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### Research Article

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# Irreversible Deformation of the Spectrin-Actin Lattice in Irreversibly Sickled Cells

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**ABSTRACT** Irreversibly sickled cells (ISC's) are circulating erythrocytes in patients with sickle cell disease that retain a sickled shape even when oxygenated. Evidence points to a membrane defect that prevents the return of these cells to the normal biconcave shape.

The erythrocyte membrane protein spectrin is believed to help control erythrocyte shape and deformability. Recent studies suggest that normally spectrin and an erythrocyte actin form a self-supporting, fibrillar, lattice-like network on the cytoplasmic membrane surface. When normal erythrocyte ghosts are extracted with Triton X-100 all the integral membrane proteins and most of the membrane lipids are removed, leaving a ghost-shaped residue composed principally of spectrin and actin.

We concentrated ISC's from patients with sickle cell anemia and compared the morphology and protein composition of ghosts and Triton-extracted ghost residues prepared from these ISC's with similar preparations of reversibly sicklable cells and normal cells. (a) Many ISC's formed ISC-shaped ghosts. (b) All ISC-shaped ghosts formed ISC-shaped Triton residues. (c) Spectrin, erythrocyte actin (Band 5), an unidentified Band 3 component, and Band 4.1 were the major protein components of the Triton residues. All membrane-associated sickle hemoglobin was removed by the Triton treatment. (d) No ISC-shaped ghosts or ISC-shaped Triton residues were formed when deoxygenated, sickled RSC's were lysed or Triton-extracted. ISC-shaped ghosts and Triton residues were never formed from normal cells.

These observations suggest that a defect of the "spectrin-actin lattice" may be the primary abnormality of the ISC membrane. Since ISC's are rigid cells, the data support the postulate that spectrin is a major determinant of membrane deformability. Finally, they provide direct evidence that spectrin is important in determining erythrocyte shape.

## INTRODUCTION

In persons with sickle cell anemia a fraction of the circulating erythrocytes are permanently deformed into a sickle or oval shape and do not resume the normal biconcave form even after vigorous oxygenation. These "irreversibly sickled cells" (ISC's)<sup>1</sup> are dense, dehydrated, viscous, relatively indeformable cells with a low affinity for oxygen and a very short life span (1-3). The short-lived, rigid ISC's make a major contribution to the hemolytic rate in sickle cell patients (4) and may be involved in the vaso-occlusive crises which characterize the disease (2, 5-7).

Despite their sickled appearance, the hemoglobin in well-oxygenated ISC's is usually not in the aggregated, "sickled" state since the microfilaments characteristic of deoxyhemoglobin S aggregates cannot be detected in electron micrographs of ISC's (8). This suggests that ISC's retain their sickled shape because a membrane defect acquired at some point in the sickling process prevents their return to the normal biconcave form. Other observations also indicate that ISC membranes are defective. ISC's are abnormally permeable to cations, particularly calcium (9) and potassium (10). As a result they accumulate membrane calcium and lose intra-

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<sup>1</sup>Abbreviations used in this paper: ISC, irreversibly sickled cell; RSC, reversibly sicklable cell; SDS-PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate.

cellular potassium and water. ISC's also have more hemoglobin bound to their membranes than normal cells or reversibly sickle cells (RSC's) (11). The relationship of these defects to the abnormal shape and deformability of the ISC is unresolved. One possibility is that the membrane-associated hemoglobin S is bound as microfilaments while the cell is deoxygenated and subsequently is unable to depolymerize. Alternatively the shape and rigidity of the ISC could be due to secondary changes in a membrane protein or proteins.

Spectrin<sup>2</sup> is a large, fibrous protein which is located on the cytoplasmic surface of the erythrocyte membrane (14). Recent observations suggest it may be organized in a meshwork (15, 16), perhaps in association with actin (13, 17) or other membrane proteins, and that this "spectrin-actin lattice"<sup>3</sup> may interact with certain membrane-spanning, integral membrane proteins (18) and immobilize them (16). The observations of Yu et al. (15) are particularly suggestive. They extracted erythrocyte ghosts with Triton X-100 in low ionic strength buffers. This nonionic detergent solubilized all of the integral membrane proteins and most of the lipids; but spectrin, actin, and a minor group of other cytoplasmic membrane proteins remained insoluble. These insoluble membrane components retained the shape of the original ghost, suggesting they may normally form a submembrane protein shell.

Such a protein shell would doubtless influence membrane shape and deformability and might be responsible for the abnormal shape and deformability of the ISC. To test this hypothesis we prepared ghosts and Triton-extracted ghosts from ISC's, RSC's, and normal cells and compared their morphology and protein composition.

