# MORPHOLOGICAL CHANGES IN ASYMMETRIC ERYTHROCYTE MEMBRANES INDUCED BY ELECTROLYTES

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SUMMARY. Washed human erythrocyte membranes are made permanently leaky to cations by EDTA. These ghosts can exhibit the stomatocyte-disc-echinocyte sequence of shape changes in response to electrolytes in the medium. The changes are instantaneous and reversible. The observations can be explained on the basis of the known effects of cations on charged phospholipids together with the bilayer couple hypothesis.

Besides the normal biconcave disc, red cells can take on a wide range of shapes. Bessis has recently classified (1) and described (2) these forms. Among them, two are of particular interest. The echinocyte is a sphere covered with spikes (crenations), resembling a sea urchin; the stomatocyte is an indented cup-shaped cell. In 1968 Deuticke (3) pointed out that many chemical agents converted normal erythrocytes into either stomatocytes or echinocytes, and, remarkably, that these compounds were antagonistic: the combination of an agent from each class left the erythrocyte as a normal biconcave disc. This suggested that the shape of the red cell depends on the balance of two opposing forces, tending to convert the cell either to a stomatocyte or to an echinocyte. These observations can be explained within the framework of a recently proposed (4) theory, the bilayer couple hypothesis. We would like to report, first, that well-washed, permeable erythrocyte ghosts can exhibit the

full range of the stomatocyte-disc-echinocyte shape spectrum simply in response to cations, and, secondly, that the observations are consistent with the bilayer couple hypothesis.

MATERIALS AND METHODS. Washed hemoglobin-free ghosts are prepared as previously described (5) and used within 3-4 h. For photography, a wet mount of a ghost suspension ($10^9$/ml) was left undisturbed in the cold for 20-30 minutes to permit the ghosts to settle. They were then photographed using Nomarski optics. Distilled freshly deionized water free of Ca and Mg by flame photometry was used for buffers. All chemicals are reagent grade.

RESULTS. The ghosts are washed twice in 1 mM EDTA, pH 7.4, during their preparation, and this treatment is reported (6,7) to render them permanently permeable to cations. This was proven for our preparation by previously described methods (5,7). Under the conditions we use for microscopy, these ghosts do not recover any appreciable impermeability to $^{22}Na$, even in the presence of divalent cations. Although no osmotic or chemical gradient can be maintained across these membranes, ghost morphology is nevertheless responsive to the composition of the buffer medium. Figure 1a shows the ghosts in 7 mM NaCl, with 5 mM Tris-Cl, pH 7.4, which is the final washing buffer. The ghosts are mostly stomatocytes and spheres. As the univalent electrolyte concentration is increased, the ghosts are converted into biconcave discs (Fig. 1b) and finally, into echinocytes (Fig. 1c). In order to demonstrate that osmotic effects are not operating, Figure 1d shows the ghosts in 250 mM sucrose. They are not crenated. NaCl, KCl and Na acetate are equally effective in crenating ghosts.

Divalent cations are also crenating agents. Table I shows the levels of $Ca^{++}$, $Mg^{++}$ and $Sr^{++}$ that bring about complete crenation of ghosts in 7 mM NaCl. It is clear that divalent cations are more effective than univalent. In addition, Table I shows that anions are not effective. The trivalent cation,

