is much more rapid than transmembrane movement in this system). As shown in Table 1, 95% of the ^{14}C-lysolecithin introduced into intact cells was initially removed by a brief exposure to albumin; this is because the transfer of lysolecithin to the cytoplasmic surface of intact cells occurs over many hours [30]. In contrast, only 80% of the label was extractable from ghosts derived from these cells. We infer that the difference represents phospholipid redistribution to the cytoplasmic surface as a consequence of hemolysis.

We suggest that hemolysis of normal red cells yields ghosts with a slight shift of lipid in favor of the outer leaflet, while the opposite shift occurs in lysolecithin-treated red cells, even when echinocytic. The pathway of transfer could be the perimeter of the hemolytic holes, since transbilayer diffusion takes many hours. While the amount of ^{14}C-lysolecithin transferred to the cytoplasmic surface was substantial, most of this might have been in exchange for cytoplasmic surface lipids, since the net movement of lipid to the cytoplasmic leaflet required to reverse the echinocytic contour originally caused by the kinetic trapping of lysolecithin at the outer surface is quite small (<1%).

If the above argument is correct, the extra lipid in the cytoplasmic leaflet of ghosts derived from echinocytic red cells should diminish their susceptibility to crenation. This hypothesis was tested in Fig. 5. It is seen that ghosts from erythrocytes pretreated with lysolecithin crenate less well than ghosts from untreated red cells when exposed to 0.022 mM lysolecithin. A similar difference was observed when 50 mM NaCl was the crenating agent (not shown). It is important to note that at sufficiently high levels of lysolecithin (0.1 mM) or salt (100 mM NaCl) the pre-treated membranes were well crenated (*see also* Fig. 4). We take these differ-

Table 1. Inaccessibility of ^{14}C-lysolecithin to extraction following red cell lysis

Expt.	Membrane	Hours of extraction	% Unextractable ^{14}C-lysolecithin
1	Cells	1	7
		2	7
	Sealed ghosts	1	19
		2	23
2	Cells	0.5	4
	Sealed ghosts	0.5	17
3	Cells	0.5	2
		1	4
	Sealed ghosts	0.5	19
		1	18

Washed red cells were labeled by incubation for 15 min at 37 °C in 27 vol 0.15 M NaCl-5 mM NaP_i (pH 8) containing 10 mM glucose and 0.06 μCi ^{14}C-lysolecithin. The red cells were pelleted by centrifugation and washed in 0.15 M NaCl-5 mM NaP_i (pH 8). Aliquots of red cells were resuspended in 0.15 M NaCl-5 mM NaP_i (pH 8) or lysed in 40 vol 5 mM NaP_i (pH 8) at 0 °C. After a 5-min incubation, concentrated saline was added to the lysed preparation to restore isotonicity and the mixtures warmed for 1 hr at 37 °C, which sealed the ghosts [24]. Cells and ghosts were pelleted. Extraction of lysolecithin was carried out in 80 vol 0.15 M NaCl-5 mM NaP_i (pH 8) containing 1% bovine serum albumin at 0 °C [30]. At the times indicated, aliquots of the mixtures were centrifuged for 1 min at 12,000 × g in a Beckman Microfuge and both supernatants and pellets assayed for radioactivity in Aquasol (New England Nuclear). The volume of red cells in these samples (6 μl) was so small that quenching by hemoglobin was negligible, as demonstrated by internal standardization.

