Journal of Supramolecular Structure 8:399–412 (1978)
Normal and Abnormal Red Cell Membranes 121–134

Triton Shells of Intact Erythrocytes

Michael P. Sheetz and David Sawyer

Physiology Department, University of Connecticut Health Center, Farmington, Connecticut 06032

About 40% of human erythrocyte membrane protein is resistant to solubilization in 0.5% Triton X-114. These components comprise a structure called a Triton shell roughly similar in size and shape to the original erythrocyte and thus constitute a cytoskeleton. With increasing concentrations of Triton the lipid content of the Triton shell decreases dramatically, whereas the majority of the protein components remain constant. Exceptions to this rule include proteins contained in band 3, the presumed anion channel, and in band 4 which decrease with increasing Triton concentration. The Triton-insoluble complex includes spectrin (bands 1 and 2), actin (band 5), and bands 3′ and 7. Component 3′ has an apparent molecular weight of 88,000 daltons as does 3; but unlike 3, it is insensitive to protease treatment of the intact cell, has a low extinction coefficient at 280 nm, and is solubilized from the shells in alkaline water solutions. Component 7 also has a low extinction coefficient at 280 nm. Spectrin alone is solubilized from the Triton shells in isotonic media. The solubilized spectrin contains no bound Triton and coelectrophoreses with spectrin eluted in hypotonic solutions from ghosts. Electron micrographs of fixed Triton shells stained with uranyl acetate show the presence of numerous filaments which appear beaded and are 80–120 Å in diameter. The filaments cannot be composed mainly of actin, but enough spectrin is present to form the filaments. Triton shells may provide an excellent source of material useful in the investigation of the erythrocyte cytoskeleton.

Key words: Triton, cytoskeleton, spectrin, actin, erythrocyte membrane

Two independent reports have shown that nonionic detergents solubilized the majority of integral membrane proteins from erythrocyte ghosts, leaving an insoluble fraction which contained the major portion of the peripheral proteins, spectrin and actin, and several other components [1–3]. Yu, Fischman, and Steck [2] showed that these proteins were organized in a shell which had a form similar to that of the original ghosts. Lux, John, and Karnovski [4] have further shown that the shape of irreversibly sickled cell ghosts is preserved in the shells obtained after Triton extraction. Therefore, the shell left after Triton extraction of the membrane may be regarded as part of the cytoskeleton of the ghosts.

Received for publication April 21, 1978; accepted June 1, 1978.

0091-7419/78/0804-0399$02.60

The shape and deformability of the erythrocyte are thought to depend upon the properties of this cytoskeleton adherent to the undersurface of the cell. For example, in the mouse model of hereditary spherocytosis the red cells are deficient in spectrin, and those cells lose membrane and lyse readily [5]. We have found that bivalent antibodies to spectrin will prevent certain shape changes from occurring in membranes isolated from normal human red cells when added at a concentration equivalent to that of spectrin [6]. At a much lower concentration of bivalent antibody the shape change of erythrocyte membranes induced by adenosine triphosphate (ATP) is accelerated. Since monovalent antibody fragments and control antisera have no effect, the cross-linking of the spectrin network underlying the membrane appears to aid the ATP-dependent shape change. A variety of other experiments also suggest that spectrin and actin have important functions in controlling membrane shape and deformability [7–9]. No doubt other proteins associated with the membrane are also important in the structure and function of the erythrocyte cytoskeleton. For example, the membrane binding site for spectrin [10] may be an oligomer ie, a protein which aids in the creation of a cohesive cytoskeleton.

We have developed a method for preparing cytoskeletons from intact erythrocytes and find that these Triton shells of whole cells are similar to the ghost shells but have several unexpected properties [11]. By working with the intact cells it is possible to maintain constant ionic conditions and to shorten the time required in preparing the shells. In this report we describe further the unusual properties of certain components of Triton shells of intact cells and show that filamentous structures are present in the shells.

MATERIALS AND METHODS

Human erythrocytes were obtained from healthy donors and used within two days. Citrate (10 mM final concentration) was used as an anticoagulant. Cells were washed three times with 146 mM NaCl, 20 mM Tris, pH 7.4 (Iso Tris) and the buffy coat was removed each time. Triton X-114 was obtained from Rohm and Haas and [^{3}H] Triton X-100 was a gift of Dr Phillip Strittmatter. All other compounds were reagent grade or better.

Detergent Extraction Procedure

Washed red cells were suspended in Iso Tris at a 20% hematocrit. In a typical experiment 6 ml of the cells were mixed on ice with 6 ml of a detergent solution containing 5 mM reduced glutathione, 1.0 mM ethylene glycoltetraectic acid (EGTA), 5 mM $MgCl_2$, 140 mM KCl, and 20 mM N-2-hydroxyethyl-piperazine-N′-2-ethane-sulfonic acid (HEPES) (pH 7.0). The mixture was layered on a 25-ml linear sucrose gradient (usually 10–60% sucrose) containing 0.2 mM dithiothreitol (DTT), 140 mM KCl, 5 mM $MgCl_2$, 20 mM HEPES (pH 7.0), and 0.5 mM EGTA. The gradients were normally centrifuged for 1 h at 120,000g max. Fractions (~1ml) were collected dropwise from the bottom of the gradient tube. The sucrose concentration of various fractions was determined from the refractive index. The protein concentration of the fractions was determined by diluting a 100- to 500-λ aliquot to 2.8 ml and precipitating the protein with 0.7 ml of 50% trichloroacetic acid (TCA). The pelleted precipitate (3,000g for 20 min) was solubilized in 200 λ of 1% sodium dodecyl sulfate (SDS) and 10 mM Na_2 HPO_4 by incubation at 37°C for 30 min. Protein was then measured by the Lowry assay [12]. In cases where [^{3}H] Triton X-100 was added to the extraction mixture, an aliquot (< 100 λ) of each fraction was added directly to 5 ml of Aqueous Counting Scintillant (ACS) (Amersham Searle) scintillation fluid and counted.

