

# Cytoskeleton of Human Platelets at Rest and after Spreading

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**ABSTRACT** The fine structure of resting and activated platelets was compared using two approaches novel to this dense cytoplasm. First, rapid lysis of platelets on carbon-coated grids was followed by negative staining of the "cytoskeletons." Second, a brief, minimal fixation of platelets in plasma was coupled with partial lysis and examination of the unstained whole mounts at 200 kV.

The results showed that the dense ground cytoplasm of discoid, fully resting platelets appeared granular or amorphous, and microfilaments were not observed. A coiled microtubule terminated in one, free, straight end.

When any slight degree of activation occurred, microfilaments could be detected in the platelets. In fully spread specimens, the amorphous character of the resting cytoplasm was strikingly altered into an interconnected network of microfilaments. Stereo views of the whole mounts showed that dense granules, 100-250 nm in diameter, appeared as if suspended in the filament nets.

The results support the view that platelet activation involves a major assembly of microfilaments from amorphous precursors. The change can only be seen convincingly when stringent precautions are taken during preparation because the platelets are very easily activated by thermal or mechanical stimuli.

Changes in the assembly state of microtubules and microfilaments correlate closely with alterations in cell shape in several instances. While studies first centered on microtubules (18), organized nets or bundles of microfilaments also appear from amorphous or disorganized states during cytokinesis (22, 20, 19), neural tube formation (7), acrosomal process formation (25), microvillus elongation after fertilization in marine eggs (2), and 3T3 cell spreading (10). The microfilaments appear at different rates but, in each case, in relation to a specific change in cell shape.

In some cases, change in the organization of cytoplasmic filaments does not require a nucleus. Cytokinesis can proceed after a certain point in mitosis even if the spindle and chromosomes are removed (12). Neither nucleus nor protein synthesis is necessary for cell spreading (21, 10). Blood platelets, enucleated fragments of cytoplasm of the megakaryocyte, can rapidly change from a flat disk to a sphere with filopodia that are micrometers long (3, 5, 27, 29). Alternatively, they can spread on surfaces and form a broad, continuous hyalomere region (1).

Although there is general agreement (3, 27, 29) that resting platelets contain a circular bundle of microtubules which appears to maintain the shape, the state of the actin is still debated

because the cytoplasmic matrix appears dense and structureless in electron micrographs of thin sections. Even in activated platelets where microfilaments are observed in some parts of the thin sections, the filaments are by no means uniformly present even though they are easily seen if washed platelets are osmotically shocked or glycerinated and then negatively stained or sectioned (4, 27, 30). There has therefore been considerable disagreement as to whether the resting platelets may also contain microfilaments that are not seen because of the method of preparation. Furthermore, there may be microfilament breakdown because of the osmium tetroxide fixation (14) used for sectioned material. The issue is unresolved. Behnke and collaborators (4) concluded that there may be an equilibrium between monomeric and polymerized forms of actin in platelets. Zucker-Franklin (30) showed that there were microfilaments in osmotically shocked platelets and suggested that plasma membrane and canaliculi were surrounded by a network of fibrils. White (27, 28) interpreted his sectioned material to show that there are submembranous filaments in resting platelets.

To resolve this problem, new methods of preparation and observation of platelets were needed. In this study, I show that by preparing resting platelets by gentle procedures followed by

either rapid lysis and negative staining, or by brief fixation, partial lysis and study of whole mounts, convincing evidence for the polymerization of microfilaments from amorphous precursors can be found. Submembranous filaments are not observed in resting platelets; only the microtubule coil is seen. A brief report of part of the findings has appeared (17).

## MATERIALS AND METHODS

### Platelet Preparation

Venous blood from healthy human volunteers was allowed to flow freely through a 19-gauge needle and butterfly unit into  $\frac{1}{10}$  vol of 3.8% sodium citrate. The first 2 ml were discarded to reduce tissue fluid contamination. Erythrocytes and leukocytes were removed by centrifugation at 180 g for 10 min at 37°C. For negative staining, 3-5 ml of platelet-rich plasma (PRP)<sup>1</sup> was passed through a column containing 50 ml of Sepharose 2B (23) pre-equilibrated and eluted with calcium-free Tyrode's solution at 37°C. The Tyrode's buffer had the following composition: 0.136 M NaCl, 2 mM KCl, 10 mM Na<sub>2</sub>CO<sub>3</sub>, 0.5 mM NaH<sub>2</sub>PO<sub>4</sub>, 4 mM MgCl<sub>2</sub>, pH 7.4. The peak containing the platelets was well separated from the plasma proteins as determined by cell counts and absorption at 280 nm. The gel-filtered platelets were then allowed to incubate in the Tyrode's buffer at 37°C with added glucose (4.4 mM) without disturbance for 20-30 min; this step was essential to obtain a large number of resting forms. For whole mounts, platelets in the freshly drawn PRP were incubated at 37°C for 20-30 min without disturbance before fixation. All procedures were carried out in plastic containers.

