

Shape and Volume Changes in Erythrocyte Ghosts and Spectrin-Actin Networks

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ABSTRACT In response to changes in electrolyte concentration and pH, erythrocyte ghosts can exhibit some of the characteristic shapes seen in the intact erythrocyte. These shape changes are accompanied by volume changes; both are reversible, not energy dependent, and not inhibited by sulfhydryl reagents. The volume reduction can also be seen in isolated Triton-free spectrin-actin lattices, showing that this network is capable of reversible contraction. The results suggest that reversible changes in size of the underlying cytoskeleton of the erythrocyte membrane can control cell shape.

The normally disk-shaped erythrocyte is able to undergo a number of morphological changes. Some of these changes are well defined, and the abnormal cells can then be recognized as belonging to one of a relatively limited number of morphological classifications (4). The best characterized of these abnormal shapes are the crenated cell, or echinocyte, and the cup-shaped cell, or stomatocyte (5, 8). These shapes can be reproducibly induced in erythrocytes by a number of agents, such as changes in pH, various drugs, and by depletion of intracellular ATP.

The erythrocyte ghost can exhibit some of the morphology seen in intact cells. For example, ghosts prepared by hypotonic hemolysis are spheres or biconcave disks. Subsequent addition of salt induces the crenated form in ghosts (14, 22, 26). We have previously reported that hemoglobin-free washed ghosts can show a sequence of shape changes from a stomatocyte-like form, to a disk, to a crenated form, in response to an increase of the electrolyte concentration of the medium (14). The shape change is reversible. This paper presents evidence that this shape change is accompanied by a reduction in volume of the isolated ghost and that the submembranous reticulum or cytoskeleton of the ghost also contracts when salt concentrations are raised. Changes in pH also alter ghost morphology (18), and we show that this morphological change is also accompanied by a volume reduction.

MATERIALS AND METHODS

Red cells were obtained from normal donors and used immediately. White cells were removed by centrifugation through Ficoll-Hypaque (Lymphoprep, Nyegaard & Co., Oslo 4, Norway) (7). The cells were washed three times in 5 mM Tris-Cl, pH 7.4, with 1 mM EDTA and 140 mM NaCl. The cells were lysed and washed three times in 20 vol of 5 mM Tris-Cl, pH 7.4, 1 mM EDTA, and 7 mM NaCl at 0°C. For divalent cation studies, the last two washes were the same

buffer without EDTA. Occasionally, ghosts were prepared in phosphate buffers (11).

Preparation of Submembranous Reticulum (Triton Residue)

The procedure of Yu et al. (28) was used. Occasionally, it was slightly modified to permit removal of Triton X-100 (Triton) from the extracted residues. 5 ml of packed ghosts in the final wash buffer were mixed at 0°C with 35 ml of the final wash buffer containing 1% Triton. After 10 min, the suspension was centrifuged at 6,000 rpm in a Sorvall SS-34 rotor (DuPont Co., Sorvall Biomedical Div., Wilmington, Del.) for 20 min. About 30 ml of the supernate was aspirated. Triton-free wash buffer was added to 40 ml, and the loose pellet was gently resuspended and centrifuged as before. This washing step was repeated four times to remove the remaining Triton and solubilized protein and lipid. The A_{275} of the final wash was zero. Triton has an approximate $E_{275}^{1\%}$ of 25.0, so that the residual Triton concentration is $<1 \mu\text{g/ml}$. As a second measure of Triton removal, 99% of [^3H]Triton X-100 is removed by these washes.

Hematocrit

About 70 μl of the ghost suspension were drawn into a microhematocrit tube and centrifuged to constant volume in a Clay Adams (Div. of Becton, Dickinson & Co., Parsippany, N.J.) microhematocrit centrifuge in the cold room. This generally took 15 min. Ghost volume was calculated using the value of ghosts per milliliter obtained from Coulter counting. We are unable to correct the hematocrit for buffer trapped between the ghosts, because these ghosts are permeable to macromolecules (13). Measurements of the amount of trapped solution in hematocrit pellets have been made using resealable hemoglobin-free ghosts, which demonstrate that the correction is not $>20\%$ (6). Because the volume contraction we observe is fivefold (150 to 30 fl), a 20% error in the determination does not significantly affect our conclusions.

