

Organization of the Cytoskeleton in Resting, Discoid Platelets: Preservation of Actin Filaments by a Modified Fixation that Prevents Osmium Damage

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ABSTRACT This study evaluates the structural organization of the cytoskeleton within unactivated, discoid platelets. Previously, such studies have been difficult to interpret because of the ease with which platelets are stimulated, the sensitivity of actin filaments to cell extraction buffers, and the general problem of preserving actin filaments with conventional fixatives, compounded by the density of the cytoplasm in the platelet. In this study we have employed a new fixative containing lysine, which protects actin filaments against damage during fixation and thin-section processing. We used thick (0.25- μm) sections and conventional thin sections of extracted cells (fixed and lysed simultaneously by the addition of 1% Triton X-100 to the initial fixative) as well as thin sections of whole cells to examine three preparations of human platelets: discoid platelets washed by sedimentation; discoid platelets isolated by gel filtration; and circulating platelets collected by dripping blood directly from a vein into fixative. In all of these preparations, long, interwoven actin filaments were observed within the platelet and were particularly concentrated beneath the plasma membrane. These filaments appeared to be linked at irregular intervals to the membrane and to each other via short, ~20- to 50-nm-long cross-links of variable width. Although most filaments were outside the circumferential band of microtubules and the cisternae of the open canalicular system, individual filaments dipped down into the cytoplasm and were found between the microtubules and in association with other membranes. The ease with which single actin filaments can be seen in the dense cytoplasm of the human platelet after lysine/aldehyde fixation suggests the great potential of this new fixative for other cells.

Platelets are extremely dynamic, responding within seconds to mechanical or chemical activation by changing shape (from a disc to a sphere), secreting the contents of storage granules, extending pseudopodia, and aggregating (1–8). Many of these responses are mediated by actin filaments and associated proteins (for review see references 9 and 10). Central to understanding the events of activation, therefore, is a knowledge of the organization of the actin filaments or cytoskeleton of the resting, discoid platelet and a knowledge of the relationships of filaments during and after platelet activation.

Recently, Fox et al. (11) reviewed the actin filament content of unstimulated platelets. They found that in washed platelets, which have the discoid shape of circulating cells and only rare pseudopodia, ~35–40% of the total actin is polymerized as measured by a DNase inhibition assay. With stim-

ulation or activation, this value rapidly increases to ~60–80% (3, 4, 10, 12–15). By the inclusion of cytochalasin D (a drug that inhibits the stimulation- or activation-induced increase in actin polymerization [14, 16]) in all stages of the isolation procedure, it was found that ~35–40% of the actin in these treated cells was polymerized (11). These two observations suggest that circulating platelets contain a similar level of polymerized actin. Although this level of actin polymerization is in agreement with other biochemical data from studies in which activation-induced actin polymerization had been suppressed (5, 14, 16, 17), it disagrees with previous morphological work on isolated and lysed discoid cells (1, 18, 19); results from these latter studies suggest that unstimulated cells contain few if any filaments. One possible explanation for the discrepancies between the biochemical and morphological

studies might be the partial activation of platelets in all of the biochemical studies. However, the removal of the cell's cytoplasm by lysis with extraction buffers, as was done in the morphological studies, can also induce changes in cytoskeletal organization as well as actin filament loss (12–15, 20).

Previously, it has proven extremely difficult to avoid both of these potential problems and to study actin filaments in whole, circulating platelets by conventional morphological techniques. The recognized sensitivity of actin filaments to damage by OsO_4 (21–25) and the cell's dense cytoplasm (which unless removed obscures fine structural features such as actin filaments, particularly single actin filaments) have made this approach impossible. However, Boyles et al. have recently introduced a modified, aldehyde fixation process that protects isolated actin filaments against damage during preparation of conventional, epoxy-embedded material for thin sections (21, 22). In the present report, this fixative has been used to study the cytoskeleton of isolated cells from portions of the same preparations of unstimulated, discoid platelets that were used in an earlier biochemical study by Fox et al. (11). The biochemical data from that study can therefore be directly correlated with the morphological information in this study. We have used the same fixative to extend our observations to the study of platelets fixed directly from the circulation, a technique that avoids any platelet activation that might be caused by the manipulation necessary to remove the cells from the plasma proteins.

MATERIALS AND METHODS

Preparation of Discoid Platelet Suspensions: In a recent study by Fox et al., discoid platelets were isolated from human blood using two different methods (11). In the first method, platelets were filtered at 37°C through a Sepharose 2B column either with or without prostacyclin (PGI_2) present. The 4 mM magnesium chloride included in the original protocol (18) was reduced to 0.4 mM because it caused changes in cell shape and induced clumping. In the second method, platelets were washed by centrifugation and allowed to regain their discoid form at 37°C.

