THE ULTRASTRUCTURE OF EARLY PLATELET AGGREGATION *IN VIVO*

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Microthrombi and emboli composed of platelets and leukocytes form within the small blood vessels of mildly traumatized tissues.[1-3] At times thromboembolism is confined to only one vessel.[3] Initially individual platelets exhibit little or no deformity and are clearly discernible in small aggregations. Depending on the severity of the trauma, augmentation occurs as further platelets, leukocytes and divers red cells become incorporated. Alternatively there may eventually be regression, the thrombi disintegrating and the vessels reverting to normal. As these stages are deemed to represent the early phase of intravascular thrombosis, an electron microscopic investigation of the platelet changes was undertaken.

In a study of the carotid body,[4] small platelet clumps were sometimes found in blood vessels. It was realized that the carotid body's rich blood supply facilitated the localization of the microthrombi and emboli caused by the minor trauma incidental to the surgical exposure of the relatively inaccessible carotid body. This technique was utilized in the present study of early platelet aggregation *in vivo*.

MATERIAL AND METHODS

One carotid body was removed from each of 16 cats. The animals were anesthetized with intraperitoneal Nembutal®. A ventral midline incision was made in the neck, the trachea was cannulated and the larynx and pharynx were reflected anteriorly to expose the medial aspect of the bifurcation of the common carotid artery. This artery was then cannulated and the bifurcation and the carotid body were perfused[4] with 5 to 10 ml of either 5 per cent glutaraldehyde in Sorensen's phosphate buffer (pH 7.2 to 7.4) or with 1 per cent buffered[5] osmium tetroxide (pH 7.3 to 7.4). The carotid body was then excised and cut into small blocks which were placed in fresh fixative. After glutaraldehyde fixation for 1 to 4 hours, the blocks were washed in phosphate buffer for 1 to 3 hours, postfixed in 1 per cent osmium tetroxide[5] for 4 to 16 hours, dehydrated in ethanol and embedded in Araldite. After osmium perfusion, fixation was continued for 16 hours. The blocks were then dehydrated in ethanol and embedded in Araldite.

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Thick Araldite sections of the carotid body were cut with an LKB microtome and stained with buffered toluidine blue. These sections were used to localize platelet aggregates. Thin sections were then cut with a diamond knife, stained with lead[6] and uranyl acetate and examined with a Siemens Elmiskop I electron microscope.

RESULTS

Light Microscopy

The general architecture and vasculature of the carotid body was as previously described.[4,7–9] Within some of the numerous vessels were isolated platelets, leukocytes and red cells. Several vessels contained small clumps of platelets (Fig. 1) occasionally associated with leukocytes which were either free in the lumen (Fig. 2) or attached to the vessel wall.

A few of the isolated platelets were closely applied to the endothelial surface. Numbers of platelets displaying little alteration in shape were loosely aggregated in both small and large clumps (Figs. 1 and 2). In the larger aggregates, the platelets were often more closely applied to one another (Fig. 3), variation in shape being more difficult to discern. A few of the small vessels were partially or completely blocked by platelet aggregates. Within the platelets, cytoplasmic granules were clearly visible but there was no evidence of degranulation[10–14] or displacement of the granulomere.[15]

Widespread thrombosis and vascular occlusion were never found. In the tissue surrounding the carotid body of one cat there were thrombi in some large vessels and also an extravascular platelet mass.

Electron Microscopy

Unagglutinated platelets were usually ovoid or elliptical in section and without pseudopodial projections. Structurally they conformed to the general description given by David-Ferreira[11] and other authors.[16] The plasma membrane consisted of a trilaminar membrane (75 to 80 Å wide) and on its surface were small accumulations of amorphous material forming strands or granules (Fig. 4) similar to those observed on endothelium.[17]

The granulomere (chromomere) contained mitochondria and dense granules. The granules (0.1 to 0.4 μ) were round or oval and rarely lobed. Usually their contents, amorphous and of uniform density, filled the space limited by the trilaminar membrane. A few contained irregular areas of varying density. Glycogen granules were scattered in the cytoplasm though occasionally they were concentrated in large aggregates in areas of reduced cytoplasmic density encircled by mitochondria and dense granules. The plasma membrane displayed several tubular invaginations (Fig. 4) more elongated than Palade's micropinocytotic

vesicles and caveolae of endothelium.[18] Each had a narrow neck and was not closed by a thin membrane as in the endothelial cell.[19] The amorphous or granular contents were of light density. The invaginations frequently branched or fused with other similar vesicular structures in the granulomere and could be regarded as elements of the agranular endoplasmic reticulum though on occasion their arrangement resembled a small Golgi apparatus.