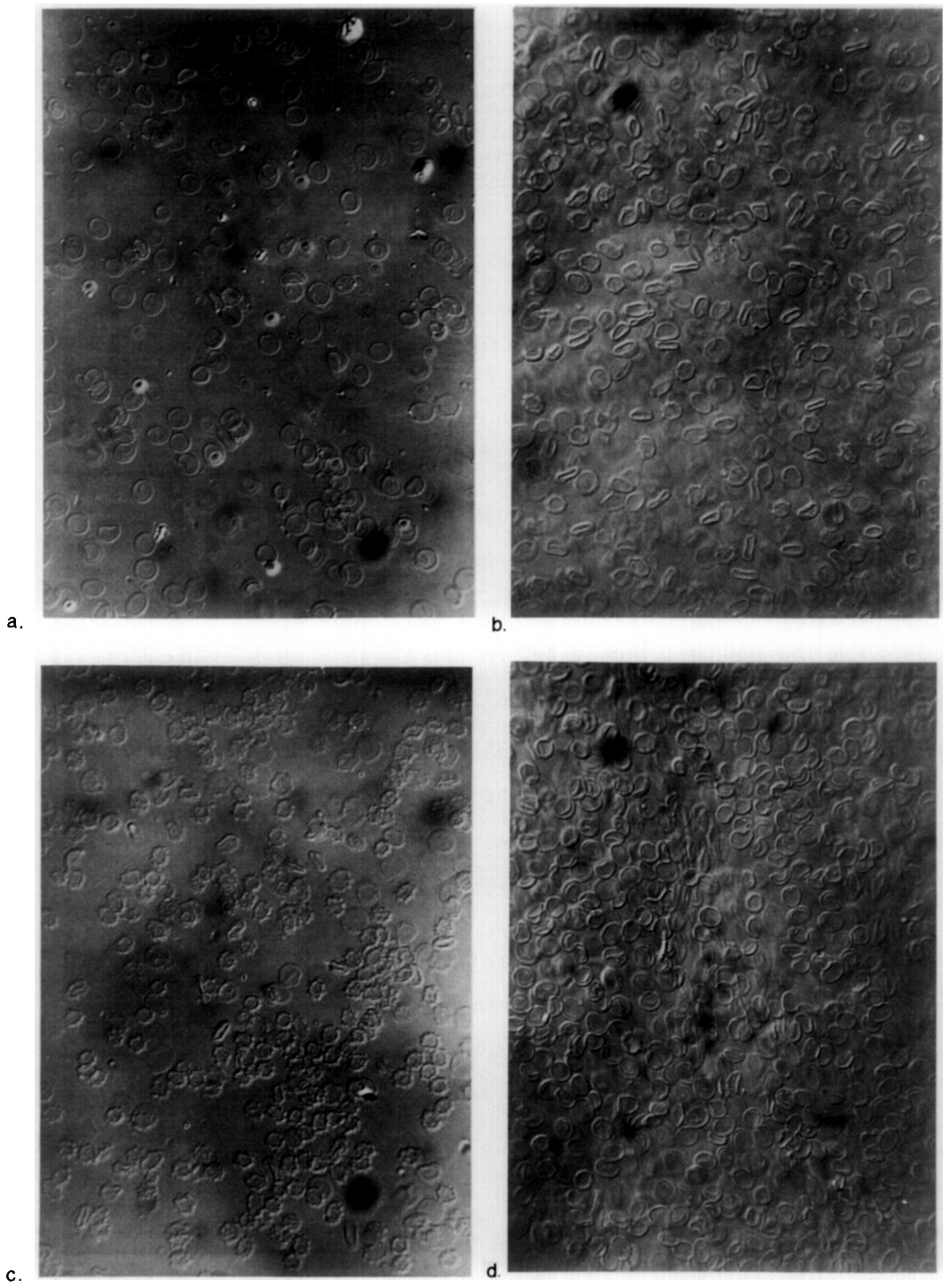


Figure 1. Nomarski phase micrographs of permeable hemoglobin-free human erythrocyte membranes. All solutions are buffered with 5 mM Tris, pH 7.4 (a) in 7 mM NaCl; (b) in 25 mM NaCl; (c) in 75 mM NaCl; (d) in 250 mM sucrose.

Table I

Wet mounts of ghosts were observed by phase microscopy immediately after being suspended in solutions of 5 mM Tris, 7 mM NaCl, pH 7.4 with electrolyte concentrations ranging from $10^{-6}$ to $10^{-1}$ M. The concentration of electrolyte which gave essentially complete crenation is listed.

| | mM |
|---|---|
| NaCl | 100 |
| KCl | 100 |
| Na acetate | 100 |
| $Na_2SO_4$ | 40 |
| sodium phosphate buffer, pH 7.4 | 50 |
| Mg $Cl_2$ | 1 |
| Ca $Cl_2$ | 1 |
| Sr $Cl_2$ | 1 |

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$La^{+++}$, caused aggregation at concentrations greater than $10^{-5}$ M. No changes were seen with $10^{-5}$ M $La^{+++}$.