Microscopy

Shape changes in ghosts were photographed with a Zeiss Nomarski interference microscope, using Kodak Panatomic film. The diameters of Triton residues

were measured by phase microscopy, using a calibrated eyepiece micrometer. The average diameter of every residue was determined in randomly selected fields until 100 residues were measured for each suspension. Occasionally only 25 diameters were determined per suspension, because the mean diameter and its standard deviation was found to be relatively unaffected when larger numbers were measured.

RESULTS

Washed erythrocyte membranes, prepared by hypotonic hemolysis, are a mixture of stomatocyte and disklike forms. The morphology changes to discocyte and then to echinocyte as salt concentration is increased (14, 26). We now report that this shape change is accompanied by a volume change, which is demonstrable by hematocrit and direct observation of the ghosts. Fig. 1 shows data for NaCl. The initial crenation of the

ghost occurs when volume is reduced ~10%, from about 160 fl to 140 fl. At high NaCl concentrations, ghost volume is reduced to a limiting value of ~30 fl. As this volume reduction proceeds, the morphology of the ghost changes. The first completely crenated ghost form has large projections, which appear as an irregular outline in photomicrographs (Fig. 1c). This resembles the erythrocyte echinocyte I form, in Bessis' terminology (4). When the volume is reduced to ~80 fl, the ghost is a round form with many fine projections (Fig. 1e). This closely resembles the echinocyte II shape of the intact erythrocyte (4, 5). With increasing NaCl, the ghost becomes smaller and tends to flatten, while retaining its crenations. Any univalent electrolyte will induce this effect. NaCl, KCl, sodium acetate, and potassium acetate are equally effective. Similar data can be obtained

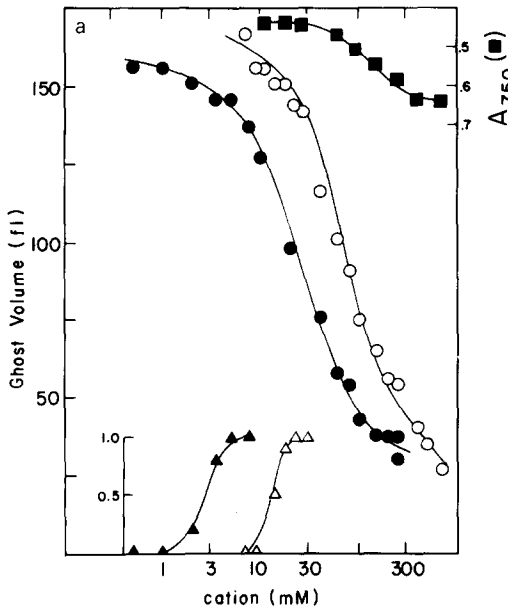
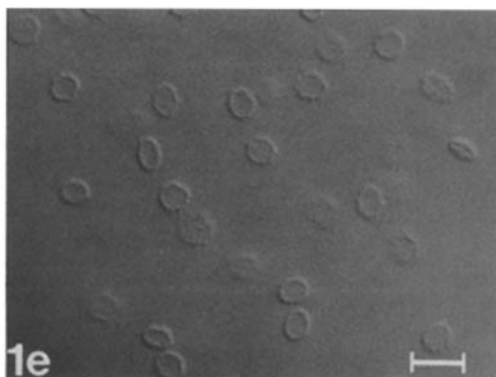
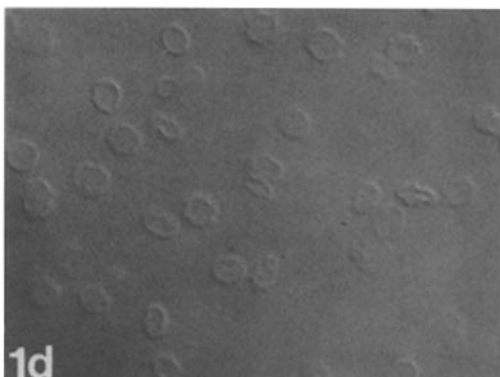
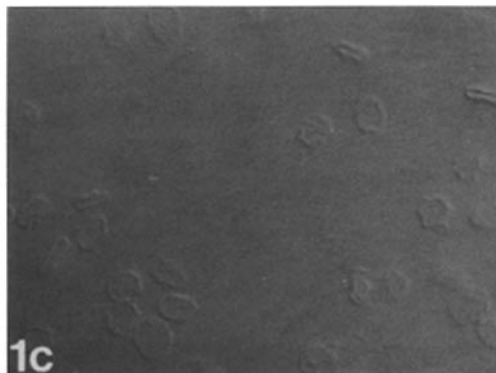
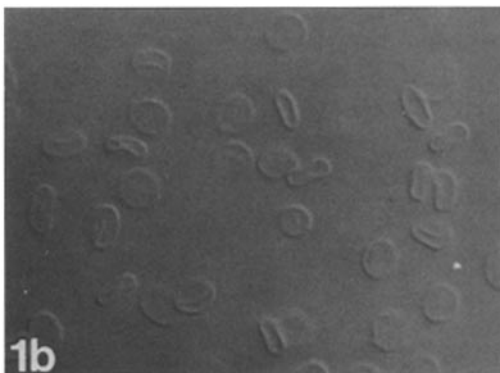