As many as 30 microtubules (200 to 250 Å in diameter) appeared at the poles of the platelet profiles (Fig. 4) in both osmium and glutaraldehyde fixed material. Most occurred as a bundle within an area of reduced cytoplasmic density but a few lay between neighboring cytoplasmic organelles and at times dense branched fibrils (30 to 50 Å wide) were situated between microtubules in the main bundle. In transverse section an occasional microtubule contained a dot in the center of its lumen.

Up to 4 siderosomes [11] were present in a single platelet profile. Dense-walled [19,20] or coated vesicles [21] and caveolae were infrequent findings. A few round vesicles (as much as 0.15 μ in diameter) contained small amounts of granular or amorphous material, and occasionally 1 to 4 small vesicles (350 to 500 Å in diameter). They were possibly small multivesicular bodies.

Single platelets in apposition with endothelium displayed little or no observable change. They were separated by a space of 200 to 500 Å and on occasion, what appeared to be villous processes from the endothelium, intervened between the platelet and the endothelial cells. Sundry dense granules had a drumstick configuration.

Small Platelet Aggregates. In small thrombi, platelets were loosely clustered in the manner of pins adhering to a magnet. The zones of contact between platelets were not extensive and deformity of platelets was minimal. A few platelets with larger areas of apposition displayed certain modifications of shape (Fig. 5). Only occasionally were pseudopodial projections seen. There was no evidence of degranulation or rearrangement of granulomere location. A few dense 'drumstick' granules appeared but for the most part cell constituents were unchanged. Only a few platelets lay in contact with erythrocytes or leukocytes. Nearly all were elliptical in shape. Flattening of the surface contour or indentation of the platelet membrane occurred (Figs. 5 to 8), however, even with the readily deformable red cells.

Small platelet aggregates were often embedded in amorphous material within the lumen. Hazy fibrillary or amorphous substance surrounded other platelets (Figs. 5 to 9), often radially arranged along the plasma membrane. At times this was a distinctive feature constituting a zone 500 to 1,000 Å wide. Not infrequently there was a noticeable increase

in the amount of material between platelets lying quite close to one another (Fig. 9) or between platelets and other cells (Figs. 6 to 8); regions of increased density were also present (Fig. 10). Evaluation of the significance of this unidentified material was complicated by the perfusion technique used in fixation.

On the platelet surface a few pseudopodia were found (Fig. 11) but these were infrequent (cf. Figs. 5 to 8). Polypoid pseudopodia containing a small amount of cytoplasmic matrix and an occasional organelle were uncommon (Fig. 12). These hydropic blebs could have been artifacts but they occurred despite good preservation of neighboring tissue. Sections through such structures were encountered close to platelet clumps; some arose from the endothelium or even from erythrocytes.

In several platelets the dense granules appeared different from those in unagglutinated platelets. These often exhibited an area of increased density, usually eccentrically situated (Fig. 13). A number of granules were elongated and the dense zone extended into these regions. Dumbbell shapes and strap-like forms (up to 1.3 μ in length) without special orientation within the platelet were seen.

The elongated areas of increased density in both the drumstick and oval forms frequently displayed cross striation (Figs. 13 and 14) consisting of thin dark bands (40 to 50 Å) alternating with broader bands (120 to 130 Å) of lighter density. The periodicity ranged from 150 to 200 Å but generally the banding ranged from 170 to 180 Å. Within an individual platelet, more than one granule exhibited this structure (Fig. 13) but it did not abut on the platelet surface.

Closely Packed Platelet Aggregates. Large aggregates of platelets were usually associated with closer packing and more noticeable alteration in shape. Platelets on the surface of such clumps were not necessarily or excessively deformed. A few exhibited pseudopodia which contained fibrillary cytoplasmic matrix but no microtubules. At times hyaloplasm in such platelets appeared unusually prominent. Fibrils were not observed in the pseudopodia or in the granulomere though they were present in the villous projections from the lumen surface of the endothelium. Platelets more centrally placed formed a closely packed mosaic (Figs. 15 and 16). Plasma membranes were intact except for occasional hydropic blebs. Platelets were usually separated by distances of 200 to 300 Å and indefinite radially arranged fibrillary material appeared to bridge the gap between some of the adjoining platelets. Small quantities of dense material or cytoplasmic fragments were also noted between platelets.

Unstriated strands of dense material (30 to 100 mμ wide) resembling incompletely polymerized fibrin [10] occasionally appeared between platelets or on the surface of thrombi (Fig. 17). Large aggregates of glycogen

granules often lay near the center of the platelet (Figs. 15 and 16) and were associated with irregular membranous profiles, though the granules frequently stained faintly. Some platelet profiles contained no granules or mitochondria. Tubular invaginations of the plasma membrane persisted and not infrequently within them was a small dense amorphous body (Fig. 16) 300 to 500 Å in diameter. Also within an occasional platelet a body measuring up to 0.35 μ in diameter, contained irregularly collapsed membranes but no myelin figures. In some platelets loosely arranged amorphous material (Fig. 17) lay within vacuolar spaces (0.5 to 2 μ in diameter). Many were not much larger than the dense granules but in large aggregates ranged up to large irregularly shaped lacunae closely resembling the phagocytic vacuoles associated with the uptake of antigen–antibody complex.[22] Less frequently they were observed within individual platelets adhering to leukocytes. Dense granules were not so numerous and many were of reduced density. No evidence of granule extrusion was seen though in a few platelets, large vacuolar spaces lighter in density than the granules communicated with the exterior. Elongated or drumstick granules were also present. Some platelet profiles appeared to be of reduced density.