Preparation of Samples for Electron Microscopy: For the fixation of whole cells, three preparations of platelets were used: suspensions of washed platelets were prepared by either of the above methods and then added to a 20-fold excess of fixative; circulating platelets were collected by allowing blood to drip directly from the cubital vein into a large volume of fixative via a 16-gauge needle. All samples were fixed in suspension for 15–90 min at ambient temperatures in 0.2 M glutaraldehyde (10% glass distilled solution; Electron Microscopy Sciences, Fort Washington, PA), 50 mM lysine (Sigma Chemical Co., St. Louis, MO), and 5 mM EGTA in 50 mM sodium cacodylate buffer, pH 7.4. To ensure that calcium-induced changes in filament structure

or associations would not occur during fixation, 5 mM EGTA was added to all fixatives. Cells were sedimented by a 15-min centrifugation in an Eppendorf microfuge, and the fixation was continued for an additional 30 min to 3 h in a solution that contained 0.2 M glutaraldehyde in 50 mM sodium cacodylate buffer, pH 7.4. The cells were washed twice in barbital buffer, pH 7.6, exposed to 0.05 M OsO_4 on ice in the same buffer for 15 min, rinsed three times in ice-cold distilled water, and stained overnight at 4°C in 0.05 M uranyl acetate in water before embedding. The protocol for making and mixing the initial fixative, as well as the procedure for all subsequent steps, was exactly as previously reported for the preservation of isolated actin filaments (21, 22). Thin, dark gray sections were cut, stained with uranyl acetate and Reynold's lead citrate, and photographed. To produce cytoskeletons for examination, suspensions of platelets were lysed and fixed simultaneously at ambient temperatures by the addition of 1% Triton X-100 (wt/vol) to the initial fixative. The resulting extracted cell ghosts were processed in the same manner as whole cells. Both conventional silver sections and thick 0.25- μm sections were cut and photographed at 80 or 120 kV.

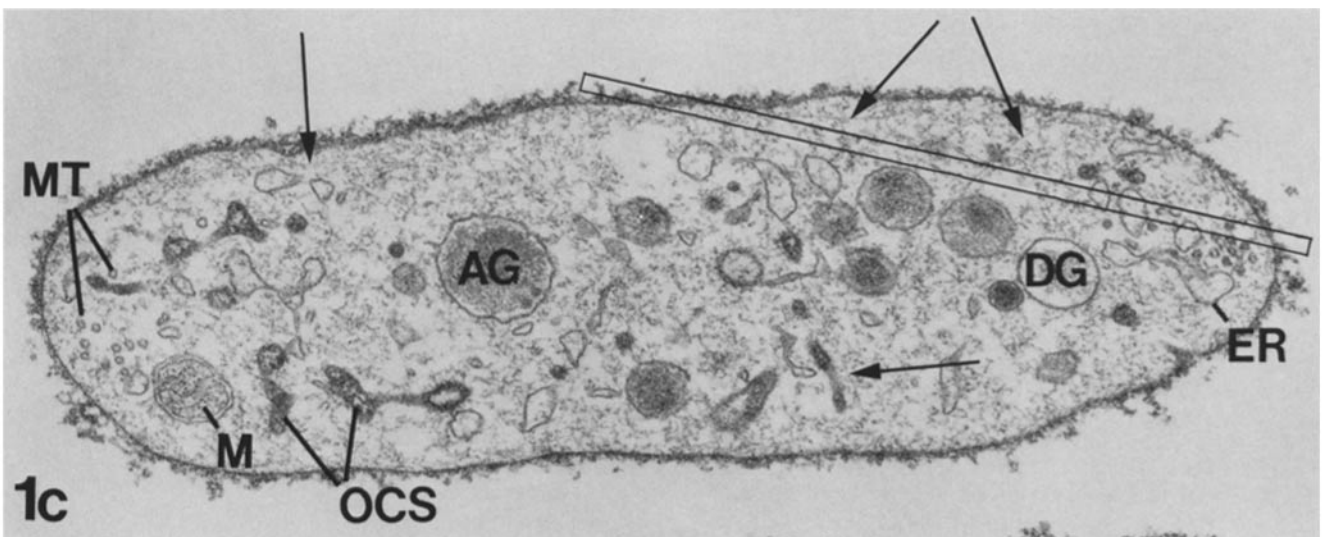
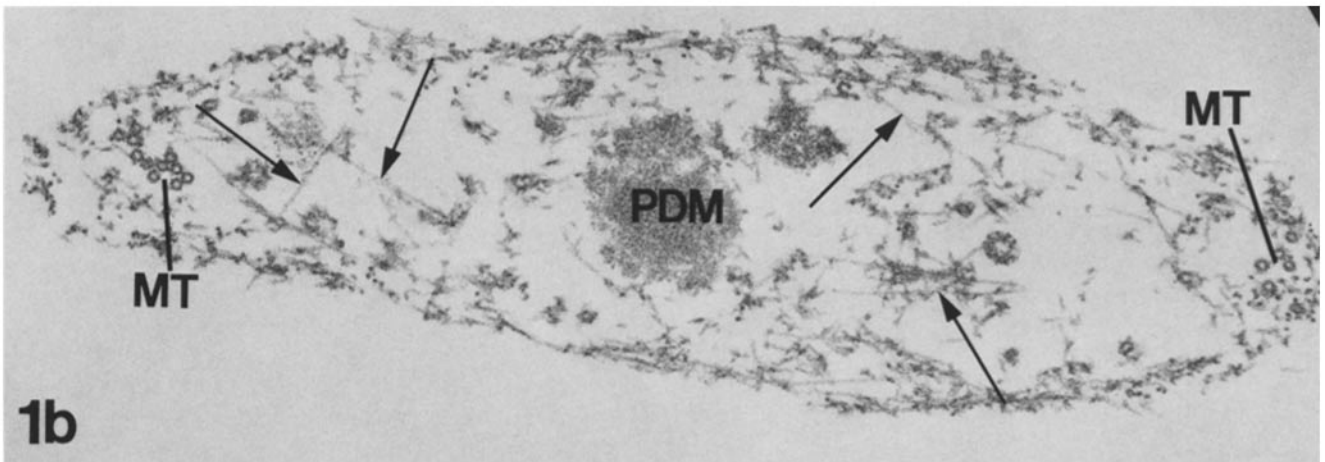
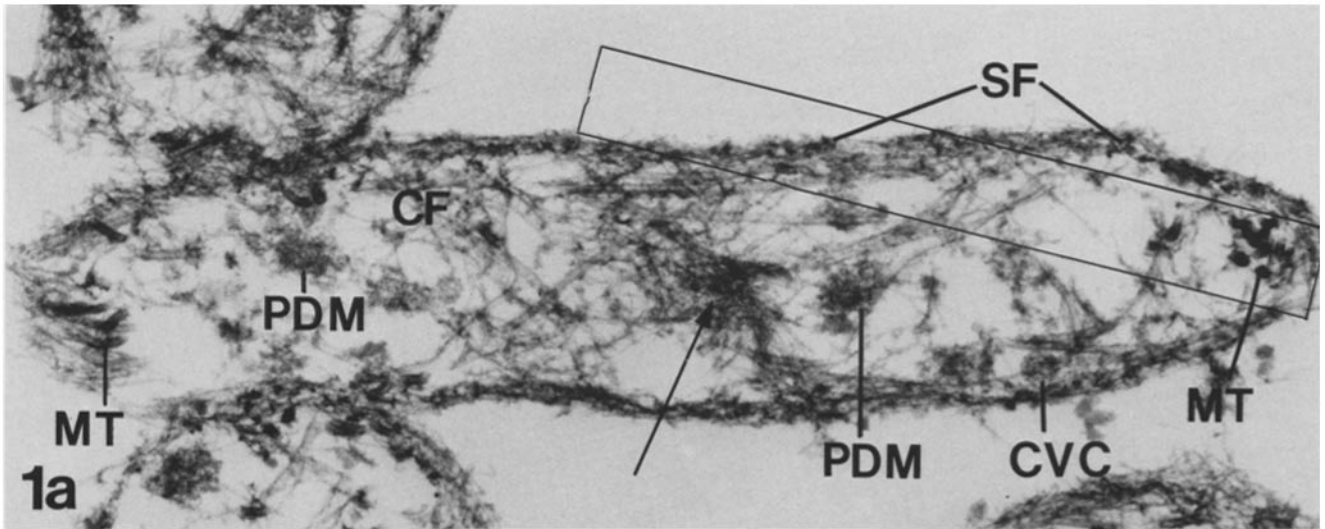
RESULTS