FIGURE 1 (a) Volume reduction in washed erythrocyte ghosts. 3 ml of a ghost suspension in 5 mM Tris, pH 7.4, mM NaCl, was titrated at 0°C with 3.0 M NaCl (○, △) or 1.0 M SrCl₂ (●, ▲) in the same buffer. Ghost volume (○, ●) was obtained as described in Materials and Methods. For NaCl, turbidity at A₇₅₀ (■) was also measured. The extent of the shape change, i.e., the fraction of the ghosts crenated (△, ▲), was estimated from counts of 25–30 ghosts by phase microscopy. The estimated error in these counts is 20%, but this does not shift the midpoint of the curve by more than a few millimolar. The shape change scored was the conversion of smooth forms to irregular forms with ~10 large crenations, resembling the type I echinocyte. (b–e) Nomarski micrographs of ghosts in increasing electrolyte concentrations: (b) 10 mM NaCl, (c) 20 mM NaCl, (d) 30 mM NaCl, and (e) 100 mM NaCl. Bar, 10 μm.



by use of divalent cations. Fig. 1*a* shows typical results for Sr. Table I lists the half-maximally effective concentrations for different cations.

Crenated shrunken ghosts in high concentrations of monovalent or divalent cations return to a smooth cup-shaped morphology upon transfer to low salt solutions. The volume change induced by NaCl or KCl is completely reversible as well (Table II). Divalent cation-induced shrinkage is not completely reversed by washing in EDTA-containing solutions, but ghost volume is restored to 75–95% of its original value. The method of ghost preparation does not seem to affect these results. Ghosts prepared in phosphate buffers (11) yield essentially identical data with those cations that can be tested: Na, K, and Mg. The shape change is also seen in ghosts prepared from neuraminidase-treated erythrocytes, suggesting that external sialic acid is not necessary for electrolyte-induced morphological change. The observed changes are not osmotic in nature, because these ghosts, prepared in EDTA, cannot be resealed to small molecules (13).

It can also be concluded from Fig. 1*a* that light scattering is not directly related to ghost volume. A_{750} does not change until ghosts are reduced to about half of their original volume. The use of light scattering as a measure of ghost volume does not seem justified.