Small myelin figures on the surface of or between platelets were infrequent and appeared to be arising from or at least to be attached to the plasma membrane (Fig. 18).

In clumped platelets, microtubules were still evident. In some instances the microtubules appeared to be coursing the periphery of the platelet in the hyalomere. In several platelets as many as 4 groups of microtubules were seen. In others, microtubules appeared to be more centrally placed within the granulomere but this could have been caused by the plane of section; for the most part, microtubules were not common in this situation. The convoluted, crisscross or radial course described in separated platelets was not observed.[23]

In an extravascular platelet mass, cell borders persisted but at the periphery numbers of platelet profiles of all sizes were empty except for amorphous cytoplasmic matrix. The platelets were more irregular in shape (Fig. 19) than in the intravascular thrombi. Dense granules were rare though large intraplatelet lacunae frequently contained granular material. In the intercellular spaces dense amorphous material was abundant but no periodicity was evident. Within neighboring vessels clumped platelets lay in contact with distinct strands of this material (Fig. 17) but again there was no cross striation.

Leukocytes. Leukocyte sticking and emigration were observed in several vessels and not infrequently platelets were adherent to them. Eosinophils participated in the reaction. These contained within their

granules bizarre lamellated structures similar to those described in cat eosinophils.[24,25]

Endothelium. The fenestrated vascular endothelium in the normal carotid body has been described elsewhere.[4,9] In these experiments, the endothelium was for the most part not demonstrably altered. Platelets were frequently in apposition with the endothelium in those vessels partially or completely blocked by thrombus. They were not invariably adherent and the endothelium was not unusual. There was no increase in the incidence of the micropinocytotic vesicles of the type described by Palade,[18] or the dense-walled [19,20] or coated variety.[21] A few leukocytes were adherent to endothelium or in the process of emigrating through endothelial stomas or lay external to the endothelium. An occasional platelet was also seen external to the endothelium and on one occasion a platelet covered a stoma. No evidence of phagocytosis of platelets by endothelium was observed.

In the tissue containing the extravascular thrombus, the endothelium of a few vessels appeared disrupted, fragmented and absent in places. Several endothelial cells contained multiple macropinocytotic vesicles.

Discussion

Unagglutinated Platelets. Platelet fine structure has been reviewed by David-Ferreira.[11] More recently, an annular bundle of microtubules analogous to the marginal band of nucleated erythrocytes [26,27] has been demonstrated in the hyalomere.[28,29]

The coated[21] or dense-walled vesicles [19,20] believed to be associated with protein uptake, have not previously been observed but their presence is not unexpected in view of the ubiquitous nature of such vesicles and the extensive metabolic activity of platelets.[30,31] A morphologic and functional relationship of the vesicles and multivesicular bodies has been proposed.[32]

Early Platelet Aggregation. In early thrombus formation in the rabbit ear chamber there is no deformity of platelets and in the present study, the earliest stage of clumping was not associated with any significant change in the shape of the platelets. The occasional pseudopodium or spike on unagglutinated platelets could have been the result of surgical trauma. In the initial adhesion of platelets, however, pseudopod formation played no role. The dendritic forms observed in *in vitro* experiments [11,33] were absent. The finding of small intraluminal platelet aggregates surrounded by a considerable quantity of amorphous material or the apparent coating by a small quantity of amorphous or fibrillary material could be significant (Figs. 5 to 10). It is conceivable that this material was concerned with the initial adhesion of platelet to platelet

or platelet to leukocyte. For some considerable time it has been thought that platelet clumping might be induced by the adsorption to the platelet surface of flocculated plasma proteins, possibly fibrin.[13]

It has been suggested that trace amounts of thrombin might lead to clotting of plasma fibrinogen adsorbed to the platelet surface.[13] It is perhaps pertinent that some materials which induce platelet clumping[3,34,35] are known to produce a fall in plasma fibrinogen.[36,37] The release of adenosine diphosphate (ADP) which causes platelet agglutination *in vitro*[38–40] and *in vivo*[3,40,41] has been regarded as an essential part or the possible "final common pathway" in the mechanism.[42] The manner in which ADP or other chemical mediators induce the stickiness of platelets is still obscure, and the significance of the amorphous material about many of the platelet clumps is also unresolved.