Platelets that are morphologically indistinguishable from those within the circulation can be isolated by a Sepharose 2B column (11). Platelets isolated by centrifugation are less regular in shape. Therefore, cells isolated by the Sepharose 2B column were chosen to establish the form of polymerized actin in the isolated but resting, discoid platelet. We compared column-isolated platelets with platelets fixed directly from the circulation in an attempt to define the cytoskeleton in these cells.

To avoid reorganization of structures and polymerization or loss of actin filaments, which can occur as a result of either the adherence of the platelets to a surface or the prolonged exposure of the cytoskeleton to extraction buffers, we worked with cells fixed in suspension. By adding isolated platelets to a fixative that contained EGTA and 1% Triton X-100, it was possible to lyse the cells in suspension, which simultaneously removed most cellular proteins and fixed the Triton X-100-insoluble residue. In this way, relatively thick sections could be used to evaluate the cytoskeleton without its being obscured by dense cytoplasm. However, solubilization of cytoplasmic components was variable, and remnants of partially solubilized granules, mitochondria, or other material frequently remained.

The cytoskeleton of the circulating platelets could be successfully investigated only in whole cells. Attempts to extract these cells resulted in cell ghosts that were so contaminated by proteins (presumably from the plasma and lysed erythrocytes) that filaments were difficult to identify. In addition,

FIGURE 1 (a) Thick (0.25- μm) epoxy section of a gel-filtered platelet fixed and extracted simultaneously by the addition of 1% Triton X-100 to the fixative. In sections cut across the plane of the discoid platelet, a few relatively long filaments can be seen within the cytosolic (CF) region. Occasionally, these may appear clustered, as in this micrograph (arrow), but this is very rare in gel-filtered platelets and may be an illusion caused by the trapping of partially extracted material. A thick sheath of apparently subplasmalemmal filaments (SF) is seen outside the circumferential band of microtubules. Many of these filaments are not easily identified, however, due to their oblique cut in this plane of the section. CVC, coated vesicle cage; PDM, partially dissolved material; MT, microtubules. The rectangle shows the approximate angle and thickness of a section that is similar to that shown in Fig. 2. (b) In conventional thin sections (silver), few filaments are recognizable in extracted ghosts of gel-filtered platelets (arrows). PDM, partially dissolved material; MT, microtubules. (c) Thin, gray section of a whole platelet fixed directly in blood. Such cells are ~90% smooth contoured, discoid forms with rare pseudopodia. Microfilaments (arrows) are present within the cytosol with a frequency similar to that of equally thin sections of isolated platelet cytoskeletons. The common platelet organelles are present as well: mitochondria (M), alpha-granules (AG), and dense granules (DG), which are pale due to the chelation of minerals by the EGTA present in the fixative. The endoplasmic reticulum (ER) is easily distinguished from the open canalicular system (OCS), which, like the surface of the cell, is darkly stained. Elements of the endoplasmic reticulum are clustered near the circumferential microtubules (MT). The rectangle shows the approximate angle and thickness of a section similar to that shown in Fig. 3. (a) $\times 45,000$; (b) $\times 50,000$; (c) $\times 42,000$.