Protease Treatment of Whole Cells

Washed erythrocytes at a 50% hematocrit were incubated with 1.5 mg/ml of chymotrypsin (Sigma) or Streptomyces griseus protease (Sigma) for 1 h at 37°C. The chymotrypsin reaction was stopped by the addition of 420 μg/ml L-1-tosylamide-2-phenylethylcholomethyl ketone (TPCK). Cells were washed twice with Iso Tris before extraction.

pH 11 Extraction

Erythrocyte ghosts were prepared by lysis in 10 mM Tris pH 7.4 (1:100) and pelleting at 25,000g for 10 min, followed by washing two times with the same buffer. Ghosts were then washed (1:100) with the spectrin extraction buffer, 0.2 mM ATP, 0.2 mM EDTA, and 0.2 mM Tris, 0.2 mM DTT (pH 8.0). After overnight extraction the membranes were pelleted and washed once with 0.6 M KCl, 0.1 mM DTT, 10 mM Tris (pH 7.5) to remove band 6. The pellet was washed one time with, and resuspended in, 10 mM glycine, 0.2 mM ATP, and 0.1 mM DTT (pH 11), incubated 16 h on ice, and centrifuged at 100,000 g max for 1 h. The final extract was concentrated by vacuum dialysis and stored as a precipitate in 50% ammonium sulfate.

SDS Gel Electrophoresis

Samples were solubilized by the addition of 2% SDS and 5% β-mercaptoethanol and incubation at 100°C for 2–3 min. The procedure of Fairbanks, Steck, and Wallach [13] was used for the gel electrophoresis in 5.6% acrylamide gels containing 0.1% SDS.

Electron Microscopy of Shells

As the gradients were being collected, some samples were collected in tubes containing 2% glutaraldehyde in 140 mM KCl, 10 mM PO_4 (pH 7.0). After fixation for 1 h on ice, samples were applied to thin carbon films on standard 300-mesh electron microscope grids for 1 min. The grids were then washed twice with Iso Tris by floating them on drops of the buffer and were finally negatively stained with 2% uranyl acetate (pH 4.5). Samples were viewed on a Phillips 300 electron microscope.

RESULTS

Triton Extraction of Whole Cells

When whole erythrocytes were extracted with 0.5% Triton X-114 and centrifuged (120,000 g for 1 h) into a 10–60% sucrose gradient in the same buffer, two light-scattering layers were found as well as a pellet. The pellet was fibrous and incompletely solubilized by SDS and reducing agents. Electron microscope analysis of this material after fixation and embedding revealed structures resembling nuclei, suggesting that the pellet contained nuclear material from contaminating lymphocytes. SDS polyacrylamide gel analysis of the material showed small amounts of protein in the component 3 and 4 region but no spectrin or actin.

The denser light-scattering layer (layer II) was found at a density of 1.185–1.203 g/ml, regardless of whether the sample was centrifugued for 1 or 18 h. The material was in a diffuse layer after 1 h of centrifugation but condensed into a gelatinous wafer after 18 h of centrifugation. Layer II was always white, with no indication of hemoglobin contamination. Layer I was found at the lower edge of (1 h spin) or within (18 h spin) the hemoglobin band at a density of 1.08–1.11 g/ml and was isolated by dilution of the material in

buffer and pelleting at 100,000 g for 30 min. Usually no redness was found with the washed pellet. The amount of material in this layer was 2–3 times greater after 18 h than after 1 h of centrifugation.

In a typical experiment 6.8 mg of membrane protein was applied to the gradient [14], 2.0–2.5 mg of protein was recovered in layer II, 0.15–0.5 mg in layer I, and 0.24 mg in the pellet. SDS polyacrylamide gel analysis of the proteins showed that layer I contained components 3 and 4 in approximately equal amounts. Layer II had these two proteins plus spectrin, actin (component 5), component 7, and several minor proteins. From densitometer analysis of the gels it was estimated that layer II was 50–70% spectrin, 6–10% actin, 7–17% component 3, 6–12% component 4, 4–5% component 7, and minor amounts of other membrane proteins. Gels stained for carbohydrate with periodic acid-Schiff reagent (PAS) [13] revealed that less than 10% of the normal content of sialoglycoproteins was present. The components in layer II were organized into shells resembling the erythrocyte in size and form as shown in Fig 1.

If the samples of layer II material were incubated on ice for 36 h, there was a change in the SDS gel pattern of the shells (see Fig 2). As previously reported, there is movement of staining intensity from higher to lower apparent molecular weight in the component 3 and 4 regions [11]. Because these changes may result from proteolysis, shells are normally solubilized with SDS immediately after preparation.