Viscous Metamorphosis. After aggregation platelets become tightly packed, the process being referred to as viscous metamorphosis. Even in loose aggregates, when adjoining surfaces of platelets come into closer apposition, there is an alteration in shape, however mild. Of the formed elements in the blood, platelets are the least and red cells the most readily deformable.[43] Yet even erythrocytes appear to indent the platelet surface or alternatively the platelet applies itself about the erythrocyte. In larger clumps the platelets become tightly packed giving the mass a hyaline appearance in light microscopy. It is as if they had been pulled or pushed together by some force. The mechanism by which platelets attain this appearance is undetermined. A physicochemical change on the surface of the platelets is a possible mechanism. Alternatively the mechanism may be related to the ability to spread, a property possessed by platelets. Recently North[44] demonstrated that spreading on glass surfaces is analogous to phagocytosis and that both processes are adenosine triphosphate (ATP) dependent. Platelets also possess remarkable phagocytic properties[11,34,45,46] and are known for their high ATP content[47,48] as well as their pronounced tendency to spread.[11] Therefore the close packing of platelets could be the result of platelets' propensity for spreading on one another. In well developed thrombosis the outlines of platelets are complex and pseudopodia are more in evidence than in early agglutination, the inference being that the relationship of one platelet to another is not static but in a state of flux.

Dense Granules. The dense granules are believed to contain platelet factor 3.[48] Recently White and Krivit[49] have indicated that the granules may be transformed into the lamellated configuration of lipid; this was not observed in the present investigation. Such structures were seen only on the surface of or between platelets (Fig. 18).

The drumstick alteration of and the periodicity observed within the

dense granules appear to be relatively early changes in platelet aggregation. The drumstick forms have been classed as one of the features of von Willebrand's disease[50] but they have been observed in 'normal' platelets separated prior to fixation.[11] The cross striation, frequently associated with dense zones in these granules whether they are round, oval or with elongated extensions, has been twice mentioned but only briefly in *in vitro* experiments.[49,51] Their occurrence *in vivo* has not been previously noted. Johnson[51] claimed that the 240 Å periodicity was consistent with that of fibrin which varies from 200 to 250 Å.[52–55] The periodicity of the crystalloid pattern observed in this study, however, was 170 to 180 Å. As the periodicity of polymerized cat fibrin is not known, the striated material within the granules cannot necessarily be regarded as fibrin. If it were fibrin, it could be argued that fibrin can be deposited outside the platelet through the mechanism of platelet degranulation. Fibrin strands about small intravascular thrombi, however, were never continuous with dense granules and were usually devoid of periodicity. Rearrangement of the fibrils to preferred positions so as to produce periodicity seems to be a late stage in fibrin production.[54,55]

Thrombosis

The study of early platelet agglutination and thrombosis is fundamental in any appraisal of the controversial role of thrombosis in the pathogenesis of atherosclerosis. Electron microscopic studies have been made of thrombi due to gross trauma[56–58] and also of platelet agglutination following the intravascular administration of thrombin[10] and sodium stearate.[59] The ultrastructure of agglutinated platelets *in vitro* has also received considerable attention[14,22,46,60–63] but the early stages of trauma-induced thrombosis have not been previously investigated. The changes have been studied in the rabbit ear chamber[3] and they appear to correspond to those observed within the vessels of the carotid body.

Since degranulation and fibrin formation were little in evidence in the initial aggregation and early packing or viscous metamorphosis of platelets, it is believed that the changes observed represent early stages of thrombosis. Although platelets may stick to endothelium[3,48] unless the endothelial cells are severely damaged,[64] sticking to leukocytes appears to be a dominant feature. The mechanism of adhesion of platelets to endothelium, to leukocytes and to one another appears to be a phase distinct from but continuous with that of packing or viscous metamorphosis but the role of plasma proteins and the surface properties of platelets need further investigation.

The later stages of viscous metamorphosis may be studied by examining the spreading properties of platelets *in vitro;* there is need, however,

for considerable caution in any interpretation of experiments on platelets *in vitro*. Electron micrographs of 'normal' platelets separated prior to fixation frequently reveal by their bizarre shape that they have already undergone considerable morphologic and possibly biochemical change as the result of environmental alterations. Eccentric displacement of the chromomere has been described as an intermediate stage of viscous metamorphosis *in vitro*.[15] This was not observed in the thrombi appearing in the carotid body; it is possible that this may be a feature of a more advanced stage than that studied.

Summary

An electron microscopic investigation was made of trauma-induced thrombosis in the carotid bodies of 16 cats. Initially platelets were loosely aggregated or adherent to leukocytes and displayed little or no distortion in shape. Amorphous or fibrillary material frequently surrounded or covered the surface of these platelets. Subsequently in the stage of viscous metamorphosis, the platelets tended to apply themselves to neighboring cells with the result that close packing and distortion of shape become evident. The dense granules exhibited localized eccentrically placed densities which often had a cross striation with a periodicity of 170 to 180 Å. Strap-like and drumstick forms of the granules were likewise cross-striated. Displacement of dense granules was not observed and degranulation and fibrin formation were little in evidence. In an advanced thrombus, platelet distortion with considerable pseudopod formation was more prominent than in earlier phases of development.

