

CYTOSKELETAL NETWORK UNDERLYING THE HUMAN ERYTHROCYTE MEMBRANE

Thin-section Electron Microscopy

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ABSTRACT

A filamentous network underlying the human erythrocyte membranes can be clearly visualized *in situ* by electron microscopy of thin sections of specimens fixed with tannic acid-glutaraldehyde. The network is composed of two layers: the first, a layer of vertical components with granular appearance, which are seen to be directly associated with the membrane proper, and the second, a horizontally disposed, anastomosing meshwork of filamentous components, ~9 nm in thickness, which are attached to the vertical components. The diameter and appearance of the filamentous components are similar to those of purified spectrin. EDTA treatment (0.1 mM, pH 8.0), which was used to extract spectrin and actin, resulted in the disappearance of the filamentous meshwork, leaving only the granular components.

The cytoskeletal network underlying the human erythrocyte membrane has often been discussed in light of its roles in determining cell shape and in constraining the lateral movement of membrane-penetrating proteins (14, 19, 23, 30). The accumulated evidence indicates that the network is mainly composed of spectrin (5, 6, 10, 18, 28), which accounts for ~75% of all of peripheral membrane proteins (31). It is now proposed that spectrin may form an anastomosing network beneath the erythrocyte membrane in combination with actin (14, 19, 20, 30, 33). Most recently, a series of proteins has been identified which connects spectrin to the erythrocyte membrane (1, 12, 37). Spectrin molecules as heterodimers have a rodlike appearance of 100 nm in length, as revealed by shadow-casting electron microscopy (26, 27). Cytoskeletal networks were observed by scanning electron microscopy (8) and by negative staining after Triton X extraction (24). In spite of

recent great progress, our understanding of the ultrastructural aspects of the network is still limited, mainly because of its poor visualization in sectioned preparations.

In the present study, we have tested various chemical fixatives in an effort to clearly visualize the cytoskeletal network, and only tannic acid fixation has revealed network structures that have not been seen before. We believe this method to be particularly useful for ultrastructural analyses of the supramolecular organization of the network, of the mode of association with the membrane proper, and of the dynamic aspects of the network during various membrane activities.

MATERIALS AND METHODS

Preparations of Erythrocyte Membranes

Human erythrocyte membranes (ghosts) were prepared from freshly drawn human blood according to the procedure of Fair-

banks et al. (7) using sodium phosphate buffer, pH 8.0. In these preparations the hemoglobin component was not detected in SDS slab gel electrophoresis with 13% polyacrylamide gel loaded with 50 μ g of membrane protein. Some erythrocyte membranes were prepared by the same method except that pH 7.5 buffer was used. Loose white pellets of the membranes were immediately processed for electron microscopy.

To avoid the use of hypotonic buffer, another method of preparation was employed. Fresh human erythrocytes were washed four times in 5 mM sodium phosphate buffer (pH 8.0)–0.15 M NaCl, and then hemolysed at room temperature by being mixed into 6 vol of 5 mM sodium phosphate buffer (pH 8.0)–0.15 M NaCl containing 1.0×10^{-4} g/ml saponin (ICN Nutritional Biochemicals, Cleveland, Ohio). After 10 min of treatment, the erythrocyte suspensions were diluted by adding 10 vol of the same buffer-NaCl solution without saponin. The erythrocyte membranes were then pelleted by centrifugation at 24,000 g for 12 min and washed three times in the buffer-NaCl solution. The resulting loose pellets were processed for electron microscopy.

EDTA Extraction of Erythrocyte Membranes

To elute membrane proteins at low ionic strength, the erythrocyte membranes were further treated with 0.1 mM EDTA (pH 8.0) at 37°C for 15 min as described by Fairbanks et al. (7). By this treatment, the erythrocyte membranes were fragmented into small inside-out vesicles, which were collected to be processed for electron microscopy. The degree of protein elution from the membranes was examined by electrophoresis.

Preparation of Spectrin

Erythrocyte membranes were prepared in 5 mM phosphate buffer (pH 8.0) from recently outdated blood, and incubated with 2 mM adenine and 20 mM inosine in 145 mM NaCl–5 mM sodium phosphate buffer (pH 7.4) at 37°C for 3 h (16). Spectrin was extracted and partially purified by the method of Marchesi (13) except that the column of Sephadex G-200 was eluted with 0.1 mM EDTA, 2.5 mM β -mercaptoethanol, 100 mM NaCl in 25 mM Tris-HCl buffer (pH 8.0). After the partially purified spectrin was concentrated by ultrafiltration (Amicon Diaflo UM-10 membrane, Amicon Corp., Scientific Sys. Div., Lexington, Mass.), spectrin dimers were purified by gel filtration on Sephadex CL-4B column in the same solution, according to the method of Ungewickell and Gratzer (34) and were immediately processed for electron microscopy. This preparation showed no trace of proteins other than spectrin after SDS polyacrylamide gel electrophoresis (see Fig. 5*d*).