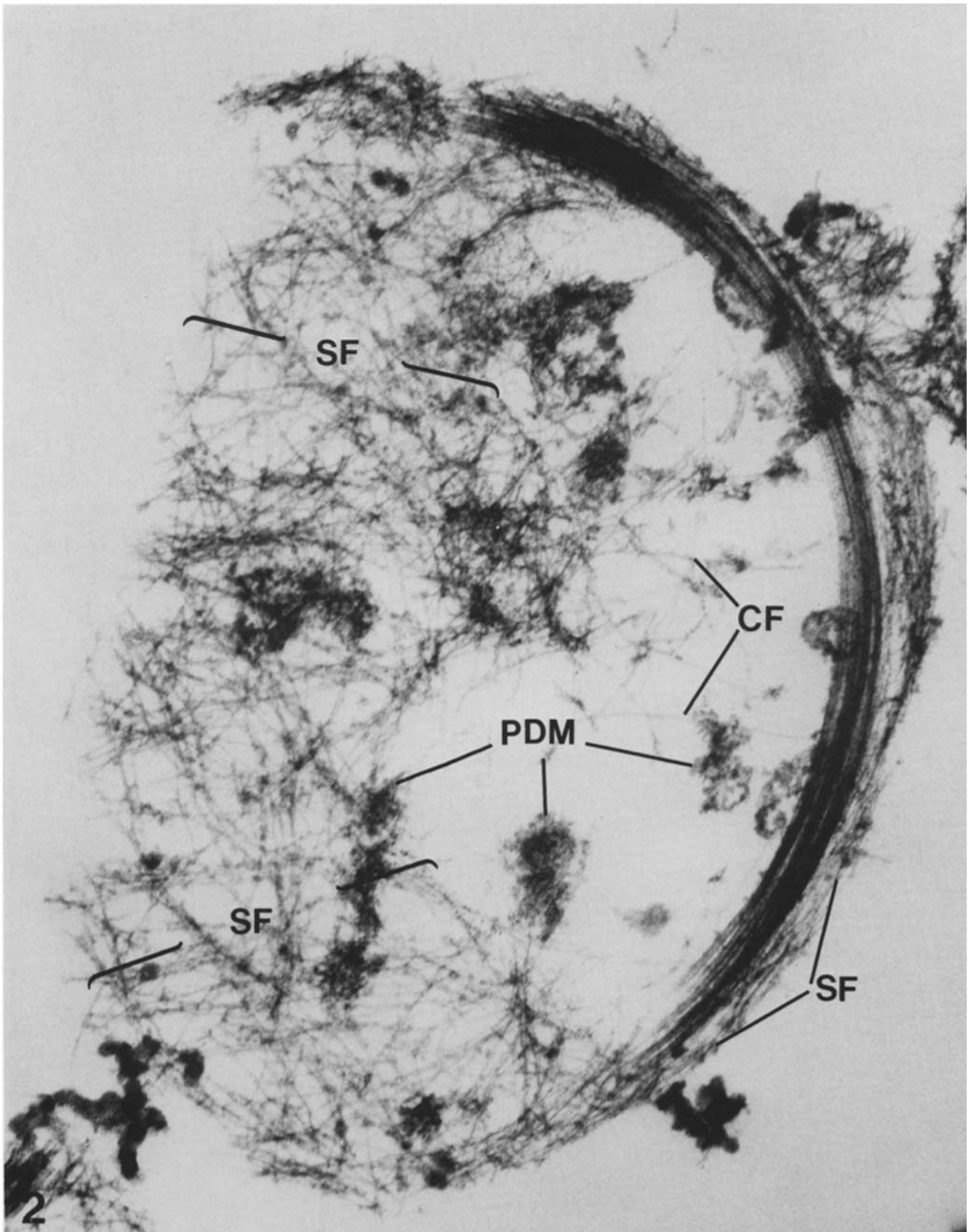


FIGURE 2 In thick sections ($0.25 \mu\text{m}$) cut obliquely across the extracted ghost of a gel-filtered discoid platelet (the rectangle in Fig. 1a shows the thickness and angle of this section), the relative density of cytosolic (CF) and subplasmalemmal (SF) filaments can be evaluated. PDM, partially dissolved material. $\times 63,000$.



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FIGURE 3 Thin, gray section of a whole platelet fixed in blood and cut obliquely (the rectangle in Fig. 1c shows the thickness and angle of this section). Microfilaments associated with the plasma membrane (*SF*) are seen at the perimeter of the cell. *MT*, microtubule; *AG*, alpha-granules; *OCS*, open canalicular system; *ER*, endoplasmic reticulum. $\times 72,000$.

