THE SUBMEMBRANOUS FIBRILS OF HUMAN BLOOD PLATELETS

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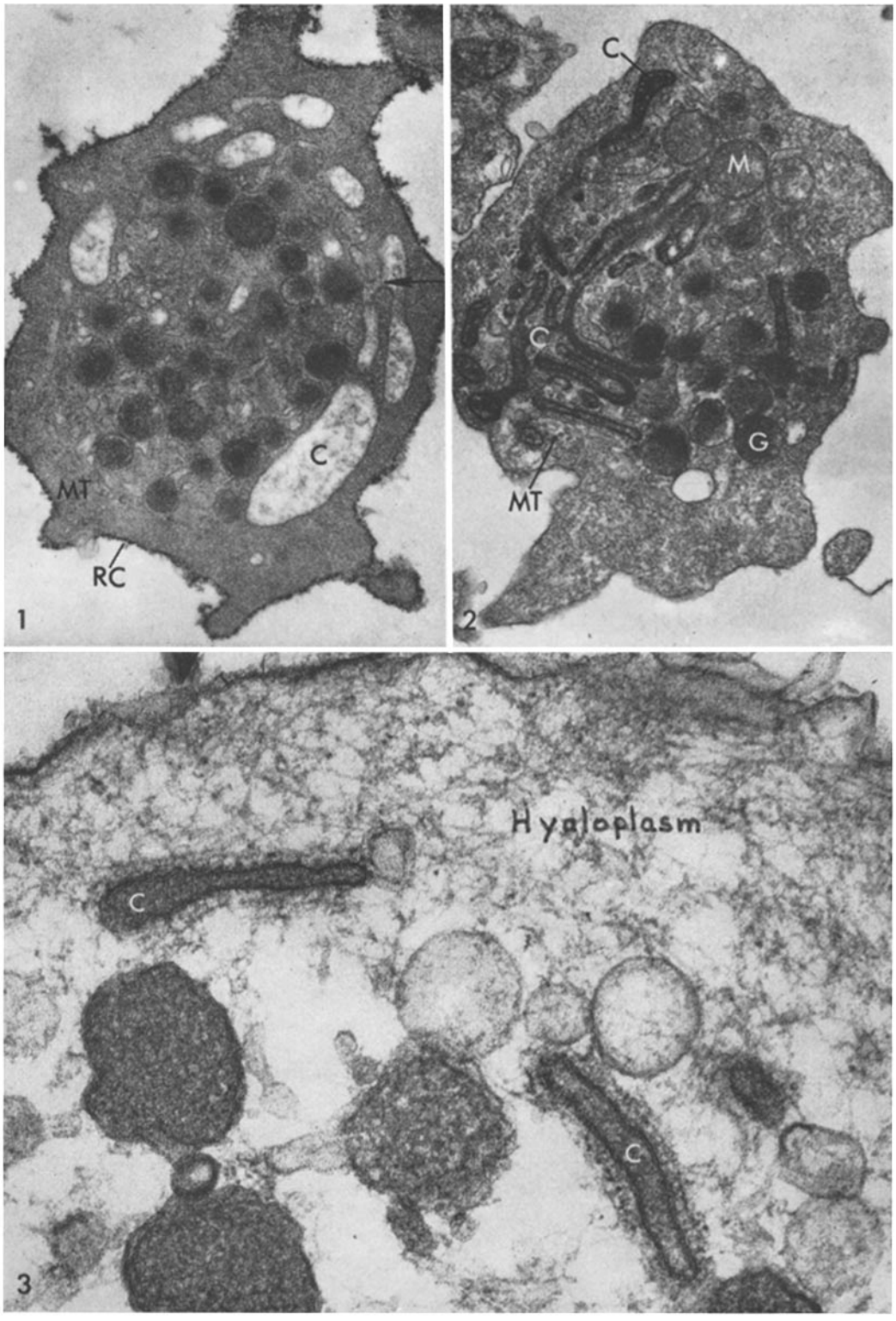
INTRODUCTION

