

# Role of the Reticulum in the Stability and Shape of the Isolated Human Erythrocyte Membrane

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**ABSTRACT** In order to examine the widely held hypothesis that the reticulum of proteins which covers the cytoplasmic surface of the human erythrocyte membrane controls cell stability and shape, we have assessed some of its properties. The reticulum, freed of the bilayer by extraction with Triton X-100, was found to be mechanically stable at physiological ionic strength but physically unstable at low ionic strength. The reticulum broke down after a characteristic lag period which decreased 500-fold between 0° and 37°C. The release of polypeptide band 4.1 from the reticulum preceded that of spectrin and actin, suggesting that band 4.1 might stabilize the ensemble but is not essential to its integrity.

The time-course of breakdown was similar for ghosts, the reticulum inside of ghosts, and the isolated reticulum. However, at very low ionic strength, the reticulum was less stable within the ghost than when free; at higher ionic strength, the reverse was true. Over a wide range of conditions the membrane broke down to vesicles just as the reticulum disintegrated, presumably because the bilayer was mechanically stabilized by this network.

The volume of both ghosts and naked reticula varied inversely and reversibly with ionic strength. The volume of the naked reticulum varied far more widely than the ghost, suggesting that its deformation was normally limited by the less extensible bilayer. The contour of the isolated reticulum was discoid and often dimpled or indented, as visualized in the fluorescence microscope after labeling of the ghosts with fluorescein isothiocyanate. Reticula derived from ghosts which had lost the ability to crenate in isotonic saline were shriveled, even though the bilayer was smooth and expanded. Conversely, ghosts crenated by dinitrophenol yielded smooth, expanded reticula. We conclude that the reticulum is a durable, flexible, and elastic network which assumes and stabilizes the contour of the membrane but is not responsible for its crenation.

The shape of the mature human erythrocyte, a biconcave disk, and its many alterations under pathological and experimental conditions have long intrigued cytologists (1–4). Since the ghost, the intact membrane recovered following hypotonic hemolysis, is itself frequently a dimpled disk and since there are no other structural systems in this cell (4), it is widely held that the membrane controls cell contour. Both membrane lipids and proteins have been implicated in this regard. In the present study, we examine the proteins; the bilayer will be considered in a subsequent paper.<sup>1</sup>

The cytoplasmic surface of the erythrocyte membrane is covered by a filamentous reticulum of proteins which conserves

its integrity by noncovalent self-associations, even when the bilayer and its integrated proteins are removed by extraction with Triton X-100 (5–7). The major components of the reticulum are polypeptide bands 1 and 2, called spectrin, and band 5, called actin (5). Isolated spectrin takes the form of heterodimers or elongated tetramers, (band 1 + band 2)<sub>2</sub>, while the actin appears oligomeric (8). The mode of association of spectrin with actin is uncertain. Bands 2.1 and 4.1 are accessory peripheral proteins which bind independently to the membrane (9) and to the reticulum (5, 7, 8). Band 4.1 appears to associate with an integral glycoprotein (10) and to promote associations between spectrin and actin in vitro (11–13). Band 2.1 associates with both spectrin (14) and the cytoplasmic pole of the major integral membrane protein, band 3 (15, 16), so as to link the

<sup>1</sup> Lange, Y., A. Gough, and T. L. Steck, submitted for publication.

reticulum to the membrane proper (see reference 8 for review).

The reticulum extracted in Triton X-100 resembles in size and shape the parent erythrocyte and ghost (5-7, 17), and the hypothesis that it is the primary effector of membrane contour has been widely discussed (see references 2, 3, 8, and 18 for review). Sheetz and Singer (19) observed that incubation with MgATP and antispectrin antibodies reversed the crenation (spiculation) of ghosts normally observed in isotonic saline. Birchmeier and Singer (20) correlated this process with the phosphorylation of band 2 and argued that membrane contour was controlled by spectrin phosphorylation. However, Fairbanks et al. (18) and Tyler and Anderson (21) found no correlation between the phosphorylation of band 2 and membrane contour. Nevertheless, Johnson et al. (22) recently interpreted their analysis of the shape and size relationship of ghosts and reticula to signify that "reversible changes in the size of the underlying cytoskeleton of the erythrocyte membrane can control cell shape."

Despite a wealth of biochemical data on its isolated components (3, 8, 23), the properties of the reticulum as a whole are obscure. When we initiated this study, for example, it was not known whether this network has the mechanical strength requisite to stabilizing the erythrocyte membrane *in vivo*, whether it is elastic (i.e., reversibly deformable), whether its breakdown *in vitro* causes the observed coincident membrane fragmentation, and whether it is mechanistic in ghost crenation. We have therefore attempted to characterize some of these properties of the reticulum. A brief account of some of this work has been presented (24).

## MATERIALS AND METHODS

### Materials

Human erythrocytes were obtained from normal donors either fresh in Na<sub>2</sub>EDTA or from outdated units generously provided by the University of Chicago Blood Bank. The age of the blood did not have a significant effect on our results.

All biochemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Glutaraldehyde stocks from Polysciences, Inc. (Warrington, PA), and Eastman Kodak Co. (Rochester, NY) were adjusted to pH 8.0 and used interchangeably without notable differences. Other chemicals were of at least reagent grade, from Fisher Scientific Co. (Pittsburgh, PA), J. T. Baker Chemical Co. (Phillipsburg, NJ), or Mallinckrodt, Inc. (St. Louis, MO).

### General Methods

Unless specified, all procedures were performed on ice at pH 8.0 and all centrifugations at 0°C for 15 min in a Sorvall SS-34 rotor at 15,000 rpm. Ghosts were prepared and maintained in 5 mM NaP<sub>i</sub> (pH 8) as described (9).

### Determination of Ghost and Reticulum Volume

Relative volumes of ghosts and reticula were determined by measuring the fractional weight of pellets after centrifugation, equating volume (cm<sup>3</sup>) with weight (g). To preweighed tubes were added different buffers, ghosts, and Triton X-100 (0.038 ml/ml packed ghosts). In most experiments with reticula, 3 μg/ml methylene blue was included to facilitate visualization of the nearly transparent pellets. The tubes were centrifuged first in a Sorvall SS-34 rotor for 30 min at 15,000 rpm and then in a swinging bucket in an HSA rotor for 15 min at 5,000 rpm to flatten the meniscus of the pellet. Fractional pellet weight was determined by weighing the tubes empty and before and after the supernatant fluid was removed. Duplicate determinations typically differed by 2-8%.