### Negative-stain Procedure

To be sure that I could lyse platelets that were discoid, I pretested the effect of carbon substrates on platelet morphology. One group of gel-filtered platelets was allowed to settle onto a carbon-coated glass slide; a second set was allowed to settle onto glass directly, and a third was fixed before examination. All were compared by oil immersion phase microscopy using Zeiss optics (N.A. 1.3). For this purpose, phase microscopy was quite adequate to distinguish between pure discoid forms and those containing one or more filopodia or activated or irregular forms. The discoid forms could be seen as they turned over in the fluid layer, deliberately kept thick. Counts of 100 platelets scored as disks, disks plus one filopodium, disks plus two and activated forms were reproducible to 5-10%. While many of the platelets on the glass surface became activated, those on the carbon-coated substrates remained discoid for at least 10-15 min, the time of observation. They were not significantly different from the platelets fixed in 2.0% glutaraldehyde in Tyrode's solution before examination. In general, 65-70% were pure disks and another 20% were disks with one filopodium. Carbon substrates were concluded to be relatively inert. For negative staining, the exact procedure was as follows: ten lambda of resting gel-filtered platelets at 37°C were gently deposited on a carbon-coated support grid using a cut-off plastic tip on a pipette. After 30 s, the grid was rinsed with two drops of calcium-free Tyrode's buffer also at 37°C. Three successive drops of 1% Triton X-100 (room temperature) in buffer B were then added followed by three drops of the buffer B alone containing 0.1 M KCl, 0.01 M imidazole, pH 7.0, and then three drops of 0.02% cytochrome c in 0.1% amyloalcohol (sometimes omitted) and three drops of 1% uranyl acetate (pH 4.5) which was rapidly removed with filter paper. For surface-spread platelets, polylysine-coated grids were used and platelets allowed to adhere for 20-30 s before lysis. The negatively stained platelets were examined in a Siemens Elmiskop 1 at 80 kV and at 10,000-20,000 initial magnifications. When counts were made of microtubule coils, 50-100 platelets were counted sequentially and negatives taken of every fifth platelet. To test the effect of calcium on the stability of the microtubule coil, discoid platelets were lysed as above and then rinsed with two drops of either 1 mM EGTA or 10<sup>-4</sup> M calcium chloride, both in buffer B, for 30 s, followed by the remaining procedure as described above.

### Whole-mount Preparation

For the whole mounts, resting platelets in PRP at 37°C were briefly fixed with 0.1% formaldehyde, freshly prepared from paraformaldehyde. This was the lowest concentration found to prevent loss of turbidity in a platelet suspension treated subsequently with Triton X-100 while monitored in a Gilford spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, Ohio) at 350 nm. In

practice, one-hundredth volume of a 10% solution of fresh formaldehyde was added to the plasma with rapid swirling. The platelets were then at once allowed to settle for 45-60 s onto polylysine-coated carbon grids. They were then treated with a single drop of 1% Triton X-100 in buffer B, rinsed with buffer B, dehydrated in ethanol, critically point dried (6) in a DCP-1 (Denton Vacuum Inc., Cherry Hill, N. J.) apparatus, and viewed in a JEOL JEM 200 A electron microscope at 200 kV. For activated platelets, a drop of PRP was allowed to spread onto polylysine-coated carbon-substrated grids for 2 or 45-60 s before treatment with the same fixation, lysis, and critical-point drying. For stereo pairs (Fig. 12), a 6° tilt was used at  $\times 20,000$ . No stain was used on these preparations.

The polylysine preparation of the grids was as follows: a drop of the polymer, 10,000-30,000 mol wt (Sigma Chemical Co., St. Louis, Mo.), was added to the grid and allowed to sit for 2 min; the grid was then rinsed with water and dried in a dust-free container. The use of polylysine was essential to keep the briefly fixed platelets adherent to the grid during the rest of the procedure.

## RESULTS

### Negative staining.

**RESTING PLATELETS:** When I used the gel filtration method strictly as described, I obtained 65-70% (and sometimes more) of resting platelets as defined by flat disk shape and absence of any filopodia. Usually, the remaining platelets were also discoid and with 1 or 2 filopodia. Maintaining the temperature at 37°C was critical. There was an increase of ~30% of platelets with filopodia when isolated at 20°C. The rest period was also essential to reduce the number of platelets with filopodia. In contrast, the best washing methods using centrifugation always resulted in a higher percentage of activated platelets. The ground cytoplasm of resting platelets prepared by this favorable procedure was amorphous or granular in character, and microfilaments were not seen. The main difficulty was the density of the cytoplasm. Many platelets had to be examined to find some in which the interior was electron-transparent enough to see detail (Fig. 1). Fig. 2 shows a higher magnification view of the same platelet: note that the microtubule coil is breaking up into apparent microfilaments. The interior (arrows) shows a wavy or irregular substructure. The very dense regions in the center probably represent dense granules; compare with Figs. 10 and 11. Fig. 3 shows another example of a platelet in which the ground cytoplasm, emerging from the coil area, is thinned out; the amorphous character is especially clear.

The microtubule coil can be seen in Fig. 1 to possess a single, straight, free end (see also Fig. 4). Rarely, a second end could be seen in the platelet cytoplasm, but never more.