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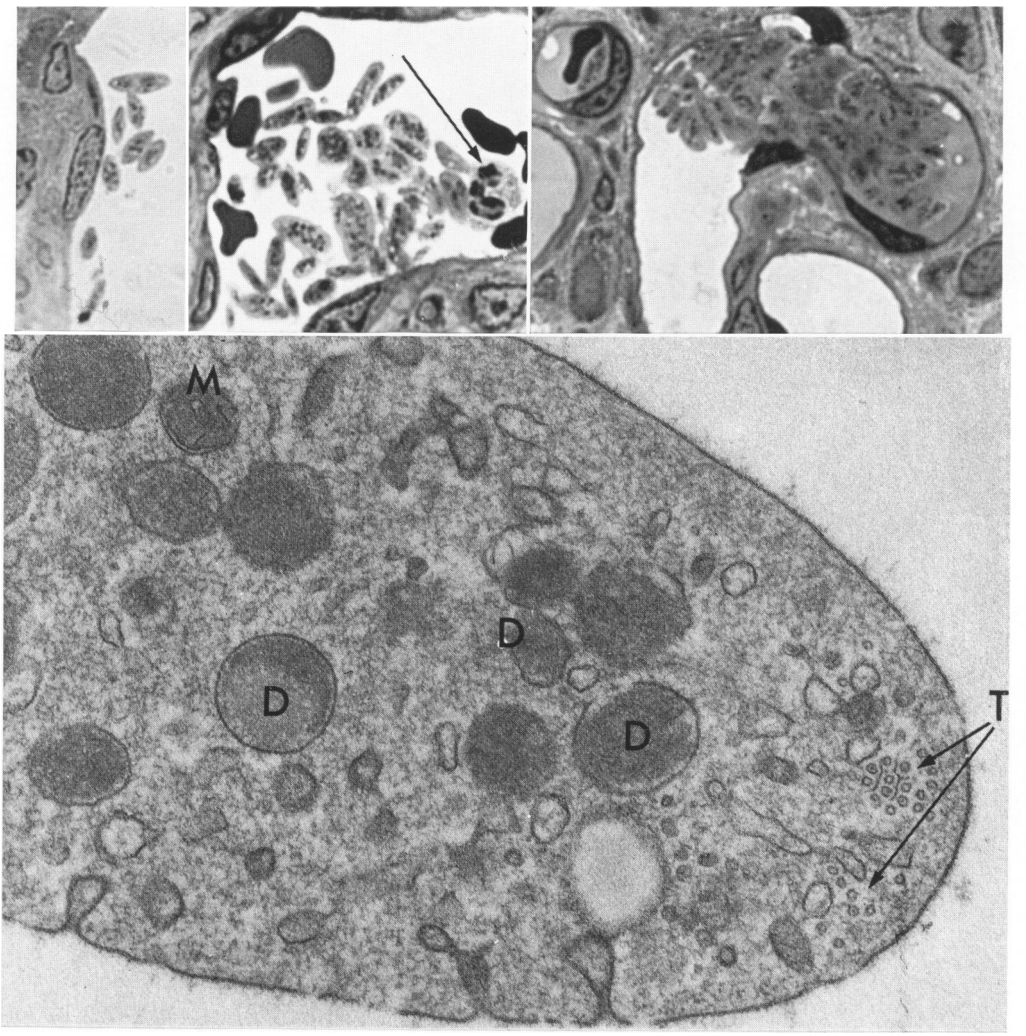


FIG. 1. A small platelet aggregate. Thick Araldite section, toluidine blue stain. × 1,600.

FIG. 2. Platelets are loosely aggregated in a vessel in the carotid body. The granulomeres of the platelets are not displaced. A leukocyte is indicated by the arrow. Thick Araldite section, toluidine blue stain. × 1,600.

