Degranulation of Discoid Platelets

James G. White, MD and Richard D. Estensen, MD

Platelet degranulation is a characteristic feature of platelet response to aggregating agents, but the mechanism and route by which secretory organelles are transferred to plasma are still uncertain. In the present study, human platelets were incubated with cytochalasin B, an agent which stabilizes discoid shape, and trypsin, which is known to cause release reaction and degranulation. Platelets treated in this manner retained their disc form, but were nearly devoid of granules and dense bodies. Electron-dense tracers indicated that degranulation was accomplished by fusion of secretory organelles with channels of the open canalicular system. The degranulated discoid platelet appears to survive exposure to cytochalasin B and trypsin and may prove to be a useful model for *in vivo* and *in vitro* experimental studies (Am J Pathol 68:289–302, 1972).

THE PLATELET RELEASE REACTION is a characteristic feature of the maximum platelet response to aggregating agents *in vitro* and *in vivo*.[1] It involves the discharge in parallel of specific substances, including ADP, ATP, serotonin and calcium, stored inside platelets to the surrounding plasma without loss of chemical constituents suggestive of cell damage.[2] Many features of the platelet release reaction have been defined, but the mechanisms by which the intracellular organelles containing the products of secretion are transferred to the exterior environment remain uncertain.[3] One of the problems involved in clarifying the mechanism of platelet secretion is the difficulty in separating the degranulation process from other physical changes occurring in the cells during viscous metamorphosis.[4]

In the course of investigations into the effects of cytochalasin B on platelet structure and function, a new approach to this problem was found.[5] Cytochalasin B is a mold metabolite with a macrolide structure in which the lactone ring is joined to a bicyclic lactum.[6,7] This unusual chemical inhibits certain functions regarded as contractile in nature in many different cells,[8,9] including the platelet-dependent process of clot retraction.[10,11] Examination of the response of cytochalasin-B-treated platelets to potent aggregating agents suggested that the drug

From the Departments of Pediatrics and Pathology, University of Minnesota Medical Center, Minneapolis, Minn.

Supported by Grants HE-11880, AI-05153, CA-08832 from the US Public Health Service and the Cardiovascular Clinical Research Program Project.

Accepted for publication Feb. 23, 1972.

Address reprint requests to Dr. James G. White, Department of Pediatrics, University of Minnesota Medical Center, Box 284 Mayo Memorial Bldg, Minneapolis, Minn 5545.

stabilizes platelet discoid form and inhibits shape change, pseudopod formation and internal reorganization without affecting stickiness.[5]

The stabilizing influence of cytochalasin B on platelet discoid shape provided an opportunity to examine degranulation as an entity separate from other features of platelet viscous metamorphosis. Trypsin was chosen as a trigger agent because it is a powerful stimulus of platelet release and degranulation, but under appropriate conditions does not cause clotting or platelet aggregattion.[12] After incubation with cytochalasin B and trypsin, platelets retained their discoid form, circumferential microtubules, filamentous matrix, mitochondria and cytochemically detectable enzymes; nearly all granules and dense bodies were extracted from the cells.

Materials and Methods

The technics used in this laboratory to obtain blood from normal donors in citrate anticoagulant, separate platelet-rich plasma (C-PRP) and prepare samples for study in the electron microscope were described in detail in recent reports.[4,13,14] Cytochalasin B (CB) was dissolved in ethanol at 2 to 4 mg ml and diluted in Hank's buffered salt solution to a concentration of 250 μg ml. A 0.1 ml aliquot of this solution was added to 0.9 ml samples of C-PRP, and the tubes placed in a constant temperature water bath at 37 C for 15 minutes. Trypsin was then added to the samples at a final concentration of 1 to 3 mg ml of C-PRP. The tubes were incubated at 37 C for intervals of 5 minutes to 1 hour, then fixed for study in the electron microscope. Horseradish peroxidase (HRP), 20 mg ml, or a 0.1 ml aliquot of thorium dioxide were added 15 minutes before fixation to some samples of C-PRP incubated with CB and trypsin for 1 hour.[13,14] Samples containing HRP were processed by the method of Graham and Karnovsky.[15] Additional platelet samples exposed to CB and trypsin were fixed by a slight variation of our usual procedure, and incubated in a modification of the Graham and Karnovsky medium adapted by others for the localization of endogenous peroxidase and catalase.[16] Details of our method for demonstrating endogenous peroxidase have been reported.[17] The effects of trypsin alone, CB alone and trypsin added to C-PRP before CB under the conditions described above were also evaluated.

Results

Effects of Cytochalasin B (CB) Alone

In previous studies we have shown that concentrations of CB up to 30 μg/ml have a minimal effect on the morphology of blood platelets.[5] After exposure at 37 C for 1 hour, CB treated platelets retained their characteristic discoid form (Figure 1). Organelles inside the cells were generally unaltered in appearance and remained randomly dispersed in the cytoplasmic matrix. The circumferential band of microtubules was unaffected by CB. Channels of the open canalicular system were somewhat dilated after exposure to CB, and elements of the dense tubular system were occasionally grouped together in the cytoplasm.