**PLATELETS AFTER SPREADING:** Fig. 4 shows a very briefly spread platelet. The microtubule coil can here be followed for several turns, while microfilaments are now visible in the interior (printed to show the coil). When platelets were allowed to spread more fully, the ground cytoplasm consisted entirely of a delicate network of interlacing fibrils (Fig. 5). The interior network is shown at higher magnification in Fig. 6. The periphery (Fig. 7) appears to be made up of a fine, closely packed network intermediate in character between the ground cytoplasm of Figs. 1-3 and the fully formed interior nets.

### Whole Mounts

**RESTING PLATELETS:** The interior of discoid platelets appears to be made up of dense globules of two size classes: one group 20-30 nm wide, the second 100-300 nm wide (Fig. 8). The curved density around the periphery probably represents the microtubule coil.

**SPREADING PLATELETS:** As platelets begin to spread, the granular matrix alters into a reticulum with short projections of filaments around the periphery (see *R*, Fig. 9). After spread-

<sup>1</sup> The plasma after erythrocytes and leukocytes have been centrifuged down at a low speed not sufficient to bring down platelets.

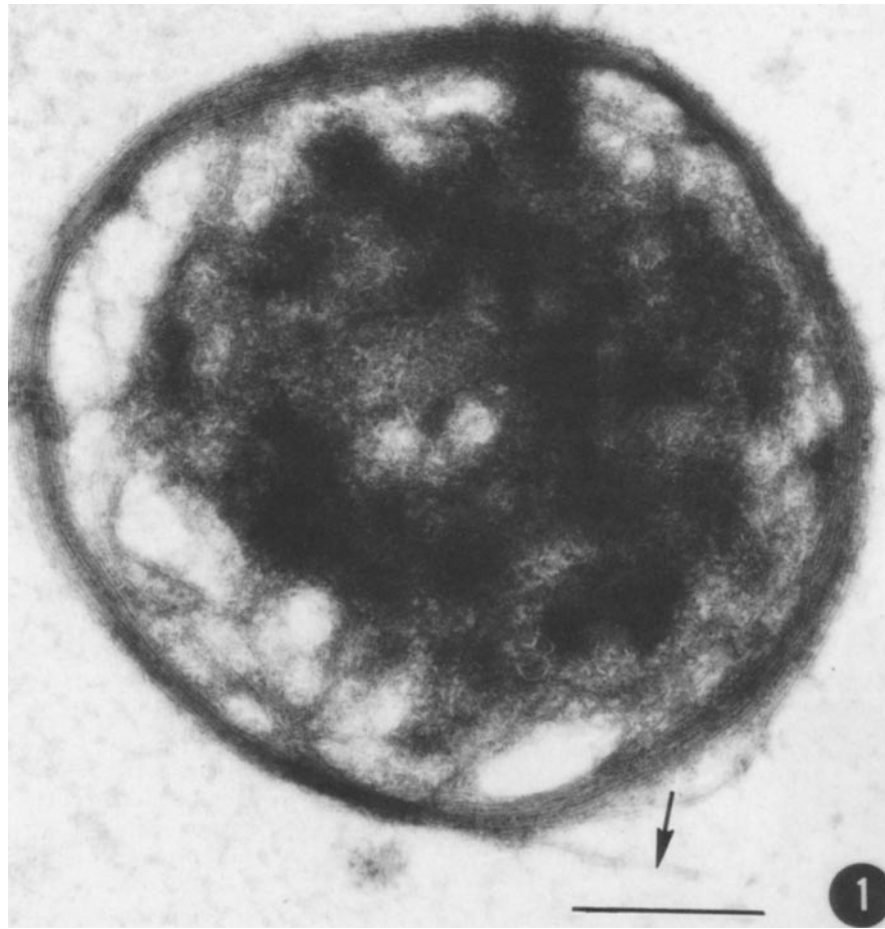


FIGURE 1 Resting, discoid platelet lysed with 1% Triton X-100 on carbon-coated grid as described and negatively stained with 1% uranyl acetate. One end of the microtubule coil is visible (arrow). The center of the platelet contains granular material and discrete regions of higher density. Compare this figure with the whole mount shown in Fig. 10. Bar, 1  $\mu\text{m}$ . X 25,000.

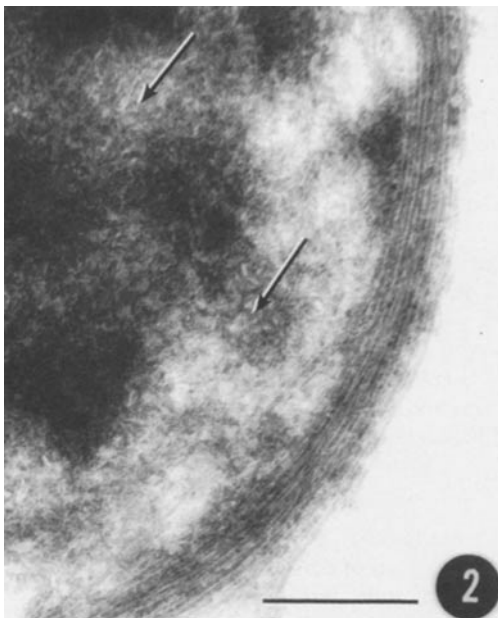


FIGURE 2 A higher magnification of the right half of the platelet shown in Fig. 1. Note the irregular nature of the ground cytoplasm (arrows) and compare with the spread platelets of Fig. 5. Bar, 0.5  $\mu\text{m}$ . X 42,500.