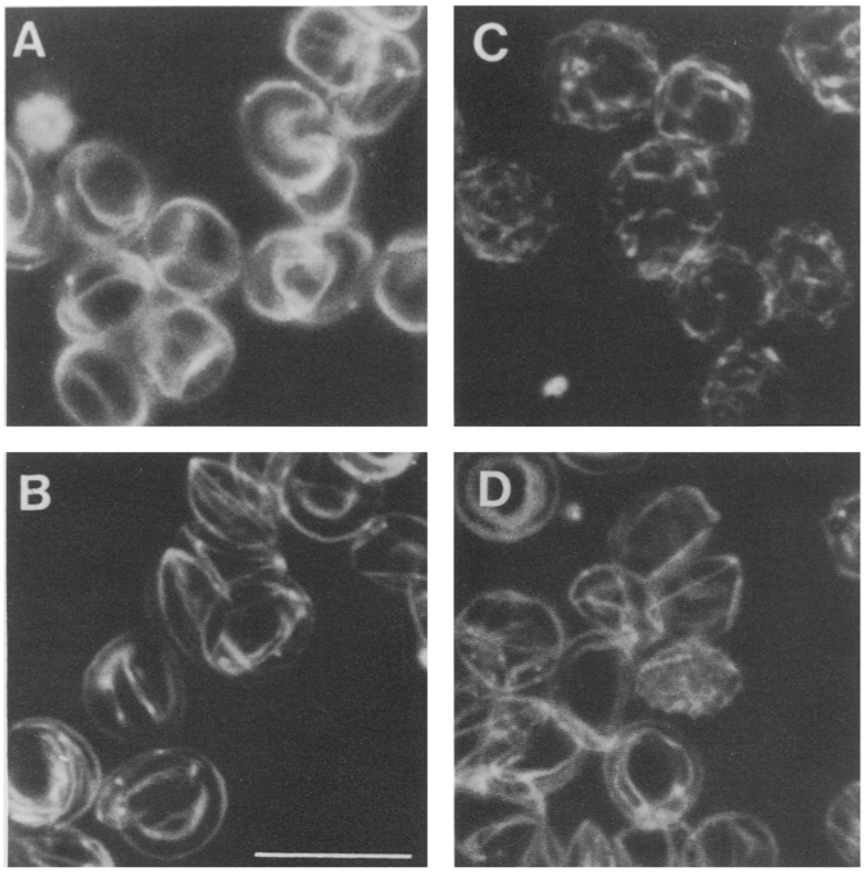

Fig. 5. Reduced crenation by lysolecithin of ghosts prepared from lysolecithin-treated erythrocytes. Washed red cells were preincubated in 7 vol saline containing or lacking 0.1 mM lysolecithin for 5 min at 37 °C and then pelleted. Standard ghosts were prepared and incubated at 0 °C for 5 min with 7 vol 15 mM NaP_i (pH 8.0) plus 1 vol water or 0.2 mM lysolecithin (0.022 mM final), as in Fig. 1. (*A*): Untreated ghosts from untreated cells. (*B*): Untreated ghosts from pretreated cells. (*C*): Treated ghosts from untreated cells. (*D*): Treated ghosts from pretreated cells