The matrix developed a "ground glass" appearance in some cells, but otherwise appeared undamaged. Aside from the minor changes, morphology of CB treated platelets was indistinguishable from normal cells incubated for the same period of time.

Effects of Trypsin Alone

Hovig pointed out the effects of various concentrations of trypsin on platelet fine structure several years ago.[12] Our results are basically similar to those he described. The changes in fixation methods, however, produce a slightly different impression of the effects of trypsin. We used a concentration of 3 mg trypsin/ml C-PRP because it caused alterations similar to those produced by 1 mg/ml, yet avoided the problem of clot formation completely. Platelets incubated for a few minutes with 3 mg trypsin had the same appearance as those which had been exposed to the agent for 1 hour.

The platelets lost their discoid form and appeared irregular or spherical with few pseudopods (Figure 2). Granules and other organelles when still present were gathered toward cell centers. The curious change in internal membrane systems which Hovig noted [12] was also apparent in our material (Figures 3, 4). In some platelets the membranes were stacked in arc-like segments, and in others they were arranged in concentric layers. Hovig recognized the similarity of the internal membranes to the platelet surface, but suggested that they probably developed from endoplasmic reticulum. However, it appears more likely that the membranes are stretched out channels of the open canalicular system, the linings of which are in continuity with the platelet cell wall. The trypsin-treated cells resembled platelets exposed to thrombin and collagen except for two features: virtual absence of aggregation and the unusual transformation of channels belonging to the open canalicular system.

Effects of Cytochalasin B and Trypsin

The appearance of platelets exposed to trypsin after incubation with CB differed strikingly from cells treated with either agent alone (Figure 5A–C). Platelets were generally discoid, although swelling of the open channel system caused some distortion. The circumferential band of microtubules usually remained peripherally located in the equatorial plane of the platelets. Mitochondria were present, but nearly all platelets were completely devoid of dense bodies and granules. The cytoplasm of the platelets often contained concentric areas of dense filamentous material (Figure 6A). The contracted masses in the matrix resembled

those seen in cells treated with trypsin alone. However, granules and microtubules were rarely associated with the filamentous zones. The appearance of these cells indicated that trypsin had caused degranulation and alterations in the cytoplasmic matrix without inducing aggregation of platelets or loss of the characteristic discoid form. Platelets incubated first with trypsin, then with CB, did not differ from platelets treated with trypsin alone.

Localization of Horse Radish Peroxidase and Thorium Dioxide in Platelets Treated with CB and Trypsin

It was important to know if an access route from the interior of the CB-trypsin-treated platelets to the surrounding plasma remained, since granules and dense bodies had apparently not emptied through the surface membrane. The electron-dense tracers, HRP and thorium dioxide, were employed to evaluate this question (Figure 6B–D). Both agents readily entered dilated channels of the open canalicular system. In some examples, thorium dioxide was present in channels which were in continuity with partially empty granules (Figure 6C).

Endogenous Peroxidase Activity of Platelets After Treatment with Cytochalasin B and Trypsin

The experiments with electron-dense tracers demonstrated that the open channel system was affected, but did not reveal whether the second system of canaliculi, the dense tubular system (DTS), was altered by the chemical treatment. Endogenous peroxidase activity resides exclusively in the DTS when platelets fixed in paraformaldehyde-glutaraldehyde are incubated at pH 6 in the medium modified from Graham and Karnovsky.[51-17] After treatment with CB and trypsin, enzyme reaction product was still detectable in channels of the DTS (Figure 6E, F). The channels were slightly dilated, in shorter segments and more dispersed than in control platelets, but the differences were minimal. Thus, the chemical treatment which radically modified the OCS had only a slight effect on the DTS and did not digest its content of enzyme with peroxidase activity.

Discussion

The terms "platelet release reaction", "platelet secretion", and "platelet degranulation" are essentially synonymous.[1-3,18] Secretion of specific chemical compounds by platelets after exposure to aggregating agents is the release reaction,[1] and the substances secreted during release are products stored in platelet granules and dense bodies.[2,3,18] Morphologic studies have indicated that the release reaction is closely related to physical changes in platelet shape and internal organization.[19] When

platelets are exposed to aggregating agents capable of triggering release, they lose their discoid form and become roughly spherical with multiple pseudopods. The circumferential band of microtubules and masses of hyaloplasmic microfilaments shift toward the central region of the platelet where they encircle closely grouped organelles. During the phase of irreversible aggregation the granular and filamentous elements fuse together, leaving a dense mass inside the cells.

Apposition, fusion and loss of substance from closely grouped granules to elements of the open canalicular system appear to be important components of the platelet release reaction. Evidence that organelles move outward to the cell surface, fuse with it and empty to the outside is meager.[20,21] The concept that individual granules dissolve in the hyaloplasm permitting their contents to diffuse through the cell and surface membrane has been largely dispelled by observations on the specificity of the substances secreted.[1,18] Ultrastructural studies employing electron-dense tracers have suggested that the open channel system of the platelet serves as the conduit for products of the release reaction; portions of granules and dense bodies have been identified in channels after aggregation.[14] Yet, the mechanism of organelle transfer and the fate of the extruded products entering channels have not been clearly defined.