Electron Microscopy

THIN-SECTIONED PREPARATIONS: Erythrocyte membranes and purified spectrin were prefixed with 2% tannic acid–2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at 4°C overnight. To replace the fixative, the samples were centrifuged at 500 g for 5 min at each step until the membranes were embedded in agar. The samples were then rinsed with the buffer and postfixed with ice-cold 1% OsO₄ in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h. Some samples were fixed with 1% OsO₄ (in 0.1 M sodium cacodylate buffer, pH 7.4) alone, or 0.6% KMnO₄ (in 0.1 M Veronal-acetate buffer, pH 7.4) alone, or 2.5% glutaraldehyde followed by 1% OsO₄ (in 0.1 M sodium cacodylate buffer, pH 7.4). After being rinsed in distilled water, pellets of the fixed membranes were embedded in 1% agar, stained en bloc with 0.5% aqueous uranyl acetate for 2 h at room

temperature, dehydrated in graded concentrations of ethanol, and embedded in Epon 812. Thin sections were cut with a diamond knife on an LKB Ultratome (LKB Instruments, Inc., Rockville, Md.), stained doubly with uranyl acetate and lead citrate, and examined in a Hitachi HU-12 electron microscope operated at 100 kV and (for stereoscopic observations) in a Hitachi H-700 electron microscope equipped with goniostage operated at 200 kV.

NEGATIVE STAINING: To obtain single-layered erythrocyte membranes for negative staining, two different procedures were applied. In some preparations, fresh human erythrocytes were washed four times in 5 mM sodium phosphate buffer (pH 8.0)–0.15 M NaCl and were added in a drop to 5 mM sodium phosphate buffer (pH 8.0). By this procedure, a small fraction of the cells was hemolysed at the air-water interface and spread flat by surface tension as originally described by Nicolson and Singer (17). The flattened membranes were picked up on carbon-coated, collodion-film grids. In the other preparations, fresh human erythrocytes were washed and attached to poly-L-lysine-coated grids, and then the tops of such attached erythrocytes were ripped off by a stream of 5 mM sodium phosphate buffer, pH 8.0, according to the method used by Clarke et al. (3). The membrane on the grid was fixed for 5 min with a drop of 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) and negatively stained with saturated aqueous uranyl acetate. Some membrane preparations were treated with heavy meromyosin (HMM) (1–3 mg/ml) on a grid for 1–10 min at room temperature before negative staining was applied.

SDS Polyacrylamide Gel Electrophoresis

Samples were subjected to electrophoresis in either 9% polyacrylamide slab gels by the method of Laemmli (11) or 5.6% polyacrylamide disc gels by the method of Fairbanks et al. (7).

RESULTS

Cytoskeletal Network in Thin Sections

After tannic acid-glutaraldehyde fixation, a characteristic structure was clearly seen directly associated with the cytoplasmic surface of the erythrocyte membrane (Fig. 1). Such a structure appeared as an anastomosing network of filamentous structures in tangentially or obliquely cut sections of the membrane. In cross section the structure underlying the membrane was resolved into two layers (Fig. 2*a*). Connected with the membrane proper was a layer of vertical components, which appeared as granular structures with sizes ranging from 10 to 13 nm. Interestingly, corresponding to the position of these granular components, the membrane proper often showed a moderate density in the middle clear zone of its trilamellar structure. In tangential section of the membrane these components were round or ellipsoidal in shape and distributed somewhat equidistantly, separated by spaces of 10 to 30 nm (Fig. 2*b*). The density of the granular components was difficult to be accurately estimated in thin sections,

