Microfibrils of Blood Platelets: Their Relationship to Microtubules and the Contractile Protein

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ABSTRACT Human blood platelets were subjected to osmotic shock, brief sonication, pressure homogenization, or treatment with adenosine diphosphate (ADP). These procedures demonstrated an abundance of cytoplasmic microfibrils. The fibrils resembled those found on electron microscopy of partially purified thrombosthenin, the actomyosin-like protein isolated from platelets, and they also appeared to resemble the myofilaments of smooth muscle. Similar fibrils were not found in leukocytes studied under identical conditions. Treatment with colchicine (2×10^{-5} mole/liter) resulted in the disappearance of microtubules but did not affect the morphology of the microfibrils or interfere with platelet-dependent clot retraction. Thus, microfibrils rather than microtubules may represent the morphologic counterpart of the contractile protein. Brief osmotic shock at low temperature or treatment with 10^{-4} M ADP caused the marginal band of microtubules to be replaced by a bundle of intertwining microfibrils. The apparent interconversion of microtubules and microfibrils under a variety of conditions led to the hypothesis that fibrils and tubules consist of similar subunits whose degree of polymerization might be dependent on local cytoplasmic forces. Furthermore, on the basis of these observations, it is postulated that the contractile properties of the cells may be vested in the microfibrils, whereas the tubules may serve to maintain the highly asymmetric shape characteristic of circulating and irreversibly aggregated platelets.

INTRODUCTION

During the past decade studies concerned with platelets and their structural alterations during blood coagulation have appeared in ever increasing numbers. In spite of this, the mechanisms by which platelets support clot retraction and hemostasis have eluded clear-cut definition. One area of uncertainty deals with the function of microtubules and microfibrils and the possible relationship of these structures to the contractile protein thrombosthenin which has been extracted from the cells (1). The microtubules which measure 250–300 A in width form a conspicuous band in the periphery of the cytoplasm (Figs. 1, 2). Their demonstration is largely attributable to the introduction of glutaraldehyde as a fixative (2, 3). Like the tubules of the mitotic spindle apparatus, platelet microtubules disappear in the cold (4, 5), in the presence of colchicine (6), and in the presence of sulfhydril inhibitors.[1] Though the structures have been thought to play a role in platelet contractility (7), it is more likely that they function as a cytoskeleton responsible for the maintenance of cellular shape (8). In this regard, it is noteworthy that electron microscopic analysis of partially purified thrombosthenin did not reveal any microtubules. Instead, this actomyosin-like protein contained an abundance of fibrils which resembled in size and structure those sometimes resolved in platelet pseudopods (9). This observation raised the possibility that microfibrils rather than microtubules could represent the morphologic counterpart of the contractile protein. Since thrombosthenin has been reported to constitute 18–20% of the total protein extractable from platelets (1, 10), one would expect microfibrils to be widely distributed throughout the platelet cytoplasm. Yet microfibrils had been resolved only in platelet pseudopods or in the periphery of lysed cells by means of the negative staining technique (11). It seemed possible that the failure to resolve these 80–100 A structures in the intact platelet might be due to the remarkable electron density of the platelet cytoplasmic matrix or to the existence of this protein in soluble form in the unaltered cell. In order to test the first hypothesis, a number of methods were

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[1] Unpublished observations.