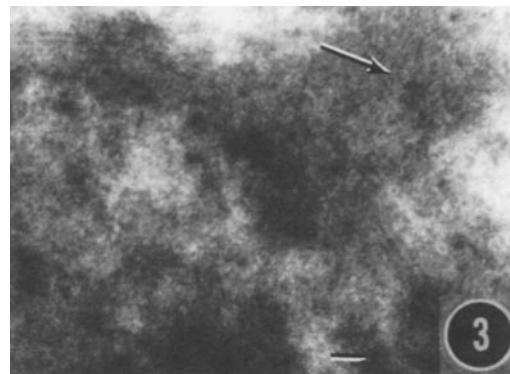


FIGURE 3 Detail of the periphery of another resting, discoid platelet. A part of the curved microtubule can be seen with amorphous ground cytoplasm (arrow) lying over and peripheral to it. Bar, 0.1  $\mu\text{m}$ . X 50,000.

ing for 30 s, the extended network or reticulum is well developed (Fig. 10). Dense granules, 150-250 nm wide, seem to be attached to the reticulum. The microtubule coil in some specimens develops angularities (Fig. 10).

In stereo views of spread platelets, the granules (*G*) appear to be suspended in the reticulum of filaments (Fig. 11). The microtubule coil projects above the surface of the grid with three visible turns at different levels (lower left, arrow).

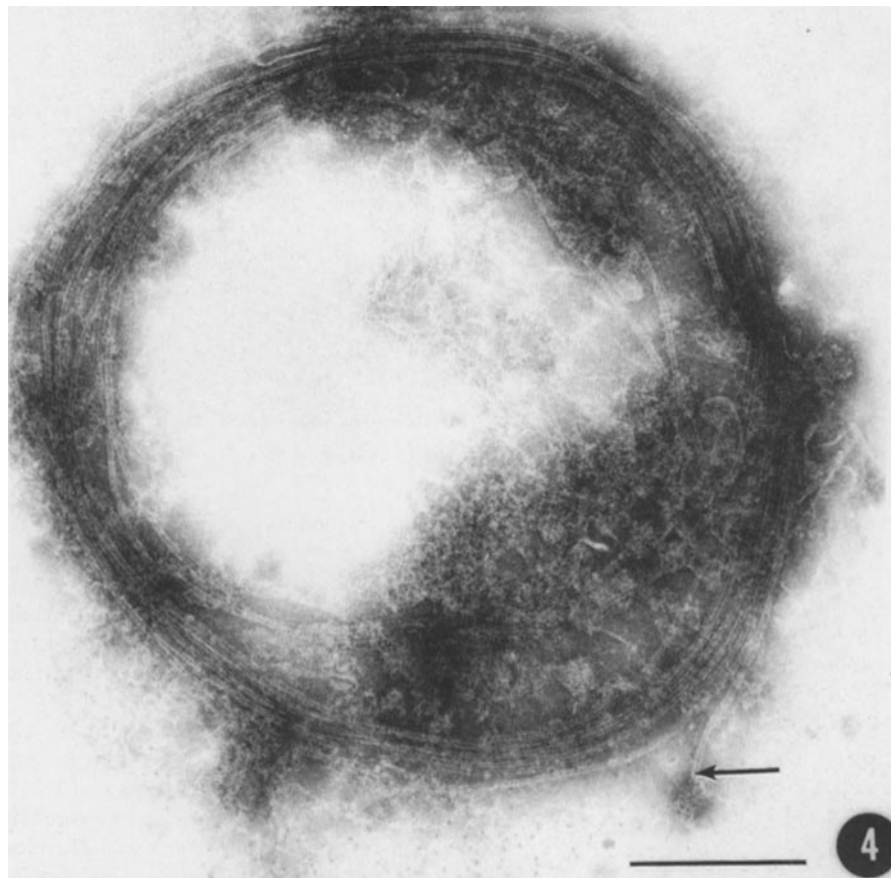


FIGURE 4 Platelet allowed to spread for a few seconds on a polylysine-coated grid. The microtubule coil has speared but has not broken down into component filaments. Note the free end (arrow). There are microfilaments present in the interior. This platelet was lysed with Triton X-100 containing  $10^{-4}$  M calcium and rinsed with the same buffer for 10 s. Bar,  $1 \mu\text{m}$ . X 24,000.