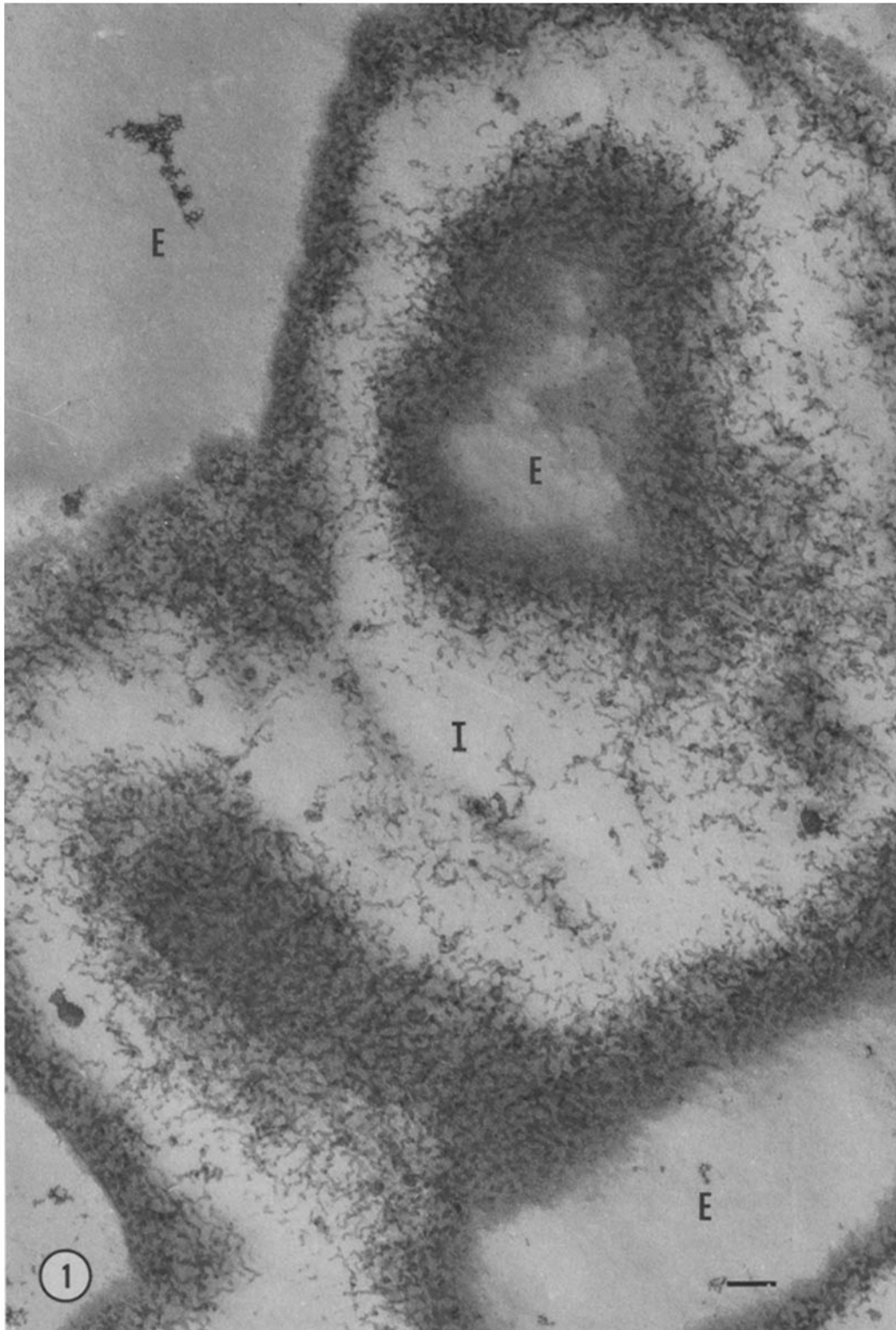


FIGURE 1 Thin section of an erythrocyte membrane fixed with tannic acid-glutaraldehyde mixture. The cytoskeletal network underlying the erythrocyte membrane is clearly visualized in obliquely or tangentially cut areas of the membrane (gray areas). *E*, Extracellular space. *I*, Intracellular space. Bar, 0.1 μm . $\times 75,000$.