When the concentration of Triton X-114 in the extraction medium is increased from 0.2 to 5%, there is no qualitative change in the SDS gels of the shells stained with Coomassie

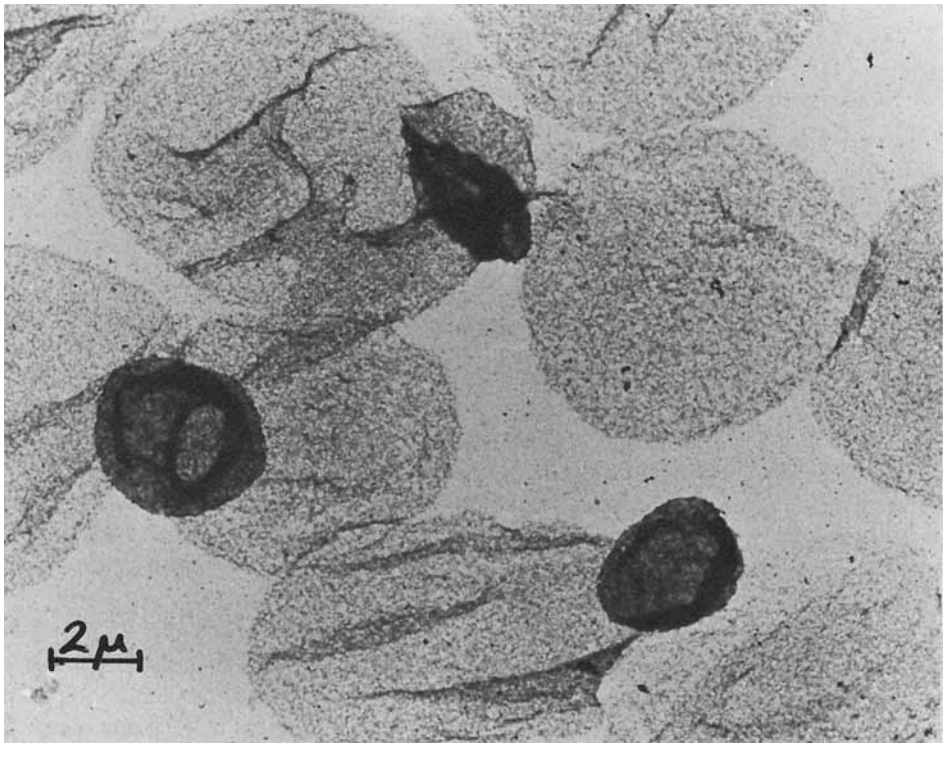

Fig 1. Electron micrograph of Triton shells negatively stained with $UO_2(Ac)_2$ (pH 4.5). magnification 4500 ×.

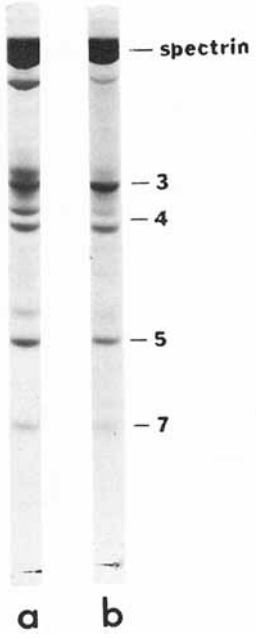

Fig 2. SDS polyacrylamide gels of 0.5% Triton shell material prepared according to the procedure described in Materials and Methods, sec, diluted (1:20) in Iso Tris, 0.5 mM Mg Cl_2, and 0.2 mM DTT, pelleted by centrifugation at 30,000 g for 30 min, and resuspended at a concentration of 1 mg/ml protein in Iso Tris, 0.5 mM $MgCl_2$, and 0.2 mM DTT. Gel a) Material immediately after preparation; gel b) the same material after 36 h on ice (25 μg of protein was applied in each case).

blue [11]. In Figure 3 the densitometer scans, however, show that the intensity of staining in the component 3 and 4 region decreases relative to spectrin and actin when the Triton concentration is increased from 0.5 to 2.0% (Fig 3b and c respectively). By integrating the peak areas through cutting and weighing, the percentages of staining in the component 3 and 4 regions are 17% and 9.5% respectively with 0.5% Triton, whereas with 2% Triton they are 12% and 7.5% respectively. The relative amounts of spectrin, actin, and component 7, however, remain constant within experimental error with a ratio of staining intensities of 1:0. 18:0.065. If we use the published values of the molecular weights of these components to compute their molar ratio [14], then for every mole of spectrin dimer there are 1.7 moles of actin and 1 mole of component 7 in the Triton-insoluble complex.

Component 3′

A component of the shells which electrophoreses in the component 3 region is not proteolyzed when intact cells are treated with trypsin, chymotrypsin, or a nonspecific bacterial protease [11]. Because this protein is distinguishable from the rest of 3 but coelectrophoreses with 3, we have designated it component 3′. Another distinguishing feature of 3′ is that it has a low extinction coefficient at 280 nm which is evident from the densitomer scans in Figure 3d and e. In the scan at 280 nm of an unstained gel of shells from chymotrypsinized cells the absorption in the component 3′ region is much less than that of component 4.2, 5, or the major proteolytic fragment of 3. When the same gel is stained with Coomassie blue and scanned at 550 nm, component 3′ staining is more intense