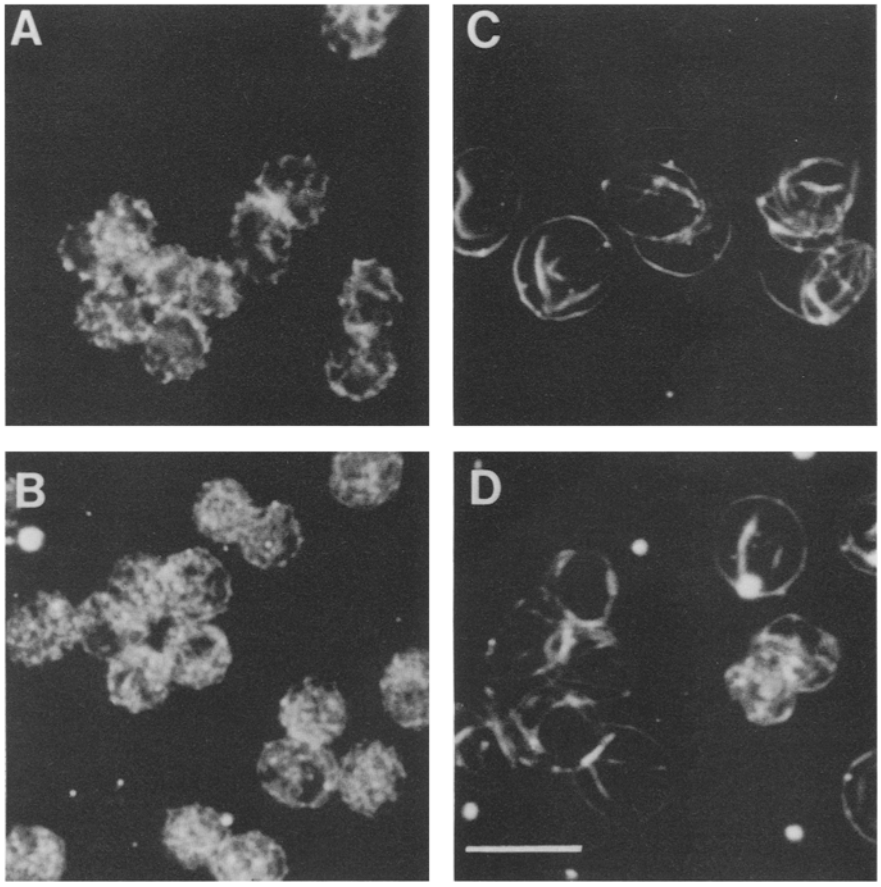

Fig. 6. Crenation of cholesterol-depleted ghosts. Freshly drawn erythrocytes were washed in saline and incubated overnight at 37 °C in the absence or presence of egg lecithin liposomes to deplete them of cholesterol, as described [22]. Standard ghosts were prepared from these cells as before. The control ghosts had 0.82 mol of cholesterol per mol phospholipid, while in the depleted membranes this ratio was 0.62. The ghosts were suspended in 7 vol 150 mM NaCl-5 mM NaP_i (pH 8) with or without 1 mM 2,4-dinitrophenol for 10 min on ice prior to fixation and microscopy. (*A*): Control ghosts in saline. (*B*): Control ghosts in saline plus dinitrophenol. (*C*): Depleted ghosts in saline. (*D*): Depleted ghosts in saline plus dinitrophenol. Calibration bar = 10 μm

ences as further evidence for a relative expansion of the cytoplasmic leaflet of the bilayer of ghosts derived from lysolecithin-treated cells.

Crenation of Cholesterol-Depleted Ghosts

Red cells depleted of cholesterol become cup-shaped stomatocytes [10][1] Furthermore, ghosts prepared from cholesterol-depleted red cells failed to crenate when suspended in isotonic saline either in the presence or absence of 1 mM 2,4-dinitrophenol (Fig. 6).

Two hypotheses could account for this dramatic effect. The first derives from previous observations that cholesterol itself is a shape-determining amphipath, partitioning at equilibrium in favor of the outer leaflet of the bilayer so as to promote evagination [22]. Cholesterol depletion thus discourages crenation. The second hypothesis holds that the redistribution of lipid accompanying hemolysis may be increased in cholesterol-depleted membranes so as to suppress crenation.

Decay of Crenatability of Ghosts

In the preceding section, we demonstrated that ghost crenation is encouraged or discouraged by conditions attending hemolysis. We interpreted these results to signify a redistribution of lipids between the leaflets of the bilayer during hemolysis. We shall now show that standard ghosts gradually become incapacitated for crenation over time. These results suggest that the metastable lipid distribution created by hemolysis eventually relaxes toward a more evenly-balanced state which does not promote spiculation.

The decay of the capacity of standard ghosts to crenate has been observed under a variety of conditions [21, 33, 34]. In our experiments, ghosts ultimately remained or reverted to smooth flat disks with large dimples even in the presence of isotonic saline, lysolecithin, or 2,4-dinitrophenol. Polyacrylamide gel electrophoresis in sodium dodecylsulfate revealed that no change in the profile of membrane polypeptides (i.e., neither aggregation nor degradation nor solubilization) correlated with the loss of crenatability (*see also* ref. 23).

Simply storing ghosts overnight in 5 mM NaP_i at 0 °C greatly reduced their crenatability. Warming accelerated this process considerably; for example, a 30-min incubation at 25 °C (Fig. 7*B*) or a three minute incubation at 37 °C in 10 mM NaP_i abolished subsequent crenation in 150 mM NaCl. Loss of crenatability increased with pH. In one experiment, a 5-min incubation at 20 °C in 5 mM NaP_i at pH 8 abolished crenation almost entirely, while 20 min was required at pH 7 and 40 min at pH 6 for the same effect.