## METHODS

**Patients.** Blood was obtained from patients with proven hemoglobin SS disease. None had been transfused in the preceding 3 mo. Blood was collected in citrate-phosphate-dextrose solution (0.15 ml/ml blood) plus ethylene diamine-tetra-acetate (1 mg/ml blood) and used within 24 h of collection.

<sup>2</sup>Spectrin is defined by its electrophoretic mobility on SDS-PAGE. It is the two highest molecular weight bands: Band 1 (approximate molecular weight, 240,000) and Band 2 (approximate molecular weight, 220,000). Previously some preparations called spectrin also included a third polypeptide, band 5 (approximate molecular weight, 45,000), which is now known to be an erythrocyte actin (12, 13).

<sup>3</sup>In this paper we will use the term "spectrin-actin lattice" as a convenient designation for this protein meshwork. We would emphasize that there is no conclusive proof that such a structure actually exists in the native erythrocyte and that, if it exists it probably contains proteins other than just spectrin and actin. Operationally this hypothetical structure is best defined as the protein residues remaining after extraction of erythrocyte membranes with Triton X-100.

**Separation of ISC's and Non-ISC's.** The plasma and buffy coat were removed after centrifugation (400 *g*, 5 min) and the erythrocytes were washed twice with 5 vol of phosphate buffered saline (15 mM NaH<sub>2</sub>PO<sub>4</sub>, 135 mM NaCl, adjusted to pH 7.4 with NaOH), containing 1% bovine serum albumin. The washed erythrocytes were oxygenated and centrifuged at room temperature in a Sero-Fuge II centrifuge (Clay Adams, Inc., Div. of Becton, Dickinson and Co., Parsippany, N. J.) for 45 min at 500 *g* followed by 60 min at 1,000 *g*. They were then divided into three fractions: the top 25% (top fraction), the middle 50% (middle fraction), and the bottom 25% (bottom fraction). Each of the three fractions was washed once with 5 vol of phosphate buffered saline, pH 7.4, containing 1% bovine serum albumin, diluted with an equal volume of the same buffer, and reoxygenated in room air.

ISC's were defined operationally as elongated, oval, or crescent-shaped cells with a length-width ratio of two or more. This definition is somewhat more stringent than that used by some previous workers as it excludes slightly elongated cells and noncrescentic poikilocytes.

**Preparation of ghosts and Triton-extracted ghost residues.** Erythrocyte ghosts were prepared by the method of Dodge et al. (19). Triton residues were prepared by washing ghosts once or twice with 10 mM Tris-HCl, pH 8, and extracting them with 5 vol of 0.5% Triton X-100 in 56 mM sodium borate, pH 8 (30 min, 0°C) as described by Yu et al. (15). Triton residues were left in suspension in the Triton-borate buffer, unless otherwise noted, as they aggregated irreversibly on centrifugation. For polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE) studies the Triton residues were separated from the Triton extract by centrifugation (50,000 *g*, 30 min) and washed once with 10 vol of the Triton-borate buffer.

**Light microscopy.** Samples of each fraction of erythrocytes, ghosts, and Triton residues were taken for fixation and light microscopy. Erythrocytes (well oxygenated in room air) were fixed in 33 vol of 2% glutaraldehyde (Polysciences, Inc., Warrington, Pa.) in 150 mM sodium phosphate buffer, pH 7.4. Ghosts were fixed in 10 vol of 0.5% glutaraldehyde in 5 mM sodium phosphate buffer, pH 8. Triton-extracted ghosts were fixed by adding 1 vol of 3% glutaraldehyde in 0.5% Triton X-100, 56 mM sodium borate, pH 8, to 12 vol of the Triton-extracted ghost suspension described above. All three preparations were fixed for 15 min at room temperature before observation. The fixed erythrocyte and ghost suspensions were observed directly by phase contrast microscopy. Under these conditions Triton residues were only barely visible. However, contrast of the fixed Triton residues was markedly enhanced by staining with an equal volume of 1% uranyl acetate.<sup>4</sup> Sodium phosphotungstate (1%) and ammonium molybdate (1%) did not produce this visual enhancement. There was no discernable effect of fixation on the morphology of normal or SS erythrocytes or ghosts. Triton residues which were fixed and stained with uranyl acetate were generally shrunken compared to Triton residues which were stained without fixation, but the morphology was otherwise unchanged. Both fixed and unfixed Triton residues showed a marked tendency to clump upon treatment with uranyl acetate. This clumping was mini-

<sup>4</sup>It is important to wash the ghosts with 10 mM Tris-HCl, pH 8, before the Triton-extraction to remove phosphate ions; otherwise an amorphous precipitate of uranyl phosphate will form at this point.

mized by working with dilute suspensions of the Triton residues and by observing them as soon after preparation as possible, but it was never completely eliminated. We did not investigate whether clumping was an intrinsic property of the Triton residues or whether it resulted from fixation or staining.