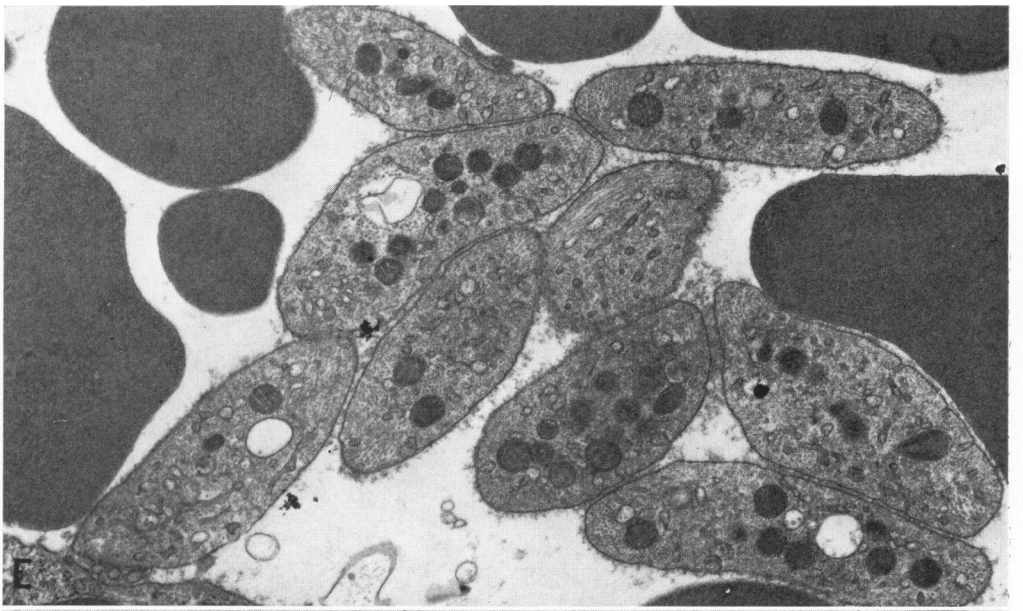
FIG. 3. Platelets are closely aggregated; a single platelet appears in the vessel at the top left. Thick Araldite section, toluidine blue stain. × 1,600.

FIG. 4. A portion of a platelet contains dense granules (D), a mitochondrion (M), invaginations of the plasma membrane and numerous vesicular structures. At the pole of the section are microtubules (T), a few of which contain a central dot. Several are separated by a thin branching line. Glutaraldehyde fixation. × 60,000.

FIG. 5. Aggregated platelets exhibit slight deformity. Amorphous material surrounds the platelets and appears in the intervening spaces. Several red cells surround the aggregate. Endothelium (E). Glutaraldehyde fixation. × 15,000.

FIG. 6. Aggregated platelets are closely applied to one another and lie close to endothelium (E). Platelets are deformed and an amorphous material is evident on their surfaces. One platelet contains many pale-staining glycogen granules. Osmium fixation. × 15,000.

FIG. 7. A platelet lies in apposition with an endothelial cell (E), a leukocyte (L) and a red cell (R). There is an accumulation of amorphous material in the intervening spaces. Drumstick granule (D). Glutaraldehyde fixation. × 29,000.



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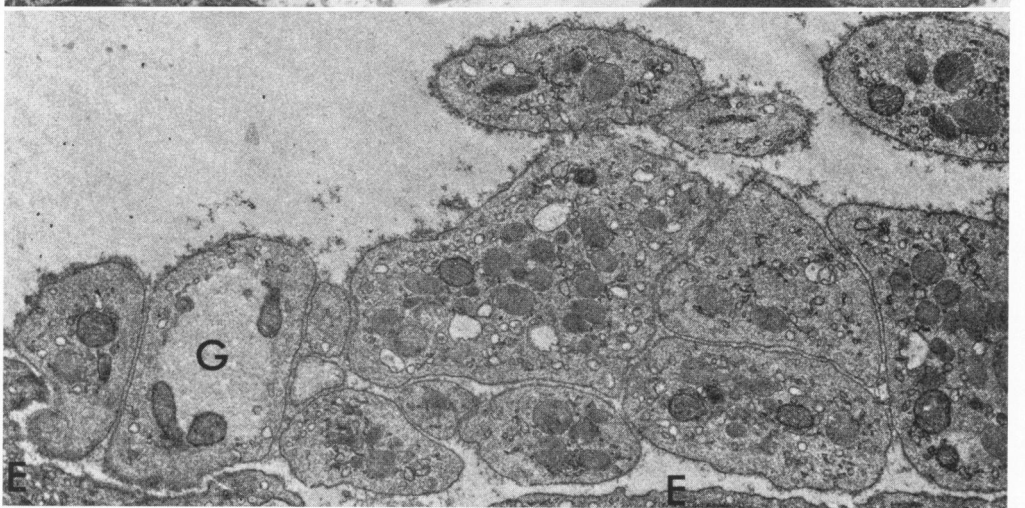