The presence of electrolytes retarded the loss of crenation. In one experiment, ghosts lost crenatability at 20 °C in less than 2 min in 0.5 mM NaP_i while 40 min was required in 5 mM NaP_i. In Fig. 7, ghosts lost crenatability within 30 min at 25 °C in 10 mM NaP_i alone (Fig. 7*B*) but not when 60 mM NaCl was present (Fig. 7*C*). Ghosts incubated in isotonic saline at 37 °C gradually lost their crenated contour, but the process took a few hours. Divalent cations (1 mM Mg^{++} or Ca^{++}) preserved crenatability; the species of anion was indifferent. In general, the ability of a cation to protect against loss of crenation paralleled its potency as a crenating agent, but this correlation could be coincidental. It is important to note that the loss of crenatability is not merely the arrest of ghosts in the smooth-contour state, since ghosts crenated in isotonic saline revert to smooth disks during incubation at 37 °C.

Time and temperature-dependent loss of crenatability was the result of a reduction in the sensitivity of ghosts to crenating agents. That is, ghosts which lost the ability to respond to one concentration of saline were still crenated by a higher concentration. Ghosts which had just lost their responsiveness to isotonic saline could be crenated if 1 mM 2,4-dinitrophenol were added. Ultimately, however, crenatability even to 1 M NaCl plus 1 mM 2,4-dinitrophenol was lost.

Chlorpromazine abolishes crenation

Chlorpromazine, which causes invagination in intact red cells [15], accelerated the loss of ghost crenatability under all conditions, particularly at elevated temperature and low ionic strength. An illustration is shown in Fig. 7. Here, 0.1 mM chlorpromazine abolished crenation during a 30-min incubation at 25 °C in 10 mM NaP_i-60 mM NaCl (*compare C* and *D*). (At zero-time, the chlorpromazine-treated ghosts were almost as crenated as the untreated control; not shown.) As discussed above for cholesterol, the effect of chlorpromazine could be direct (i.e., through preferential partition into and expansion of the cytoplasmic leaflet) or indirect (i.e., through its effect on the redistribution of other bilayer constituents).

Surfactants Abolish Crenation

That the bilayer and not the submembrane reticulum is responsible for the loss of crenatability is

1 Lange, Y., Slayton, J.M. Interaction of cholesterol and lysophosphatidylcholine in determining red cell shape. (*submitted*)