**Electron microscopy.** Ghosts and Triton residues were negatively stained with either uranyl acetate (0.3 or 4%) (pH 4.5) or ammonium molybdate (1%) (pH 7.4) and viewed by transmission electron microscopy.

**Photography.** Erythrocytes, ghosts, and Triton residues were photographed on High Contrast Copy Film 5069 (Eastman Kodak Co., Rochester, N. Y.) developed with Kodak D-19 developer.

**SDS-polyacrylamide gel electrophoresis.** SDS-PAGE was performed as previously described (20). The bands were numbered according to the system instituted by Fairbanks et al. (21) and extended by Steck (20). Additional bands (e.g., Band 8 and globin), not designated by these authors, were assigned numbers in sequence. The proportion of various bands was assessed by densitometry of the stained gels using a densitometer (E-C Apparatus Corp., St. Petersburg, Fla.) connected to an integrating recorder (model 252A, Linear Instruments Corp., Irvine, Calif.).

**Experimental design.** The experimental design of the present studies is outlined in Fig. 1. Normal or SS erythrocytes were separated into three fractions (top, middle, and bottom) of increasing density by centrifugation. Ghosts and Triton-extracted ghosts were prepared from each fraction, and the morphology and protein composition of these membrane preparations were examined.

## RESULTS

**Morphologic studies: ISC-shaped ghosts.** After centrifugation of oxygenated SS erythrocytes, SS reticulocytes and RSC's concentrated in the top fraction and ISC's migrated to the bottom (Fig. 2, panels 1-3; Fig. 3) (3). With the low speed centrifugation technique used in these experiments the top fraction contained more than 95% of the total reticulocytes in the sample and less than 5% of the total ISC's. Less than 1% of the total reticulocytes and 55-65% of the total ISC's were found in the bottom fraction.

Many of the SS erythrocyte ghosts retained the ISC-shape of their precursors (Fig. 2, panels 5 and 6). The proportion of ISC-shaped ghosts in the top, middle, and bottom fractions correlated with the percent ISC's in each of these fractions (Fig. 3) though the transformation from ISC's to ISC-shaped ghosts was incomplete [average =  $55.5 \pm 1.0\%$  (SEM)], particularly in the bottom fractions (Fig. 4). The reason for this discrepancy was not examined. One possibility is that the "ISC's" which formed normal-shaped ghosts were really incompletely reoxygenated RSC's. This is plausible since the hemoglobin in many SS erythrocytes has an unusually low oxygen affinity (1). Alternatively, the membrane lesion may have been reversed in some ISC's by the hemolysis procedure.

In control experiments ISC-shaped ghosts were not generated when RSC's were sickled ( $> 95\%$ ) and hemolyzed under nitrogen. Normal erythrocyte fractions contained no detectable "ISC's" ( $< 0.1\%$ ) and no ISC-shapes were produced by hemolysis of normal erythrocytes. We concluded that the membranes of many circulating ISC's were permanently deformed into a sickle shape.

**Morphologic studies: ISC-shaped Triton-extracted ghost residues.** When SS erythrocyte ghosts were extracted with Triton X-100 some of the ghost residues retained a sickle shape (Fig. 2, panel 8 and panel 9a-c). In contrast, sickle-shaped Triton residues were never seen after extraction of normal ghosts. The proportion of sickle-shaped Triton residues found in any fraction correlated closely with the number of ISC-shaped ghosts present before extraction (Fig. 3). Unlike ISC's, which are incompletely transformed into ISC-shaped ghosts, all [ $102 \pm 1.9\%$  (SEM)] of the ISC-shaped ghosts formed ISC-shaped Triton residues (Fig. 4). There are two possible explanations of these observations. Either the ISC-shaped Triton residue contributes to membrane shape and exists in the sickle shape before extraction with Triton X-100 or the membrane shape determinants reside in the Triton soluble protein components and the Triton extraction procedure artifactually fixes the protein residue in the sickle shape at the moment of extraction.

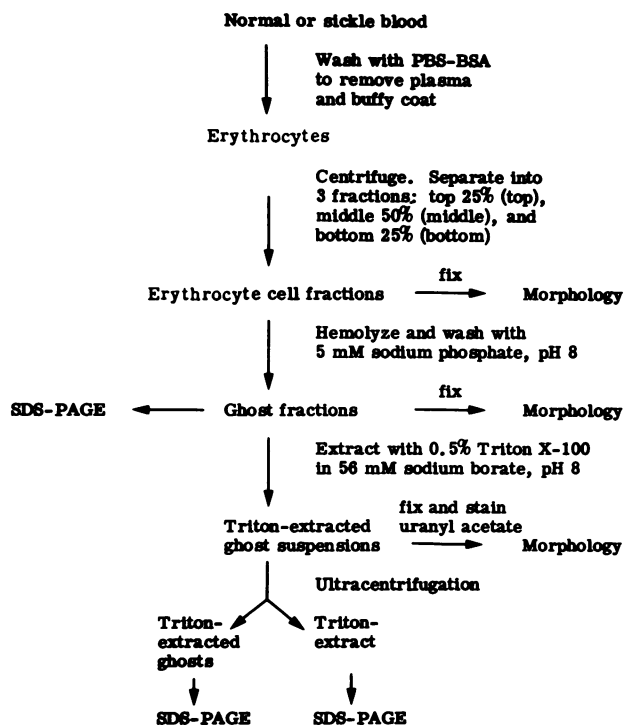


FIGURE 1 Experimental design.