To determine the volume of ghosts and reticula above an ionic strength of 0.005, 1 vol of ghosts was suspended in 2 vol of buffer. To bring the suspensions to lower ionic strength without prior washing (which aggregates and disrupts the reticula), the proportion of ghosts to buffer was reduced. In the extreme,  $\mu = 0.001$ , ghosts in 5 mM NaP<sub>i</sub> were suspended in 14 vol of water. Because the volume of ghosts and reticula increased with decreasing ionic strength, the decrease in the proportion did not diminish appreciably the accuracy of the assay.

The effect of variation in the proportions of ghosts, Triton X-100, and buffer

on the apparent fractional pellet volume of the reticula was studied in control experiments. It was found that at a constant Triton X-100/ghost ratio, the apparent reticulum volume remained invariant as the proportion of ghosts in the mixture was increased from 0.06 to 0.33 ml/ml.

**GEL ELECTROPHORESIS:** PAGE was performed as described (25), except that SDS was reduced to 0.1% to enhance the resolution of bands 4.1 and 4.2 (reference 9). In fractionation experiments (e.g., Fig. 3), gel pairs were loaded with the equivalent amount of supernatant and pellet material, derived from the same number of ghosts (as in references 5, 9, and 25). Recovery was complete, and there was no indication of proteolysis. To estimate the fraction of bands 1, 2, 4.1, and 5 solubilized during an incubation, gels were compared visually by two independent observers to a set of calibration gels prepared from the input mixture. This method proved more reliable than densitometry (although both gave comparable results) because of the variable degree of overlap between closely spaced components in densitometric tracings.

**LABELING OF MEMBRANES:** Fluorescent labeling of ghosts was adapted from a general method (26). 1.2 vol of 0.2 M NaHCO<sub>3</sub>-Na<sub>2</sub>CO<sub>3</sub> buffer (pH 9.2) was added to 1 vol of freshly-prepared ghosts in 7 vol of 5 mM NaP<sub>i</sub> containing, in some experiments, 1 mM MgSO<sub>4</sub>/1 mM dinitrophenol or 0.15 M NaCl. Fluorescein-5-isothiocyanate (7 mg) was dissolved in 1 ml of dioxane and then 0.5 ml of acetone was added; 0.5 vol of this solution was added to the ghosts. After incubation for 15 min, the mixture was diluted 3- to 6-fold in the incubation buffer, centrifuged, and the ghosts were washed once or twice more in 40 vol of the same buffer until the supernatant fluid no longer was fluorescent under ultraviolet light. The fluorescent ghost pellet was kept on ice and examined within 2 h.

Labeled ghosts were suspended in 7 vol of various buffers. Aliquots were extracted with 0.5% Triton X-100 for 2-5 min at 0°C before fixation in 1% glutaraldehyde for 30 min. Isolated reticula could not be labeled directly because of their disruption and aggregation during centrifugation. Fluorescent ghosts and reticula were more labile than controls, presumably because of the exposure to the solvent and high pH and/or their covalent modification, but otherwise exhibited all of the properties observed in unmodified preparations by dark-field microscopy. Fluorescence microscopy employed a Zeiss microscope, epi-illumination, and 1,000-fold magnification under oil. Ektachrome (ASA 400) film was used with exposure times of 60 s.

## RESULTS AND DISCUSSION

In the studies described below, three types of preparations were compared: (a) intact isolated ghosts; (b) reticula released from ghosts with Triton X-100 following experimental manipulation; and (c) reticula released from ghosts before experimental manipulation. In this way, the characteristics of the reticulum could be assessed both within the ghost and after isolation. We avoided attempts to separate the reticula from the dissolved membrane lipids and integral proteins, since manipulations such as centrifugation can cause aggregation and breakdown of the network. We have no evidence that the presence of the detergent solution of bilayer constituents affected our results.

### Stability of the Ghost and Reticulum

Under favorable conditions, both hemoglobin-free membranes and the reticula liberated therefrom by dissolution of the bilayer in Triton X-100 were indefinitely mechanically stable. For example, continuous brisk magnetic stirring overnight at 0°C in 2-8 vol of isotonic saline ( $\pm 0.5\%$  Triton X-100) caused no detectable breakdown of either preparation, as judged by dark-field microscopy and by the constancy of their pellet volumes. These simple observations signify that the reticulum is highly stable without the bilayer. Results compatible with these were recently reported by Liu and Palek (27).

Both ghosts and reticula became highly unstable as the ionic strength was reduced and the temperature was raised (see also references 9 and 23). For example, ghosts and reticula incubated at 37°C broke down after 1 min in 0.5 mM NaP<sub>i</sub> and after 5-10 min in 5 mM NaP<sub>i</sub> (both pH 8) but were indefinitely stable in isotonic saline. We analyzed for physical instability using four types of assay. Each assay had limitations, but satisfactory agreement was found among them. They were (a)

visualization of breakdown by dark-field microscopy after glutaraldehyde fixation, (b) estimation of the packed ghost or reticulum volume after centrifugation, (c) electrophoretic analysis of the loss of sedimentability of polypeptides by low-speed centrifugation, and (d) the disruption and loss of adherence to the wall of a slanted glass centrifuge tube of a ghost or reticulum pellet during a postcentrifugation warming step. (This last assay, while indirect, was most convincing in establishing that reticula disintegrate in the absence of shear stress.)

Analysis of reticulum breakdown was not straightforward. Light microscopy was hampered by the poor visibility of the reticula and the intriguing failure of reticula to be fixed by glutaraldehyde in very low ionic strength alkaline buffer (e.g., 0.5 mM NaPi, pH 8). As reticula became labile, their disintegration was promoted by simple maneuvers such as the centrifugation step used in some assays. Furthermore, the dispersion of fragment size during disintegration made the assay of breakdown dependent on the integrated centrifugal force applied.