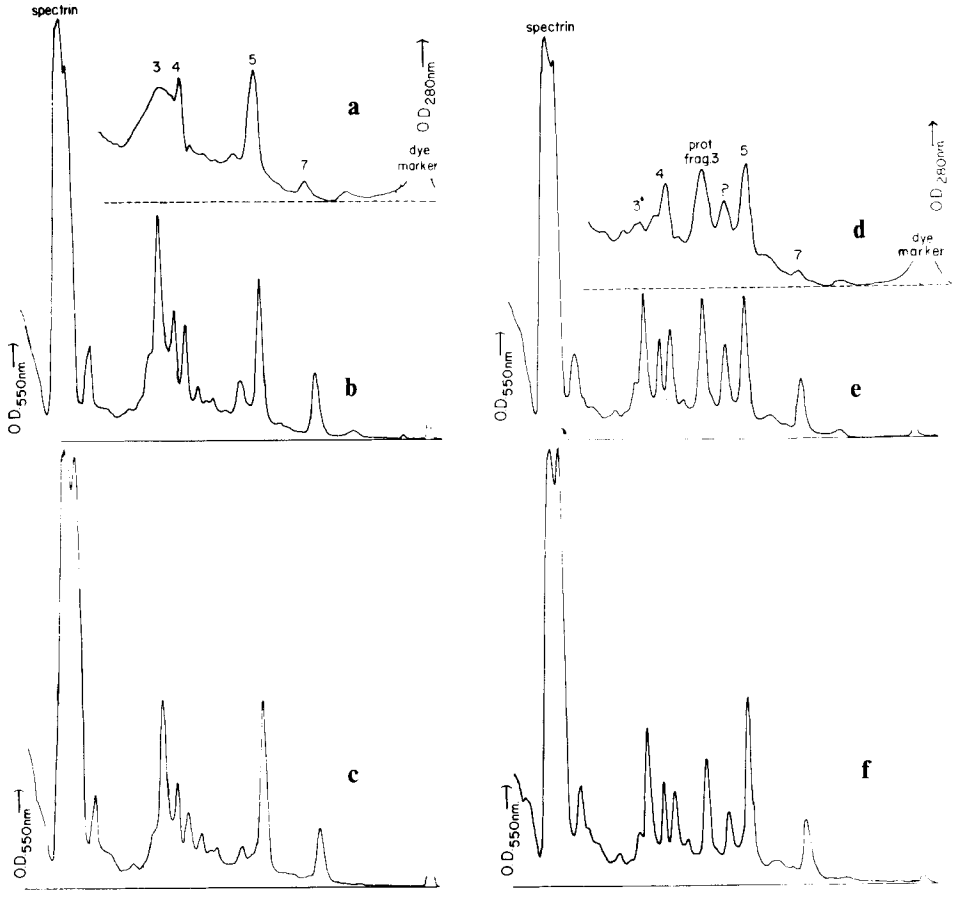

Fig 3. Densitometer scans of SDS polyacrylamide gels of Triton shells SDS-solubilized immediately after isolation and washing (as described in Fig 2). a,b) Gels of 0.5% Triton shells from normal erythrocytes: a) 160 μg of protein was applied and the gel was scanned immediately after electrophoresis at 280 nm; b) 20 μg of protein was applied and the gel was stained with Coomassie blue before scanning at 550 nm. c) Gel of 2% Triton shell material treated analogously to scan b. d, e, f) Shells prepared from chymotrypsinized erythrocytes: d, e) 0.5% Triton shells run as described for the gels a and b; f) 2% Triton shells of chymotrypsinized cells (20 μg of protein applied).

than 4.2, as in Figure 3e, which is a scan of a gel loaded with 20 μg of the same material. If 3′ is a different protein than 3, it may have different solubility properties. Indeed 3′ is released from the shells in 0.5 mM glycine, 0.5 mM EDTA, 0.2 mM DTT (pH 9.0) and is present in the supernatant after centrifugation at 150,000 g for 2 h. Since 3′ may be solubilized from the shells as a complex with Triton, a method was found for isolating 3′ from ghosts. In Figure 4 are densitometer traces at 280 nm and 550 nm before and after Coomassie blue staining, respectively, of the pH 11 extract from membranes which had been preextracted to remove the majority of spectrin and actin. The scan at 280 nm shows absorbing material at the top of the gel which is removed upon fixation with 10% isopropanol and 10% acetic acid. Again the absorbance at 280 nm of the protein which

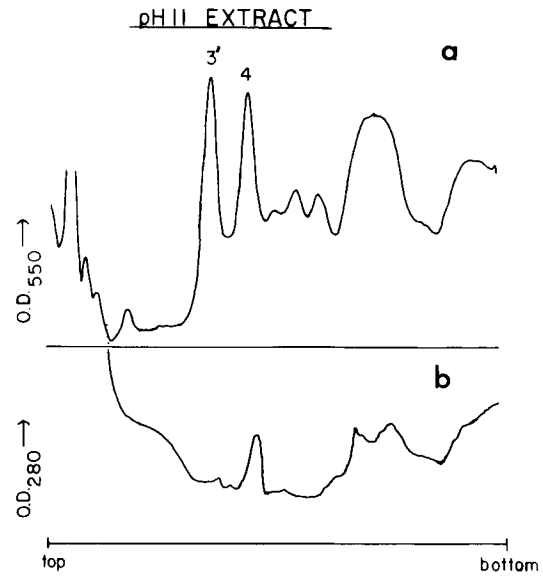

Fig 4. Densitometer scans of the same SDS polyacrylamide gel on which 100 μg of the pH 11 extract (prepared as described in Materials and Methods) was run. Scan b was recorded at 280 nm immediately after electrophoresis and scan a was recorded at 550 nm after staining with Coomassie blue.

electrophoreses in the component 3 region is significantly less than band 4, and this protein has been tentatively called 3′. The lower-molecular-weight components in this extract have not been characterized. Component 3′ is then defined as a protein which coelectrophoreses with band 3 but has a low absorption coefficient at 280 nm and is solubilized in alkaline glycine solutions. Its apparent molecular weight is 88,000 daltons.

Component 3

Since the major chymotryptic fragment of band 3 coisolates with the shells of chymotrypsinized cells, component 3 as well as 3′ may be present in Triton shells normally (Fig 3d). When the concentration of Triton X-114 is increased from 0.5% to 2%, the amount of the chymotryptic fragment of band 3 decreases by 25% in the shells of chymotrypsinized cells whereas the content of 3′ drops only 5% (Fig 3 e,f). As might be expected, higher detergent concentrations removed more of the band 3 fragment than 3′. The decrease in the amount of stain in the 3 region in gels of shells of normal cells prepared by 2% versus 0.5% Triton extraction suggests that band 3 is present (Fig 3b and c). Further indication that 3 is normally in the shells is given by the densitometer scan at 280 nm, which shows that the component in the 3 region absorbs more than 3′ does (Fig 3a).