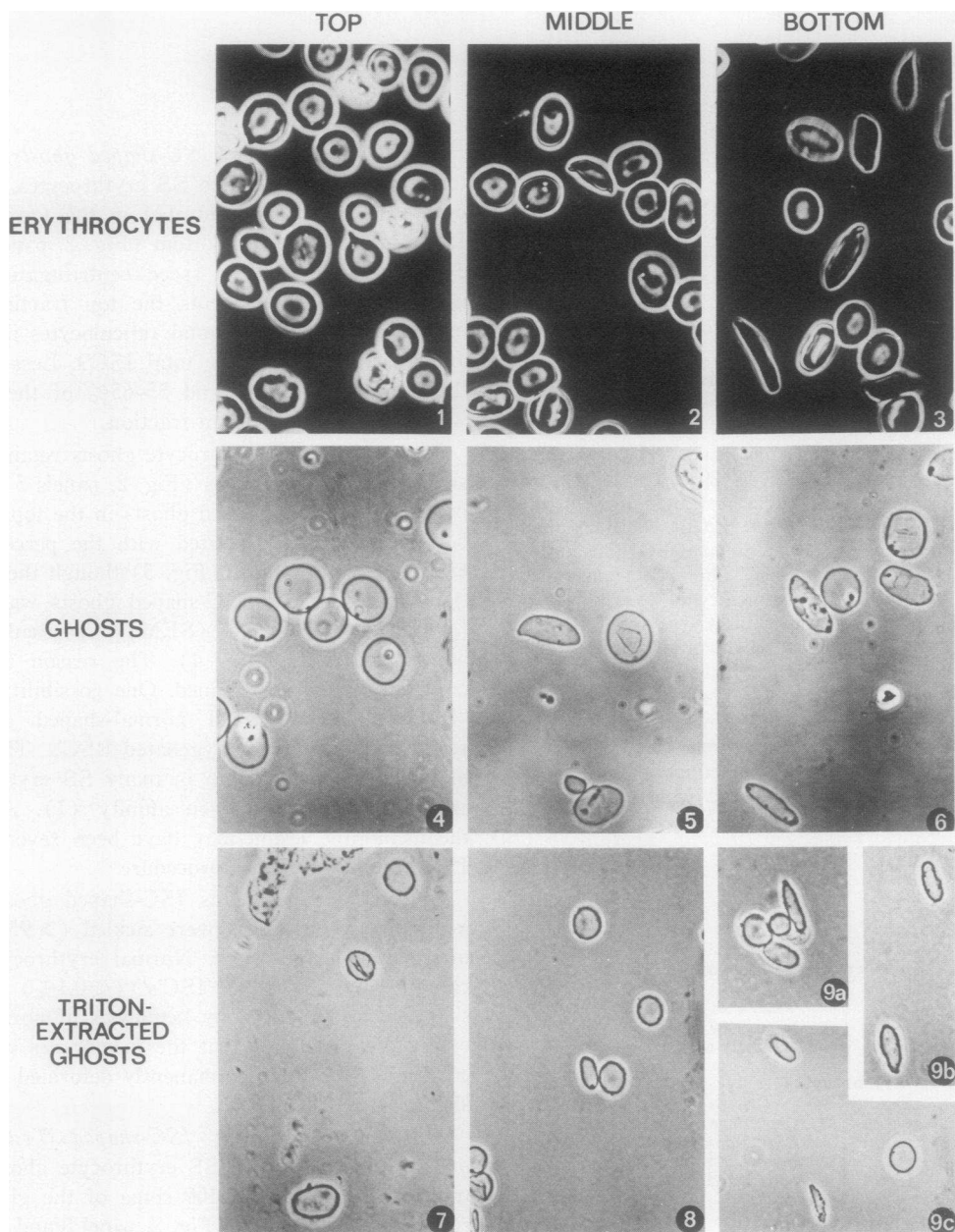


FIGURE 2 Phase contrast photomicrographs of intact erythrocytes, ghosts, and Triton-extracted ghost residues representative of the top, middle, and bottom fractions of a centrifuged blood sample from a patient with sickle cell anemia. All samples were observed as "wet" preparations after fixation. The Triton residues were treated with 1% uranyl acetate to enhance contrast. Three fields of the Triton residues from the bottom fraction are shown to illustrate the ISC-like deformation present in this preparation. Reticulocytes (erythrocytes, top fraction) are distinguished by their "puckered" appearance (22). ISC's (erythrocytes, bottom fraction) are defined as elongated, oval, or crescent-shaped cells with a length/width ratio of 2 or more.  $\times 1,200$