In all four assays, the time-course of breakdown was characterized by a lag period during which the structures appeared normal, followed by relatively rapid disintegration. The breakdown of ghosts was usually by endocytic vesiculation (28). Reticula merely fell to pieces of progressively smaller size. Breakdown was immediately arrested at any point by increasing the ionic strength. A typical experiment is shown in Fig. 1B. During an incubation at 30°C in 5 mM NaPi, isolated reticula broke down abruptly after a lag of about 20 min. In contrast, the reticula extracted from standard ghosts at various times during the same incubation period were stable, with no breakdown apparent until some vesiculation was observed at 80 min, the decrease in pellet volume at 10 min notwithstanding. (We note in passing that ghosts gently warmed in a commonly used hypotonic buffer are not stable for long.)

The pattern of breakdown during incubation in 2 mM NaPi was strikingly different (Fig. 1A). After 20 min at 20°C, the ghosts and the reticula within them disintegrated. In contrast,

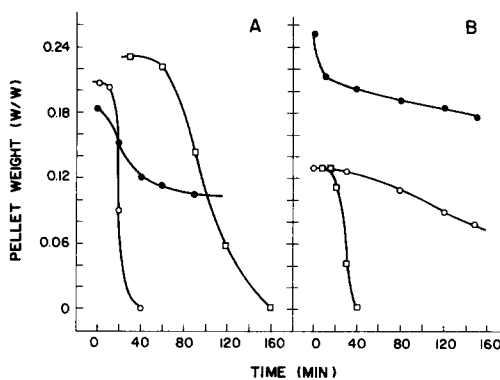


FIGURE 1 Time-course of breakdown of ghosts, isolated reticula, and reticula within ghosts. Panel A: ghosts (●) packed in 5 mM NaPi (pH 8) were mixed with 2 vol 0.5 mM NaPi to make the solution 2 mM NaPi, incubated at 20°C, and then chilled on ice. 0.25 vol of the same buffer was added and the mixtures were centrifuged to determine the fractional pellet weight (hence volume) of the membranes as described in Materials and Methods. Isolated reticula (□): the experiment was performed as with ghosts (above), except that 1% Triton X-100 was present in the buffers. Reticula within ghosts (○): the experiment was performed as with ghosts, except that the 0.25 vol of buffer added after the incubation contained concentrated Triton X-100 to make a final concentration of 1%. Panel B: as in panel A, except the experiment was performed in 5 mM NaPi (pH 8) at 30°C.

isolated reticula were stable for >1 h. Thus, at very low ionic strength, the reticulum was more stable when freed of the bilayer than inside it, while the opposite was true at higher ionic strength. (This result was obtained at all temperatures tested.) The stabilizing effect of the bilayer on the reticulum in Fig. 1B is easily rationalized; the destabilizing effect at very low ionic strength (Fig. 1A) is surprising, but might be ascribed to increased electrostatic repulsions between the highly anionic reticulum and phospholipids at the cytoplasmic surface of the bilayer when charge screening is reduced.

The time-course of breakdown was highly temperature-dependent, as shown in Fig. 2 for ghosts and isolated reticula in 0.5 mM NaPi. The time for 50% breakdown, assayed as the failure of spectrin to sediment, was found to vary from ~1 min at 37°C to ~500 min at 0°C. It is of particular importance that the kinetics of breakdown of ghosts paralleled that of isolated reticula over nearly three orders of magnitude (Fig. 2), since this finding provides the most direct evidence to date that disruption of the reticulum is mechanistic in ghost disintegration. However, these data do not distinguish between two mechanisms: (a) that the disintegration of the reticulum simply left the bilayer destabilized, and (b) that the disintegration of the reticulum operated on the bilayer, forcing its endocytic vesiculation (see, for example, Fig. 6 in reference 28).

There was a systematic discrepancy between the disappearance of the visible reticulum pellet and the recovery of spectrin and actin at the top of the column of supernatant fluid. This represented the partial sedimentation of fragments of intermediate sizes. For example, in one experiment, ghosts were incubated for 50 min at 20°C in 0.5 mM NaPi without agitation and were then extracted with 0.5% Triton X-100 (in isotonic saline to prevent further breakdown of the reticulum). The pelletable residue of reticulum proteins essentially disappeared during this period, yet only 50% of the spectrin was recovered at the meniscus after a centrifugation of  $0.9 \times 10^6 g_{\text{avg}} \cdot \text{min}$ . A subsequent recentrifugation of the supernatant fluid at  $8 \times 10^6 g_{\text{avg}} \cdot \text{min}$  cleared this sample of 95% of the spectrin. We conclude both from these data and from microscopy that the

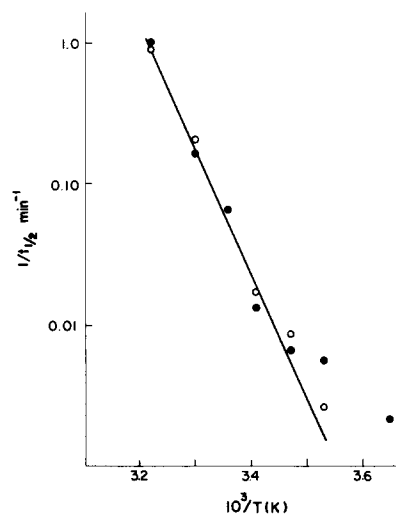


FIGURE 2 Temperature dependence of the rate of breakdown of ghosts and isolated reticula. Packed ghosts in 5 mM NaPi (pH 8) were added to 7 vol of 0.5 mM NaPi (pH 8) preequilibrated to the indicated temperature containing (●) or lacking (○) 0.5% Triton X-100. After incubation for different times, the mixtures were centrifuged and supernatant fluids analyzed by gel electrophoresis. The half-

time of breakdown,  $t_{1/2}$ , was the interval (in minutes) at which one half of the total spectrin (bands 1 and 2) present was released into the supernatant. The data are plotted according to Arrhenius. The slope of the line corresponds to an energy of activation of 41 kcal/mol; however, this value has no clear-cut interpretation in the face of such a complex kinetic process.

pathway of breakdown of the reticulum entailed neither the stepwise release of individual spectrin and actin molecules nor its all-or-none disintegration into soluble forms, but rather repeated fragmentation to progressively smaller complexes until dissolution was complete. This pattern of progressive fragmentation would of itself, create sigmoid time-courses such as observed in Fig. 1, since reticula and ghosts must undergo a series of breakdown steps before they become unsedimentable. However, a distinct lag and abrupt upheaval are also seen both by microscopy and during the warming of pellets (assays *a* and *d*, discussed above) where no particle size threshold operates.