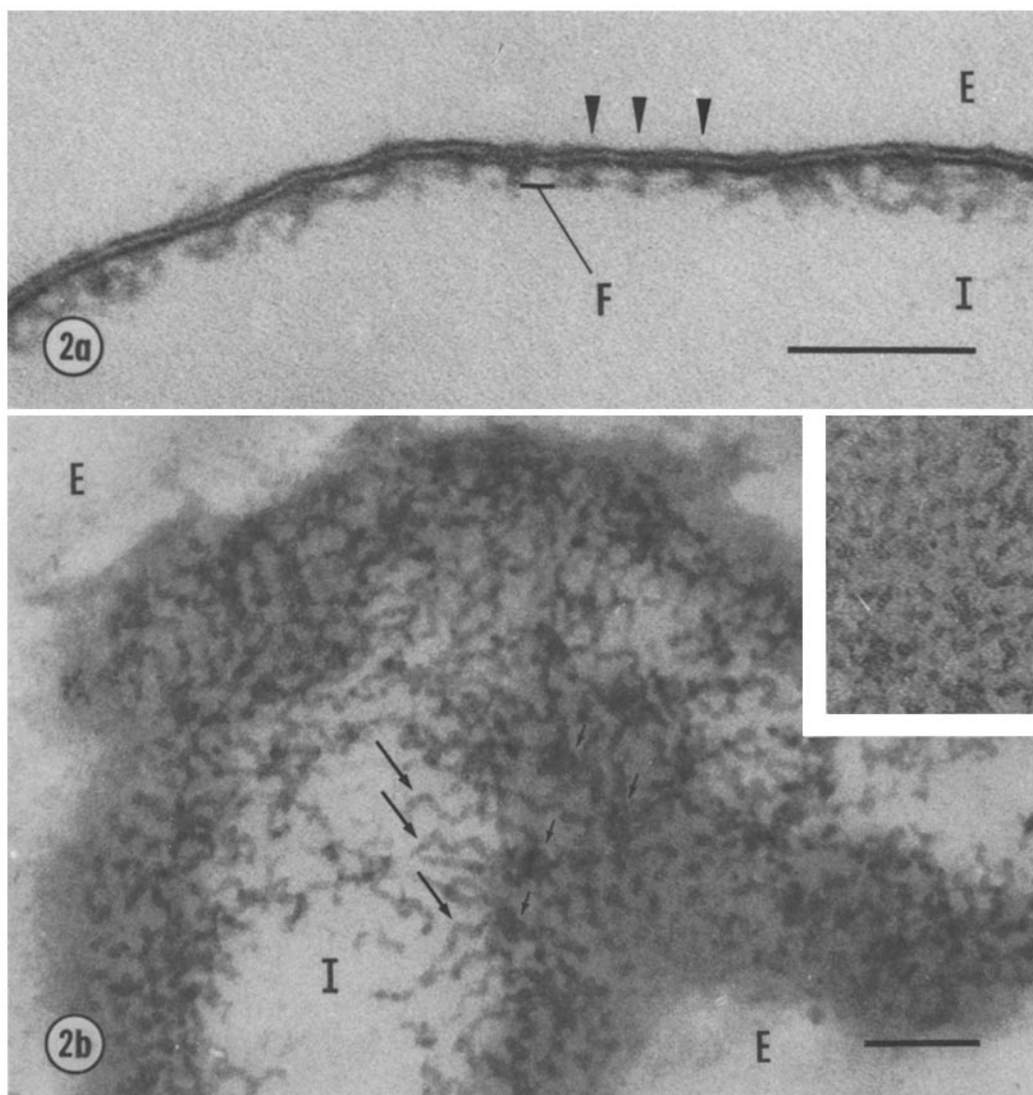


FIGURE 2 High-power electron micrographs of erythrocyte membranes. *E*, Extracellular space. *I*, Intracellular space. (*a*) In cross section, the cytoskeletal network is clearly visible on the cytoplasmic surface of the membrane. The structure is resolved into two layers: vertical granular components (arrowheads) and horizontally arranged filamentous components (*F*). Note the moderate densities in the middle clear zone of the trilamellar structure, often corresponding to the positions of the granular components of the network. Bar, $0.1 \mu\text{m}$. $\times 250,000$. (*b*) In obliquely cut section, the appearance of the layered structure of the cytoskeletal network is gradually changed. Next to the homogeneous layer of the membrane proper (gray area) is seen the granular layer (see *inset*), then there appears the filamentous meshwork superimposed on the granular layer, and finally only the filamentous meshwork is observed. The diameter and the appearance of the filamentous components (large arrows) are very similar to those of purified spectrin. Note the scattered occurrence of round spots (small arrows), to each of which several filamentous components appear to attach. Bar, $0.1 \mu\text{m}$. $\times 150,000$.

for the area showing only the granular components is limited and some larger granules are likely to be aggregates of the unit components. Our rough

estimation indicated that the density of the granular components ranged from $1,600$ to $2,300 / \mu\text{m}^2$ depending on the membranes, with the mean of

1,800 / μm^2 . Abutting directly on this granular layer was a second layer composed of a horizontally arranged filamentous meshwork (Fig. 2a). This meshwork extended parallel to the membrane all over the cytoplasmic surface. The meshwork was made up of filamentous components of a relatively uniform thickness, 9 ± 1 nm SD (No. of measurements: 100), which were connected with each other to form angular meshwork units. The arrangement of the meshwork was very irregular. The distance between different filaments of the meshwork ranged from 9 to 50 nm. One could often recognize the characteristic configuration of the filamentous components as possible repeating units (Fig. 2b), similar to that of purified spectrin (27). Scattered in the filamentous meshworks were seen round spots of 25 ± 3 nm SD (No. of measurements: 100) in diameter, to each of which several filamentous components appeared to attach (Fig. 2b). The distance between adjacent spots ranged from 80 to 280 nm. Because their counterparts could not be unequivocally identified in cross sections or in negatively stained membranes, more work is needed to clarify their involvement in network formation.