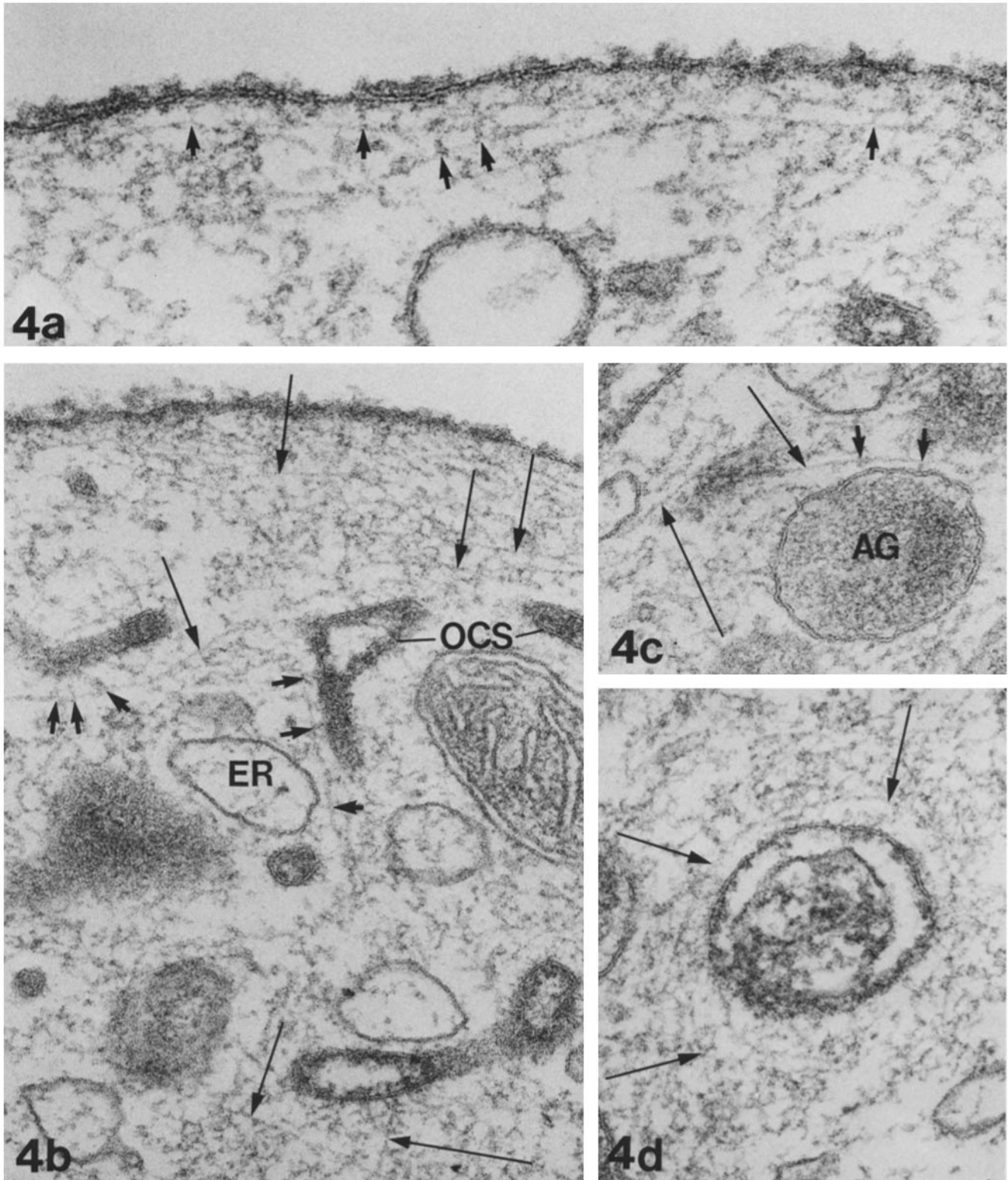


FIGURE 4 Thin sections of platelets fixed in whole blood. (a) The filaments beneath the membrane appear to be connected to it via irregular links (short arrows). Filaments are associated with one another by similar links. (b) Within the cytosol, microfilaments (arrows) can be found near the elements of the open canalicular system (OCS) and endoplasmic reticulum (ER). These filaments may be joined to these membranes via short links (short arrows). (c) Filaments (arrows) may also associate with elements such as the alpha-granules (AG) by short, irregular links (short arrows). (d) Occasionally, filaments (arrows) follow the contour of an organelle's membrane, which suggests that the membrane and the filaments are linked. (a) $\times 200,000$; (b) $\times 130,000$; (c) $\times 130,000$; (d) $\times 130,000$.

exceptionally thin sections were required to visualize filaments in the dense cytosol of the whole platelet.

In thick, 0.25- μm sections of extracted washed platelets, actin filaments were primarily seen in a 50- to 100- μm -thick sheath beneath the plasma membrane, with the ring of circumferential microtubules occurring just interior to this layer of filaments (Fig. 1*a*). In conventional thin sections, the density of filaments appeared substantially less (Fig. 1*b*), which was expected because fewer filaments paralleled the plane of these thinner sections for a great enough distance to be recognizable and fewer filaments were present in the smaller volume of these sections. The still thinner sections of whole circulating platelets displayed a similar small number of filaments (Fig. 1*c*).

As seen in oblique cuts of extracted isolated platelets, the filaments that made up the subplasmalemmal filament sheath were long elements apparently arranged randomly (Fig. 2). It was impossible to measure the entire length of any given filament because of the limited thickness of a section, which did not necessarily include the full length of any filaments. However, single elements $>1\text{-}\mu\text{m}$ long were observed, and elements of 500 nm were common. At least some of the filaments at the plasma membrane extended into the depth of the cytoplasm (Figs. 1 and 2). Filaments within the interior of the platelet, however, were not as densely arrayed as those beneath the plasma membrane. No other filament patterns were observed. Isolated circlets of microtubules, which might be expected if platelets without actin cytoskeletons existed in these preparations, were never found. When occasional filopodia were seen on some discoid cells, the long filaments of these filopodia flared and joined the subplasmalemmal filaments. The basic cytoskeletal pattern remained unchanged, with the filopodial filaments appearing as additional elements.