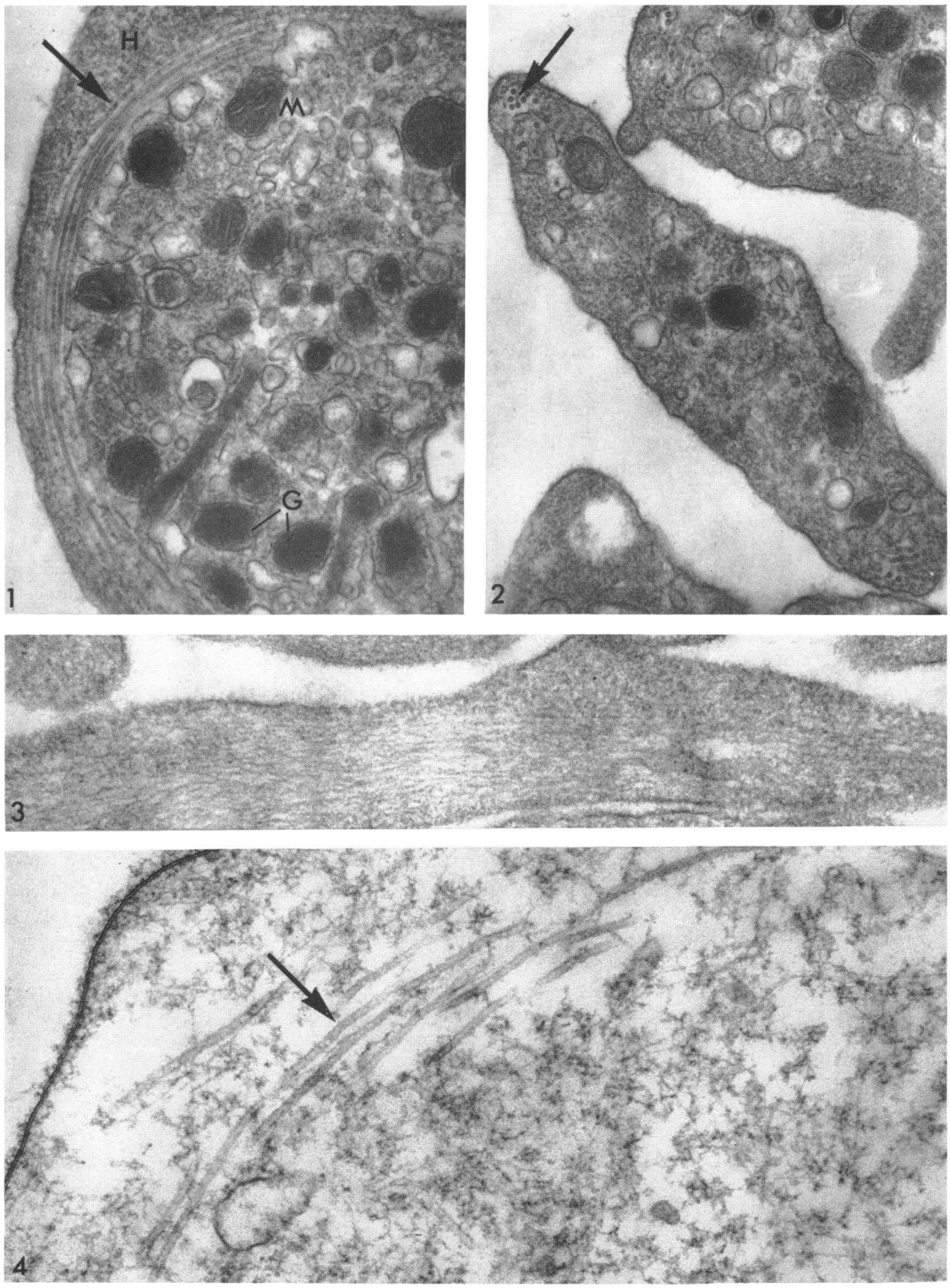


FIGURE 1 Detail of human blood platelet showing the marginal band of microtubules (arrow), granules (*G*), and mitochondria (*M*). The area peripheral to the microtubules is usually referred to as hyalomere (*H*). × 37,000.

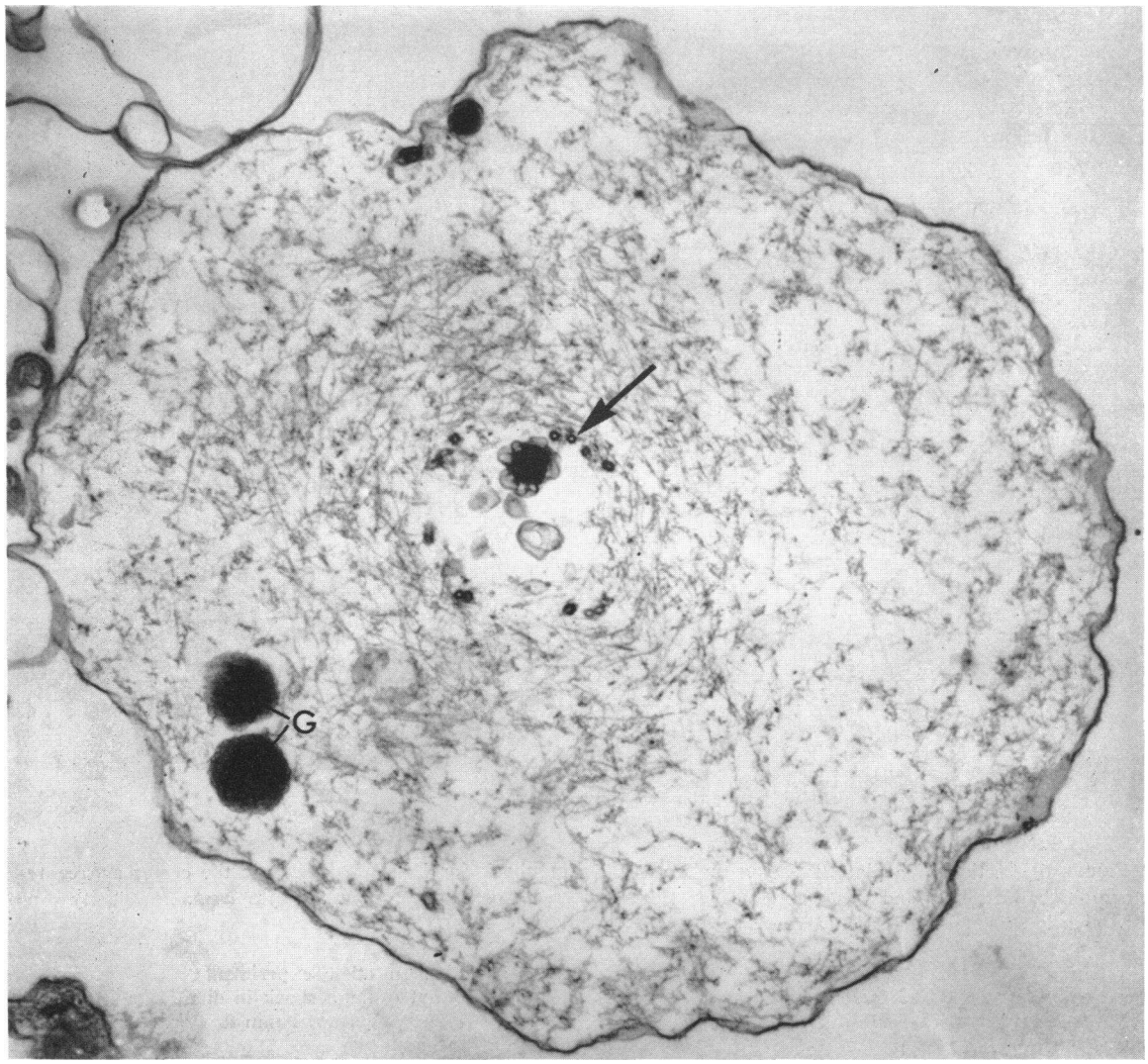


FIGURE 5 Thin section of a platelet which has been incubated in distilled water at 37°C for 1 hr. The cytoplasm is replete with fibrils. The irregularly shaped dots probably represent fibrils in cross-section. Most granules (*G*) are located beyond the plane of the section or have been extruded. Cross-sections of at least 12 microtubules are seen in the center (arrow). × 36,000.

introduced to render the cytoplasm less dense and to increase the contrast of smaller structures. These methods have resulted in the demonstration of microfibrils throughout the platelet cytoplasm and, in addition, provided an opportunity to show the relationship between microfibrils and microtubules under a variety of conditions.