In sections cut oblique to the membrane, the appearance of the layered structure was gradually changed: next to the homogeneous layer of the membrane proper was seen the granular layer where the filamentous meshwork was not visible (Fig. 2b). Then, there appeared an area where the granular components were superimposed vertically over the filamentous components. Careful analysis of both obliquely and transversely cut sections showed that the granular components were cross-bridged between the membrane proper and the filamentous meshwork. Beyond the filamentous meshwork, some fuzzy or fine filamentous materials extended from the network. However, such materials were too irregular in configuration and occurrence to be seen as structures of the erythrocyte membrane. Stereoscopic observations of obliquely or tangentially cut sections showed the cytoskeletal network in three dimensions, confirming the spatial relationship described above (Fig. 3).

The overall structure of the network underlying the erythrocyte membrane was essentially the same among the different membrane preparations examined: hypotonic preparations at pH 8.0 and pH 7.5, and the isotonic preparations at pH 8.0 by use of saponin (data not shown). In saponin-lysed preparations, many holes ~ 10 – 15 nm in diameter

were found in tangential views of the membranes (data not shown). With respect to the protein composition in different preparations, the electrophoretic pattern turned out to be the same in erythrocyte membranes prepared hypotonically at both pH 8.0 and 7.5, showing the typical pattern obtained by Fairbanks et al. (7) (see Fig. 5a). A similar protein composition, except for a complete lack of band-6 protein, is recognized in the saponin preparation.

When erythrocyte membranes were fixed with OsO_4 alone, KMnO_4 alone, or glutaraldehyde- OsO_4 , a layer of fluffy material was observed on the cytoplasmic surface. In tangential section, this layer only rarely revealed a filamentous network (see reference 33) in contrast to that seen in the tannic acid-glutaraldehyde preparation.

When erythrocyte membranes were treated with 0.1 mM EDTA (pH 8.0) to extract spectrin (bands 1 and 2) and actin (band 5), the membranes were fragmented into small vesicles, which were inverted inside-out with the cytoplasmic leaflet facing outside (Fig. 4). These residual membranes varied in size up to $1 \mu\text{m}$ in diameter and took more or less spherical shapes, but usually did not form closed vesicles. The SDS gel electrophoresis of this preparation indicated that $\sim 91\%$ of the spectrin and almost all of the actin was extracted from membranes (Fig. 5). In thin-section electron microscopy, the layer of filamentous meshwork was not visible on the vesicular fragments, and only the granular components were left behind (Fig. 6). The granular components had a round or ellipsoidal appearance, ranging from 10 to 13 nm for the shorter diameter. The distribution density and pattern of the granular components did not seem to be changed significantly from the unextracted membrane, though they could not be estimated exactly, because of the vesicular configuration of the fragments.

Purified Spectrin in Thin Sections

In thin sections, spectrin appeared as a densely packed aggregate, in which filamentous structures could often be discerned as unit constituents (see Fig. 6). They showed a relatively uniform thickness of 9 ± 2 nm SD (No. of measurements: 100), similar to that of the *in situ* filamentous structures; their length could not be determined because of aggregation.