To test the latter possibility we performed the experiment shown in Table I. Washed SS erythrocytes were divided into two portions. One was deoxygenated and then extracted, under nitrogen, with Triton X-100; the other was deoxygenated and then reoxygenated before extraction. The number of ISC-shaped Triton residues in the two extracted samples was comparable even though the deoxygenated sample contained 95% sickled cells (33.0% ISC-shaped) at the moment of Triton extraction. This experiment permits the quali-

fied conclusion that ISC-shaped Triton residues are not produced artifactually by the Triton extraction procedure and must exist in an ISC-shape before extraction. The problem is that the Triton extraction in this control was performed on intact cells rather than ghosts. The ideal experiment would have been Triton extraction of RSC ghosts while they were in the sickle shape, but we could not devise a suitable scheme for producing such ghosts. Despite this limitation we believe the conclusion is valid since hemoglobin is readily solubilized

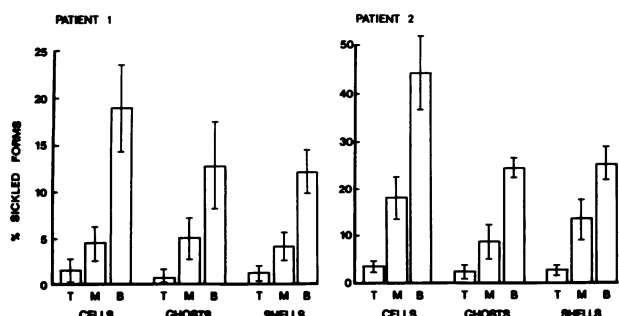


FIGURE 3 The proportion of sickled forms in oxygenated intact erythrocytes, ghosts, and Triton residues ("shells") prepared from top (T), middle (M), and bottom (B) fractions. The vertical line at the top of each bar is the SD of 5-10 separate counts of 100 cells each. Two independent experiments are shown.

by the Triton extraction (as shown in the following section). As a consequence, the Triton residues produced by extraction of intact cells are almost identical to the Triton-extracted ghosts. Both contain the same limited spectrum of membrane proteins although there are small differences in the proportion of some of the minor components (data not shown).

**Protein composition of ghosts and Triton-extracted ghosts.** SDS-PAGE patterns of the ghosts and Triton residues are presented in Fig. 5. The gel patterns of the Triton extracts are also shown. Several points are notable. (a) The distribution of ghost proteins in the top, middle, and bottom fractions was identical except for hemoglobin which, as expected (11), was more con-

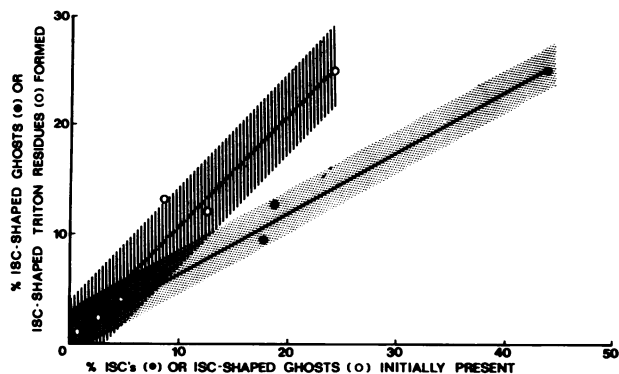


FIGURE 4 A comparison of the transformation of ISC's to ISC-shaped ghosts (●—●) and ISC-shaped ghosts to ISC-shaped Triton residues (○---○). The proportions of ISC's or ISC-shaped ghosts in various fractions are plotted on the X-axis. The corresponding proportions of ISC-shaped ghosts formed from the ISC's and ISC-shaped Triton residues formed from the ISC-shaped ghosts are plotted on the Y-axis. The hatched areas represent a range of 2 SEM about the line fitted to these points. Only  $55.5 \pm 1.0\%$  (SEM) of the ISC's formed ISC-shaped ghosts, but all  $[102 \pm 1.9\% \text{ (SEM)}]$  of the ISC-shaped ghosts generated ISC-shaped Triton residues.