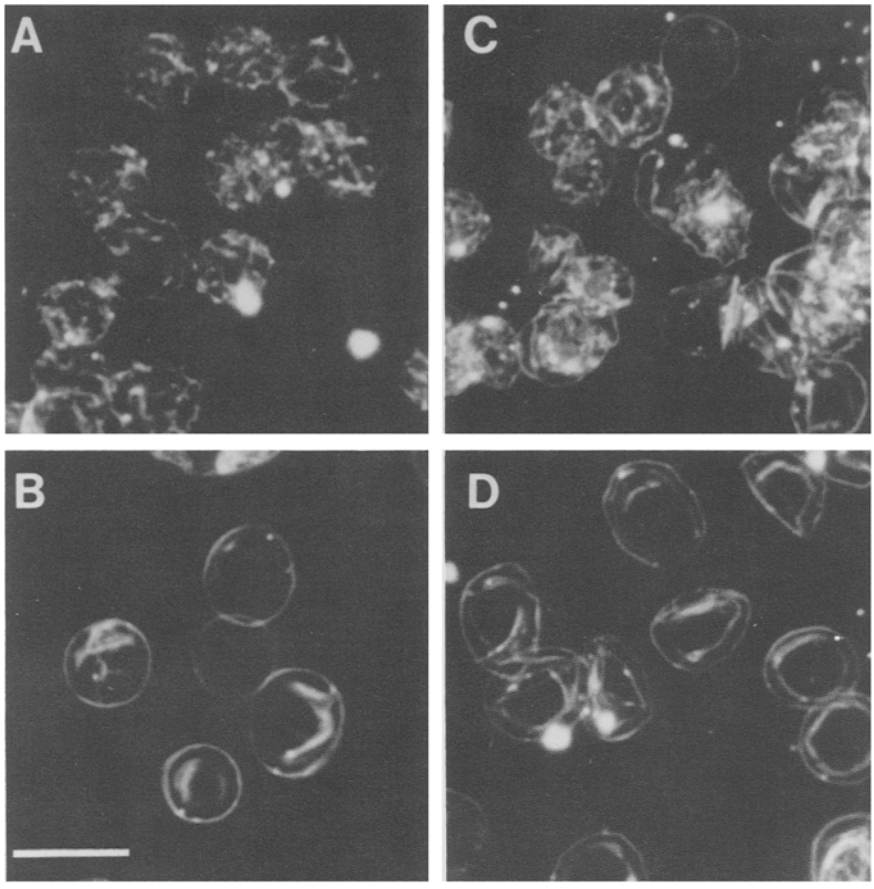

Fig. 7. Loss of crenatability of ghosts after pre-incubation with low ionic strength buffer and chlorpromazine. 100-μl aliquots of packed, freshly-prepared ghosts were mixed with 800 μl buffer (10 mM NaP_i, pH 8.0, final) plus additions. The suspensions were pre-incubated for 30 min at 0 or 25 °C, then chilled. The NaCl concentration was brought to 0.15 M in a final vol of 1.0 ml. After 10 min on ice, the specimens were fixed and photographed. Pre-incubations were: (*A*) 0 °C in buffer alone; (*B*) 25 °C in buffer alone; (*C*) 25 °C in 60 mM NaCl; and (*D*) 25 °C in 60 mM NaCl plus 0.1 mM chloropromazine. Calibration bar = 10 μm