In whole cells fixed directly from the circulation and cut obliquely to the disc of the platelet, a layer of filaments was also found just beneath the plasma membrane (Fig. 3). These filaments, which appeared to associate with the open canalicular system, as well as with the plasma membrane, usually appeared to be parallel to the membrane and linked to it by short 20- to 50-nm-long structures at irregular intervals (Fig. 4*a*). Filaments also appeared to be laterally linked to one another (Fig. 4*a*). Therefore, many of the filaments in the subplasmalemmal sheath were at some distance from the plasma membrane. Within the cytoplasm, occasional filaments were found near granules and tubules of the canalicular system, as well as near the endoplasmic reticulum (Fig. 4*b-d*); some of these filaments may have had no true association with these organelles, but may, in the crowded interior of the platelet, have been randomly crossing the cytosol. Apparent links between these filaments and the membranes of organelles were seen, however. The concentration of filaments associated with cytosolic membranes was never as great as that seen beneath the plasma membrane. When very thin sections of whole cells from isolated platelet preparations were compared with sections of platelets in blood, they appeared essentially identical. In short, we could detect no differences between the cytoskeletons or the general morphology of the gel-filtered isolated discoid platelets and those of circulating platelets.

In addition to actin filaments, the platelet's cytoskeleton contains microtubules. Associations between filaments and microtubules as well as between microtubules and other mi-

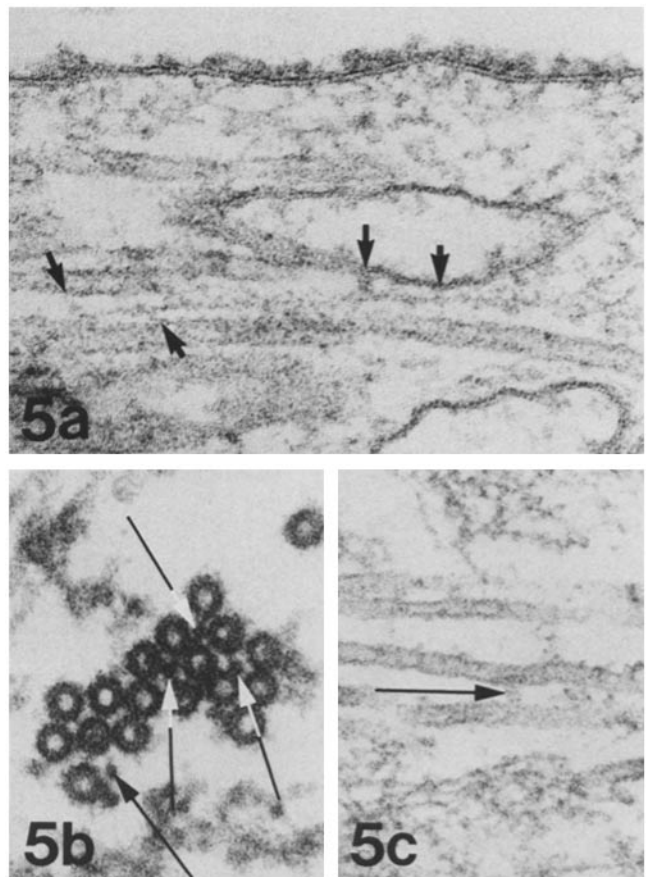


FIGURE 5 (a) In thin sections of whole cells, filaments are found that parallel the microtubules for some distance and that are linked by short arms (arrows) not only to adjacent tubules but also to elements of the endoplasmic reticulum that weave between tubules. (b) In extracted cells, the microtubules collapse into a tight bundle, and dots (arrows) that may represent filaments cut in cross-sections are found. (c) As seen in this micrograph of a whole platelet, direct microtubule-to-microtubule links (arrow) are seen occasionally. (a) $\times 170,000$; (b) $\times 165,000$; (c) $\times 130,000$.

cro-tubules were observed. In whole cells, single actin filaments with links to microtubules were found (Fig. 5*a*). The same filaments often appeared to be linked to elements of the endoplasmic reticulum, which also extended between microtubules. In extracted cells, where the microtubules collapse together, these same filaments appeared in cross-sections as dark dots with arms extending to adjacent microtubules (Fig. 5*b*). In unextracted cells, additional direct tubule-to-tubule links were occasionally observed (Fig. 5*c*).

Because platelets isolated by centrifugation have a collapsed canalicular system (Fig. 6*a*), they were not used to establish the nature of the cytoskeleton of the resting, discoid platelet. The cytoskeletons of these cells were distinctive as well. In thick (0.25- μm) sections of extracted cells, centers of radiating filaments and less defined material could be seen. As many as three or four of these centers may exist in each cell (Fig. 6*b*). Comparison of the cytoskeletons with images of whole cells and with images of poorly extracted cells suggested that these centers were associated with the collapsed open canalicular system. In whole cells, amorphous areas were present along regions of these membranes (Fig. 6*a*).