Our data suggest that the disintegration of the reticulum, hence ghost, at low ionic strength simply reflects the progressive rupture of a repeated stabilizing link in the network. At first, such breaks are not manifest in the overall structure but after a sufficient number accumulates, pieces of the reticulum of varied size are released. The pieces continue to break down, sedimenting more slowly, and, eventually, not at all. Although it too has a high temperature dependence, the dissociation of spectrin tetramers into dimers is not the likely cause of the breakdown of the reticulum since it does not proceed to an appreciable degree at 0°C (compare Fig. 2 and reference 29). Instead, the dissociation of oligomeric actin or of band 4.1 from spectrin may well be the rate-determining step.

#### Release of Polypeptides during Reticulum Breakdown

The principal proteins associated with the Triton X-100 insoluble reticulum at low ionic strength are bands 1, 2, 4.1, and 5; however, variable amounts of bands 2.1, 3, 4.2, 6, and 7 and lipids are also present (5, 7). By monitoring the time-course of breakdown of the pelletable reticulum at very low ionic strength (0.5 mM NaP<sub>i</sub>), further dissection of this complex structure was revealed (Fig. 3). In these experiments, the su-

pernatant and pellet fractions always summed to equal the input; that is, the original components were recovered quantitatively and no proteolysis or aggregation was detected.

Fig. 3 shows that most of band 2.1 was solubilized from the reticulum at the very low ionic strength. (While band 2.1 is obscured by band 2 in gels of the pellet, gels of the supernatant fraction contain the bulk of the input band 2.1.) Thus, while band 2.1 binds spectrin (14), it is clearly not essential for the integrity of the reticulum (see also reference 7). These experiments also show that the residual portion of band 3 retained in the pellet is not linked to spectrin by band 2.1 (although such complexes have been observed [15, 16]). Furthermore, band 4.2 is retained in the pellet out of proportion to band 3 (see also reference 5), suggesting a preferential association with the subfraction of band 3 associated with the reticulum.

The solubilization of the four major reticulum polypeptide species during incubation at 0°C in 0.5 mM NaP<sub>i</sub> was not synchronous. In the experiment shown in Fig. 3, more than half of band 4.1 was rapidly solubilized before any significant release of bands 1, 2, and 5. The reticula, which appeared intact microscopically at this time, were stable over the next few hours in the absence of half of their band 4.1. The remainder of band 4.1 was then progressively solubilized along with bands 1, 2, and 5 over the remainder of the 15-h incubation, either because of its association with spectrin (8) or its attachment to the residual patches of bilayer adherent to the reticulum (5, 10).

We infer from these data that the integrity of the reticulum is not dependent on the full complement of band 4.1 molecules, i.e., that band 4.1 does not provide an essential link in the network. (While it is possible that the reticulum could remain intact after some links were broken, it is hard to imagine that the structure could survive when 50–70% of an essential component was removed, as in Fig. 3). This inference is consistent with reconstitution studies, which suggest that band 4.1 pro-

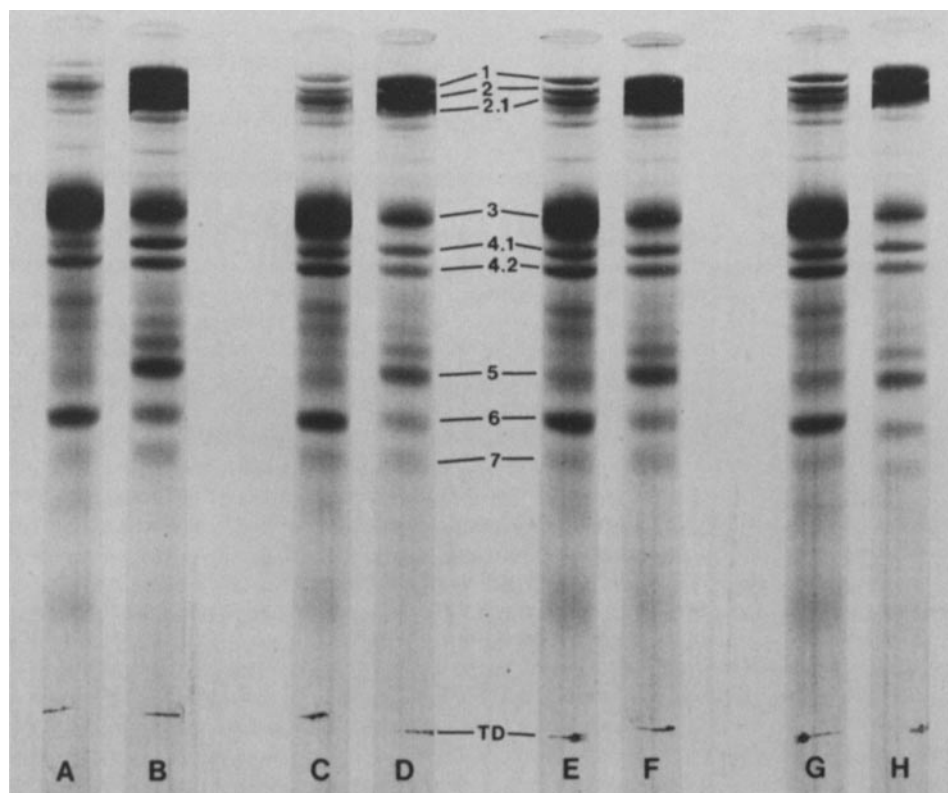


FIGURE 3 Release of polypeptides during low ionic strength incubation. Ghosts were incubated in 7 vol of 0.5 mM NaP<sub>i</sub> (pH 8) at 0°C for the times indicated. Then Triton X-100 was added to 0.45% and NaP<sub>i</sub> to 5 mM (to stabilize the residual reticula). The samples were centrifuged for 30 min and equivalent aliquots of supernatants and pellets analyzed by gel electrophoresis. The left- and right-hand gels of each pair show supernatant and pellet fractions, respectively. TD, tracking dye. (A and B) 0 h; (C and D) 0.5 h, (E and F) 1.0 h, and (G and H) 1.5 h.

notes but is not required for spectrin-actin association (8, 11-13).