Although the photographs in Figure 1 were taken 1/2 hour after the ghosts were suspended in the various solutions, the morphological changes can be seen immediately. All the effects are reversible by changing the salt concentrations. The method of ghost isolation does not appear to affect the results, since ghosts prepared in 5 mM phosphate, pH 8.0 (8), respond in the same way as ghosts prepared in Tris buffer.

DISCUSSION. The results demonstrate that the stomatocyte-discechinocyte spectrum of red cell morphology in fact depends on intrinsic membrane properties. A plausible explanation can be proposed, based on known properties of phospholipids. (a) The erythrocyte membrane phospholipids are asymmetrically distributed (reviewed in 9). The choline-containing lipids are located on the outer leaflet of the bilayer, while other phospholipids, notably phosphatidylserine, compose the inner, cytoplasmic, leaflet. As a result, the outer leaflet has a net charge of zero, while the inner leaflet has a net negative charge. (b) Electrolytes can affect the packing of charged phospholipids. For

example, when phosphatidylserine monolayers are exposed to $Ca^{++}$ or $Mg^{++}$, the average area per head group declines, presumably by a reduction of charge-charge repulsion (10). Similarly, in bilayers made with negatively charged phospholipids, factors that reduce charge repulsion favor closer packing of the phospholipids. This was shown (11) by demonstrating that such factors (pH and cations) promote the transition from the loosely packed fluid phase to the more tightly packed ordered phase, at constant temperature. Moreover, a reduction in the surface potential of monolayers of acidic phospholipids by univalent electrolytes has been directly measured (12). Therefore, the area of the inner lipid leaflet of the erythrocyte membrane will diminish, as a result of tighter phospholipid packing, as electrolyte concentration is increased. (c) The ratio of the areas of the inner and outer leaflets affects ghost morphology. Cationic anesthetics convert erythrocytes to stomatocytes (3). Sheetz and Singer (4) proposed that positively charged compounds preferentially insert in the negative inner leaflet, increasing its area. Similarly, anionic anethetics crenate erythrocytes (3); these drugs preferentially enter the outer leaflet. Calculations of the area ratio changes required to bring about the morphological changes were made (4) and are consistent with this picture.

Taken together, these statements lead to the prediction that an increase in electrolyte concentration in a buffer bathing both sides of the membrane will preferentially diminish the area of the inner leaflet, by a shielding mechanism, and crenate the cell. On the other hand, if electrolyte concentrations are reduced, the area of the inner leaflet should expand relative to the outer uncharged leaflet, to yield a spherical indented stomatocyte. At some intermediate concentration, the ghost will

be a biconcave disc. This is exactly what we observe.

In addition, we tentatively propose that our observations may help explain the selectivity of Steck's procedures (13) for generating right-side-out and inside-out vesicles. Without Mg or Ca, ghosts in dilute buffers are stomotocytes (Fig. 1a); i.e., they tend to invaginate. Upon ghost disruption, the resulting vesicle would be expected to be inside-out, as observed (3). In the presence of divalent cations, however, the ghosts are crenated (Table I) and evaginated and would be expected to yield right-side out vesicles. This is also observed (13).

This explanation advanced here for the observed shape changes may require subsequent modification, since it is unlikely that it will be sufficient to account for all the morphological changes of erythrocyte membranes. For example, the spectrin-actin complex plays some role in controlling cell shape (14) and presumably interacts with the phospholipid bilayer. Nevertheless we have shown that isolated membranes can respond to small changes in cation concentration, that these changes are reversible, and that the isolated ghost can exhibit the full range of the stomatocyte-disc-echinocyte shape sequence. If the phospholipids of other biological membranes are asymmetrically distributed, the proposed mechanism for shape changes in the presence of divalent cations may be of general importance.

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