The present investigation has examined the influence of two chemical agents, Cytochalasin B and trypsin, on blood platelets in order to gain new insights into the process of degranulation. Trypsin at low concentrations causes platelet aggregation and stimulates release.[22] At higher concentrations the agent initiates the release reaction but renders platelets inaggregable.[1,2,12,18,22,23] Both high and low concentrations appear to produce similar effects on platelet morphology.[12] Alterations in platelet shape and internal organization are similar to those initiated by other potent aggregating agents, except for the changes noted in the open channel system. Conversion of the tortuous fenestrated channels into long, arc-like or circular tubes is peculiar to trypsin. Ethylenediaminetetracetic acid (EDTA) is the only other chemical agent with a somewhat similar effect on platelets.[24]

Cytochalasin B causes only minor changes in platelet morphology,[10] but strongly influences the capacity of the cells to undergo shape change and alters the pattern of internal reorganization induced by aggregating agents.[5] It does not affect the capacity of platelets to become sticky or secrete substances essential for development of irreversible aggregation, but delays the onset of the release reaction in a concentration dependent manner. The resistance of CB-treated cells

to shape change, pseudopod formation, internal reorganization and onset of release appear to underlie the inhibitory effect of this agent on clot retraction.[5,10,11]

Examination of platelets treated with CB prior to trypsin confirmed the impression gained in previous studies.[5] Despite the potent effects of the proteolytic enzyme, most CB-treated platelets retained a relatively discoid shape. Circumferential bundles of microtubules remained in their usual location in the equatorial plane under cell surfaces. Channels of the open canalicular system were dilated and straightened after CB and trypsin, but were not converted into the concentric or arc-like arrangements seen in cells exposed to trypsin alone. Filamentous elements and mitochondria were well preserved in the hyaloplasmic matrix of treated cells. Granules and dense bodies, however, were virtually absent.

Thus, CB appeared to protect platelets from the drastic changes in form and internal reorganization usually caused by trypsin, but did not prevent contraction of the matrix, disappearance of the storage organelles or alterations in the open canalicular system. Previous investigations have indicated that trypsin acts on the outside surface of platelets and does not enter the hyaloplasm.[1,22,23,25] The excellent preservation of filaments and mitochondria, and the cytochemical demonstration of endogenous peroxidase activity in the dense tubular system after treatment with CB and the proteolytic enzyme indicate that trypsin did not seriously damage the interior of platelets observed in this study. The contraction of the matrix into dense spots is not an indication of platelet cytoplasmic injury, but a peculiar effect of CB on internal reorganization. An identical condensation of filamentous material occurs in CB treated platelets after exposure to all other aggregating agents tested.[5]

Trypsin might have caused granules to move to the platelet surface, fuse with it and empty to the exterior, but no evidence supporting this possibility was encountered. An unusual influence of trypsin on channels of the open canalicular system and interaction between the altered channels and organelles in CB-treated cells seemed more likely. Addition of electron-dense tracers to the treated cells verified this hypothesis. Neither horseradish peroxidase nor thorium dioxide entered the cytoplasm of CB-trypsin-treated platelets, indicating that the cell membrane was intact. Both agents, however, penetrated into channels of the open canalicular system. In some cells the channels containing thorium dioxide were in continuity with incompletely dissolved granules. These findings suggested that trypsin-induced degranu-

lation of platelets is accomplished by fusion of granules and dense bodies with channels of the open canalicular system. The paucity of granular debris in the open channels may have been due to proteolysis of the extruded material by trypsin or to lysis of granules on contact with plasma.

In conclusion, the stabilizing influence of cytochalasin B on disc form and the capacity of trypsin to alter the open canalicular system and selectively remove dense bodies and granules from platelets have been employed in the present investigation to prepare discoid platelets without secretory organelles. Mitochondria, glycogen, elements of the dense tubular system, filaments, microtubules and the cell membrane appeared essentially unchanged after chemical dissection by the two agents; a specific enzyme activity, platelet peroxidase, could still be detected cytochemically in its usual location. Trypsin, in the concentration used, renders plasma incoaguable and causes such profound changes in the exterior coat that the platelets no longer aggregate. However, the platelets appear in excellent morphologic condition despite the loss of secretory organelles and susceptible components of the exterior surface, and it is possible that they survive the drastic treatment as intact, metabolically active cellular units. If so, the degranulated discoid platelet can serve as a useful model for a variety of biochemical, physiologic and immunologic studies *in vitro* and *in vivo*.