These findings bear on the solubilization of spectrin and actin from detergent-free ghosts at low ionic strength. Under those conditions, the retention of bands 1, 2, and 5 in the ghost should not be a function of spectrin binding to band 2.1, since we show here that such associations are broken at low ionic strength. The release of bands 1, 2, and 5 from ghosts must await both the breakdown of the reticulum *in situ* and the dissociation of spectrin from band 4.1 or, less likely, of band 4.1 from the membrane proper. A small fraction of solubilized spectrin and actin is usually recovered as high molecular weight aggregates containing band 4.1 (reference 8); these may be the counterpart of the stable complexes seen in Fig. 3 (e.g., gel *H*). Since it is this material which stimulates actin polymerization, it may be taken to be the most native component of the solubilized reticulum.

### Effect of Ionic Strength on Ghost and Reticulum Volume

Both the membrane phospholipids and the reticulum proteins bear a strong net negative charge at neutral pH. It was not surprising, therefore, that the volumes of both ghosts and reticula varied inversely with ionic strength (hence charge-shielding) under conditions selected to minimize breakdown (Fig. 4). Johnson et al. (22) have recently reported similar observations.

The decrease in ghost volume with increasing ionic strength (from 87 fl at  $\mu = 0.014$  to 64 fl at  $\mu = 0.164$ ) was not caused by breakdown but by shrivelling and/or flattening, readily apparent in the dark-field microscope. On the other hand, the drop in ghost pellet volume noted at very low ionic strength ( $\mu = 0.001$ ) was associated with vesiculation. This breakdown was promoted by the centrifugation step, since it was not observed in the unspun input ghosts.

The ionic strength dependence of the packed volume of the reticulum showed several interesting features (Fig. 4). (a) The reticulum assumed a volume less than half that of the ghosts at

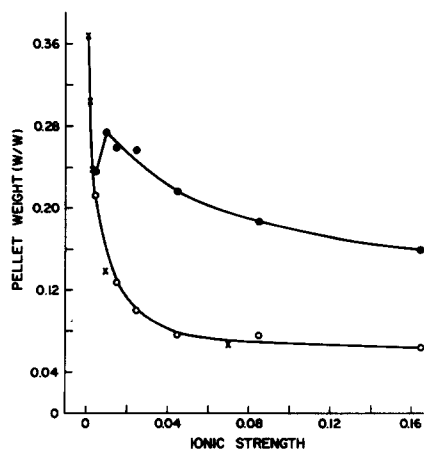


FIGURE 4 Dependence of ghost and reticulum volume on ionic strength. Ghosts (●): packed ghosts in 5 mM NaPi were mixed with 2-9 vol of water or NaPi (pH 8) to the indicated ionic strengths. After brief incubation on ice, the mixtures were spun to determine the fractional pellet weight as described in Materials and Methods. Reticula (○): as above, except in the presence of 0.038 ml of Triton X-100/ml of packed ghosts. Reticula in MgSO<sub>4</sub> (×): as above, except in the presence of 0.038 ml of Triton X-100/ml of packed ghosts plus 0.1 mM MgSO<sub>4</sub> (final).

$\mu = 0.164$ . (b) The reticulum assumed a volume 50% greater than the ghosts at very low ionic strength. The volume excursion of the reticulum was approximately three times greater than that of the ghost. The reticulum is thus capable of much larger volume changes than the ghost itself, suggesting that the bilayer normally constrains it and not vice versa. (c) The volume of the reticulum continued to rise at low ionic strengths where ghosts broke down. This differential stability of the isolated reticulum at low ionic strength was previously demonstrated in Fig. 1A. The dramatic expansion of the reticulum beyond the limits of the inextensible bilayer might account for the accelerated ghost breakdown at very low ionic strength (Figs. 1A and 4). That is, the charge repulsions in the polyanionic network cannot be reduced by expansion while trapped within the ghost and are relieved only by its disintegration, accompanied by ghost destabilization and vesiculation. (d) The presence of 0.1 mM MgSO<sub>4</sub> or CaCl<sub>2</sub> (not shown) had no effect on reticulum volume.

The reticulum has been visualized by light and electron microscopy (5-7, 17, 22, 27). However, these studies ran the risk of distorting the contour of the reticulum by employing positive or negative staining with heavy metal ions, drying on supports, etc. While we observed that wet-mounts of untreated reticula were visible in the dark-field microscope (cf. also reference 22), they were too diaphanous at low ionic strength to be well-photographed. However, by prelabeling ghosts with fluorescein isothiocyanate, the reticula isolated therefrom could be visualized by fluorescence microscopy.

Fluoresceinated reticula were photographed at varied ionic strength (Fig. 5). In all cases, the reticula assumed a discoid rather than globoid shape (seen best during their initial tumbling before they aligned parallel to the slide and cover slip). At high ionic strength, we frequently observed in the reticula depressions, clefts or even bowl-shapes reminiscent of stomatocytes (Figs. 5-7).

The diameter of the profiles decreased with increasing ionic strength. The dependence on ionic strength of the square of the diameter of the profiles paralleled the curve for the reticulum shown in Fig. 4, providing confirmation of those data. (If the profiles represented globes rather than disks, the variation would be roughly proportional to the cube rather than square of their diameter.)

The effect of ionic strength on the size and contour of the reticulum was reversible. That is, residues prepared in 5 mM NaPi ( $\mu = 0.014$ ) and then brought to 10 times or one-tenth that ionic strength closely resembled reticula prepared directly at those final ionic strengths.