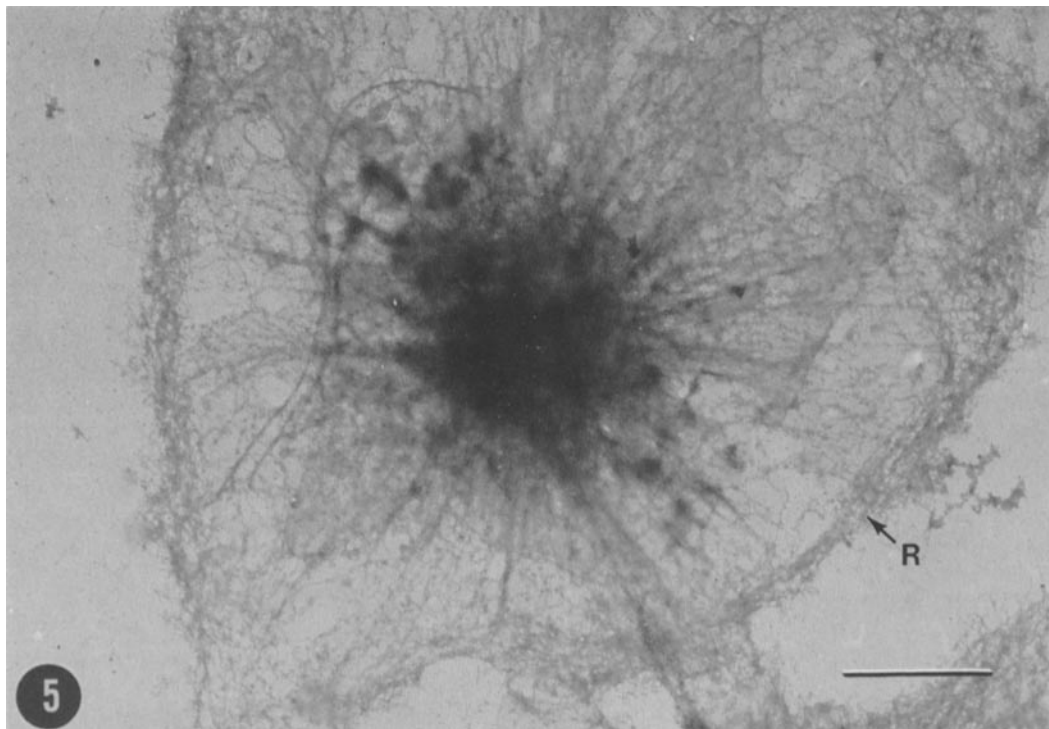


FIGURE 5 Platelets treated with  $10 \mu\text{m}$  ADP, then allowed to spread on polylysine-coated grid for a minute before lysis. Note the netlike arrangement or reticulum in the body of the platelet, and the thicker edge (R). Bar,  $1 \mu\text{m}$ . X 20,000.

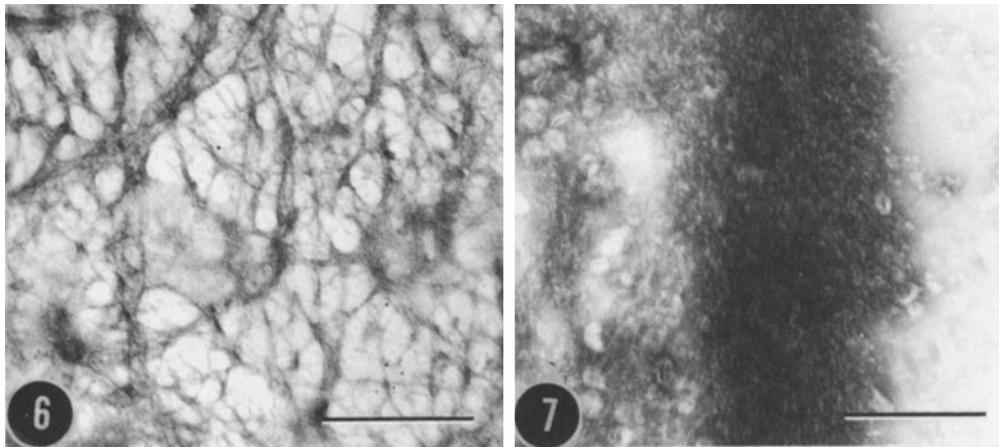


FIGURE 6 Higher magnification of a spread platelet. The strands of filaments making up the network are thicker and more separate than in the periphery. Bar, 0.5  $\mu\text{m}$ . X 40,000.

FIGURE 7 Detail of the border of a partly spread platelet. At this magnification it appears to be composed of a fine meshwork. Bar, 0.5  $\mu\text{m}$ . X 38,000.

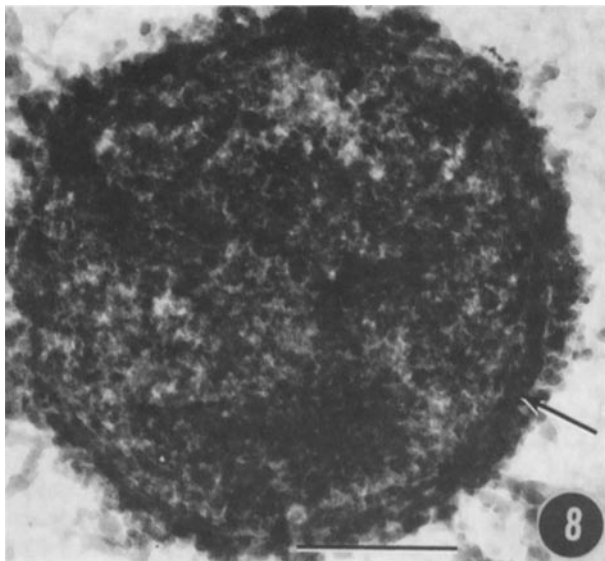


FIGURE 8 Resting platelet fixed in plasma, allowed to adhere to the grid, lysed with 1% Triton X-100, and critically point dried. No stain was used. Interior of platelet appears to be composed of globules; a circumferential rim (arrow) represents microtubule coil. Note the great difference in size of platelets; compare this with Fig. 2. Bar, 0.5  $\mu\text{m}$ . X 41,000.