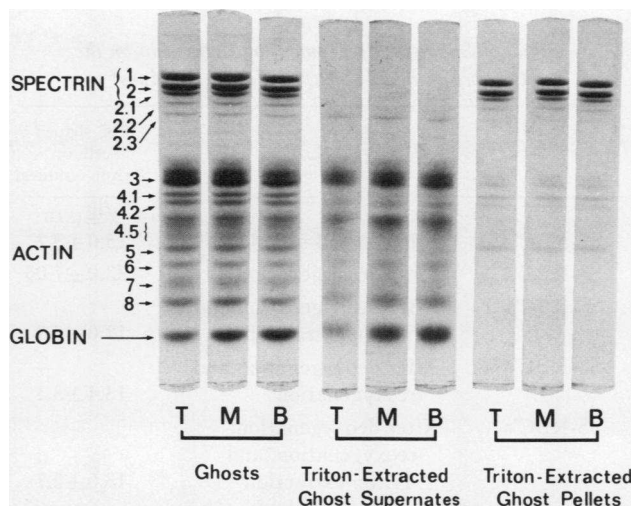


FIGURE 5 SDS-PAGE of ghosts (left), Triton-extracted ghosts (right) and the Triton supernates (middle) from the top (T), middle (M), and bottom (B) fractions of sickle erythrocytes.

centrated in the ISC-rich bottom fractions. (b) Spectrin was the major component of the Triton residues. Together, spectrin ( $75.9 \pm 6.1\%$  SD)<sup>5</sup> and actin ( $4.8 \pm 1.7\%$ ) accounted for over 80% of the total residue protein. Other proteins in the Triton residue included Bands 2.2 and 2.3 ( $3.4 \pm 1.1\%$ ), Band 4.1 ( $5.2 \pm 1.2\%$ ), and portions of Band 4.2 ( $1.7 \pm 0.6\%$ ), and Band 3 ( $8.9 \pm 3.4\%$ ). The distribution of proteins in the Triton extracts and Triton residues of SS and normal erythrocyte ghosts was indistinguishable. Similar patterns were observed in six different SS patients. (c) All membrane-associated hemoglobin S was solubilized by the Triton extraction. Specifically, no hemoglobin S was present in the fractions rich in ISC-shaped Triton residues.

**Electron microscopy of Triton residues.** Fresh, uncentrifuged Triton residues were examined after negative staining with uranyl acetate (0.3 and 4%) and ammonium molybdate (1%). RSC- and ISC-shaped Triton residues were easily distinguished by this technique (Fig. 6a). Triton-extracted RSC's (Fig. 6b), ISC's (Fig. 6c), and normal cells (not shown), all contained

<sup>5</sup> This number includes Band 2.1 which could not be resolved well enough to permit separate quantitation. The reader may notice that although all of the spectrin and actin in the ghosts remains in the Triton residue, the ratio of spectrin to actin in the residues (15.8:1) is much higher than the spectrin-actin ratio reported in ghosts ( $\sim 6:1$ ) (21). We can't explain this discrepancy with certainty but suspect it may reflect inadequacies of densitometry as a means of quantitating erythrocyte membrane proteins. In densitometric scans of SDS gels of ghosts the actin band is superimposed on a high background created by Bands 4.5 and 6. This may lead to overestimation of the actin concentration which is revealed when the interfering proteins are removed by Triton extraction.

TABLE I  
Effect of Shape at the Moment of Extraction on the  
Proportion of ISC-shaped Triton Residues\*

|          |                                                                 | ISC-shaped<br>cells or<br>Triton residues† |
|----------|-----------------------------------------------------------------|--------------------------------------------|
|          |                                                                 | % $\pm$ (SD)                               |
| SS-RBC's | Initial blood sample                                            | 15.0 $\pm$ 3.2                             |
| SS-RBC's | After deoxygenation                                             | 33.0 $\pm$ 7.0§                            |
| SS-RBC's | After deoxygenation and<br>Triton extraction                    | 12.0 $\pm$ 3.9                             |
| SS-RBC's | After deoxygenation and<br>reoxygenation                        | 13.4 $\pm$ 3.1                             |
| SS-RBC's | After deoxygenation,<br>reoxygenation, and<br>Triton extraction | 13.0 $\pm$ 2.7                             |

\* Fresh sickle erythrocytes (5 ml) were washed three times in phosphate buffered saline, pH 7.4, containing 1% bovine serum albumin and suspended in the same buffer at a 50% hematocrit. The sample was divided equally into two flasks. Both were deoxygenated (100% nitrogen, 30 min, 37°C) and one was then reoxygenated (100% oxygen, 30 min, 37°C). Small samples from both flasks were fixed and examined by phase contrast microscopy. The remaining cells were extracted with 5 vol of 0.5% Triton X-100 in 56 mM sodium borate, pH 8, at 4°C for 30 min. The deoxygenated cells were extracted under nitrogen with a carefully deoxygenated Triton solution. After extraction the Triton residues were fixed by adding 1 vol of deoxygenated 6% glutaraldehyde in 0.5% Triton X-100, 56 mM sodium borate, pH 8 to each flask. The fixed Triton residues were stained with an equal volume of 1% uranyl acetate and examined by phase contrast microscopy.