Our results indicated that the reticula changed size but not shape with ionic strength. In contrast, ghosts underwent significant shape changes as ionic strength was varied. At very low ionic strength ( $\mu < 0.002$ ), ghosts were spheroids. At physiologic ionic strength, ghosts were typically either crenated (spiculated) or flattened disks. This dissociation of ghosts and reticulum shapes suggests that the reticulum is not a determinant of ghost crenation; this hypothesis is directly tested below.

### The Role of the Reticulum in Ghost Crenation

We used the fluorescence microscope to examine the shape of the reticulum from ghosts of varied contour. Fluoresceinated ghosts were induced to crenate by incubation with 2,4-dinitrophenol plus MgSO<sub>4</sub> and with isotonic saline<sup>1</sup> and aliquots of these specimens were extracted with Triton X-100 before examination. Standard ghosts yielded reticula of corresponding contour (Fig. 6, panels A1 and B1). Dinitrophenol/Mg-treated

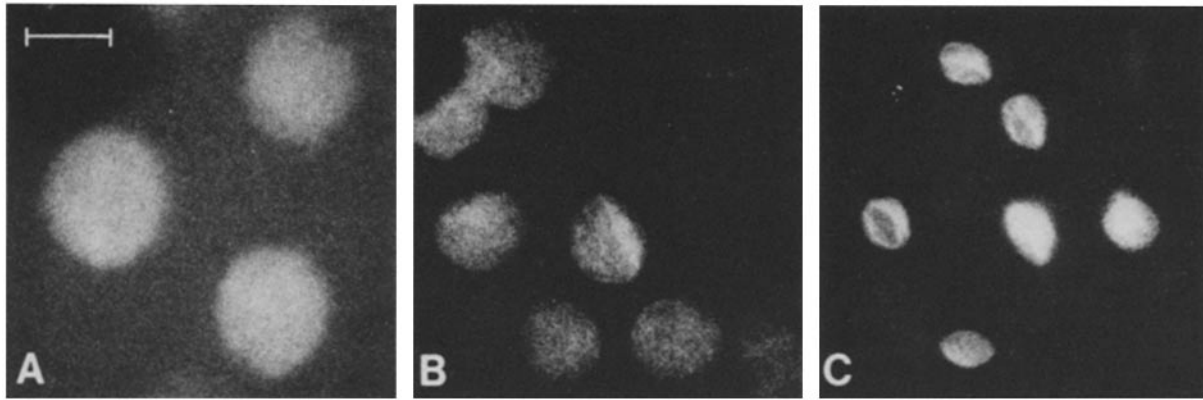


FIGURE 5 Effect of ionic strength on the appearance of the fluoresceinated reticulum. Ghosts labeled with fluorescein isothiocyanate as described in Materials and Methods were suspended in 7 vol of 0.5 mM  $\text{NaP}_i$  (A), 5 mM  $\text{NaP}_i$  (B), or 5 mM  $\text{NaP}_i$ /150 mM NaCl (C) all at pH 8.0 at  $0^\circ\text{C}$ . The bilayer was dissolved by making the suspension 0.5% in Triton X-100. Samples were then fixed in 1% glutaraldehyde and visualized by fluorescence microscopy. Final buffer ionic strengths were: (A) 0.003, (B) 0.014, and (C) 0.164. Bar, 10  $\mu\text{m}$ .