References

1. Grette K: Studies on the mechanism of thrombin-catalyzed hemostatic reactions in blood platelets. Thromb Diath Haemorrh 56 (Suppl 195):1–93, 1962
2. Holmsen H, Day HJ, Stormorken H: The blood platelet release reaction. Scand J Haematol (Suppl) 8:1–26, 1969
3. White JG: Origin and function of platelet dense bodies. Series Haematol 3:17–46, 1970
4. White JG: Fine structural alterations induced in platelets by adenosine diphosphate. Blood 31:604–622, 1968
5. White JG: Platelet microtubules and microfilaments: Effects of cytochalasin B on Structure and Function. Platelet Aggregation. Edited by J Caen. Paris, Masson et Cie, pp 15–52, 1971
6. Carter SB: Effects of cytochalasin B on mammalian cells. Nature (Lond.) 213:261–264, 1967
7. Aldridge DC, Armstrong JJ, Speake RN, Turner WB: The structure of cytochalasins A and B. Chem Com 1:26–27, 1967
8. Schroeder TE: The contractile ring. I. Fine structure of dividing mammalian (HeLa) cells and the effects of cytochalasin B. Z Zellforsch Mikroskop Anat 109:431–449, 1970

9. Estensen RD: Cytochalasin B. I. Effect on cytokenesis of Novikoff hepatoma cells. Proc Soc Exp Biol Med 136:1256–1260, 1971
10. Shepro D, Belamarich FA, Robblee L, Chao F: Antimotility effect of cytochalasin B observed in mammalian clot retraction. J Cell Biol 47:544–547, 1970
11. Wessells NK, Spooner BS, Ash JF, Bradley MO, Luduena MA, Taylor EL, Wrenn JT, Yamada KM: Microfilaments in cellular and developmental processes. Science 171:135–143, 1971
12. Hovig T: The effect of various enzymes on the ultrastructure aggregation and clot retraction ability of rabbit blood platelets. Thromb Diath Haemorrh 13:84–113, 1966
13. White JG: The interplatelet zone. Am J Pathol 58:19–29, 1970
14. White JG: A search for the platelet secretory pathway using electron dense tracers. Am J Pathol 58:31–49, 1970
15. Graham RC, Karnovsky MJ: The early stages of absorption of injected horse-radish peroxidase in the proximal tubules of kidney. Ultrastructural cytochemistry by a new technique. J Histochem Cytochem 17:675–680, 1969
16. Novikoff AB, Goldfischer S: Visualization of peroxisomes (microbodies) and mitochondria with diaminobenzidine. J Histochem Cytochem 17:675–680, 1969
17. White JG: Interaction of membrane systems in blood platelets. Am J Pathol 66:295–312, 1972
18. Day HJ, Holmsen J: Concepts of the blood platelet release reaction. Series Haematol 4:3–27, 1971
19. White JG: Platelet morphology. The Circulating Platelet. Edited by SA Johnson. New York, Academic Press, Inc, pp 45–121, 1971
20. White JG, Krivit W: The ultrastructural localization and release of platelet lipids. Blood 27:167–186, 1966
21. Weber AJ, Johnson AA: Platelet participation in blood coagulation: Aspects of Hemostasis. Am J Pathol 60:19–27, 1970
22. Haslam RJ: Role of adenosine diphosphate in the aggregation of human blood platelets by thrombin and by fatty acids. Nature 202 765–769, 1964
23. Schmid HJ, Jackson DP, Conley CL: Mechanism of action of thrombin on platelets. J Clin Invest 41:543–549, 1962
24. White JG: Effects of ethylenediaminetetracetic acid (EDTA) on platelet structure. Scand J Haematol 5:241–254, 1968
25. Markwardt F: Studies on the release of biogenic amines from blood platelets. Biochemistry of Blood Platelets. Edited by E Kowalski, S Niewiarowski. New York, Academic Press, Inc, pp 105–116, 1967

[Illustrations follow]