Electrolyte-induced Contraction of Spectrin-Actin Lattices

The membrane residue that remains after Triton extraction (28) has been shown to be a network or lattice of bands 1 and 2 (spectrin) and 5 (actin) with a few other proteins, notably band 2.1 (ankyrin) and 4.1 (3, 16, 25) (Fig. 2). There is good evidence that this residue represents the cytoskeleton of the red cell, i.e., a protein network, attached to the cytoplasmic surface of the membrane, that confers mechanical stability (9, 23). Conditions that lead to a volume reduction of the ghost also reduce the size of this lattice. This can be shown by the turbidity and hematocrit techniques used for ghosts. The significance of these measurements is doubtful, however, because the lattices tend to aggregate, making the values time dependent. A better measure of contraction is the diameter of the lattice, observed microscopically. We have not been able to find conditions that permit measurements with the electron microscope because negative stains are themselves concentrated electrolyte solutions. The lattices can, however, be observed in wet mounts by phase or Nomarski interference, and diameter measured with a calibrated eyepiece micrometer. The contrast in unstained lattices is so poor that we do not present any photographs. The optical contrast of Triton residues can

TABLE I
Half-Maximally Effective Concentrations

	Shape*		Volume		Light scattering	
	mM	\sqrt{I}	mM	\sqrt{I}	mM	\sqrt{I}
Na	14	4.2	72	8.7	140	12.0
K	14	4.3	75	8.9	145	12.2
Mg	2.3	3.3	37	10.7	20	8.0
Ca	2	3.2	29	9.5	25	8.9
Sr	2.7	2.7	21	8.2	30	9.7
Ba	2.5	3.4	15	7.0	24	8.7

The cation concentration required to induce a 50% change in the parameter measured was estimated from data in Fig. 1 and three similar experiments. \sqrt{I} , ionic strength.

* The shape change scored was appearance of large crenations.

TABLE II
Reversal of Ghost Volume Changes

Salt addition	mM	Percent of the original ghost volume	
		Before washing	After washing
Na	50	79	93
	100	61	95
	500	38	94
K	50	88	100
	100	77	98
	500	36	93
Mg	10	89	89
	30	70	87
	200	33	80
Ca	10	83	83
	30	70	83
	200	37	79
Ba	10	86	97
	30	72	77
	200	36	75
Sr	10	88	99
	30	80	86
	200	52	72

Ghosts in 5 mM Tris-Cl, pH 7.4, 7 mM NaCl, were mixed with the indicated salt solutions and hematocrit and Coulter counting was performed. The ghosts were then washed twice in 5 mM Tris, 7 mM NaCl, 1 mM EDTA, pH 7.4, and hematocrit and Coulter counts were repeated. The ghost volumes are given as a percentage of the volume of control ghosts not exposed to salt (usually 155–170 fl). All procedures were done at 0°C.

be enhanced by uranyl acetate or ammonium molybdate (17), but the high ionic strength of the stains contracts the residues.

The midpoint for NaCl-induced cytoskeleton contraction is 85 mM (Fig. 3), in reasonable agreement with the result for ghost contraction. KCl gives a similar curve. For divalents, the midpoint is around 25 mM, again in agreement with the ghost data. Triton does not affect the result, because a similar lattice contraction can be observed in Triton-free residues. As for ghost volume changes, the lattice contraction is reversible upon dilution (Table III).

Effect of ATP and Inhibitors

The volume contraction of both ghosts and lattices is not energy dependent. Addition of ATP, GTP, ADP, or AMP (0.1–1 mM) has no effect on the observed electrolyte response. Incubation with 1 mM Mg-ATP at 37°C for 15 min (22) has no effect on the volume. Shape is also not affected, as noted earlier (22). Incubation with 0.5 mM *N*-ethylmaleimide, under conditions known to inactivate myosin is without effect.

pH-Induced Ghost Contraction

Ghosts appear as irregularly shaped crumpled forms at pH 5.5 (cf. Fig. 3 of reference 18). The morphology is highly reminiscent of the earliest stage of ghost crenation in response to electrolytes (Fig. 1*b*), resembling the echinocyte I form of the intact erythrocyte. Because this degree of shape change in salt is associated with a 10–15% reduction in ghost volume, it might be predicted that ghosts at pH 5.5 would also show a volume reduction of 10–15%. Fig. 4*a* shows that this prediction is verified.