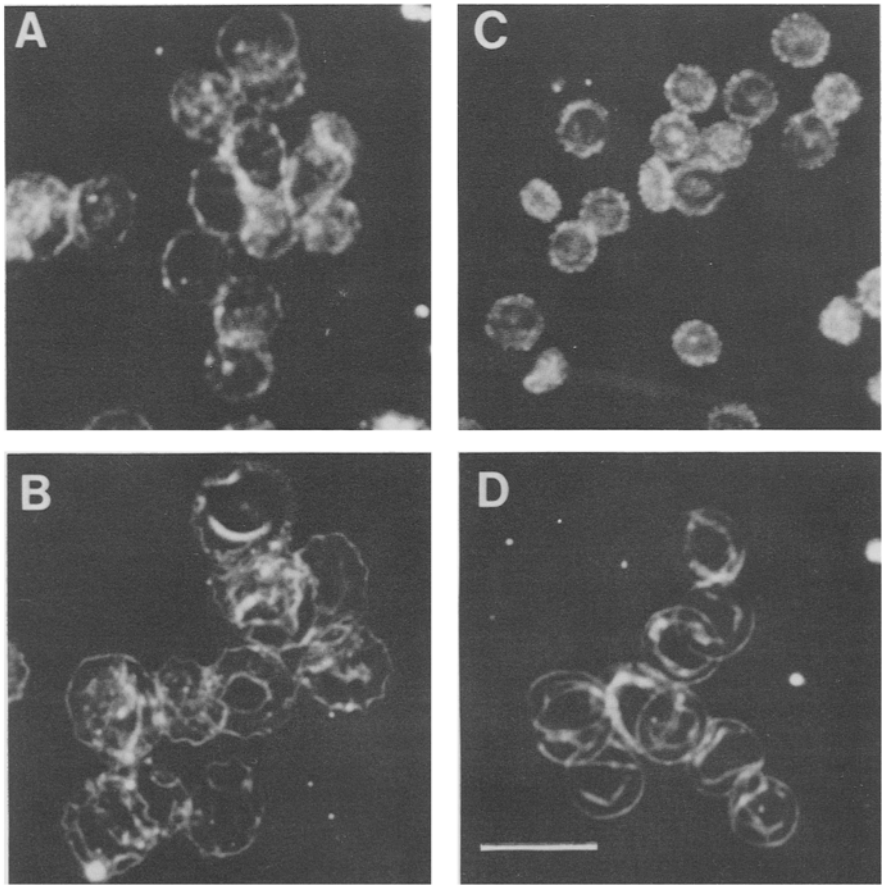

Fig. 8. Lysolecithin both enhances and reverses crenation. Freshly-prepared ghosts were suspended in isotonic saline lacking or containing 0.1 mM lysolecithin and incubated at 0 or 37 °C for 4 min, then chilled and fixed for microscopy. (*A*): Minus lysolecithin at 0 °C. (*B*): Minus lysolecithin at 37 °C. (*C*): Plus lysolecithin at 0 °C. (*D*): Plus lysolecithin at 37 °C. Calibration bar = 10 μm

strongly suggested by the fact that a series of surfactants at concentrations too low to dissolve the membrane blocked or reversed crenation. Among these were 0.01 to 0.1% saponin, sodium deoxycholate, Triton X-100, and lysolecithin. Lysolecithin, like some others of this group, also induces echinocytosis in erythrocytes [15] and crenation in ghosts (Fig. 1). An experiment which highlights this dual effect is shown in Fig. 8. Ghosts were incubated in saline in the presence and absence of 0.1 mM lysolecithin. At 0 °C, the presence of lysolecithin caused increased crenation (*compare* Fig. 8*A* and *C*). However, during a 4-min incubation at 37 °C, the presence of lysolecithin accelerated the loss of crenation (*compare* Fig. 8*B* and *D*). We suggest that these surfactants accelerate the redistribution of lipids in the bilayer so as to relax the bias toward evagination engendered by the hemolytic event. We view their promotion of echinocytosis of intact cells as a separate action, caused by their transient (kinetic) trapping at the surface to which they were added [30].

Conclusions

What determines the contour of the isolated red cell membrane? Clearly, the ghost appears to remember the shape of the parent erythrocyte. The contour of the red cell itself is probably constrained by three major factors: (i) minimization of bending energy [9]; (ii) asymmetry in the area of the two membrane surfaces; and (iii) the physiologically-regulated, submaximal cytoplasmic volume [14]. We have argued that the submembrane reticulum of spectrin and actin is assembled on the bilayer and thus passively assumes its contour [23]. This network is flexible and elastic and can stabilize cell shape against shear stress [17, 38]. However, it neither imposes nor retains the contour of the crenated membrane *in vitro* [23]. Instead, we place primary emphasis on a bilayer couple mechanism.

If the areas of the two bilayer leaflets were precisely conserved upon hemolysis, the contour of the ghost might match that of the parent cell. (It could also be argued that, freed of the constraint of a fixed intracellular volume, there are many alternative forms the asymmetrical membrane could achieve while still minimizing curvature. Retention of a shape resembling the red cell might therefore, to some degree, reflect the elasticity of the submembrane reticulum.) However, if even a minute fraction of the lipid in the bilayer were transferred between leaflets as a result of hemolysis – for example, by flowing across the perimeter of the hemolytic hole [24, 25] – the shape of the ghost would be altered accordingly.

We suggest that the contour of standard ghosts resembles that of the parent cell because of two nearly balanced effects. One is the differential, electrostatically-driven expansion of the inner surface at low ionic strength; the other is a net transfer of mass from the inner to the outer leaflet of the bilayer during hemolysis. It is this shift of lipid to the outer surface which makes the standard ghosts monoconcave rather than biconcave and crenatable in isotonic saline. But these shifts are highly conditional. For example, minimizing the stress of hemolysis yields ghosts which are not susceptible to conventional crenating agents, presumably because they are minimally deranged. In cells pretreated with lysolecithin, the net transfer of mass between leaflets may also be minimal or even reversed; as a result, the ghosts are not as susceptible to crenation as controls.