## DISCUSSION

### *Ground Cytoplasm*

The problem of determining the state of microfilaments in cells treated with fixatives has recently been emphasized, and it is clear that it is difficult to fix pure actin with osmium tetroxide without causing some breakage (14). The microfilaments in platelets are composed at least in part of actin, because they decorate with heavy meromyosin (HMM) (15, 16). In the case of the resting platelets, the situation is even more difficult because of the great density of the cytoplasm. Sections of platelets are therefore poor material to use for study of the state of the actin. By lysing platelets without any fixation, or with only brief formaldehyde fixation, one can approach the problem in a different way and, by removing the membrane, obtain

enough reduction in density in some cases to sample the platelet interior. But what are the possible artifacts of these procedures and could they cause the disappearance of preexisting filaments?

In the negative-stain procedure, the cells are lysed, contrasted (here at low pH), and dried, with tremendous flattening caused by the surface tension, within 10 s. This is a short time compared to the time for surface spreading (45-50 s). The lysis itself is almost instantaneous. Therefore, although internal changes during processing may occur, a complete conversion of F- to G-actin during or after the cell is opened is unlikely. In addition, the consistent differences between the ground cytoplasm of resting platelets and that of activated platelets prepared in exactly the same way strengthen the conclusion that the results are not caused by random variations. Indeed, the more gently and carefully the resting platelets were prepared, the more frequently were large numbers of platelets observed to contain only the amorphous material shown in Figs. 1-3. Nevertheless, even with the best treatment, some platelets contained microfilaments. It may be that a continuous alteration between filaments and amorphous material occurs in resting platelets or it may be that some degree of activation has occurred despite all precautions. Some platelets completely lack any microfilaments. An array of submembranous microfilaments (described in osmotically shocked platelets) are not seen in the resting platelets. Finally, there is no reason to think that the flattening that occurs during negative staining could collapse microfilaments: both single actin filaments and filaments within platelets are well preserved. The conclusion that the amorphous material represents a real precursor seems inescapable.

The evidence from the whole-mount approach supports this conclusion. Here, the method involves prior fixation so that selective removal or disassembly of filaments is unlikely. The three-dimensional structures seen in stereo views give added confidence that the preservation is good. Here, too, the differences between resting and activated platelets are consistent.

The contents of resting platelets in Figs. 1-3 can be resolved into irregular subunits 20-30 nm in diameter. In the whole mounts, round densities of  $\sim 10$ -30 nm are seen. This agreement is encouraging, considering how different the preparations are. A complex of actin and a small molecule named profilin has

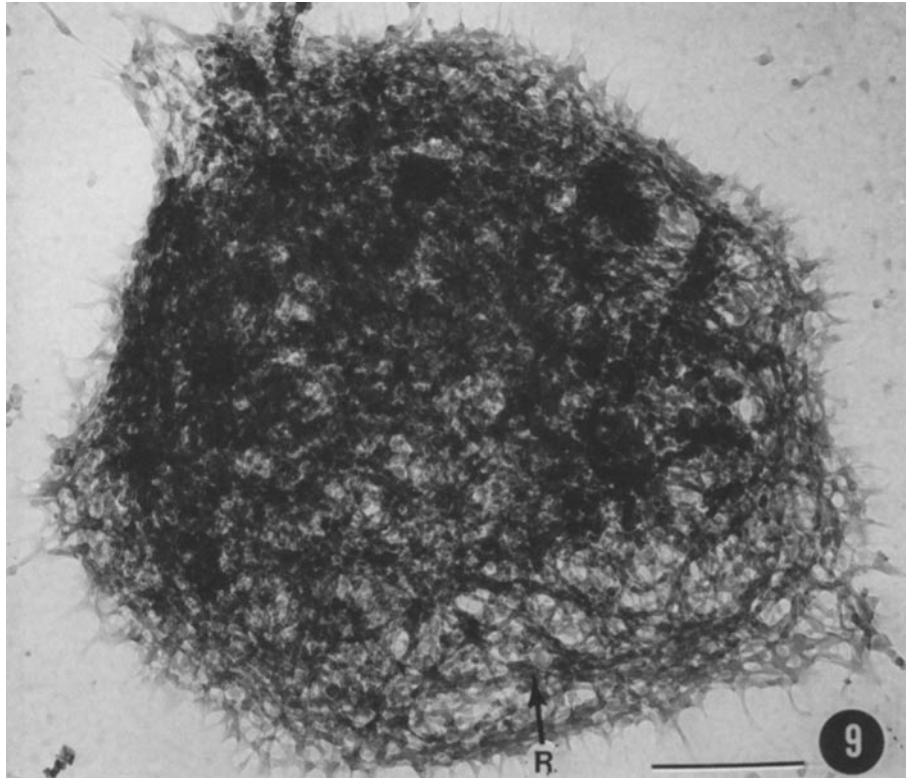


FIGURE 9 Platelet fixed as in Fig. 8 after 6 s on a polylysine grid; lysed after another 5 s. One side (see R, arrow) appears to be developing a netlike arrangement. Note also the presence of large, dense globules in the upper third, ~200 nm in diameter. Bar, 0.5  $\mu\text{m}$ . X 40,000.