METHODS

Blood obtained by venipuncture from normal human subjects was anticoagulated with heparin (5 μ/ml), 3.8% trisodium citrate (0.1 ml/ml), or a 10% solution of disodium ethylenediaminetetracetate (EDTA, 0.01 ml/ml). Initially, polystyrene syringes and tubes were used, but these were abandoned since neither the type of glassware nor the type of anticoagulant seemed to affect the experimental results.

FIGURE 2 Platelet showing microtubules in cross-section (arrow). × 34,000.

FIGURE 3 A platelet pseudopod showing microfibrils running parallel with its long axis. × 66,000.

FIGURE 4 Section of a platelet fixed after incubation in distilled water at 37°C for 5 min. A fibrillar substance has become apparent in the cytoplasm. Microtubules (arrow) are intact. A coating is seen on the external surface of the trilaminar plasma membrane. × 60,000.

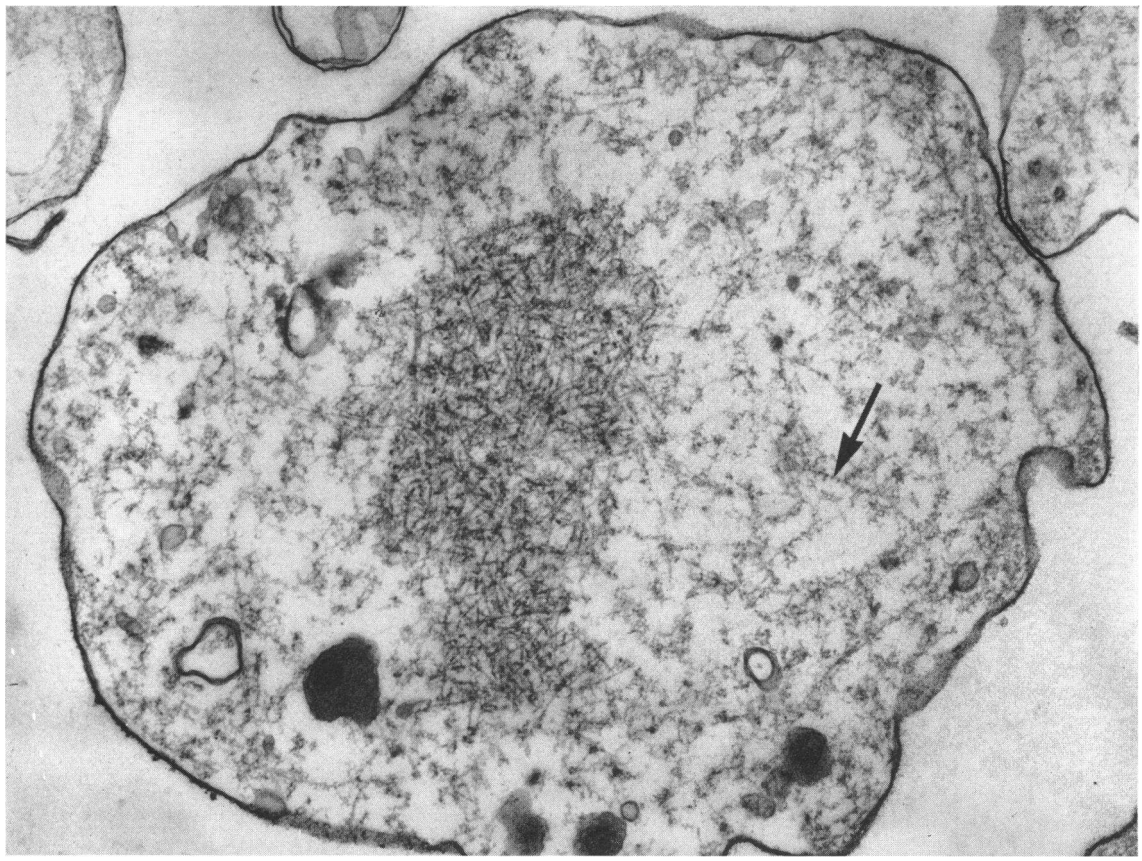


FIGURE 6 Section of a platelet having undergone osmotic shock for 1 hr. The fibrils in the center aggregate are generally thicker and better defined than those scattered loosely throughout the cytoplasm. Periodicity can be seen in some of the fibrils (arrow). × 36,000.

When preservation of microtubules was the object, specimens were processed at 35–37°C. Erythrocytes were sedimented at 200 *g* for 8–10 min in a table model clinical centrifuge, and platelet-rich plasma (PRP) was separated with Pasteur pipettes. A fairly large number of buffy coat cells was collected with the PRP in order to afford an opportunity to study leukocytes under the same conditions as platelets.

Osmotic shock. Platelets were sedimented at 1500 *g*, after which they were washed in Puck's saline (12) at 37°C and resuspended in 2 ml of distilled water or their own platelet-poor plasma containing 5% deuterium oxide (D_2O) by volume. Heavy water was used because of reports that this isotope has a stabilizing effect on spindle microtubules (13). The volume of the final suspension corresponded to 10% of the volume of blood from which the platelets were derived. In several experiments platelets were resuspended without prior wash followed by the immediate addition of an equal volume of 0.1% glutaraldehyde (5). This procedure halted osmotic swelling and prevented complete lysis of cells. The partially fixed platelets were then sedimented and resuspended in 3% glutaraldehyde at 37°C for 2 hr. When platelets were subjected to osmotic shock for prolonged time periods, they were placed in distilled water or plasma-D_2O at 37°C for 15 min, 30 min, 1 hr, and 2 hr. They were sedimented and fixed as a pellet with 3% glutaraldehyde for 2 hr at 37°C. In other experiments, platelets prepared at 37°C underwent osmotic shock in distilled water at 4°C for time periods ranging from 1 min to 1 hr. They were fixed in a similar fashion.