† A cell or Triton residue was considered to be ISC-shaped if its length/width ratio was  $>2$ .

§ 95% of the cells in this sample were sickled but most were "holly-shaped" and did not meet the morphologic criteria above.

a prominent, dense network of fine, randomly oriented, fluffy, filamentous material. This material must represent spectrin or a complex of spectrin and other protein(s) since spectrin is the only component of the Triton residues which is sufficiently abundant to form such a network. Most residues also contained large, somewhat vesicular structures (not seen in Fig. 6) probably due to unextracted lipid (15). Small (ca 200 Å) doughnut-shaped structures were seen in some fields. A typical example is marked by the arrow in Fig. 6c. These structures are very similar to the "hollow cylinder" protein of erythrocyte membranes described by Harris several years ago (23). They also resemble the spectrin-like actin-binding protein found on macrophage membranes (24). The true identity and significance of these interesting structures remains to be determined. Although the Triton residues all contained

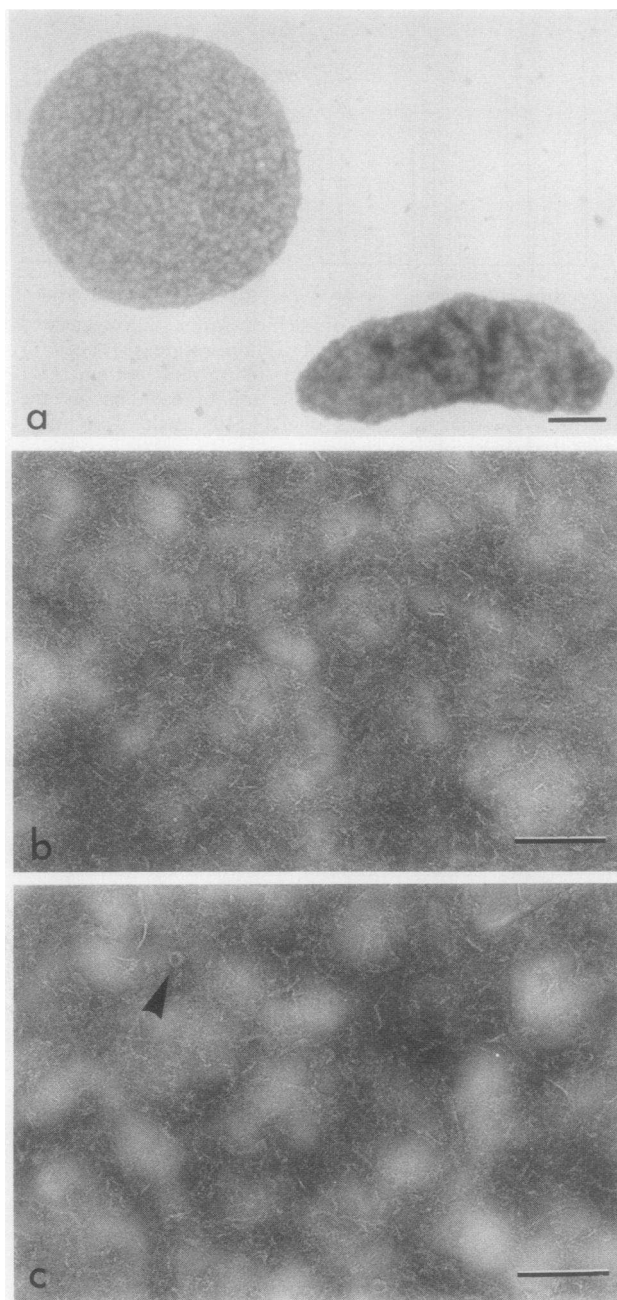


FIGURE 6 Electron micrographs of Triton residues prepared from ghosts of the top and bottom fractions of sickle erythrocytes and negatively stained with 1% ammonium molybdate, pH 7.4. (a) Low power view of RSC (left) and ISC (right). Bar = 1  $\mu$ m; (b) High power view of a RSC. Bar = 0.1  $\mu$ m; (c) High power view of an ISC. Bar = 0.1  $\mu$ m. The arrow marks one of the 200 Å doughnut-shaped structures referred to in the text. The major structural feature in the high power views is a meshwork of randomly oriented, fluffy, filamentous material, which is presumed to be spectrin. The organization of this material is similar in RSC's and ISC's.



Band 5, an erythrocyte actin (12, 13, 17) (Fig. 5), we did not detect the typical double helical filaments characteristic of polymerized actin. Since erythrocyte actin can form such filaments (13, 17) it must be largely unpolymerized and/or associated with other proteins in the Triton residues.