Similarly, we interpret the time-dependent loss of crenatability following ghost preparation as a relaxation of the potential for evagination imparted by the hemolytic event. This process may involve a redistribution of lipid from the outer to the inner leaflet. The pathway of movement might again be across the edge of the hemolytic hole. The loss of crenatability induced by MgATP [33] could similarly represent the relative expansion of the cytoplasmic surface of the bilayer through the phosphorylation of lipids at that surface [2].

Recently, Alhanaty and Sheetz [1] reported that red cells lose their sensitivity to crenation by dinitrophenol during a prolonged incubation in its presence. They suggest that the mechanism may be a metabolically-driven redistribution of membrane components which serves to maintain normal cell shape. It may help in evaluating this appealing hypothesis to keep in mind that similar phenomena can be observed with isolated ghosts in the absence of a source of metabolic energy.

We conclude that crenation of the isolated red cell membrane, while complex, is explicable in terms of a bilayer couple mechanism. The profound effects of the redistribution of a fraction of a percent of the mass of the bilayer between its two leaflets may complicate and even dominate shape-behavior of ghosts unless properly defined and controlled. Transfer of mass between the leaflets of the bilayer *in vitro* may provide a useful analogue to physiological shape-defining events in the intact cell.

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References

1. Alhanaty, E., Sheetz, M.P. 1981. Control of the erythrocyte membrane shape: Recovery from the effect of crenating agents. *J. Cell Biol.* **91**:884–888
2. Allan, D., Thomas, P., Michell, R.H. 1978. Rapid transbilayer diffusion of 1,2-diacylglycerol and its relevance to control of membrane curvature. *Nature (London)* **276**:289–290
3. Beck, J.S. 1978. Relations between membrane monolayers in some red cell shape transformations. *J. Theor. Biol.* **75**:487–501
4. Bennett, V. 1982. The molecular basis for membrane-cytoskeleton association in human erythrocytes. *J. Cell. Biochem.* **18**:49–65
5. Bessis, M., Weed, R., Leblond, P.F. (editors) 1973. Red Cell Shape: Physiology, Pathology, Ultrastructure. Springer Verlag, New York
6. Birchmeier, W., Singer, S.J. 1977. On the mechanism of ATP-induced shape changes in human erythrocyte membranes. II. The role of ATP. *J. Cell Biol.* **73**:647–659
7. Branton, D., Cohen, C.M., Tyler, J. 1981. Interactions of cytoskeletal proteins on the human erythrocyte membrane. *Cell* **24**:24–32
8. Bretscher, M. 1972. Asymmetrical lipid bilayer structure for biological membranes. *J. Mol. Biol.* **71**:523–528
9. Canham, P.B. 1970. The minimum energy of bending as a possible explanation of the biconcave shape of the human red blood cell. *J. Theoret. Biol.* **26**:61–81
10. Chailley, B., Giraud, F., Claret, M. 1981. Alterations in human erythrocyte shape and the state of spectrin and phospholipid phosphorylation induced by cholesterol depletion. *Biochim. Biophys. Acta* **643**:636–641
11. Conrad, M.J., Singer, S.J. 1979. Evidence for a large internal pressure in biological membranes. *Proc. Natl. Acad. Sci. USA* **76**:5202–5206
12. Conrad, M.J., Singer, S.J. 1981. The solubility of amphipathic molecules in biological membranes and lipid bilayers and its implications for membrane structure. *Biochemistry* **20**:808–818
13. Danielli, J.F. 1967. The formation, physical stability, and physiological control of paucimolecular membranes. *In:* Formation and Fate of Organelles, Symposium of the International Society of Cell Biologists. Vol. **6**:pp. 239–253. Academic Press, New York
14. Deuling, H.J., Helfrich, W. 1976. Red blood cell shapes as explained on the basis of curvature elasticity. *Biophys. J.* **16**:861–868