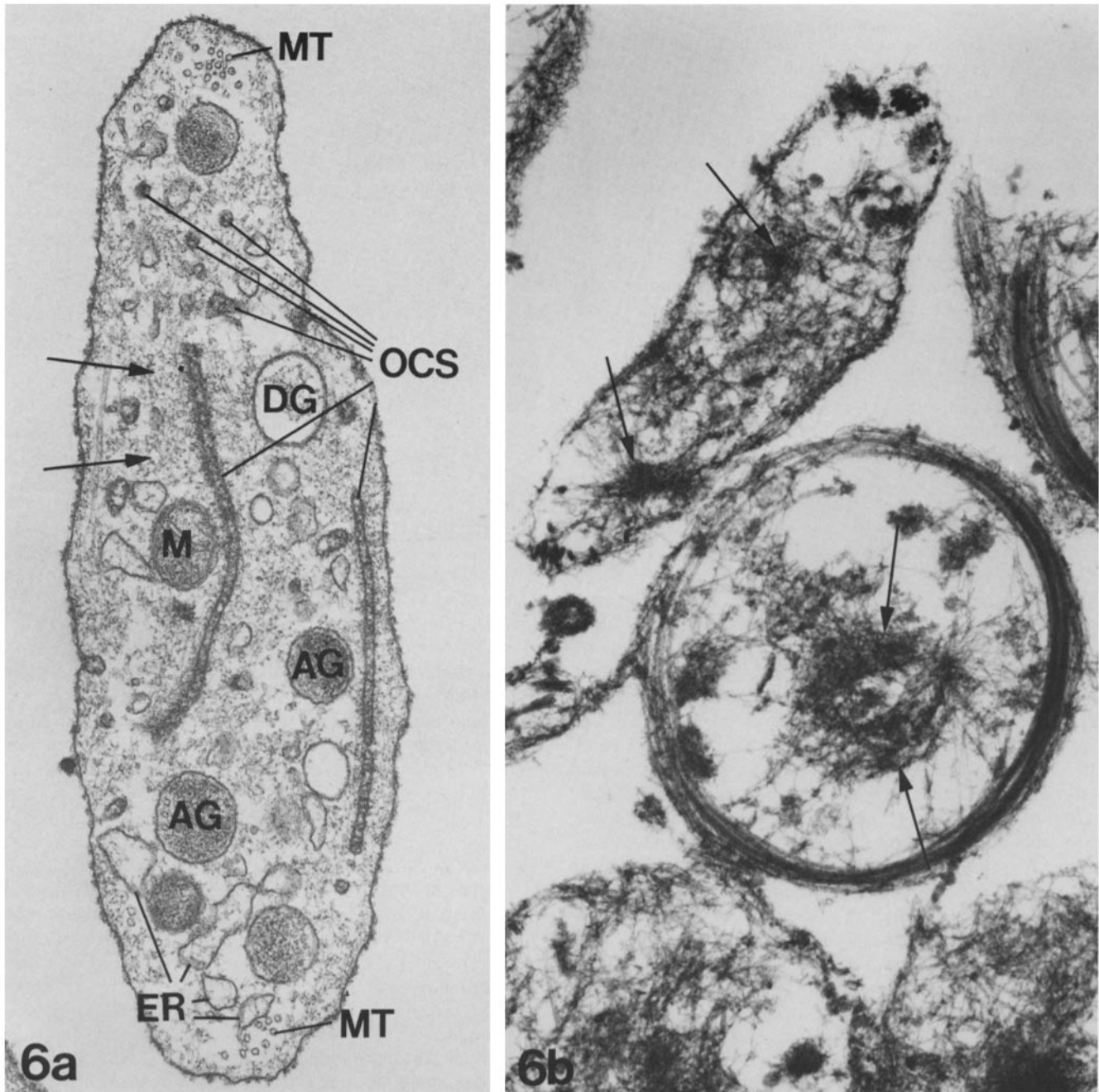


FIGURE 6 (a) Platelets isolated by repeated centrifugation and allowed to rest for 3 h at 37°C in the presence of 1 mM EDTA are more irregular and have an open canalicular system (OCS) that has collapsed to form small tubules and flat sheets. Areas of organelle exclusion and increased density are frequently associated with the open canalicular system (arrows). Because of the presence of EDTA in the fixative, the mineral of the dense granules (DG) has been dissolved. The other cell organelles, mitochondria (M), alpha-granules (AG), endoplasmic reticulum (ER), and microtubules (MT) appear normal. (b) In thick sections (0.25 μm) of the extracted ghosts from these cells, there are numerous clusters of filaments (arrows) and dense material within the interior of the cell. (a) $\times 45,000$; (b) $\times 30,000$.

DISCUSSION

Our results, which demonstrate an extensive actin network in both isolated discoid platelets and circulating platelets, agree with biochemical studies that have found that ~35–40% of the actin is polymerized in unstimulated platelets (11), cytochalasin D-treated platelets (11, 14, 16), and anesthetic-treated cells (17). Because ~15–30% of the protein in platelets consists of actin (26, 27), one would expect ~6–12% of the protein in discoid platelets to be polymerized actin. If polymerized actin,

as variously measured in these biochemical studies, is in fact filamentous, one should observe a relatively large or extensive network of filaments (which is doubled by activation) in the platelet. We have found just such an extensive array of filaments in the unactivated platelet. These are clearly actin filaments, because in the study by Fox et al. (11), which used the same preparations as the present study, actin was the major protein present in the isolated platelet cytoskeletons, as demonstrated by polyacrylamide gel electrophoresis.