Cytoskeletal Network in Negative Staining

In single-layered membrane preparations, we

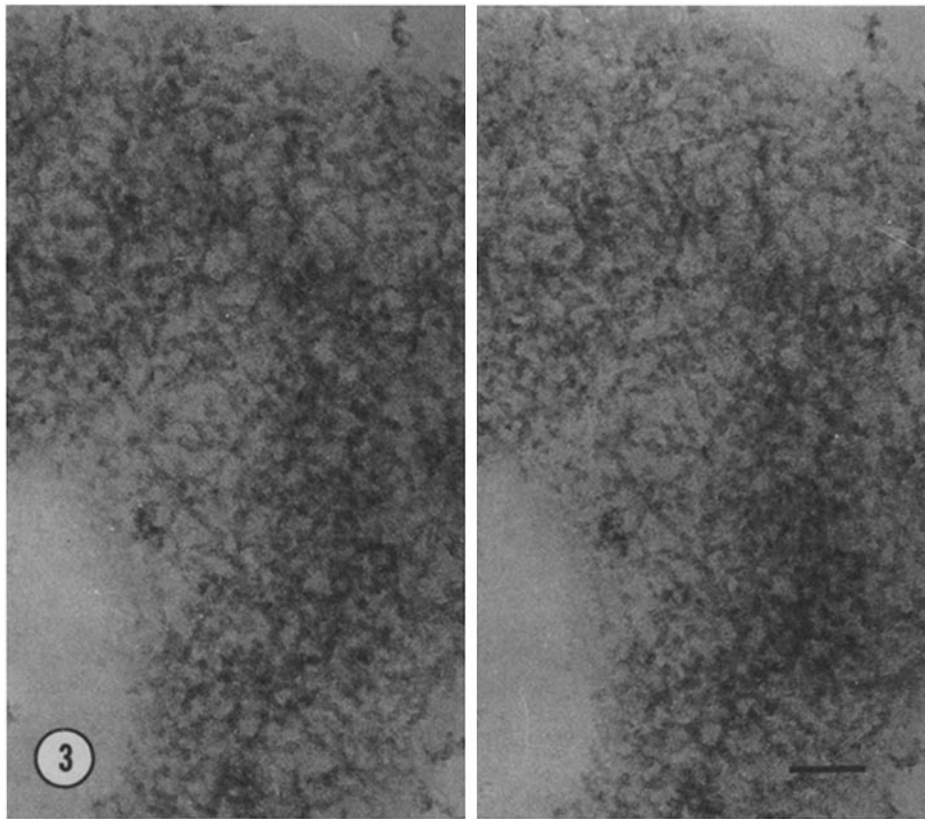


FIGURE 3 Stereo pair of electron micrographs of a tangentially cut erythrocyte membrane. The section was tilted at + and -8° . Bar, $0.1 \mu\text{m}$. $\times 100,000$.

expected to directly observe the *en face* structures of the cytoplasmic surface of the disrupted membranes. The network was clearly observed on the cytoplasmic surface that faced upward on a grid, confirming the observations made on thin sections (Fig. 7). The networks were irregular in pattern and composed of filamentous structures, $8 \pm 2 \text{ nm}$ SD (No. of measurements: 100) in diameter, somewhat finer than those in thin sections. At the vicinity of the edges of such a disrupted membrane, the network was seen to be stretched in one direction so that it appeared as parallel-arranged filaments, similar in diameter to the filamentous components of the meshwork. In HMM treatment of the disrupted membranes, these filamentous components of the meshwork were never decorated with HMM to form arrowhead complexes.

DISCUSSION

In the present report, we have demonstrated that tannic acid fixation is useful for visualizing the

cytoskeletal network underlying the erythrocyte membrane *in situ* in thin-section electron microscopy. Since it was first introduced into electron microscopy by Mizuhira and Futaesaku (15), tannic acid has been widely used to obtain an improved fixation, which permits observations of the ultrastructure of a variety of cells, cell organelles, and protein polymers. Indeed, tannic acid enhances preservation and contrast for many cellular structures by acting as both fixative and mordant (15, 29).

The present result confirms the observations made by other different approaches: the network seen in thin section appears to be equivalent to the submembrane reticulum (8) and Triton shell (24) observed, respectively, by scanning electron microscopy and negative staining. Taken together, it can be concluded that a cytoskeletal network no doubt exists beneath the human erythrocyte membrane. In addition, the visualization of the cytoskeletal network in thin section has the advantage

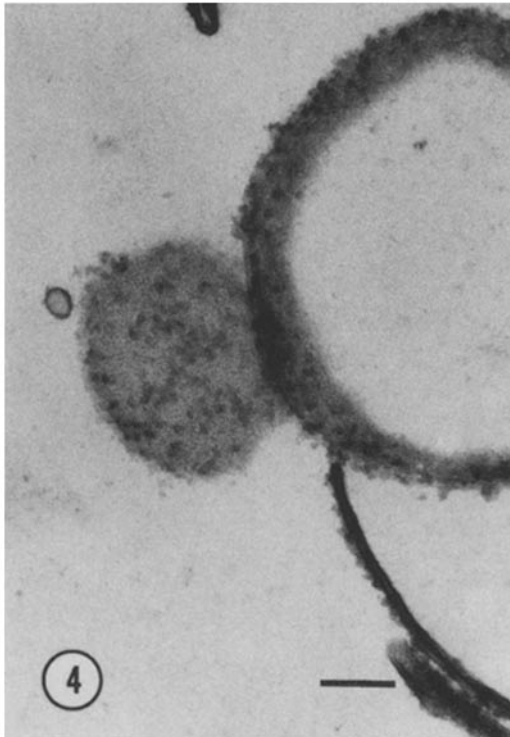


FIGURE 4 Erythrocyte membranes treated with 0.1 mM EDTA (pH 8.0). The membranes are fragmented into small inside-out vesicles, in which the filamentous meshwork has disappeared and only granular components are left behind. Bar, 0.1 μ m. \times 100,000.

of studying the relationship with the membrane proper, and thus of analyzing dynamic aspects of the network during various membrane activities such as change in cell shape and lateral movement of membrane-penetrating proteins.

The observations described here are open to several interpretations. The cytoskeletal network was composed of two layers: a granular layer and a filamentous meshwork. The filamentous meshwork appeared to be connected with the membrane proper through vertical granular components. When spectrin and actin were extracted by EDTA treatment, the horizontally arranged filamentous meshwork disappeared, whereas the granular components were left on the cytoplasmic surface of the fragmented membranes. This suggests that the filamentous meshwork is mainly constructed of spectrin and actin.