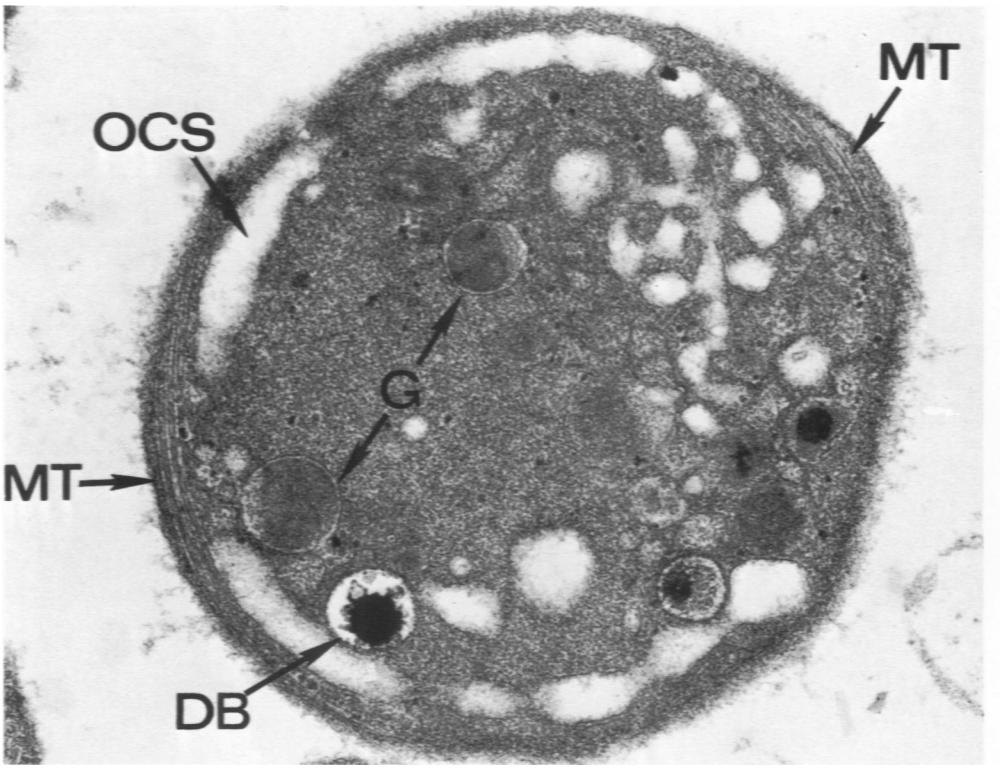
Fig 1—Cytochalasin B (*CB*)-treated platelet. The cell is from a sample of normal C-PRP incubated 30 minutes with CB at a concentration of 25 μg/ml. Platelet discoid shape is preserved, despite some dilatation of the open canalicular system (*OCS*). The circumferential bundle of microtubules (*MT*) remains intact and in its usual location under the cell surface. Granules (*G*), dense bodies (*DB*), and other cytoplasmic organelles appear relatively unaltered. The matrix of the platelet develops a "ground glass" appearance after exposure to CB. Aside from these mild changes, the morphology of CB-treated platelets did not differ significantly from normal cells (× 39,900).

Fig 2—Trypsin-treated platelets. The cells are from a sample fixed 60 minutes after addition of trypsin at 3 mg/ml C-PRP. After exposure to this concentration the sample does not clot, and the platelets no longer aggregate. Trypsin causes platelets to lose their discoid form and become irregular. Organelles are shifted toward the cell centers where they may fuse or disappear. In addition, to these alterations, which are similar to those produced by potent aggregating agents, trypsin causes striking changes in the open canalicular system. Several types of membrane alterations are apparent in the cells of this illustration (× 18,500).

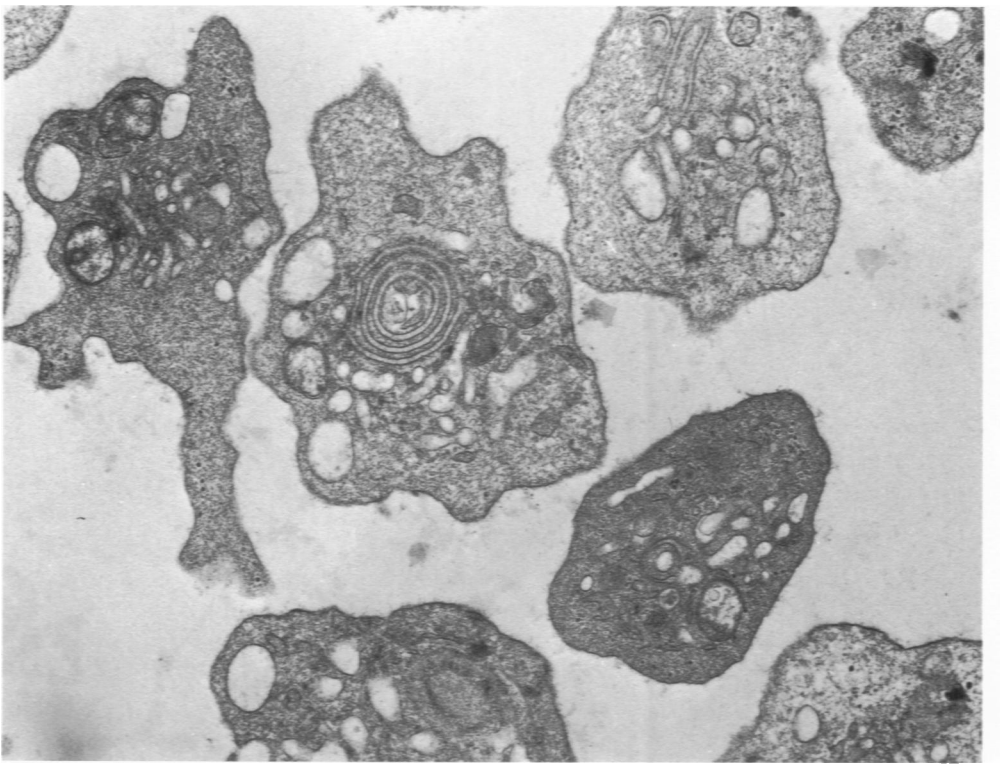
Fig 3 and 4—Trypsin-treated platelets. The cells are from samples exposed to 3 mg trypsin/ml of C-PRP for 30 minutes. In Figure 3 the altered channels of the open canalicular system are arranged concentrically, while in Figure 4 the channels are stacked in an arc-like segment. The arrangement suggests a relationship between the altered channels and membranes enclosing granules (**3,** × 41,500; **4,** × 41,500).