Among several functions attributed to blood platelets is a contractile one which is believed to play a significant role in hemostasis. In addition, platelets are known to take up a large variety of substances which are transported interiorly by means of surface-connected channels (1). Since propulsion of substances through this canalicular system is known to be energy dependent (13, 14), it has also been suggested that contractile mechanisms are operative here. Moreover, during clot formation or in vitro aggregation following treatment of platelets with thrombin or adenosine diphosphate (ADP), the canaliculi move toward the center of the cell where they appear to fuse (18, 20). Lastly, an actomyosin-like protein, thrombosthenin, has been isolated from human (2, 3) and porcine (6) platelets, and examination of partially purified thrombosthenin by electron microscopy has disclosed an abundance of microfibrils (21). This has raised the question whether the microfibrils seen in the cytoplasm of platelets represent the contractile protein. In intact platelets, microfibrils run in parallel bundles within pseudopods, and under certain conditions the entire cytoplasm can be shown replete with these organelles (20). However, even the assumption that microfibrils consist of thrombosthenin would not explain how these randomly oriented structures could be instrumental in displacing the plasma membrane or in moving the canaliculi through their various formations. Therefore, specimens used in previous studies (20) were reviewed and additional experiments were conducted to determine whether microfibrils are also attached to platelet membranes. The observation that


C
MT
RC
1
C
M
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G
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Hyaloplasm
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microfibrils are associated with the inner surface of the plasma membrane and the cytoplasmic aspect of the canaliculi seems worthy of brief report.

MATERIALS AND METHODS

Blood was obtained from normal subjects and anticoagulated with heparin (5 units/ml) or a 10% solution of disodium ethylenediaminetetracetate (EDTA, 0.01 ml/ml). Platelet-rich plasma (PRP) was prepared as previously reported (20).

Osmotic Shock

Platelets were sedimented from 10–20 ml of PRP at 1500 *g* for 10 min, after which they were washed in Puck's saline (15) and resuspended in 2 ml of distilled water at 37°C for 15 min–2 hr. The final volume of the platelet suspension corresponded to 10% of the volume of blood from which the platelets were derived. Following incubation the platelets were sedimented and fixed for electron microscopy.

Peroxidase

In order to delineate the canalicular system to good advantage, portions of 10 ml of PRP were incubated with 15 mg of peroxidase at 37°C for 1 hr with gentle agitation. It was necessary to add EDTA (0.01 ml/ml) to these specimens prior to sedimentation, to prevent irreversible aggregation of such platelets during centrifugation. On some occasions platelets which had taken up peroxidase were subsequently treated with adenosine diphosphate (ADP) (Sigma Chemical Co., St. Louis, Mo.) to yield final concentrations of 10^{-4} to 10^{-6} mole/liter. Incubation was continued for 2–4 min or until aggregation was detected with the naked eye.

Electron Microscopy

Sedimented platelets were fixed with 3% glutaraldehyde (16) for a minimum of 2 hr, or overnight. Some specimens were treated with ruthenium red in the fixative (9, and personal communication) for better delineation of the platelet "coat". Platelets which had been incubated with peroxidase were processed with the substrate H_2O_2 and 3,3′-diaminobenzidine according to the method of Karnovsky (7). Postfixation with osmium tetroxide for 1–2 hr was followed by staining "en bloc" with 0.5% uranyl acetate in saline for 1 hr. Dehydration and embedding in Epon 812 were carried out according to the procedure of Luft (8). An LKB or Huxley ultratome was used to obtain thin sections, which were contrasted with uranyl acetate (17) and lead hydroxide (12). A Siemens Elmiskop I electron microscope equipped with a decontamination device was used at accelerating voltages of 60–80 kv.

RESULTS AND DISCUSSION

The platelet depicted in Fig. 1 was obtained from a specimen anticoagulated with EDTA. This chelating agent causes some increase in platelet volume (5) and apparently distends the canalicular system, making these channels more conspicuous. A band of microtubules is present peripheral to the canaliculi. It is postulated that the canaliculi form by invagination of the plasma membrane, and that they communicate with the exterior (1, 19). However, since ruthenium red can rarely be demonstrated on the inner aspect of the canalicular membrane, it is unlikely that the canaliculi are in continuity with the outside at all times. On the other hand, communication between one canaliculus and another can often be demonstrated (arrow, Fig. 1). When living platelets are incubated with peroxidase at 37° C, the reaction product may delineate the entire canalicular system within minutes (Fig. 2). Prolonged incubation results in the formation of large cisternae filled with the enzyme. This process is not limited to peroxidase, but has been demonstrated for many other soluble and par-

FIGURE 1 Platelet obtained from specimen anticoagulated with EDTA shows distended canalicular system (*C*) with communication (arrow). Though under these conditions the cells assume a somewhat irregular shape, the organelles are in their normal location. Microtubules (*MT*) are barely discernible in the periphery. The ruthenium red-reactive surface coat is seen (*RC*). × 13,000.