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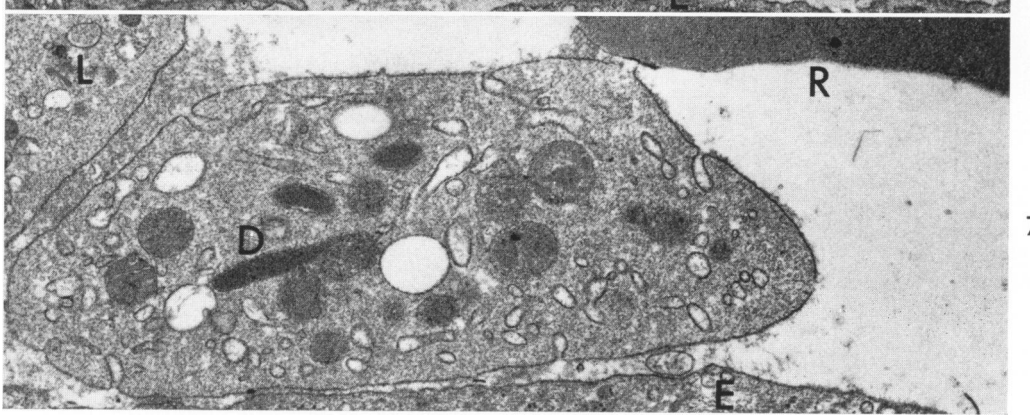

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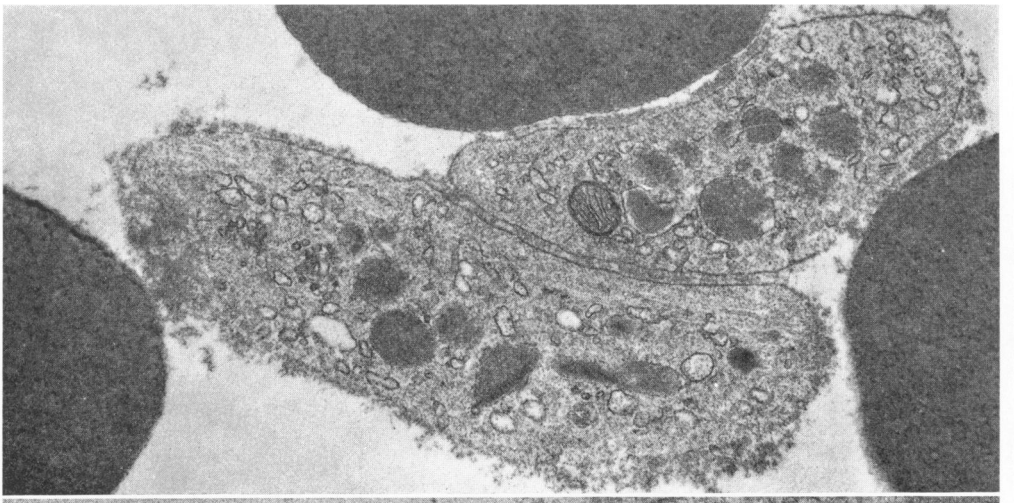
FIG. 8. Two platelets adhere to one another and to neighboring red cells. There is dense amorphous material in the intervening spaces. The platelet above appears to be applying itself to an erythrocyte. Osmium fixation. × 25,000.

FIG. 9. Fibrillary and amorphous material is applied to the surface of and lie between aggregated platelets. Pale-staining glycogen granules (G), mitochondria (M), and dense granules (D) with patchy areas of reduced density are shown. Osmium fixation. × 60,000.



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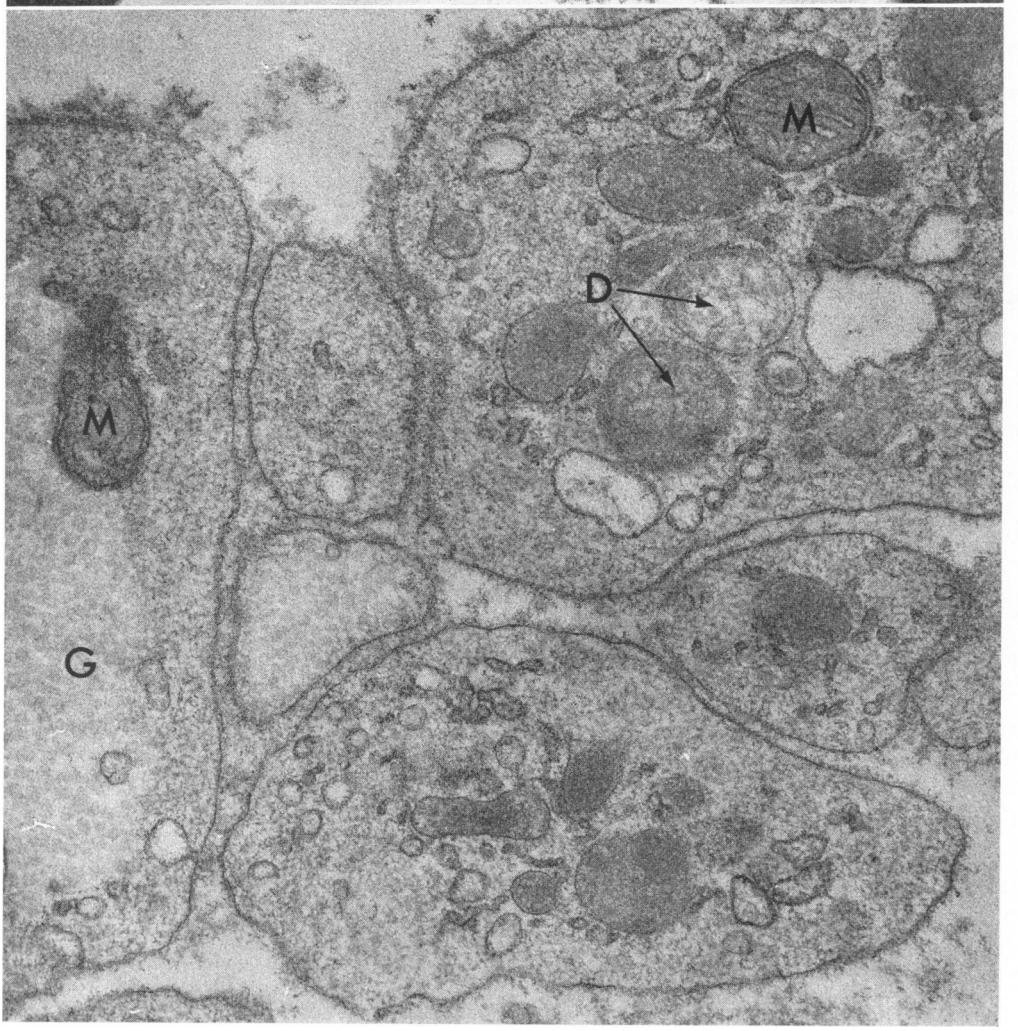