Most filaments in the discoid platelet lie beneath the plasma

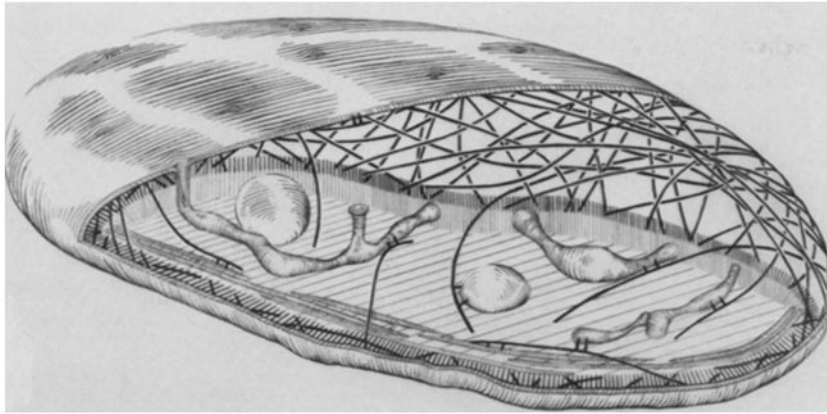


FIGURE 7 An artist's conception of the cytoskeleton of the platelet.

membrane, where they form an open, loose, network of long, interwoven filaments apparently held to the membrane and to each other by short lateral links. Individual filaments of the subplasmalemmal layer leave the surface of the cell and dip down into the cytoplasm, where they form a sparse lacework of filaments throughout the cell and possibly join the opposite membrane surface. Within the cytoplasm, filaments may associate with the tubules of the open canalicular system and endoplasmic reticulum, the cell's numerous granules, and the microtubules at the periphery of the cell. Thus, the entire cell is criss-crossed by long filaments that can act as organizational elements. Fig. 7 demonstrates our current understanding of the cytoskeletal organization of the discoid platelet.

The use of the lysine/glutaraldehyde fixative in this study has made it possible to preserve and visualize long, single actin filaments in the isolated Triton X-100-insoluble cytoskeleton as well as within the dense cytosol of the platelet. Whereas lysine in combination with glutaraldehyde can preserve pure, isolated actin filaments (21), lysine alone or in combination with glutaraldehyde in the buffers used for fixation does not cause filament polymerization from monomeric or g-actin. These characteristics suggest that this fixative or other amine fixatives may be generally useful for the fixation of cellular actin.

Three major obstacles have previously frustrated attempts to define the cytoskeleton of the platelet. First, many extraction buffers, particularly those in which calcium has not been chelated, have been shown in biochemical studies to be deleterious to actin filaments and to the integrity of the cytoskeleton of the platelet (12–15, 20). The unstimulated platelet, with its large stores of immobilized calcium, may be particularly sensitive to the lysis buffer composition. Second, the poor preservation of actin by conventional fixation and thin-section procedures (21–25), coupled with the problems caused by the platelet's dense cytoplasm, has prevented the demonstration of filaments by these techniques in all but activated, whole cells in which filaments tend to bundle together. In the present study, we have carefully avoided the use of extraction buffers with living cells and have, instead, fixed and lysed cells simultaneously or worked with whole cells. In addition, we fixed all preparations in the presence of calcium chelators by a new procedure capable of preserving single purified actin filaments.

A third difficulty has been the sensitivity of the platelet to activation-induced cytoskeletal changes. Therefore, studies of circulating cells dripped directly from an arm vein into fixative

were used to confirm our results from studies of the isolated cells that had been biochemically characterized. These circulating platelets, which showed no signs of activation, represent the normal condition of platelets. Therefore, we do not believe that the extensive filamentous cytoskeleton seen in the present study is the result of cell activation.

The validity of images that show linkages between filaments and between filaments and membranes or tubules is open to question. Associations between filaments are seemingly relatively rare when unstimulated, discoid platelets are extracted with Triton X-100 and the cytoskeletons are fixed after lysis. In addition, few actin filaments from Triton X-100-extracted platelets sediment by low-speed centrifugation, which indicates that the filaments of these cytoskeletons are not cross-linked into networks (11). However, linkages between filaments are frequently seen in whole fixed cells. Some of the filament–filament and filament–membrane linkages seen in unextracted platelets may actually be only artifactual precipitates of cytosolic protein. Alternately, these cross-links may be structures rapidly solubilized by the lysis buffers used for biochemical analyses. Just as the circumferential band of microtubules is lost in these buffers, so may other structures be lost.

The primary location of actin beneath the plasma membrane of the platelet is clearly significant to platelet activation. The presence of these filaments indicates that the plasma membrane is not accessible to granules for fusion, thus supporting those studies that have concluded that exocytosis of granules occurs by fusion with the open canalicular system (28–30). In addition, after these filaments lose their plasma membrane association and are contracted by myosin in the activated or adherent platelet (3, 31–33), they may form the knot of filaments that surrounds the cell's organelles and remnant microtubules (32–36). This supposition is in keeping with results from previous biochemical work that has suggested that the preformed filaments of platelets participate in this structure but not in the formation of pseudopodia (5).

The existence of associations between microtubules and between actin filaments and microtubules suggests that they are linked together in the resting or discoid platelet. These associations may help stabilize the shape of the platelet. The presence of associations between tubules would also explain why they are isolated as circlers from lysed platelet preparations (18, 37). The first morphologically defined event of activation is a loss of discoid shape and the assumption of an irregular round shape. This event precedes new actin filament formation (3). This rapid change may be partially due to the

loss of associations that bind the microtubules together and to their subsequent splaying apart under the initial tension from a contraction of the primarily subplasmalemmal filaments of the cytoskeleton.

Only a careful study of the time course of activation and changes in the cytoskeleton during activation can determine the role of both actin filaments and microtubules in platelet activation. This report should help provide the necessary morphological basis to continue that work.

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