Sonication. For sonication, 2 ml of 10-fold concentrated PRP was diluted with 8 ml of Puck's saline in order to accommodate the supersonic probe. A probe-type Branson Sonifyer (Heat Systems, Inc.) with the current set at $\frac{1}{2}$ maximum was used. The suspension was sonicated for a total of 12 sec with three interruptions of about 1 min. The sonicated platelets were sedimented at 800 rpm and fixed in 3% glutaraldehyde as above.

Pressure Homogenization. On two occasions, platelets which had been disrupted in a pressure homogenizer as part of unrelated experiments were examined.[2] For this purpose, platelets were washed and resuspended in buffer in a pressure homogenizer supplied by Artisan Industries, Inc., Waltham, Mass. and subjected to 900 psi of nitrogen pressure for 30 min or 1500 psi for 1 hr. After release, the disrupted platelets were sedimented and fixed as before.

Colchicine. Platelet suspensions containing 2.5×10^{-5} mole/liter of colchicine were incubated for 2 hr at 37°C,

[2] These experiments were conducted in the laboratory of Dr. Aaron Marcus, Manhattan Veterans Administration Hospital, and we are indebted to him for allowing us to examine these specimens.

after which they were fixed in the manner described. No attempt was made to wash out this chemical. Clot retraction in the presence of colchicine was tested on whole blood as well as PRP by standard qualitative clinical methods (14).

Adenosine diphosphate (ADP), (Sigma Chemical Co., St. Louis, Mo.) dissolved in Puck's saline was added to 2 ml of 10-fold concentrated PRP to yield final concentrations ranging from 10^{-11} to 10^{-4} mole/liter. Incubation with gentle manual agitation was allowed to proceed until aggregation of platelets could be detected with the naked eye. When EDTA was the anticoagulant, aggregation could not be detected grossly, and incubation with the nucleotide was continued for 10–20 min.

Electron microscopy. Fixation in 3% glutaraldehyde (2) was allowed a minimum of 2 hr and a maximum of 20 hr. Postfixation with osmium tetroxide was carried out for 1–2 hr. The fixed specimens were washed and resuspended in 0.5% uranium acetate in saline for 1 hr. Dehydration and embedding in Epon 812 was accomplished by the procedure of Luft (15). Thin sections were obtained with a Huxley or LKB ultrotome. After the sections were stained with lead hydroxide (16) or double stained with uranyl acetate (17) and lead hydroxide, they were viewed with a Siemens Elmiskop I electron microscope at instrument magnifications ranging from 2500 to 40,000.

RESULTS

Platelets prepared at 37°C and fixed in glutaraldehyde showed the peripheral band of microtubules (Fig. 1) which has been abundantly illustrated in the literature (3–5, 18). In cross-section (Fig. 2), the tubules appeared circular with a diameter of about 250 A. Microfibrils could not be resolved in the cytoplasm of the unaltered platelet. However, under a variety of conditions platelets form pseudopods which show bundles of microfibrils running parallel with the long axis of the pseudopod (Fig. 3) (9). Under optimal conditions of fixation, only a small percentage of platelets form such processes. When platelets were suspended in distilled water at 37°C for less than 2 min, they underwent slight swelling accompanied by some dislocation of their organelles. However, the cytoplasmic matrix of these platelets seemed to have become less dense and began to show a fibrillar substance (Fig. 4). Exposure of platelets to warm distilled water for 15 min–2 hr resulted in progressive distortion of the cell with random clumping and (or) dispersion of the organelles, "empty"-appearing cytoplasmic spaces, broken membranes, and granule extrusion into the extracelular medium (Figs. 5 and 6). Unexpectedly, this maneuver succeeded in demonstrating that the entire cytoplasm is replete with microfibrils (Figs. 5 and 6). At first glance, these fibrils seemed to fall roughly into two categories: *a*) fibrils whose diameter ranged between 50 and 70 A (Fig. 5) and which were scattered throughout the cytoplasm, and *b*) fibrils whose thickness ranged between 80 and 120 A which were seen in bundles or dense aggregates (Fig. 6). In most platelets, the concentration of thick fibrils was higher in the periphery of the cytoplasm, but occasionally, the opposite state pertained (Fig. 6). The length of none of the fibrils could be established, since they criss-crossed into and out of the plane of section. At times a periodic substructure was suggested but could not be determined with certainty because of the background grain (Fig. 6, arrow). Some of the thicker fibrils were frayed at the end or terminated in a thin wisp. In general, they bore a striking resemblance to the fibrils found in thrombosthenin extracted from human platelets which also varied in thickness and length (Fig. 7) (9). It is noteworthy that lymphocytes, monocytes, and granulocytes present in these specimens and thus subjected to the same treatment never showed the type of fibrils seen in platelet cytoplasm. Occasional 50–60 A filaments were attached to the inner aspect of the plasma membrane of leukocytes, but these were not well defined, and their number was small compared to those seen in platelets.

Platelets incubated in warm distilled water for more than 15 min frequently showed clumps of microtubules and microfibrils (Figs. 8 and 9). In such aggregates the number of microtubules and pieces of microtubules seemed particularly high, a finding which raises the possibility that more tubules had formed during the incubation procedure. As a rule, intact and fragmented microtubules ended abruptly, but occasional tubules were seen in