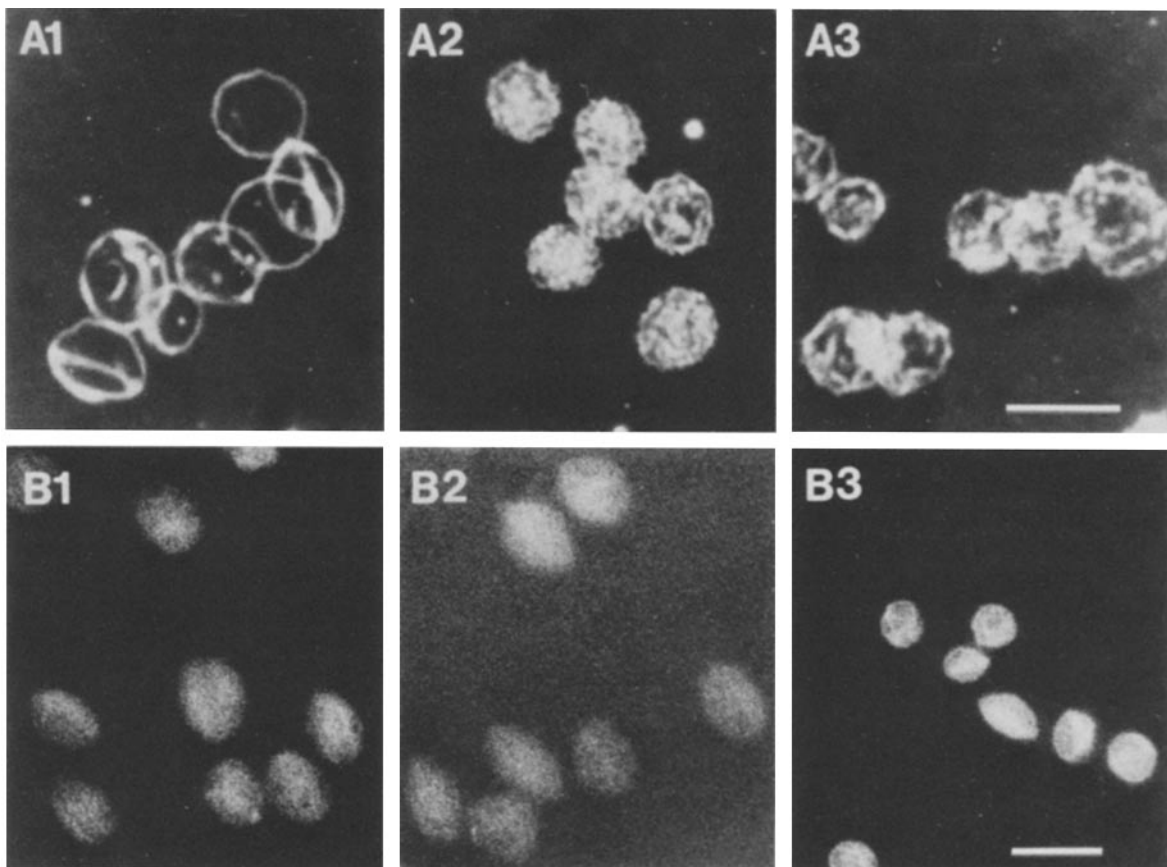


FIGURE 6 Effect of 2,4-dinitrophenol and saline on the shape of ghosts and reticula. Ghosts labeled with fluorescein isothiocyanate as described in Materials and Methods were suspended at  $0^\circ\text{C}$  in 5 mM  $\text{NaP}_i$  (pH 8.0), plus additions, in the presence (B) and absence (A) of 0.5% Triton X-100. After a brief incubation at  $0^\circ\text{C}$ , the samples were fixed and photographed by dark-field (series A) or fluorescence (series B) microscopy. The additions were (1) none; (2) 1 mM  $\text{MgSO}_4$ /1 mM 2,4-dinitrophenol, and (3) 0.15 M NaCl. Bar, 10  $\mu\text{m}$ .

ghosts yielded smooth reticula, indistinguishable from the control, even though the parent ghosts were highly spiculated (panels A2 and B2). Ghosts treated with isotonic saline also were markedly crenated (panel A3) but yielded smooth, compact reticula (panel B3). (A similar result was reported by Johnson et al. [22].) In all three cases, there was no evidence of a free reticulum within the ghosts (panels A1-A3); we must presume that the reticulum conformed to the membrane con-

tour (see reference 30). The fact that the reticulum assumed an independent contour after liberation by detergent suggests that it was not the cause of the ghost shape change but accommodated to it.

We next performed a control experiment to ascertain whether the crenation of ghosts in saline (panel A3) might be caused by the condensation of the reticulum inside (panel B3), as suggested by Johnson et al. (22). For this purpose, we

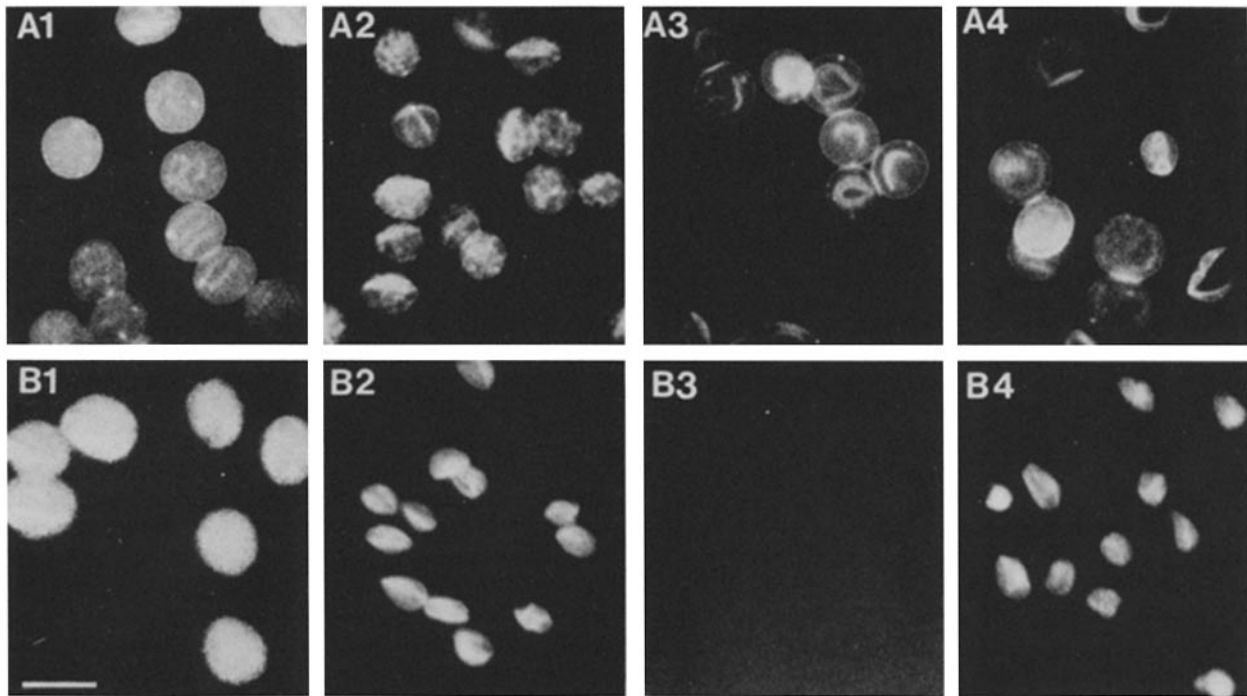


FIGURE 7 Visualization of reticula isolated from ghosts which had lost the ability to crenate. Ghosts were labeled with fluorescein isothiocyanate as described in Materials and Methods. Aliquots were suspended in 7 vol of 5 mM NaP<sub>i</sub> (pH 8.0) (i.e., 5P8) and preincubated for 3 min at 0°C (panels 1 and 2) or 37°C (panels 3 and 4), then chilled. Concentrated Triton X-100 was added to 0.5% (series B), and NaCl to 0.15 M (panels 2 and 4). After 5 min at 0°C, the samples were fixed and photographed by dark-field (series A) or fluorescence (series B) microscopy. (A1) 0°C, ghosts in 5P8; (B1) 0°C, reticula in 5P8; (A2) 0°C, ghosts in saline; (B2) 0°C, reticula in saline; (A3) 37°C, ghosts in 5P8; (B3) 37°C, reticula in 5P8; (A4) 37°C, ghosts in saline; (B4) 37°C, reticula in saline. Bar, 10 μm.

preincubated ghosts for 3 min at 37°C to abolish their ability to crenate<sup>1</sup> and examined the contour of their reticula by fluorescence microscopy. As before, reticula in 5 mM NaP<sub>i</sub> resembled the parent ghosts in size and shape (Fig. 7, panels A1 and B1). Again, exposure to saline caused the control (i.e., unwarmed) ghosts to crenate and their reticula to form condensed disks (panels A2 and B2). After a 3-min incubation at 37°C, the ghosts in 5 mM NaP<sub>i</sub> appeared normal (panel A3); however, their reticula, while initially visible as large, pale disks, were so labile that they disintegrated before they could be photographed (panel B3). (This observation, made many times under various conditions, signifies that reticula warmed in low ionic strength buffers become unfixable in glutaraldehyde.) Finally, prewarmed ghosts did not crenate in saline (panel A4). Nevertheless, their reticula were no different from the controls which had not been warmed (compare panels B2 and B4). We conclude that the loss of crenatability caused by brief warming is not attributable to an alteration in the shape of the reticulum.