Morphologically, ghosts crenate at pH 5.5 but, at lower pH

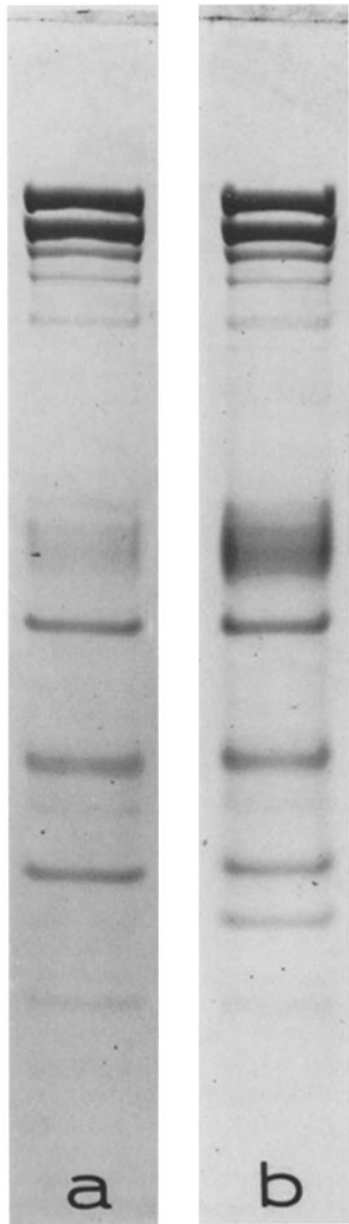


FIGURE 2 (a) SDS-gel slab electrophoresis of Triton-free spectrin-actin lattice. (b) SDS-gel slab electrophoresis of the ghosts in Tris-EDTA used as starting material for lattice isolation. The slabs had a 4–12% acrylamide gradient, with the buffer system of Fairbanks et al. (16).

TABLE III
Reversal of Lattice Contraction

Cation mM	Lattice diameter	
	Before washing	After washing
	μm	
Na 30	5.24 \pm 0.39	5.88 \pm 0.45
Na 200	4.52 \pm 0.35	5.78 \pm 0.47
Ca 10	3.76 \pm 0.33	6.10 \pm 0.46
Mg 10	4.00 \pm 0.46	6.24 \pm 0.36
Ba 10	3.90 \pm 0.32	6.21 \pm 0.30
Sr 10	4.08 \pm 0.37	6.40 \pm 0.37

The indicated concentrations of cation chlorides were added to Triton residues in 5 mM Tris-Cl, pH 7.4, 7 mM NaCl. 20 diameters were measured as described. The residues were then washed twice in 5 mM Tris-Cl, pH 7.4, 7 mM NaCl, 1 mM EDTA, and diameters again measured. All procedures were done at 0°C.

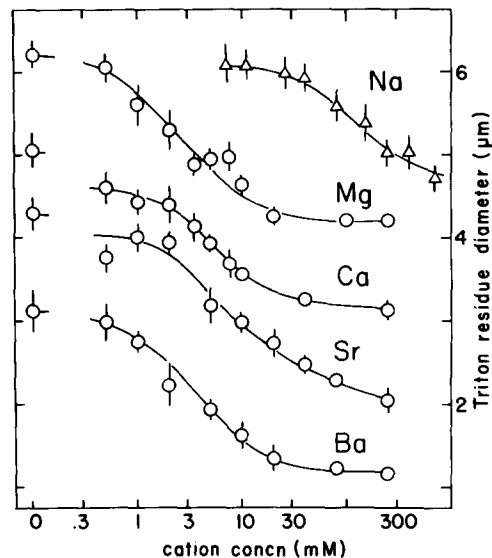


FIGURE 3 Size reduction in spectrin-actin lattices in response to salt. Triton residues of ghosts were titrated with 3.0 M solutions of the indicated cation chlorides in 5 mM Tris, 7 mM NaCl, pH 7.4, on ice. Diameters were determined by phase microscopy. These lattices were not freed of Triton before the addition of NaCl. Some of the curves are moved down on the graph for clarity: Ca by 1 μm , Sr by 2 μm , Ba by 3 μm .

values, become rounded spheres with fine projections. Shape and volume changes are reversible, as long as pH does not go below 5.5.