Fig 5A, B, C—Platelets from samples of C-PRP incubated initially with cytochalasin B (25 μg/ml) for 15 minutes, then with trypsin (3 mg/ml) for 30 minutes at 37 C. After exposure to the two agents platelets are slightly irregular due to swelling of the open channel system, but most cells retain a relatively discoid form. The platelets in Figure 5A reveal normal mitochondria (*M*) and occasional lipid droplets (*L*), but are nearly devoid of granules and dense bodies. In some cells remnants of partially dissolved granules (*G*) can be identified. The platelets in Figure 5B and C are discoid and contain circumferential bundles of microtubules in cross section at opposite poles in each cell. Granules and dense bodies are entirely absent in this plane of the section (**A,** × 15,300; **B,** × 42,300; **C,** × 41,500).

Fig 6—The platelet in Fig 6A is from C-PRP exposed to CB and trypsin and demonstrates the contraction of cytoplasmic gel into a filamentous mass (arrow) without loss of disc form. A residual granule (*G*) remains in the treated cell. Platelets in Fig 6B and C are from C-PRP treated with CB and trypsin to which a small amount of thorium dioxide (*TD*) has been added. Particles of electron-dense TD appear in the open canalicular system of the platelets. In Fig 6C TD particles are also present in a partially dissolved granule (arrow) which appears to communicate with a channel of the OCS. The platelet in Fig 6D is from a sample incubated with CB and trypsin to which horseradish peroxidase has been added before fixation. After fixation the sample was incubated for peroxidase activity. Peroxidase is apparent covering the cell surface and lining the OCS. The platelets in Fig 6E and F were treated with CB and trypsin, and incubated for the demonstration of endogenous platelet peroxidase. The sections were not stained with uranyl acetate or lead citrate. At either pH 9 (Fig 6E) or pH 6 (Fig 6F) endogenous platelet peroxidase is demonstrable in the treated cells, and is confined to channels of the dense tubular system as it is in normal cells (**6A,** × 42,000; **6B,** × 37,300; **6C,** × 33,200; **6D,** × 37,000; **6E,** ×33,200; **6F,** × 41,500).