FIGURE 2 Platelet following incubation with peroxidase at 37°C for 1 hr. The canalicular system (*C*) is filled with reaction product. Microtubules, *MT*; mitochondria, *M*; granules, *G*. × 17,000.

FIGURE 3 Detail of a platelet following osmotic shock. The canaliculi (*C*) are surrounded by a network of fibrils which are seen in longitudinal and cross-section. Microfibrils are also seen in the hyaloplasm. The granules are swollen. × 98,000.

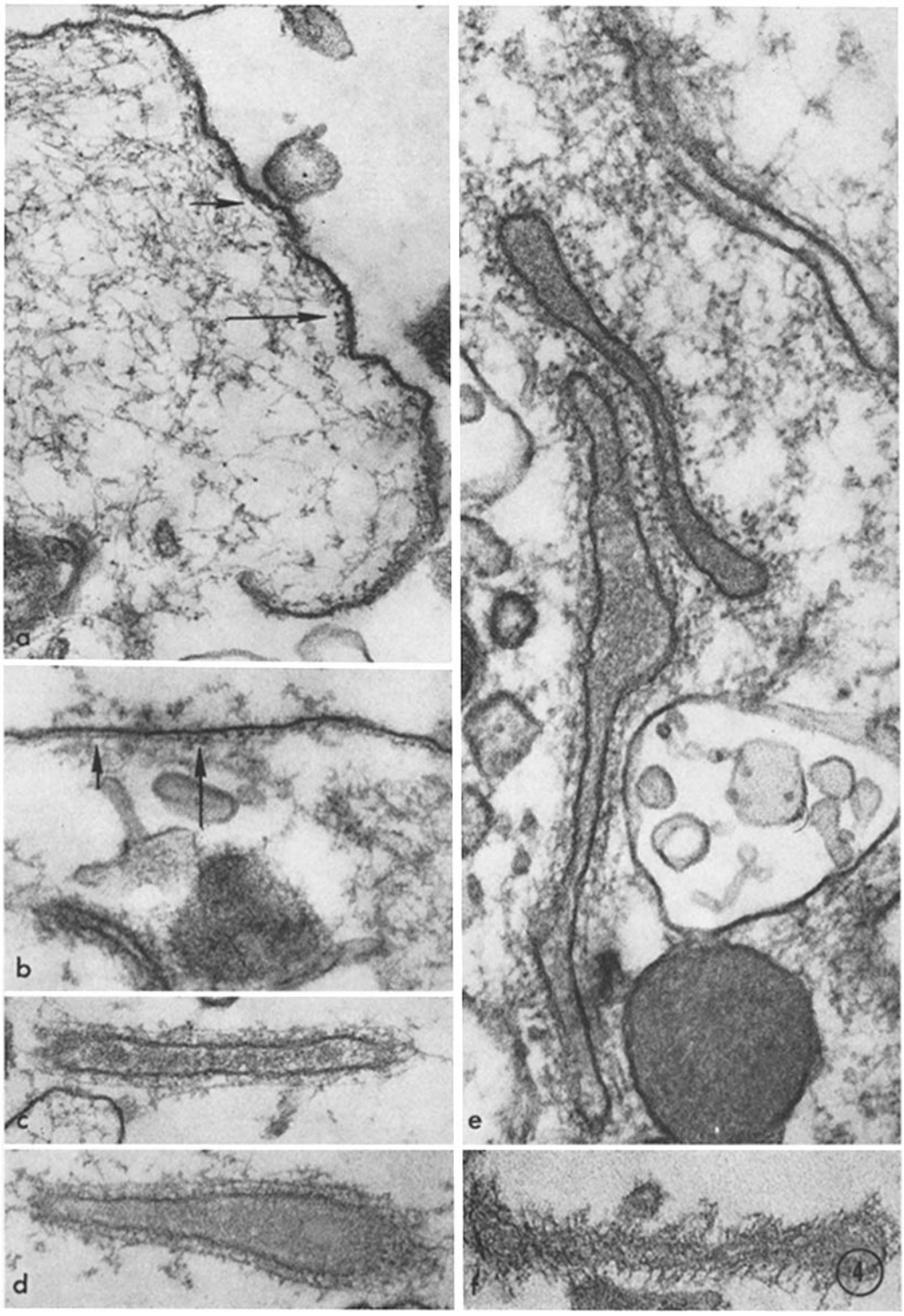
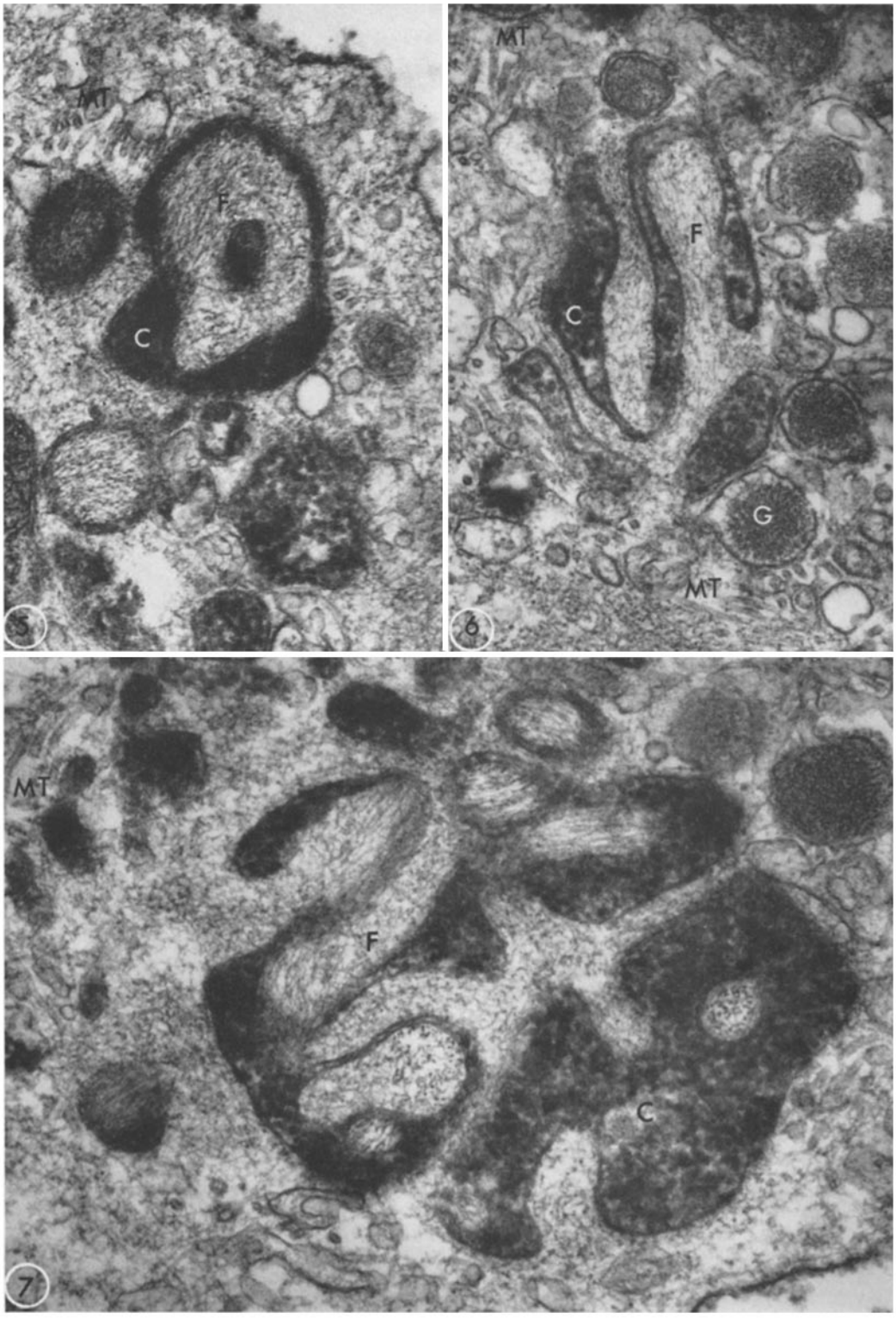


FIGURE 4 *a–f* Details of platelets which have been subjected to osmotic shock. In Figs. 4 *a* and *b*, fibrils in close association with the plasma membrane are seen in longitudinal (short arrow) as well as in cross-section (long arrow). Fig. 4 *f* shows a grazing section of a canaliculus. Figs. 4 *a,b,c,d,* and *f*, × 81,000. Fig. 4 *e*, × 104,000.