The pH experiments were extended to pH 4.8 to include the reported isoelectric point of the spectrin-actin lattice (10). At this pH, the ghosts are still able to respond to electrolytes by a further reduction in ghost volume (Fig. 4b). This result suggests that even at the isoelectric point, the spectrin-actin lattice will contract in response to electrolytes. We have not been able to test this point directly with isolated spectrin-actin lattices because they aggregate and precipitate at pH values of 6 or less.

Effect of 37°C Incubation on the Salt-induced Shape and Volume Changes

The volume and shape changes can be uncoupled. Incubation of ghosts for 15 min at 37°C in low ionic strength buffers abolishes their ability to crenate upon subsequent addition of salt, but does not affect the volume change (Table IV). The salt response of the Triton residue is also unchanged, suggesting that cytoskeleton contraction is not affected by 37°C incubation. The heat-treated ghosts assume a crumpled appearance in salt but do not crenate, suggesting that 37°C incubation has disrupted the link between cytoskeleton contraction and crenation. SDS gels of ghosts show very little proteolysis (1) or cross-linking (15) occurs during 37°C incubation, which is done in 1 mM EDTA. In addition, at the ionic strength used in these experiments, no significant amount of spectrin is released from the membrane.

DISCUSSION

There have been frequent suggestions that a contractile protein is present in erythrocytes (reviewed in reference 12). The results described here show that isolated ghosts can reduce their volume in response to electrolytes or pH, and this volume reduction is also a property of the isolated spectrin-actin lattice.

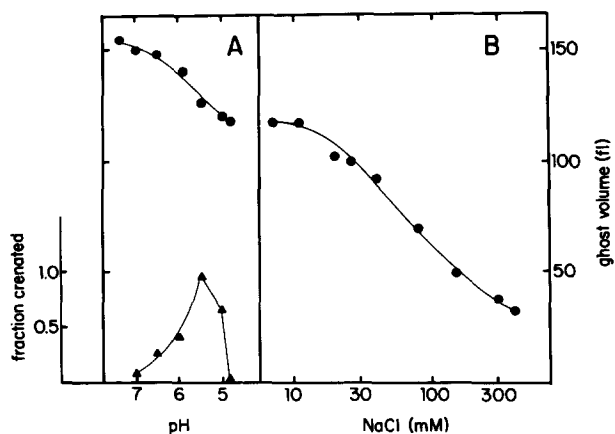


FIGURE 4 (A) Size reduction of ghost in response to pH. A 20-ml suspension of ghosts in 5 mM Tris, pH 7.4, 7 mM NaCl, 1 mM EDTA was titrated at 0°C with 5- μ l additions of 0.10 N HCl. At the pH indicated, measured volumes were taken for hematocrit and microscopy. (B) Ghost electrolyte response at pH 4.8. The same ghost suspension was titrated with 3.0 M NaCl at 0°C. Ghost volume was calculated from hematocrit and Coulter counting.

TABLE IV
Effect of 37°C Incubation on Electrolyte-induced Changes in Ghosts and Cytoskeletons

NaCl mM	Ghosts		Triton residues			
	Volume fl	Percent crenated	Percent crenated		Diameter μ m	
			0°C	37°C	0°C	37°C
7	162	160	0	0	7.07 \pm 0.54	6.50 \pm 0.38
30	131	129	100	0	5.98 \pm 0.35	5.14 \pm 0.30
80	87	84	100	0	5.10 \pm 0.28	5.02 \pm 0.30
150	57	53	100	0	5.02 \pm 0.33	4.56 \pm 0.67
300	42	41	100	0	4.45 \pm 0.42	4.64 \pm 0.67

Hemoglobin-free ghosts were prepared in 5 mM Tris-Cl, 7 mM NaCl, 1 mM EDTA, pH 7.4. An aliquot was incubated at 37°C for 15 min. Control (0°C) and incubated (37°C) ghosts were titrated with 3.0 M NaCl on ice, and volume and shape were determined as described for Fig. 1. Triton residues were prepared in the same buffer from control ghosts; an aliquot was incubated at 37°C for 15 min, and diameters were determined as described for Fig. 3.