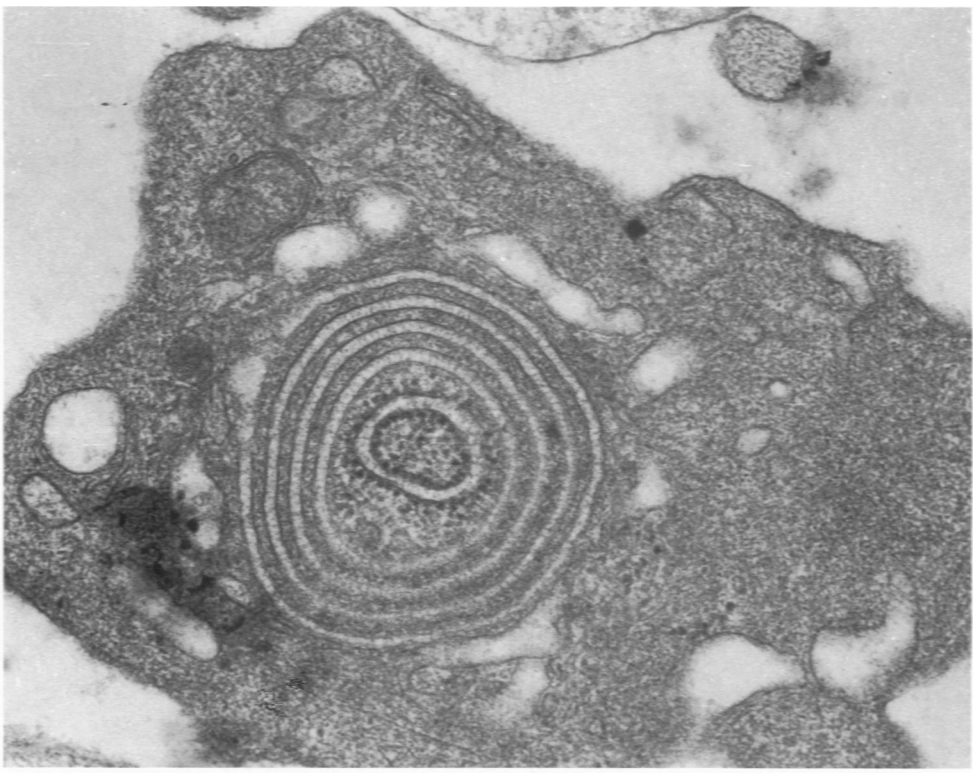


1

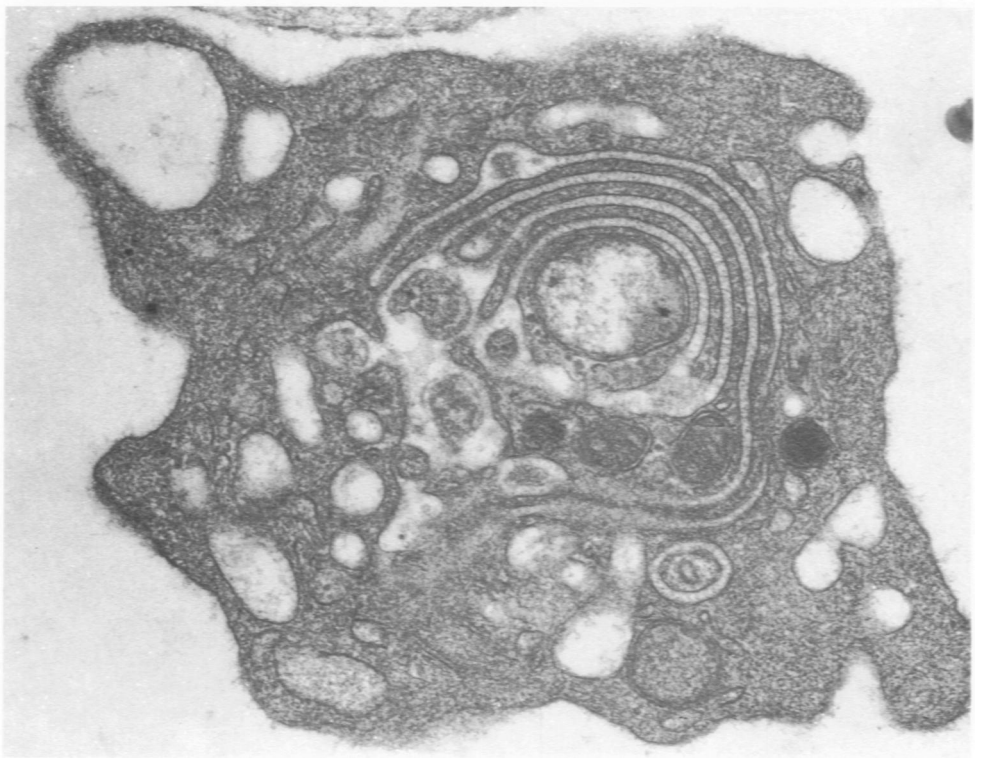