been isolated from platelets (13, 11). This complex should be considerably smaller than 30 nm, as it would be little larger than the actin monomer itself, i.e., ~6 nm. Partially purified profilactin prepared by the methods of Harris and Weeds (11) consisted of subunits much finer than those seen in Figs. 2 and 3 (Gonnella and Nachmias, unpublished observations). It is important also to point out that it is unlikely that we are seeing residual glycogen. Although glycogen is present in platelets and is about the right size, it is very soluble and also localized in discrete clumps (27). Furthermore, the amorphous material disappears on activation while glycogen should remain. At present, I can only conclude that nonfilamentous precursor material is present and seems to be in the form of complexes larger than profilactin which are also not extremely soluble. It has recently been shown that thrombin stimulation causes an increase in the amount of filamentous actin in platelets as measured by the DNase I technique (8). This is in agreement with my observations of fine structure.

### Microtubule Coil

It is not possible to follow the coil in platelets for more than a few turns even in the clearest preparations (Fig. 4, reference 16). Typically, one (Figs. 1 and 4) and rarely two ends of the coil are seen. The conclusion that the structure is composed of a single long coil is the most likely explanation of this finding. Behnke (3) came to a similar conclusion after studying shadowed preparations.

### Nature of the Changes in Activated Platelets

The changes in the platelet cytoplasm are similar to those in other systems (24, 2, 25, 9), especially the coelomocyte petaloid-

filopodia change and the acrosomal reaction, although they differ from both. Platelets can either spread on surfaces, as here, and form an extensive microfilament network or, after ADP activation, form long filopodia containing actin bundles (3, 26, 27, 29, 15, 16). In a recent work, Allen et al. (1) showed that platelets initially spread on siliconized surfaces by extending filopodia from the disk. Broad hyalomere regions then develop between the filopodia. This is a mirror image of the change in coelomocytes (9) where the petaloid form transforms into the filopodial form. The initial change from the disk form also resembles the irreversible acrosomal reaction (25), but platelets are able to retract filopodia and subsequently reextend them (1, 16). Platelets seem to be partially reversible systems that can change from a nonfilamentous ground cytoplasm to bundles (filopodia) or nets (spreading) or from bundles to nets. It should be noted that the microfilaments described here have been decorated with HMM under different conditions in several studies (4, 31, 15, 16). The location of myosin in the spread platelets is unknown, as distinctive filaments are not resolved.

### Stimulus

Experiments designed to induce change in platelet shape by an alteration in pH or ions as reported in other systems (25, 2) using methylamine, ammonium chloride, or nigericin were all negative. It is easy to induce shape change with the calcium ionophore A-23187 even in 10 mM EGTA. Therefore, experiments were also run to see if assembly of nets could be caused, or microtubule coils disassembled, by calcium ( $10^{-5}$  to  $10^{-4}$  M) in the buffers used to rinse resting "cytoskeletons." The results were inconclusive. Platelets rounded by lidocaine with disassembly of microfilament nets (15) were also gel-filtered in