Band 7

Band 7 is the low-molecular-weight component of the shells, which is present in constant amounts relative to spectrin. The molar ratio of band 7 to spectrin is estimated to be 1:1 from the Coomassie blue staining intensities. But under hypotonic extraction conditions which solubilize spectrin from ghosts, band 7 remains in the ghost. The densitometer tracings at 280 nm reveal that band 7 has a strikingly low absorbance at 280 nm (Fig 3a and b or d and 3).

Spectrin

Washed shells suspended in isotonic media will release a significant but variable fraction of their protein with time. SDS gels of the solubilized protein reveal that it is > 90% spectrin, [11, 15]. As seen in Figure 5 the isotonically solubilized spectrin coelectrophoreses with hypotonic spectrin, and within the precision of the gels (differences of 4,000 daltons could be resolved) the two proteins are identical in molecular weight. The protein may be released as a complex with detergent. To determine if this was the case, spectrin was purified by velocity sedimentation. As seen in Figure 6, the isotonic spectrin sediments as a single species that has the same sedimentation velocity as does hypotonic spectrin in the same ionic conditions. There is no Triton peak associated with the protein peak and from the Triton specific activity there is less than a mole of Triton bound per 2 moles of spectrin dimer. Thus spectrin was released from the shells without detectable proteolysis and free of detergents.

Another parameter of spectrin is its phosphorylation, which occurs in vivo as well as in vitro [16]. We have prelabeled spectrin in intact erythrocytes by incubating with $^{32}P_i$ and have observed that the isotonically solubilized spectrin has a significantly lower specific activity than spectrin in Tris ghosts or hypotonic spectrin [11, 15]. Concomitant with the release of spectrin from shells there is a rise of phosphate label in the non-TCA-precipitable fraction.

Fig 5. Coomassie blue-stained SDS polyacrylamide gels (3.25% acrylamide) of spectrin prepared a) by hypotonic elution from 10 mM Tris ghosts in 0.1 mM ATP, 0.1 mM EDTA, 0.1 mM DTT, and 0.2 mM Tris (pH 8.0) and b) by isotonic elution from 0.5% Triton shells in Iso Tris, 0.5 mM $MgCl_2$, and 0.2 mM DTT incubated 36 h on ice (20% of the shell spectrin was solubilized in this experiment).

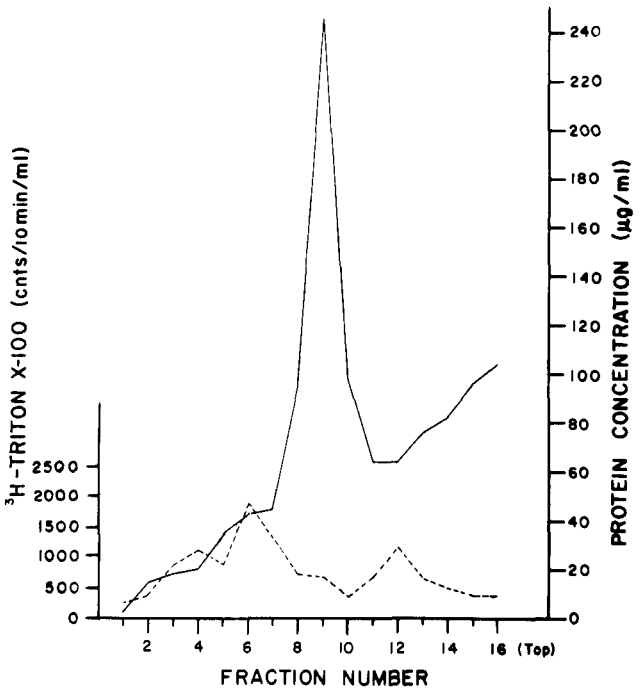

Fig 6. Graph of a velocity sedimentation experiment showing the protein concentration (—) and (^{3}H) counts (- - -) versus gradient fraction for isotonic spectrin after centrifugation for 16 h at 150,000 g in 10–40% sucrose gradient made with Iso Tris, 0.5 mM $MgCl_2$, and 0.2 mM DTT. The specific activity of the Triton was 461 cpm/µg of Triton, and 0.5 ml of a 500 µg/ml solution of isotonic spectrin was applied to the 4.5-ml gradient.

Electron Microscopy

Earlier electron micrographs of Triton shells of erythrocyte ghosts [8, 10] have not revealed a well-defined reticulum such as has been observed by scanning electron microscopy [17]. A definite reticulum is observed, however, in the shells of whole cells. Special care was taken to shorten the time from lysis until the samples was fixed with glutaraldehyde. After fixation, the samples were negatively stained with uranyl acetate (pH 4.5). As seen in Figure 7a at low magnification the reticulum from a single cell appears unordered over most of its area, particularly where two layers of the network are superimposed. In certain regions, however, where shear has oriented the fibrous elements of the network, a definite order appears in the reticulum. This order is particularly evident where a single layer of the reticulum is viewed, as in Figure 7b. In that figure the shell was torn open and the tearing forces also aligned the fibrous elements of the shells. The fibers are beaded and are 80–120 Å in diameter. Similar structures have been observed in shells stained with phosphotungstic acid (pH 7.4) but the contrast is not as great. From the composition of the shells we can estimate the possible length of actin filaments in the area of the micrograph to be sufficient to go from the top to the bottom of the micrograph four times (Fig 7b); therefore, there is insufficient actin to account for the fibers.