Negative staining clearly shows the presence of filamentous structures, which appeared beaded and are 8–12 nm in diameter in the Triton shells

(24) and 8 ± 2 nm in diameter in our single-layered membrane preparations. These values coincide well with those (8–10 nm) of constituent filaments of the meshwork in thin section. In solution, spectrin exists as a heterodimer or as a tetramer of two heterodimers (9, 22, 34). Low-angle shadowing reveals that the heterodimer is an asymmetric flexible molecule \sim 100 nm in length, and that its monomer polypeptides may lie side by side (26, 27). Our observations demonstrated that after tannic acid fixation, purified spectrin showed a relatively uniform thickness of 9 ± 2 nm, similar to that of the filamentous components of the erythrocyte membrane, though the exact length of the filamentous components was not determined *in*

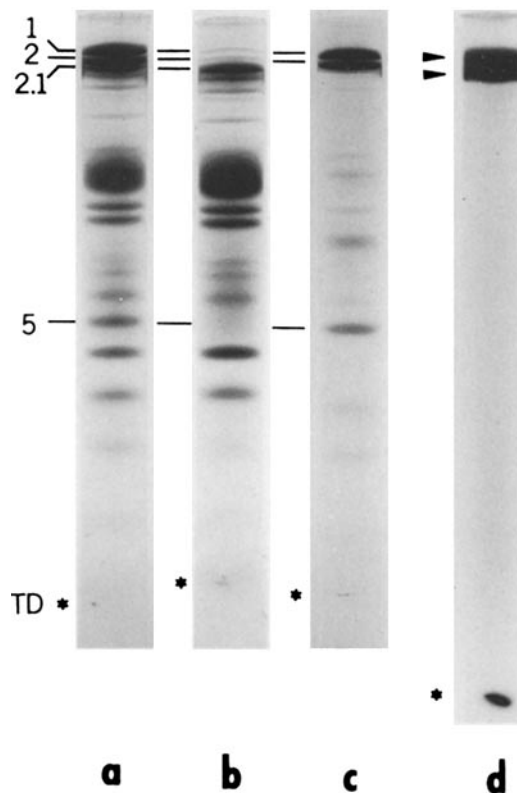


FIGURE 5 SDS polyacrylamide disc gels. (a) Original erythrocyte membrane. (b) EDTA-extracted membrane. (c) Extract of EDTA treatment. The electrophoretic pattern indicates that \sim 91% of the spectrin (bands 1 and 2) and almost all of the actin (band 5) are extracted by this EDTA treatment, whereas virtually all the band-2.1 protein is left unextracted in the membrane. (d) Purified spectrin. Spectrin (arrows) was heavily loaded on the gel for confirming the purity. TD, Final position of tracking dye.

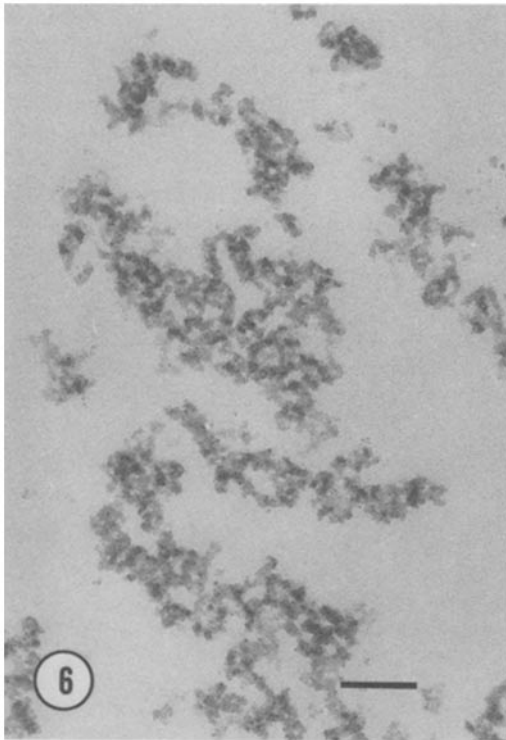


FIGURE 6 Purified spectrin fixed in tannic acid-glutaraldehyde mixture. Spectrin shows a relatively uniform thickness, similar to that of the filamentous components of the erythrocyte membrane, though its unit length could not be determined because of aggregation. Bar, 0.1 μm . $\times 100,000$.

situ. Furthermore, the characteristic wavy configuration of the filamentous components was often recognized as possible repeating units. These morphological similarities lead us to the speculation that the filamentous components themselves represent spectrin.