15. Deuticke, B. 1968. Transformation and restoration of biconcave shape of human erythrocytes induced by amphiphilic agents and changes of ionic environment. *Biochim. Biophys. Acta* **163**:494–500
16. Evans, E.A. 1974. Bending resistance and chemically induced moments in membrane bilayers. *Biophys. J.* **14**:923–931
17. Evans, E.A., Hochmuth, R.M. 1977. A solid-liquid composite model of the red cell membrane. *J. Membrane Biol.* **30**:351–362
18. Fairbanks, G., Steck, T.L., Wallach, D.F.H. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry* **10**:2606–2617
19. Jacobs, M.H., Parpart, A.K. 1938. Osmotic properties of the erythrocyte: X. On the permeability of the erythrocyte to ammonia and the ammonium ion. *J. Cell Comp. Physiol.* **11**:175–192
20. Johnson, R.M., Robinson, J. 1976. Morphological changes in asymmetric erythrocyte membranes induced by electrolytes. *Biochim. Biophys. Res. Commun.* **70**:925–931
21. Johnson, R.M., Taylor, G., Meyer, D.B. 1980. Shape and volume changes in erythrocyte ghosts and spectrin-actin networks. *J. Cell Biol.* **86**:371–376
22. Lange, Y., Cutler, H.B., Steck, T.L. 1980. The effect of cholesterol and other intercalated amphipaths on the contour and stability of the isolated red cell membrane. *J. Biol. Chem.* **255**:9331–9337
23. Lange, Y., Hadesman, R.A., Steck, T.L. 1982. Role of the reticulum in the stability and shape of the isolated human erythrocyte membrane. *J. Cell Biol. 92:* 714–721
24. Lieber, M.R., Steck, T.L. 1982. A description of the holes in human erythrocyte membrane ghosts. *J. Biol. Chem.* (*in press*)
25. Lieber, M.R., Steck, T.L. 1982. Dynamics of the holes in human erythrocyte membrane ghosts. *J. Biol. Chem.* (*in press*)
26. Lin, G.S.B., Macey, R.I. 1978. Shape and stability changes in human erythrocyte membranes induced by metal cations. *Biochim. Biophys. Acta* **512**:270–283
27. Lux, S.E. 1979. Spectrin-actin membrane skeleton of normal and abnormal red blood cells. *Semin. Hematol.* **16**:21–51
28. Lux, S.E., John, K.M., Karnovsky, M.J. 1976. Irreversible deformation of the spectrin-actin lattice in irreversibly sickled cells. *J. Clin Invest.* **56**:955–963
29. Matayoshi, E. 1980. Distribution of shape-changing compounds across the red cell membrane. *Biochemistry* **19**:3414–3422
30. Mohandas, N., Greenquist, A.C., Shohet, S.B. 1978. Bilayer balance and regulation of red cell shape changes. *J. Supramol. Struct.* **9**:453–458
31. Mohandas, N., Shohet, S.B. 1978. Control of red cell deformability and shape. *Curr. Top. Hematol.* **1**:71–125
32. Op den Kamp, J.A.F. 1979. Lipid asymmetry in membranes. *Annu. Rev. Biochem.* **48**:47–71
33. Patel, V.P., Fairbanks, G. 1981. Spectrin phosphorylation and shape change of human erythrocyte ghosts. *J. Cell Biol.* **88**:430–440
34. Sheetz, M.P. 1977. Cation effects on cell shape. *In:* Cell Shape and Surface Architecture. J.P. Revel, U. Henning, and C.F. Fox, editors pp. 559–567 Alan R. Liss, New York
35. Sheetz, M.P., Painter, R.G., Singer, S.J. 1976. Biological membranes as bilayer couples: III. Compensatory shape changes induced in membranes. *J. Cell Biol.* **70**:193–203
36. Sheetz, M.P., Singer, S.J. 1974. Biological membranes as bilayer couples. A molecular mechanism of drug-induced interactions. *Proc. Natl. Acad. Sci. USA* **71**:4457–4461
37. Sheetz, M.P., Singer, S.J. 1977. Equilibrium and kinetic effects of drugs on the shapes of human erythrocytes. *J. Cell Biol.* **70**:247–251
38. Steck, T.L. 1974. The organization of proteins in the human red blood cell membrane. *J. Cell Biol.* **62**:1–19
39. Weed, R.I., La Celle, P.L., Merrill, E.W. 1969. Metabolic dependence of red cell deformability. *J. Clin. Invest.* **48**:795–809

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