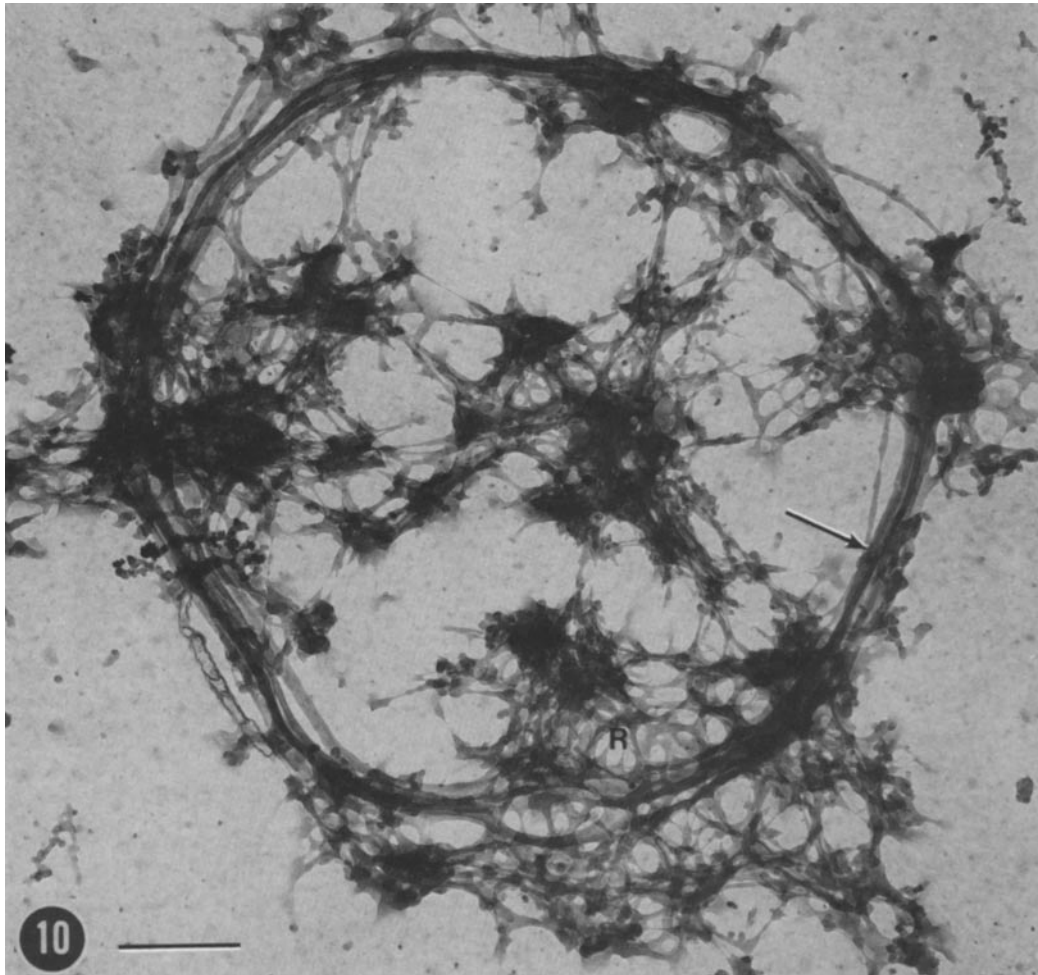


FIGURE 10 Platelet allowed to settle onto a polylysine-coated grid for 45 s before brief, minimal fixation and lysis. This typical view shows a circumferential density that represents the microtubule coil (arrow). The coil appears to be developing angles at intervals. Note strands of the reticulum (R) and dense granules 150-250 nm in diameter. Bar, 0.5  $\mu\text{m}$ . X 32,000.

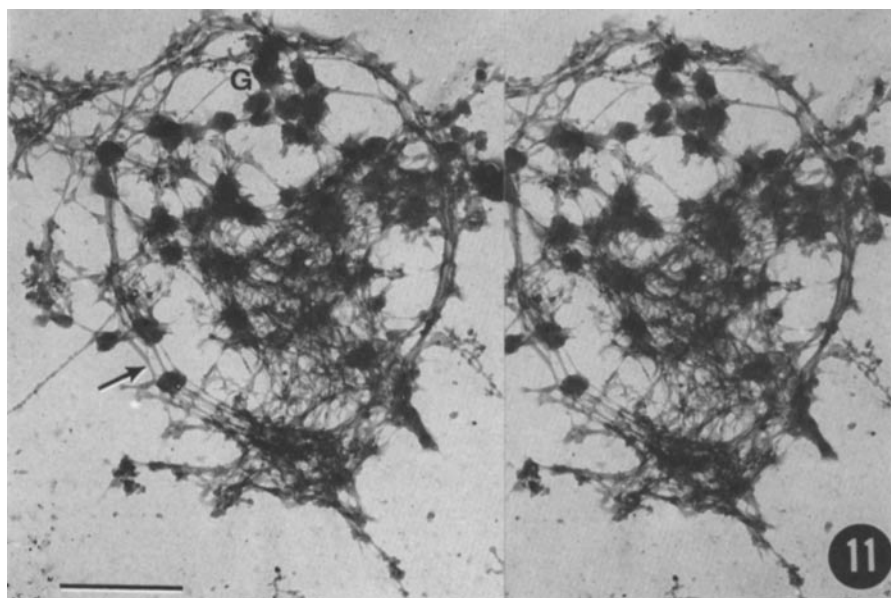


FIGURE 11 Stereo view of platelet: a second example of platelet prepared as in Fig. 10. The pair were taken at  $\pm 6^\circ$ . Using a table stereo viewer, it can be observed that the coils of the microtubule project above the surface (arrow) and that the dense granules (G) appear as if suspended in the net or reticulum of filaments. Bar, 1  $\mu\text{m}$ . X 16,000.

Tyrode buffer containing potassium instead of sodium; return of filopodia was indistinguishable from that of platelets gel-filtered in sodium buffer. At present, the intracellular stimulus for change remains elusive. However, my results do show clearly that major microfilament assembly can be largely uncoupled from the disassembly of microtubule coils (Figs. 4, 10, and 11). Therefore, there are either two different internal signals or two separable thresholds for these processes.

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Note Added in Proof: Phillips and colleagues recently reported (*J. Cell Biol.*, 1980, 86:77-86) that the cytoskeleton of washed platelets was filamentous. It is likely that the washing partially activated the platelets.

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