On the other hand, EDTA treatment of the erythrocyte membrane invariably elutes actin together with spectrin. Since the first demonstration of the presence of actin in the erythrocyte membrane by Tilney and Detmers (33), the association of actin with the cytoskeletal network has often been proposed (20, 25, 36). However, it is not at present clear whether actin plays an essential role in forming the cytoskeletal network (4). We could not find any microfilaments corresponding to actin filaments. Our efforts to decorate the actin with HMM have failed so far in negative staining as well as in thin section (also see reference 33). If actin exists in the nonfilamentous state as often

suggested (20, 33), there may be no way, at present, to visualize it directly by electron microscopy.

We have no direct evidence for identifying the vertical components of the granular layers. However, our observations showed that these vertical components apparently connected the membrane proper and the filamentous meshwork. Furthermore, the vertical components appeared spatially related to some intramembrane densities. It has been demonstrated that the band-3 protein, a major integral protein, has a cytoplasmic projection as visualized by freeze-etch study (see reference 32). It is probable that the vertical component comprises the cytoplasmic projection of the band-3 protein. Recently, the spectrin-binding protein(s) (band 2.1 and its derivatives) has been identified (1, 12, 37), which provides a high-affinity membrane attachment site for spectrin. The band 2.1 is shown to be associated with 10–15% of the total band-3 protein (2). On the other hand, the density of the vertical components in thin sections was roughly estimated to be 1,600–2,300 $/\mu\text{m}^2$, though the value is likely to be underestimated. This value corresponds to 55–75% of the density of the intramembrane particles as seen by freeze-fracture (21), each of which is believed to include the band-3 protein (35). This discrepancy may suggest that the band-2.1 protein is not associated with every individual vertical component. If this is the case, not all the vertical components may be involved in cytoskeletal network formation.

In thin-section electron microscopy, the dense materials underlying the plasma membranes can be observed in a limited number of cell types. Such “undercoating” structures have often been discussed with an emphasis on their possible roles in cell phenomena characteristic of the particular areas of the plasma membranes. Now the cytoskeletal network of the erythrocyte membrane can be included in the same category of the undercoating structures. With improved methods for specimen preparation, similar undercoating structures may be visualized, however thin, in many other cell types.

In conclusion, the erythrocyte membrane is one of few examples that has been extensively studied in terms of molecular organization (for reviews, see references 14 and 31). The *in situ* visibility of the cytoskeletal network may lead to further investigation of the identity of its molecular architecture as well as the direct association between it and the membrane proper. A study along these lines is being conducted in our laboratory.

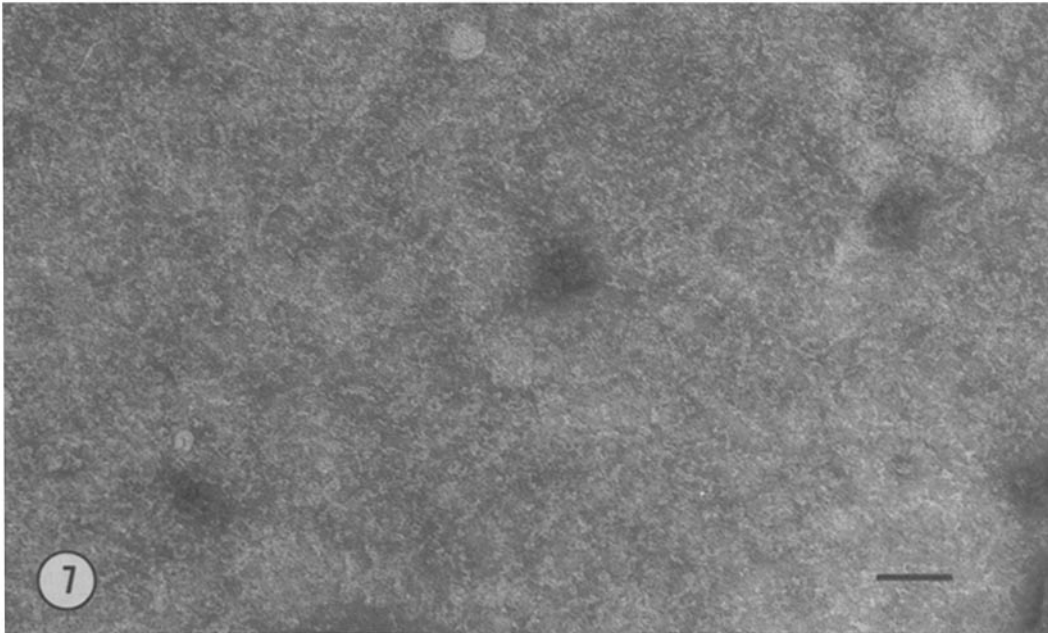


FIGURE 7 Negatively stained erythrocyte membrane. The cytoskeletal network on the cytoplasmic surface is observed *en face* in the single-layered preparation. The network is irregular in pattern and composed of filamentous structures, somewhat thinner than in thin sections. Bar, 0.1 μm . $\times 100,000$.

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