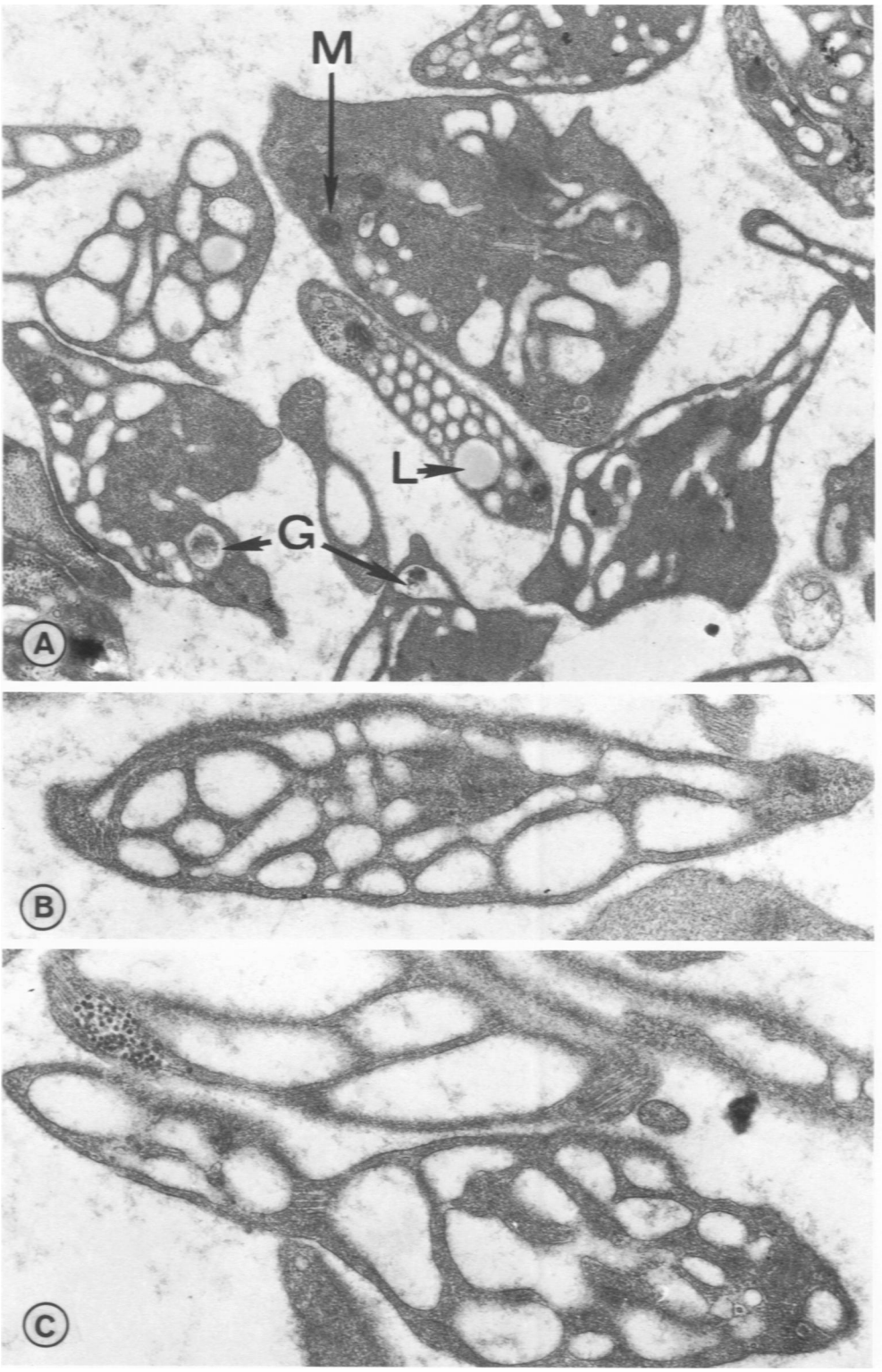
2

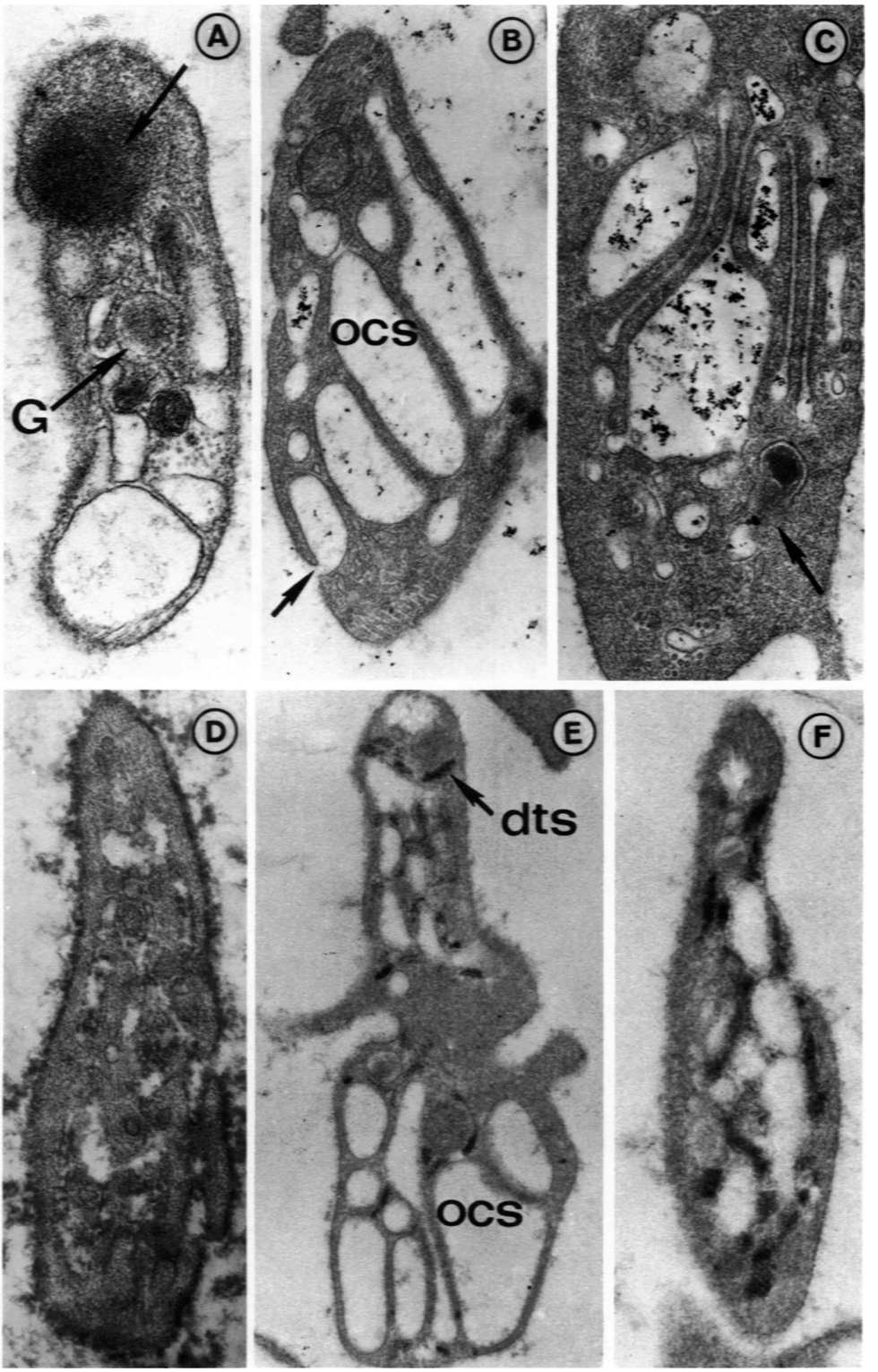
3



4




M
L
G
A
B
C



6