All of the phenomena reported in Figs. 5–7 were also seen in unfluoresceinated controls by dark-field microscopy; however, the images were too pale to be properly recorded photographically. Thus, fluorescein labeling did not alter our results. We conclude from the experiments in Figs. 6 and 7 that shape changes seen in ghost crenation do not reflect the contour of the underlying reticulum, contrary to the notion advanced previously (19, 20, 22).

## CONCLUSIONS

We suggest a hypothesis for the role of the reticulum in the shape and mechanics of the erythrocyte, emanating from pres-

ent and previously published data. The elements of the reticulum are assumed to be in a slow association equilibrium with each other and with binding sites on the membrane (as suggested by association studies on isolated reticulum components; e.g., 11–16, 27, 29). In the long term (hours or days), the reticulum takes the shape of its container by continuous reassembly on the cytoplasmic surface of the membrane. (For example, the reticulum may already be in place in the erythroblast [31] and hence be remodeled repeatedly during erythrocyte maturation.) Over short intervals (seconds or minutes), the highly deformable reticulum may be distorted by both mechanically and chemically induced changes in the contour of the bilayer. The strength of the reticulum stabilizes the bilayer against distortion while its elasticity helps to restore cell shape. Thus, in the short term the reticulum is active in maintaining cell shape while in the long term it is passive. The reticulum can assume and then preserve but cannot initiate membrane contour. Figuratively speaking, it is “plastic” in the long-term and “elastic” in the short term.

Our current observations on the properties of the isolated reticulum support this hypothesis. (a) The isolated reticulum is mechanically stable and is resistant to shear in physiologic saline. (On the other hand, it is chemically unstable in very low ionic strength, alkaline media, presumably because of the electrostatically driven dissociation of the proteins within.) The fluid bilayer is believed to lack tensile strength and, by itself, should succumb to mild shear; the durability of the reticulum can provide the observed mechanical stability to the membrane (32). Breakdown of the reticulum at low ionic strength is highly correlated with the breakdown of ghosts to vesicles (Fig. 2). Mild proteolysis of ghosts (33) and an inborn deficiency in the spectrin of *sph* mice (3) are also accompanied by a mechanical

fragility of the membrane. Finally, one has only to observe the process of stomatolysis to appreciate the role of the reticulum in stabilizing (as opposed to determining) the contour of the membrane. In this phenomenon, unfixed ghosts in very low ionic strength buffer become distorted by gentle shearing forces encountered between the microscope slide and cover slip, especially as the temperature rises (i.e., the conditions promoting reticulum breakdown). The membranes are pulled into thin tubes or chains of vesicles many times longer than the diameter of the ghost.

(b) The isolated reticulum is highly deformable (flexible) yet elastic. We noted previously in scanning electron micrographs that the reticulum was extended on stretched ghost surfaces and condensed in relaxed ghosts (6). It expands reversibly in response to electrostatic forces at low ionic strength and shrinks reversibly at high ionic strength. Unlike Johnson et al. (22), we do not interpret these volume changes as suggesting a physiological contractility, but only high deformability and elasticity. These properties allow for both the membrane distortion and recovery.

(c) The reticulum conserves its long-term, cell-specified contour and imprints it on the native ghost. Isolated Triton X-100 residues (like normal ghosts) were not globoid but discoid, even when expanded at low ionic strength. Reticula were typically cupped and dimpled, as are stomatocytes and ghosts. These features were not altered by short-term shape changes induced by variation in ionic strength. Moreover, the shape of the reticulum was not altered by ghost crenation, presumably because crenation arises from forces within the bilayer which the reticulum cannot overcome but from which it can recover upon isolation.

Consider the shape of the reticulum isolated from cells with a stable and distinctive long-term contour: the biconcave disk (5-7, 17, 22), the hereditary elliptocyte (3), and the irreversibly sickled cell (17). In each case, the network remembers the cell shape. While the genesis of the shape is uncertain in the first two cases, it seems clear that the contour was imposed on the irreversibly sickled reticulum by a prolonged exposure to tactoids of polymerized hemoglobin. In contrast, Lux et al. (17) showed that a brief period of induced sickling of hemoglobin S cells *in vitro* did not leave ghosts or reticula distorted. We have made similar observations with echinocytes; cells crenated *in vitro* with dinitrophenol or lysolecithin yield ghosts and reticula indistinguishable from controls (not shown). In Figs. 6 and 7, we have demonstrated that the isolated reticulum does not preserve the contour of the parent ghosts in short-term crenation experiments. Since it seems clear both in intact erythrocytes (34) and in ghosts (Figs. 6 and 7 and reference 30) that the reticulum follows the contour of the membrane *in situ*, its regular appearance when isolated from distorted membranes must reflect its elastic properties.

Thus, the reticulum *in vitro* is durable, flexible, extensible, and elastic. *In vivo*, these properties would serve to restore long-term membrane contour after short-term distortions, thus stabilizing the red cell against the rigors of repeated mechanical deformation in the circulation without preventing such shape changes. The properties of the reticulum do not, however, account for the normal erythrocyte shape which it conserves; this seems to be an attribute of the lipid bilayer, as discussed in a subsequent report.<sup>1</sup>

We thank Dr. R. J. Dinerstein for his advice on fluorescence microscopy.

This work was supported by National Institutes of Health grant HL 20303 (to Y. Lange) and grant BC-95 from the American Cancer Society (to T. L. Steck). Y. Lange is the recipient of Research Career Development Award HL 00481 from the National Institutes of Health. T. L. Steck is the recipient of an American Cancer Society Faculty Research Award.

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Received for publication 3 April 1981, and in revised form 31 August 1981.

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