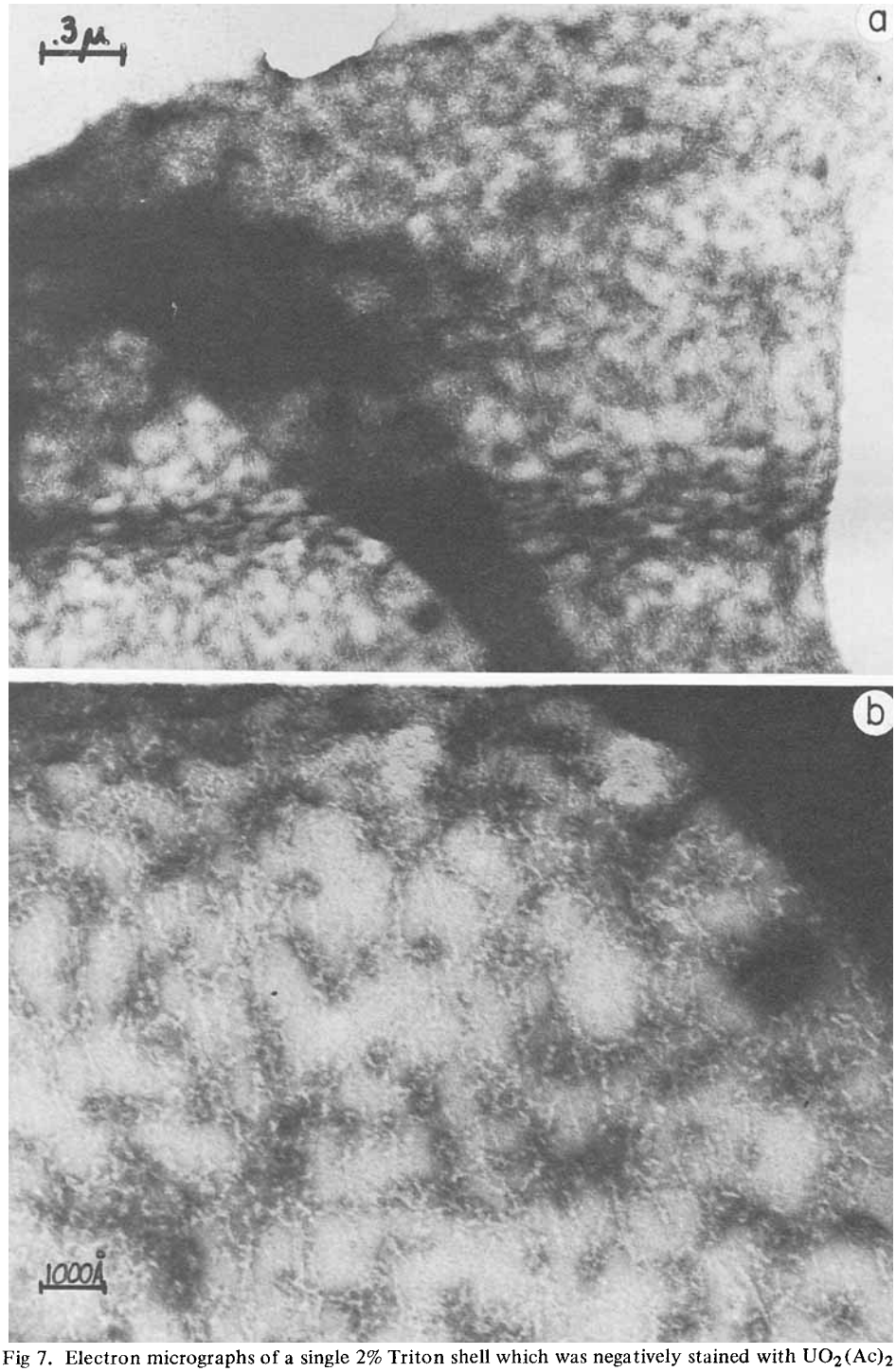

Fig 7. Electron micrographs of a single 2% Triton shell which was negatively stained with $UO_2(Ac)_2$ at pH 4.5 after fixation with glutaraldehyde as described. a) Majority of shell is shown; b) portion of the shell at higher magnification. The second layer of the Triton-insoluble material appears to be torn away in this region, which affords a clearer view of the filamentous structures in the shell.

DISCUSSION

Through the careful dissection of the erythrocyte membrane with nonionic detergents we can understand which complexes exist among the many possible combinations. With this information it will be easier to design reconstitution experiments to understand the bases for the component interactions. In order to isolate complexes which may reflect in vivo structures, we have approximated the ionic environment of the cell cytoplasm during extraction. The nonionic detergent Triton was chosen because it denatures relatively few enzymes [18]. Intact cells rather than ghosts were employed so that the proteins would not be exposed to hypotonic solutions. Because protease contamination was found with whole cell preparations, the proteins of the shells were separated from the detergent, cytoplasm, and solubilized components by sedimentation into a sucrose density gradient of the same ionic composition as the extraction solution. These steps were taken to assure that the shell components would suffer the least possible insult in preparation and to allow for systematic variation of the extraction conditions.