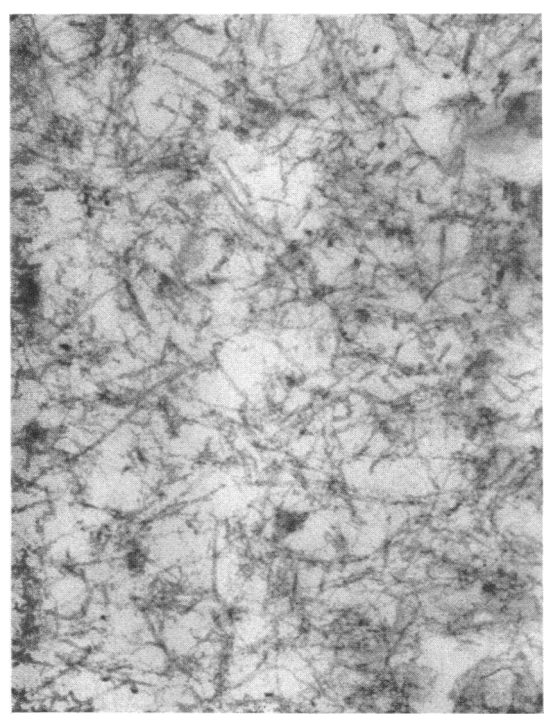
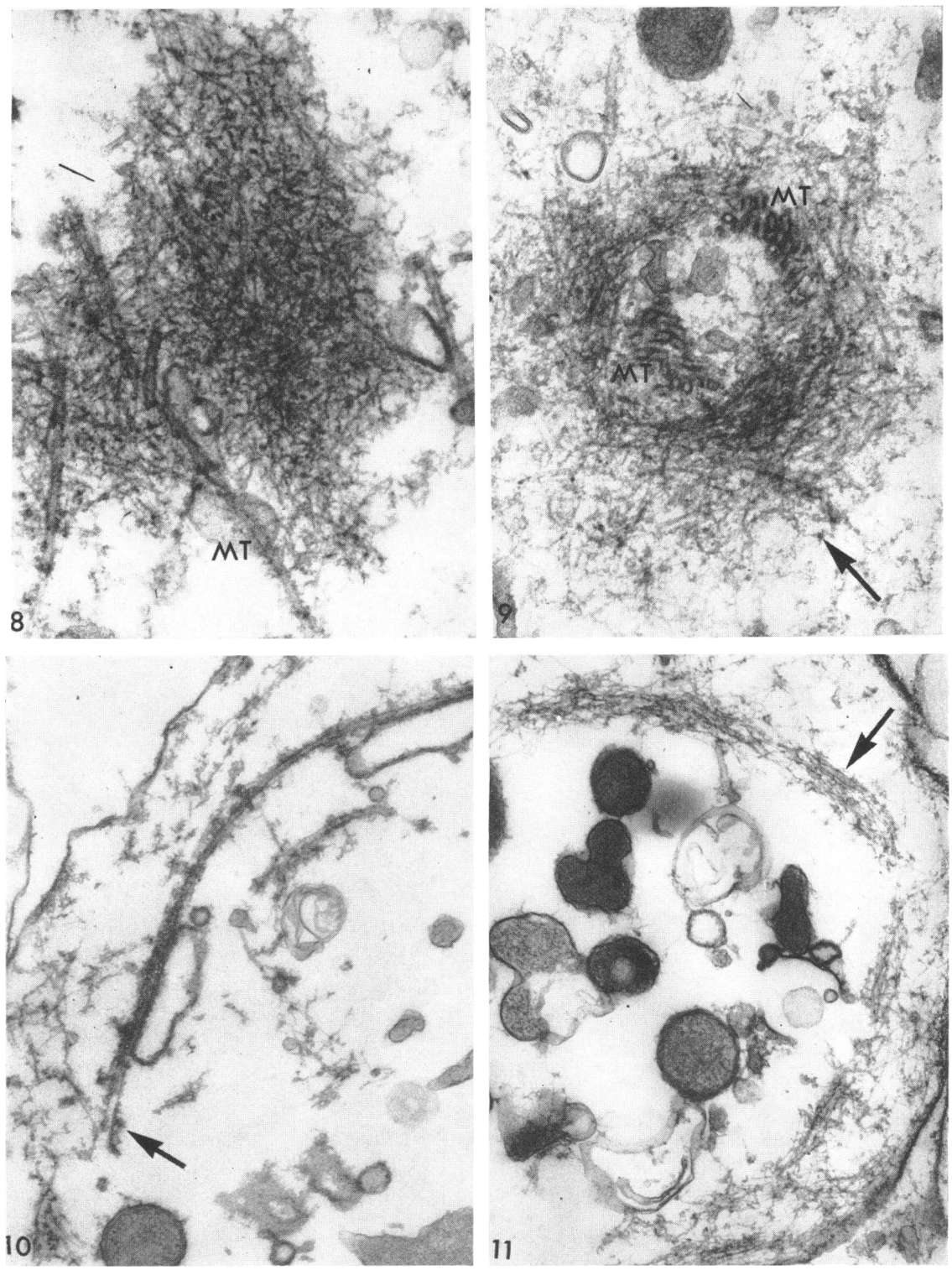


FIGURE 7 Electron micrograph of partially purified thrombosthenin extracted from human platelets showing fibrils similar in structure to those seen in swollen intact platelets. × 62,000.



FIGURES 8 and 9 Clumps of microtubules and microfibrils seen in platelets which had been incubated in distilled water at 37°C for 1 or 2 hr. A close relationship between the tubules and fibrils seems to exist. In Fig. 8, the tubules vary in diameter and direction. Polymerization or depolymerization could have taken place here.