FIGURES 5–7 Details of coalescing canalicular system (*C*). Incubation with peroxidase followed by treatment with ADP. Microfibrils (*F*) extend between various compartments of condensing channels. Microtubules, *MT*; granules, *G*. Figs. 5 and 6, × 60,000. Fig. 7, × 64,000.

ticulate substances (for review see 1, 11). In the intact platelet no fibrils can be resolved either on the plasma or on the canalicular membranes. However, when the cells are subjected to osmotic shock or other procedures that cause rarifaction of the cytoplasmic matrix (20), fibrils become visible throughout the cell (Fig. 3). In the hyaloplasm the fibrils appear to have a random distribution, but those associated with the plasma or canalicular membranes seem more organized (Figs. 3–7). In cross-section, the fibrils present as irregularly shaped particles which measure about 75 A in diameter. They are separated from each other by a space of about 100 A and are located at a distance of 200–250 A from the inner aspect of the plasma membrane (Figs. 4 *a* and *b*) or the cytoplasmic aspect of the canalicular membrane (Figs. 3 and 4 *e*). When sectioned longitudinally, they appear at about the same distance from these membranes (Figs. 4 *b–d*). Thus, the impression is gained that the plasma membrane and the canaliculi as well are surrounded by a network of fibrils. The assumption that the particles represent fibrils in cross-section is supported in grazing sections (Figs. 4 *d* and *f*).

If such fibrils would indeed be contractile, it is not difficult to conceive how their orientation could be translated into propulsive movements along the canaliculi in a manner analogous to the mechanism afforded by the longitudinal and circular layer of smooth muscle constituting the wall of the mammalian gastrointestinal tract. Moreover, the finding that the cytoplasmic surfaces of both the plasma and the canalicular membranes are equipped with a similar network of fibrils furnishes additional support for the hypothesis that the canaliculi are formed by invaginations of the plasma membrane.

Treatment of platelets with ADP causes aggregation and clumping of organelles in the center of the cell, eventually leading to complete obliteration of all recognizable structures (18, 20). To date, the mechanism by which this centripetal movement of the organelles occurs has remained unexplained. When platelets are treated with ADP after they have taken up peroxidase, various stages of canalicular movement and fusion can be analyzed (Figs. 5–7). Though the cytoplasm does not seem rarified, microfibrils are seen clearly in association with the canaliculi. The channels are distended with reaction product and have undergone distortion and/or fusion in the process of moving toward the center of the cell. Prolonged incubation with ADP results in the formation of a central, electron-opaque area from which most membranes and structures have disappeared, although a clump of fibrils can often still be resolved. Whether the fibrils exert a centripetal pull on the canaliculi or whether these structures are representative of "stress fibers" (4) which are merely incidental to the movement of the canaliculi cannot be ascertained by morphologic means. It should be noted, however, that at no time have fibrils been seen to radiate from the canaliculi outward, nor have fibrils been seen in association with mitochondria or the platelet granules. In the center of the platelet where coalescence occurs (Fig. 7), the fibrils are seen to extend between the various compartments of the system. This is best seen in grazing sections, where the membrane of the channels cannot be delineated and the fibrils are sectioned longitudinally (Figs. 6 and 7). It is noteworthy that, so far, similar microfibrils have not been observed in association with the invaginating cell membrane of phagocytic blood cells nor around the membrane delimiting phagocytic vacuoles. However, the structures described here bear a superficial resemblance to the ones observed on the inner aspect of red cell ghosts, particularly after treatment with ATP (10). In erythrocyte ghosts, it was possible to localize ATPase activity in this area though the specificity of the enzyme could not be determined. Thus, the concept that the submembranous fibrils in platelets may serve a contractile function must be regarded as merely hypothetical at the present time. Definite proof may have to await the development of immunochemical and histochemical techniques that give higher resolution than those currently available.

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