Although the detergent concentration had dramatic effects on the lipid content of the shells, the protein components remained relatively constant. The lipid content was reflected by the shell density and phosphate-to-protein ratio. In shells prepared with 0.5% vs 2.0% Triton X-114 the density was 1.19 vs 1.27 g/ml and the phosphate-to-protein ratio was 0.60 vs 0.04 μmoles P/mg protein [11]. As seen in Figure 3, however, the protein composition of shells extracted with 0.5% vs 2.0% Triton did not change qualitatively. There were significant decreases of staining intensity in the band 3 and 4 region, but those changes can be explained as an increased extraction of the integral membrane protein band 3 and concomitant loss of band 4. In support of this the shells of chymotrypsinized cells extracted with 0.5 vs 2.0% Triton show significant decreases only in the amount of the band 3 proteolytic fragment and band 4. Because we might expect that a complex of peripheral proteins would be relatively insensitive to Triton, it is significant that the amounts of spectrin, actin, band 7, and band 3′ remain roughly proportional in a molar ratio of 1:1.7:1:0.8 in shells prepared with 0.5% or 2% Triton assuming that the staining with Coomassie blue is a true measure of protein concentration. Although the addition of detergent may induce artifactual protein associations, the shell complex is a relatively stable unit and provides a basis to study further the protein components contained in it.

Component 3′ is of particular interest because of its unusual properties. As reported earlier component 3′ is the protein which electrophoreses in the band 3 region, isolates with the shells of intact cells, and is insensitive to chymotrypsin, trypsin, and a nonspecific bacterial protease in the intact erythrocytes [11]. Although a certain portion of the integral protein, band 3, normally appears to coisolate with the shells, band 3′ can be clearly differentiated from that protein. Component 3′ is a water-soluble, ie, peripheral, protein which has a low extinction coefficient at 280 nm. Because 3′ is insensitive to a variety of proteases in the intact cell, it is most likely on the cytoplasmic face of the membrane. In ghosts the amount of 3′ is normally less than the amount predicted from the shells but is variable (Sheetz and Sawyer, unpublished results). The unusual behavior of component(s) 3′ on SDS gels is not understood and raises the possibility that 3′ is several proteins or a major proteolytic fragment of a larger protein. In accord with the latter possibility is the fact that the light meromyosin fragment of myosin has a low absorbance at 280 nm [19]. But the presence of 3′ in ghosts of chymotrypsinized cells does not correlate with the loss of another component nor does 3′ increase appreciably when proteolysis occurs in ghosts or shells. Thus if 3′ is a proteolytic produce of spectrin, then the specific protease must be

washed away or inactivated in ghosting and in shell preparation. There is no single high-molecular-weight protein band other than spectrin that is present in enough copies to produce 3′. Again, we assume that Coomassie blue staining gives a true measure of all the proteins present.

Several possible functions for 3′ have been suggested and these functions may be interrelated. Our data suggest that the stimulation of actin polymerization by spectrin is modulated by the erythrocyte casein kinase [20] when 3′ is present (Sheetz and Sawyer, manuscript in preparation). Perhaps related to the actin polymerization activity White and Ralston [21] have solubilized a Mg^{2+} ATPase under conditions which extract 3′ (Sheetz and Sawyer, unpublished results, 1978) and the Mg^{2+} ATPase has an apparent molecular weight similar to 3′. Goodman and Branton [21a] have found that spectrin binds to the vesicles which are left after hypotonic elution of spectrin and actin from the shells; therefore 3′ may form part of the spectrin attachment site in the membrane. Certainly all of these functions are consistent with the present data and indicate that 3′ may have an important role in the functions of the erythrocyte cytoskeleton.

The portion of band 4 associated with the shells immediately after Triton extraction is predominently component 4.1 and with time the staining intensity moves from the 4.1 to the 4.2 region [11]. Since protease will catalyze the same apparent transition in ghosts (Sheetz and Sawyer, unpublished results, 1978), we feel that the conversion is the result of proteolysis. Bennett [22] has observed that proteolysis of inside-out vesicles releases a 72,000-dalton component which coelectrophoreses with 4.2 and competes with the spectrin-binding component on inside-out vesicles. This component may account for the increase in staining in the 4.2 band with time. The amount of band 4, like the amount of band 3, in the shell decreases significantly with higher detergent concentrations in the shells. The content of these two proteins may be coupled since it has been noted previously that they do interact [23, 24].

Another component of the shells which is not well understood is band 7. It is interesting that band 7 is present in stoichiometric amounts with spectrin and that it has a low extinction coefficient at 280 nm. Several calcium-binding proteins – the parvalbumins, troponin C, bovine brain S-100 protein, and bovine intestinal calcium-binding protein – all are low in tyrosine and tryptophan [25]. The calcium-binding proteins are all lower in molecular weight than band 7, but studies are under way to determine if 7 has calcium-binding properties similar to those proteins.

Spectrin is the major component of the shells on the basis of mass, and it is logical to assume that it plays a major role in maintaining the structure of the shell. When spectrin is solubilized either under isotonic conditions or by hypotonic extraction, the shell structure is lost. In isotonic media spectrin alone is solubilized and the reticular structure of the shells disappears [11]. Only vesicles and filaments resembling actin filaments remain after spectrin removal [11]. This suggests that spectrin either constitutes or induces the 80- to 120-Å filaments. Indeed, similar filaments have been observed in spectrin preparations treated with divalent cations [26, 27]. We do not know if shear forces are responsible only for aligning preexisting filaments in the shells or if the filaments are formed as a result of shear stress on the reticulum. The latter possibility raises the point that some bridging structures if not filaments must exist to maintain shell integrity. Numerous attempts have been made to visualize filaments on the cytoplasmic face of the erythrocyte membrane, and only the recent studies of Steck and Hainfeld [17] have revealed an ex-

tensive fibrillar network. Again the fibers were visualized best in regions where the network appears to be sheared. The spectrin released from the shells in isotonic media is a dephosphorylated spectrin but does not appear proteolyzed [11, 15]. The phosphorylation of spectrin has been implicated as an important factor in erythrocyte membrane shape changes [28] and in the polymerization of actin stimulated by spectrin [20]. If the dephosphorylation of spectrin causes it to release from the network, then the observed shape changes and loss of actin-polymerizing activity with dephosphorylation could be explained. Rephosphorylation experiments, however, have not resulted in spectrin reaggregation, but certain components necessary for aggregation may be missing.