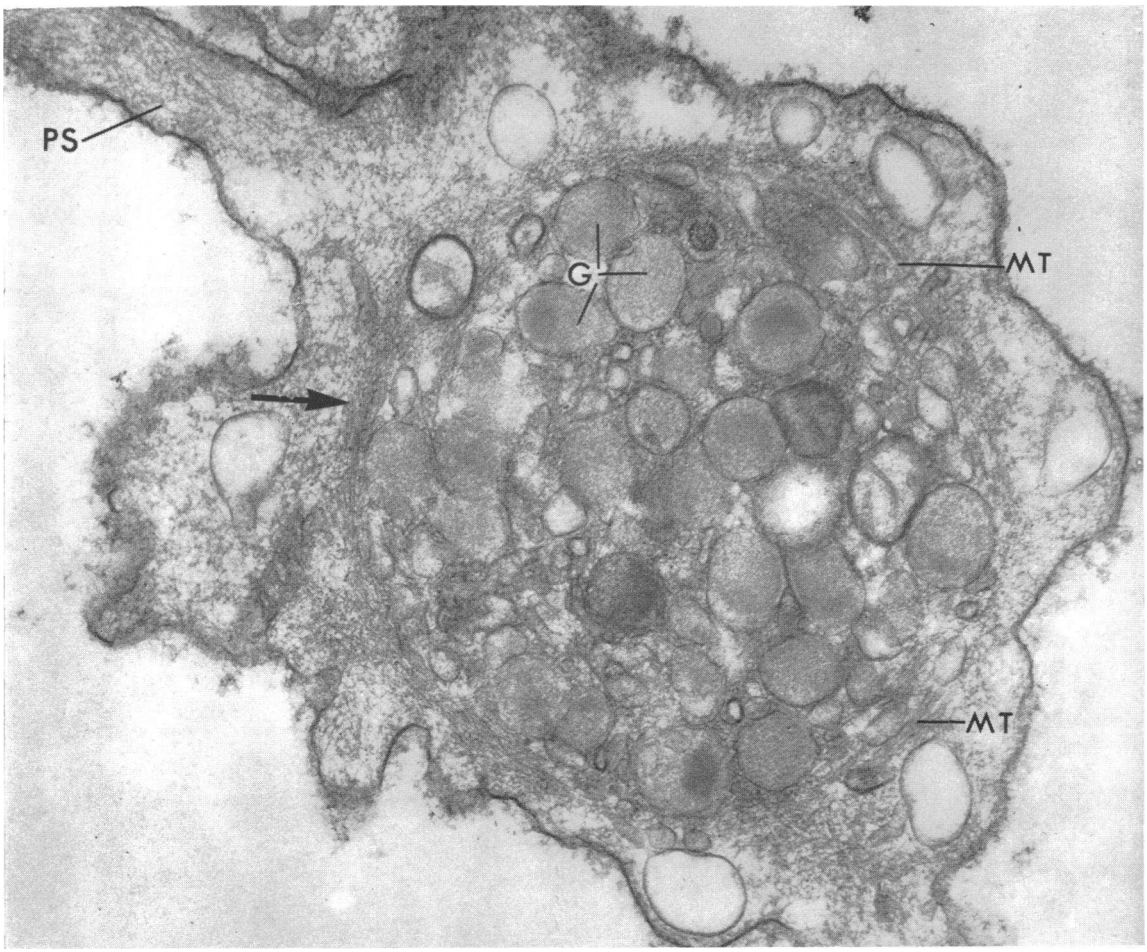


FIGURE 12 Platelet obtained from specimen briefly incubated with 10^{-5} M adenosine diphosphate (ADP). Microfibrils have become apparent throughout the hyaloplasm and particularly in the pseudopod (*PS*). Granules (*G*) have come to lie in close apposition. The marginal band of microtubules (*MT*) has in part been replaced by fibrils (arrow). × 45,000.

continuity with one or several fibrils (Fig. 10), an observation supporting the evidence obtained by negative staining techniques that the tubules have a filamentous substructure (11).

When platelets were exposed to cold distilled water for 1 min or less or if fixation in cold glutaraldehyde was carried out after osmotic shock at room temperature, the microtubules disappeared completely. In such platelets a bundle of intertwined microfibrils was often seen in the location usually occupied by the marginal band of microtubules, giving the impression that the latter structures had depolymerized (Fig. 11). It may be significant that in leukocytes, the microtubules associated with the centriole did not disappear when the cells were fixed at 4°C.

Treatment with colchicine (2.5×10^{-5} mole/liter for 2 hr) also resulted in the disappearance of microtubules without any effect on the morphology of the microfibrils. However, in the colchicine-treated platelets, a bundle of intertwining microfibrils in the location commonly oc-

Fig. 9 shows microtubules (*MT*) in cross- and tangential section. Arrow points to dot probably representing a fibril running at right angles to the plane of section. × 27,000.

FIGURE 10 Detail of a platelet taken from specimen which was exposed to hypotonic shock at 37°C showing a microfilament in continuity with microtubule (arrow). × 46,000.

FIGURE 11 Detail of a platelet exposed to cold distilled water for 1 min. The marginal band of microtubules is replaced by a band of intertwined microfibrils (arrow). Additional fibrils are seen in the hyaloplasm. Granules and mitochondria are swollen and distorted. The remainder of the cytoplasm appears "empty." × 38,000.