In a careful search we did not find any consistent difference in the fine structure or organization of the filamentous network and its associated structures in the Triton residues of RSC's, ISC's, and normal ghosts. In particular there was no tendency for the filamentous strands in the ISC-shaped residues to align in parallel, like filaments of deoxygenated hemoglobin S (8).

## DISCUSSION

These studies provide new insight into the pathogenesis of ISC's. The observation that most ISC's yield ISC-shaped ghosts confirms and extends the preliminary report of Jensen et al. (25) and strongly supports the concept that the ISC retains its shape because of an acquired membrane defect. Even more important, the filamentous protein residues left after Triton X-100 extraction of these ISC-ghosts also retain a sickle shape. These protein shells are largely composed of spectrin and actin and do not form if spectrin and actin are selectively eluted from ghosts before Triton treatment (15). As noted in the Introduction, other indirect evidence (13, 16-18) supports the concept that these two proteins are normally organized in a self-supporting meshwork which laminates the inner surface of the erythrocyte membrane. Since ISC-shaped ghosts and protein shells were not produced when deoxygenated, sickled RSC's were lysed or treated with Triton X-100, there is no reason to suspect that the sickle deformation of ISC-derived ghosts and protein shells is an artifact of the lysis or Triton-extraction procedures. Instead, we believe, these observations indicate that the primary membrane abnormality in the ISC is a defect involving a protein or proteins in the Triton residues, the operational equivalent of the hypothetical "spectrin-actin lattice."<sup>3</sup>

Since sickle cell disease is caused by an inherited defect in hemoglobin and not in spectrin, actin, or the minor proteins of the Triton residues, it is important to learn how the spectrin-actin lattice becomes irreversibly deformed. A logical hypothesis is that initially this structurally normal protein network is passively deformed by the oriented microfilaments of hemoglobin S and then becomes fixed in the deformed configuration by some presently undefined process. Electron micrographs (Fig. 6) are compatible with this hypothesis since they show that the fundamental structure of the ISC- and RSC-shaped protein shells is the same. The primary candidates for this process include: (a) in-

creased membrane calcium, (b) decreased ATP, (c) cellular dehydration, or (d) a direct interaction between hemoglobin S and spectrin or actin.

Sickle erythrocytes are abnormally permeable to calcium, particularly when deoxygenated (9, 26, 27). This leads to very high levels of calcium in the membrane of sickle cells, especially ISC's (9). Spectrin reportedly binds calcium avidly (28) and sometimes aggregates (29, 30) in the presence of low concentrations of calcium. Calcium also modifies the specific phosphorylation of spectrin by membrane protein kinases and ATP (31, 32) and is necessary for the formation of ISC-like cells in vitro from deoxygenated, ATP-depleted sickle erythrocytes (33). ATP-depleted normal erythrocytes show changes in membrane deformability (34) and permeability (35) analogous to those of ISC's. These alterations have been attributed to increased membrane calcium (34) and are associated with a change in the physical state of spectrin (36). Recent measurements indicate that in vivo ISC's are also ATP-deficient (37, 38). Thus, for a variety of reasons, either increased membrane calcium or decreased cellular ATP or both could lead to "fixation" of the spectrin-actin lattice in the ISC shape.

Glader and Müller have recently shown that ISC-like cells produced in vitro by deoxygenation and incubation with propranolol and calcium will not form if the incubation is conducted in a high potassium buffer (39). Since the high potassium buffer prevents the loss of intracellular potassium and water, these investigators concluded that cellular dehydration per se might produce the membrane defect in the ISC. It will be important to determine whether the ISC-like cells produced by this and other (33, 40) in vitro methods exhibit the same deformation of the spectrin-actin lattice seen in true ISC's.

ISC's have more hemoglobin bound to their membranes than normal cells or RSC's (11). This and the observation that ISC-like cells formed during prolonged hypoxia of ATP-replete cells also accumulated excess membrane hemoglobin (40), have led to suggestions that the membrane lesion in the ISC is due to an interaction between hemoglobin S and the ISC membrane (40). The present studies appear to exclude the possibility that the membrane deformation of ISC's is the direct result of the accumulation of hemoglobin S on the membrane since the defect persists in Triton residues which contain no detectable hemoglobin. They do not exclude the possibility that the abnormal organization of the spectrin-actin lattice is a secondary result of the interaction of one or both of these proteins with deoxygenated hemoglobin S. To date, however, no such specific interaction has been discovered.

In conclusion these studies point to a defect of the spectrin-actin lattice in the ISC. Since ISC's have rigid



membranes (2) they also reinforce the postulated role for spectrin in membrane deformability. Further, they provide direct evidence that spectrin is important in determining erythrocyte shape.

## ACKNOWLEDGMENTS

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