Although filamentous actin would form a natural bridging structure in the cytoskeleton, several reports suggest that erythrocyte actin may be largely in a globular form [26, 29]. Evidence, however, for the presence of filamentous actin in the erythrocyte is strong. We have repeated the observations of Pinder, Bray, and Gratzer [20], that crude spectrin extracts promote actin polymerization, and Birchmeier and Singer [7] find that actin will polymerize inside the erythrocyte ghosts at concentrations below the critical polymerizing concentration. After spectrin solubilization in isotonic media, actin is always in the pelletable fraction, where numerous 60- to 80 Å-diameter filaments are found. Further we have observed that actin filaments will interact with inside-out but not right-side-out vesicles of erythrocyte membranes (Sheetz and Hom, unpublished results). Figure 7b shows that numerous filaments can be observed in the erythrocyte cytoskeleton which cannot all be accounted for by actin. However, a portion of them which are smaller could be actin filaments. Considerable inferential evidence exists for the presence of globular and filamentous actin in the erythrocyte but neither form has been shown to be involved directly in erythrocyte membrane function.

Since the Triton shells can be simply prepared from intact erythrocytes, they provide a good source of cytoskeletal material which has not been exposed to hypotonic salt solutions. Many of the presumed erythrocyte cytoskeletal components are highly anionic and exposure to hypotonic solutions may alter their native conformation and their subsequent behavior. The Triton-insoluble complex in the shells which is composed of spectrin, actin, and components 3′ and 7 may be a detergent-induced aggregate, but reconstitution studies should test for possible interactions of these components. Whether the presence of component 3 in the shells reflects incomplete extraction or a meaningful interaction with the cytoskeleton is yet to be determined. The isotonic elution of spectrin from the shells provides a means of preparing this protein in physiologic salt conditions, although the protein is in a dephosphorylated form and may have undergone an undetected proteolytic cleavage. Since electron microscopic studies of protein structure are fraught with possible artifacts, we do not assert that the structures in Figure 7b do exist in vivo. The micrograph does depict the cytoskeleton as we imagine it, and it therefore provides a form of model for the cytoskeleton. However, we have refrained from identifying specific proteins with specific structures. The Triton shell of intact erythrocytes provides an excellent starting material for studies of the cytoskeletal structure and components.

ACKNOWLEDGMENTS

We thank Su Ming Hom for his help in the original phases of this work. This project was supported by NIH grant HL-18317.

REFERENCES

1. Steck TL, Yu J: J Supramol Struct 1:232, 1973.
2. Yu J, Fischman D, Steck TL: J Supramol Struct 1:220, 1973.
3. Kirkpatrick FH, Gordesky SE, Marinetti GV: Biochim Biophys Acta 345:154, 1974.
4. Lux SE, John KM, Karnovski MJ: J Clin Invest 58:955, 1976.
5. Lux SE, Pease B, Tomaselli MB, John KM, Bernstein S: J Supramol Struct 6:194a, 1978.
6. Sheetz MP, Singer SJ: J Cell Biol 73:638, 1977.
7. Birchmeier W, Singer SJ: Biochem Biophys Res Commun 77:1354, 1977.
8. Palek J, Liu SC, Snyder LM: Blood 51:385, 1978.
9. Kirkpatrick FH: Life Sci 19:1, 1976.
10. Bennett V, Branton D: J Biol Chem 252:2753, 1977.
11. Sheetz MP, Sawyer D, Jackowski S: In Brewer G (ed): "Erythrocyte Metabolism and Function," 1978, vol 4, p 431.
12. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: J Biol Chem 193:265, 1951.
13. Fairbanks G, Steck TL, Wallach DFH: Biochemistry 10:2606, 1971.
14. Steck TL: J Cell Biol 62:1, 1974.
15. Sheetz MP, Sawyer D: In Pepe F (ed): "Motility in Cell Function." (In press.)
16. Wolfe LC, Lux SE: Blood 48:963, 1976.
17. Steck TL, Hainfeld JF: In Porter KR (eds): "International Cell Biology, 1976–1977, p6.
17. Steck TL, Hainfeld JF: In Brinkley BR, Porter KR (eds): "International Cell Biology, 1976–1977," 1977, p6.
18. Tanford C, Reynolds JA: Biochim Biophys Acta 457:133, 1976.
19. Lowey S, Slayter HS, Weeds AG, Baker H: J Mol Biol 45:1, 1969.
20. Pinder JC, Bray D, Gratzer WB: Nature 270:752, 1977.
21. White RD, Ralston GB: Biochim Biophys Acta 436:567, 1976.

21a. Goodman SR, Branton D: J Supramol Struct 8:455–463, 1978.

22. Bennett V: J Biol Chem 253:2292, 1978.
23. Yu J, Steck TL: J Biol Chem 250:9176, 1975.
24. Wang K, Richards FM: J Biol Chem 249:8005, 1974.
25. Demaille J, Dutruge E, Caponey JP, Pechere JF: In Drabikowski W et al (eds): "Calcium Binding Proteins," 1974, p 643.
26. Tilney LG, Detmers P: J Cell Biol 66:508, 1975.
27. Kirkpatrick FH, Woods GM, Weed RI, and LaCelle PL: Arch Biochem Biophys 175:367, 1976.
28. Birchmeier W, Singer SJ: J Cell Biol 73:647, 1977.
29. Ralston GB: J Supramol Struct 6:190a, 1978.