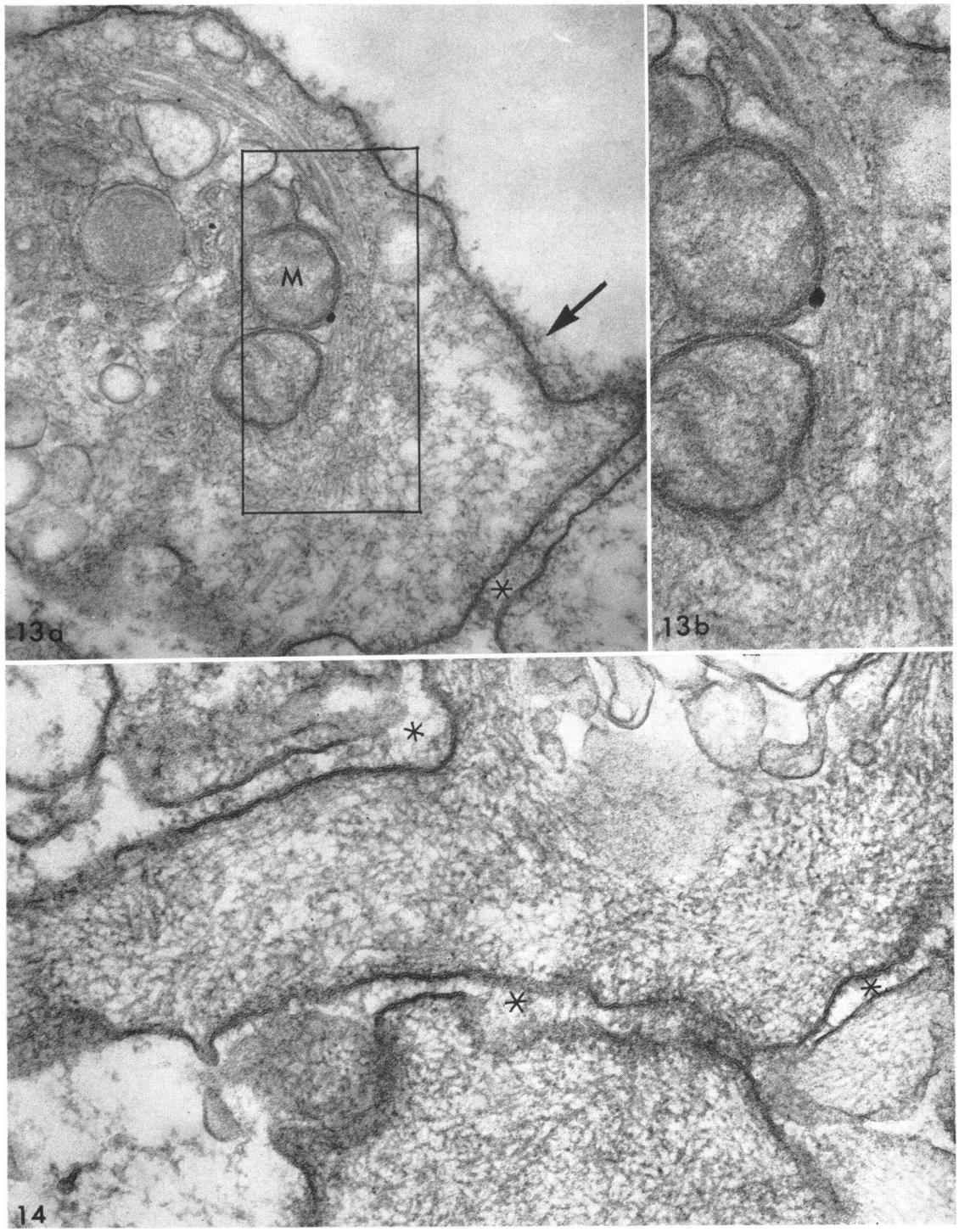


FIGURE 13 *a* and *b* Detail of platelet obtained from specimen treated with ADP shows transition of microtubules to fibrils to better advantage. Area in inset is shown at higher magnification in Fig. 13 *b*. The surface coat (arrow) and bridges between the plasma membranes of two adjacent aggregated platelets (asterisk) are also observed. Mitochondria (*M*). Fig. 13 *a*, × 55,000; Fig. 13 *b*, × 102,000.

FIGURE 14 Detail of several ADP-aggregated platelets showing the entire cytoplasm filled with fibrils seemingly running in random directions. The irregularly shaped dots are presumed to represent fibrils running at right angles to the plane of the section. Bridges (asterisks) formed between aggregated platelets and the intact surface membranes are noteworthy. × 91,000.

cupied by microtubules was not seen. Since the colchicine-treated platelets were only examined after 2 hr exposure to the chemical, it is possible that intermediate stages of microtubule depolymerization were missed. The concentration of colchicine used in these studies did not interfere with clot retraction of whole blood or PRP, a finding which suggests that microtubules per se may not play a role in this platelet function.

Sonication and pressure homogenization were carried out in isotonic media to eliminate the possibility that the fibrils formed only in hypotonic solutions. Damage to the platelet membrane by these methods also resulted in rarifaction of the cytoplasmic matrix and the appearance of microfibrils. Since, of necessity, these procedures had to be carried out in the cold, microtubules could usually not be demonstrated in these specimens. Platelets which had been briefly sonicated and were subsequently incubated at 37°C for 1–2 hr always showed some microtubules, an observation suggesting that these structures had reformed. Despite the stabilizing effect of deuterium oxide on the tubules of the mitotic spindle (13), no morphologic differences were seen in the presence or absence of this isotope as part of the suspension medium.

An abundance of microfibrils was also seen in platelets which had been treated with ADP (Figs. 12–14) in autologous plasma in the absence of additional osmotic or mechanical shock. In the concentrations used, ADP is known to cause platelet aggregation (19, 20) and sphering (21, 22) of the normally lenticular-shaped cell. It has also been observed that in the presence of low concentrations of this agent platelet aggregation is reversible, whereas high concentrations lead to viscous metamorphosis i.e., irreversible aggregation of a high proportion of the cells, with release of platelet contents (23, 24). As observed by others (25), the earliest morphologic changes induced by ADP was the crowding of the organelles to the center of the cell so that granules came to lie in close apposition, while the distance between the marginal band of microtubules and the surface membrane had increased (Fig. 12). It could not be established on the basis of these observations alone whether microtubules had actually moved in a centripetal direction thereby condensing the granulomere, or whether the effect could be attributed to preferential swelling of the peripheral hyalomere. When fixation was delayed several minutes after gross aggregates had formed in the presence of 10^{-4} M ADP, the microtubules often appeared to have been replaced by microfibrils (Figs. 12 and 13). In some platelets, the marginal band seemed to consist of tubules in some areas and of fibrils in others (Figs. 13 *a* and *b*). In addition to the strands of microfibrils surrounding the granulomere, an abundance of fibrils had also become apparent elsewhere in the cytoplasm. A striking example of this phenomenon can be seen in Fig. 14, which shows details of several ADP-aggregated platelets. These fibrils appeared more uniform in size belonging mostly to the 80–120 A variety. The bridges which form between the plasma membranes of platelets treated in this manner are also seen to good advantage. In platelets which had become irreversibly aggregated in that granule lysis and membrane damage had reached a far advanced stage, the microtubules and (or) fibrils consituting the marginal band could no longer be resolved, and the granulomere was surrounded by a homogeneously gray area which was more electron dense than the remainder of the cytoplasm. A detailed description of the sequential morphologic alterations induced by ADP is not relevant to the subject under discussion.