Under some circumstances, therefore, a form of contractility can be directly demonstrated in ghosts and in isolated spectrin-actin networks.

This contraction of the spectrin-actin lattice is probably electrostatic in nature, as it is not energy-dependent and is not inhibited by agents that affect myosin. It is plausible that as the effective charge on the components of the lattice is reduced, they will approach more closely. Effective charge can be lowered by small ion shielding, which is dependent on the square root of the ionic strength (\sqrt{I}) for polyelectrolytes (cf. reference 24). Table I is consistent with this, showing that all solutions tested are effective at similar \sqrt{I} values. The electrostatic repulsion between the components of the lattice will also decline as pH approaches their isoelectric point. As expected, ghost volume declines with pH. Even when pH = 4.8 (the pI for the spectrin-actin lattice), however, volume reduction is only ~20%, and ghosts still respond to electrolytes. Because intermolecular electrostatic repulsion is minimized at the isoelectric point, the observation of further electrolyte-dependent

contraction at pH 4.8 suggests that the dimension of the ghost and cytoskeleton depend on both intermolecular and intramolecular forces. In fact, it is known that the hydrodynamic radius of spectrin declines as NaCl or KCl concentrations are raised above 20 mM (2, 20). In related work, it was shown (27) that the Triton residue of the *Tetrahymena* nucleus also reversibly contracts in response to cations, suggesting that non-myosin-dependent contractile networks may be a general feature of biological membranes. The relation between this cytoskeleton contraction and membrane shape changes is consistent with a model of membrane structure in which contraction of the lattice attached to the inner surface leads to bending of the incompressible lipid bilayer. The model requires lattice attachment to the inner surface at discrete points, and spectrin is known (3) to bind to band 2.1. Moreover, any treatment that releases spectrin from its binding site should eliminate the shape change, and it is known that spectrin binding to inside-out membrane vesicles is weak at 37°C in low salt (2), conditions that eliminate the shape change (Table IV). Significantly, 37°C incubation does not affect the isolated cytoskeleton, implying that binding interactions within the lattice are stable under these conditions. It therefore appears that the effect of a brief 37°C incubation is to dissociate spectrin-membrane links, without affecting binding interactions within the lattice itself.

A similar model was proposed by Elgsaeter et al. (10) to explain the appearance of protein-free lipid vesicles in ghosts treated with the strongly basic molecule, polylysine. The electrolyte- or pH-induced shrinkage of the lattice postulated here differs from that caused by polylysine, in that it is less extreme and is reversible. A role for contractile protein in the control of ghost volume was also proposed earlier by Palek et al. (19), who noted ghost shrinkage in response to Ca^{++} . Our results generalize this observation to show that Ca^{++} is not unique in this ability, and that the effect is not energy dependent or related to known contractile proteins.

Other mechanisms have been proposed to explain ghost shape changes. For example, crenation in intact erythrocytes and in pink ghosts in response to drugs has been elegantly rationalized by the bilayer couple hypothesis (21). The bilayer couple hypothesis has earlier been invoked to explain electrolyte effects on white ghosts (14), using the known effects of ionic strength and divalent cations on phospholipid monolayers. It remains possible that bilayer couple effects are relevant to ghost shape changes in response to electrolytes, but the observed submembranous lattice contraction and ghost volume reduction in response to electrolytes must also be included in any explanatory model. It is of course, reasonable to assume that both the lipid and protein components of the membrane will participate in morphological changes.

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