DISCUSSION

The importance of platelets in hemostasis and blood coagulation has been attributed in part to the fact that the cells are contractile. Consolidation of a platelet "plug" at the site of a cut vessel and retraction of a clot are evidence of this platelet function. More recently, the extraction of an actomyosin-like protein from human (1, 26) and pig (10) platelets has lent further support to the hypothesis that platelets may function as miniature muscles in approximating fibrin strands or walls of small vessels to which they have adhered. However, to date electron microscopic studies of platelets have not disclosed any structures reminiscent of myofilaments in numbers large enough to account for thrombosthenin, the contractile protein which reportedly constitutes 18–20% of the cell's extractable protein (1, 10). At times, it was suggested that the band of microtubules played a role in the contractile phenomena exhibited by platelets (7). This concept stemmed primarily from the fact that microtubules are found in cilia and flagella of many plant and animal cells (27, 28), and that spindle tubules, which are morphologically similar organelles, seem to be involved in moving chromosomes to opposite poles of the metaphase plate. However, the concept that microtubules are responsible for platelet contractility has been dispelled by the observation that colchicine, an agent which depolymerizes these structures, does not interfere with clot retraction. In addition, electron microscopic analysis of thrombosthenin (Fig. 7) did not reveal tubules but showed primarily microfibrils, a finding which suggests that fibrils rather that tubules are responsible for the contractile properties. Therefore, it is of interest that under the conditions of the present study platelets show an abundance of fibrils similar to those seen in isolated thrombosthenin. These fibrils also bear a striking resemblance to the myofilaments of smooth muscle (29, 30) particularly when they are oriented in parallel, as can be seen in pseudopods (Fig. 3). Only a very

small number of similar fibrils were found in leukocytes studied under identical conditions. Thus, it would seem that platelet fibrils deserve serious consideration in the search for a morphologic counterpart of the contractile protein.

The apparent variation in thickness of the fibrils also warrants some comment. The thicker fibrils were mostly found in clumps or in the periphery of the cell particulary in the area of microtubule depolymerization, whereas the thinner filaments were always widely scattered throughout the cytoplasm. It is possible that the thin and thick fibrils represent two entirely different proteins comparable to actin and myosin obtained from muscle. This theory would be compatible with the report that thrombosthenin also consists of two components which resemble actin and myosin (31). On the other hand, it is also conceivable that the thicker fibrils are composed of aggregates of thinner filaments. The latter hypothesis was suggested by the fact that the thicker fibrils seemed to have a filamentous substructure as indicated by the branching, splitting, and wisping at their ends and the frequent occurrence of helical, braided, and periodic arrays. The presence of filaments in some aggregated platelets has been reported previously by Behnke who was able to resolve 50 A structures in experimentally induced platelet thrombi (32). These filaments had a tendency to run parallel with microtubules in platelet pseudopods. Though there is a discrepancy in the diameter of the filaments reported by Behnke and those described here, this may be explained by the contrast-enhancing methods used in this laboratory. The use of uranium salts during the fixation procedure tends to increase the width of membranes and fibrils. Since platelet aggregation during thrombus formation is associated with and, in part, dependent on platelet-released ADP (24, 33), the occurrence of fibrils in some of the platelets observed by Behnke and those seen in ADP-treated platelets illustrated in Fig. 14 is probably not coincidental. It may be significant that in platelets treated with ADP, the fibrils seemed more numerous and more uniform in diameter (Fig. 14) than in platelets subjected to osmotic or mechanical shock. However, since ADP also causes platelets to swell (23, 34), the question whether this nucleotide per se induces fibril formation or whether the structures only become visible as a result of cytoplasmic rarifaction remains unanswered.

The suggestion that the microfibrils represent the morphologic counterpart of the contractile protein does not preclude a close relationship between microfibrils and microtubules. Though in tissue sections microtubules seem to have solid walls (Figs. 1 and 2), with negative staining techniques they have been shown to possess 13 subfilaments measuring about 50 A in diameter (11). Depolymerization of the tubules into filaments does not seem to be due to the hypotonic media which are often used in conjuction with negative staining. As has been demonstrated here, platelet microtubules are well preserved in distilled water provided they are maintained at 37°C. Depolymerization takes place at low temperatures and as a consequence of treatment with agents which lead to irreversible platelet aggregation. It should also be noted that the interconversion of microtubules and filaments does not constitute a newly observed phenomen in biology. Microtubules as well as microfibrils have been observed in regions of cytoplasmic streaming in many nonmammalian cells. The organelles appear and disappear as axial structures in bacterial flagella, in the slender extensions of protozoa (35), and in spermatids during cell elongation (36). In general the fibrils are present during the formation of cytoplasmic protrusions, whereas the tubules seem to serve as cytoskeletal structures associated with the maintenance of highly assymmetrical shapes (37–39).

It has been suggested by others studying nonmammalian cells (40–42) that fibrils and tubules are alternate states of the same material, the former being organized when motive force is prevalent, the latter when structural rigidity is essential. Thus it would be reasonable to postulate the existence of a cytoplasmic pool of contractile protein which supplies the subunits for the fibrils as well as for the tubules. Indeed there is recent evidence that the protein which forms the spindle tubules in sea urchin eggs and neurotubules in porcine brain (43) exists as a monomeric unit in the cytoplasmic pool. The extent to which such molecules can polymerize may be dependent on local cytoplasmic conditions (44).

In view of these studies, it is possible that similar mechanisms for the formation of fibrils and tubules are operative in platelets. Fibrils seem to be associated with the "active" state of the cells when participation in hemostasis or coagulation necessitates changes in shape. For reasons discussed above fibrils may subserve the contractile function. Fully polymerized microtubules on the other hand are present in disc-shaped circulating platelets or in the pseudopods of irreversibly aggregated cells (7) when the maintenance of a rigid but highly assymmetric shape seems desirable.

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