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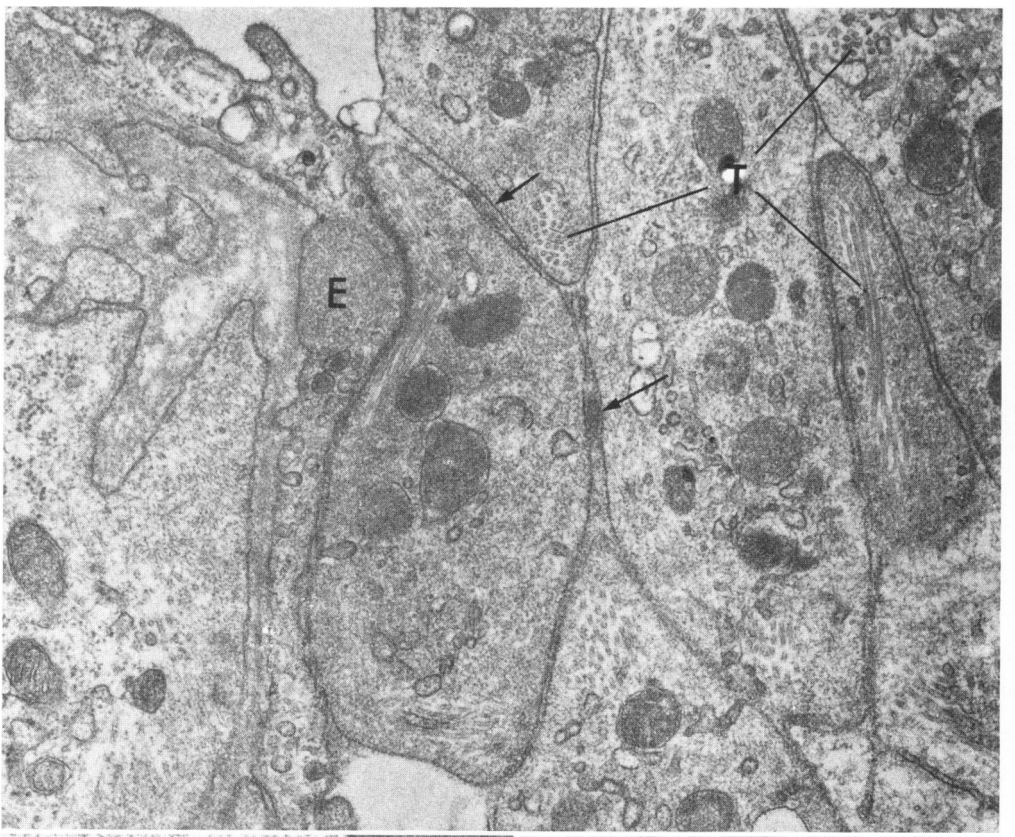
FIG. 10. Aggregated platelets lie in apposition with endothelium (E). Microtubules (T) appear in the platelets. An amorphous material is evident between platelets and between a platelet and the endothelium. Increased density of the material is indicated by the arrows. Glutaraldehyde fixation. × 30,000.

FIG. 11. Pseudopod formation on the surface of a platelet consists of hyalomere only. Glycogen granules are indicated by arrows. Glutaraldehyde fixation. × 25,000.

FIG. 12. A hydropic bleb on a platelet surface. Glutaraldehyde fixation. × 45,000.



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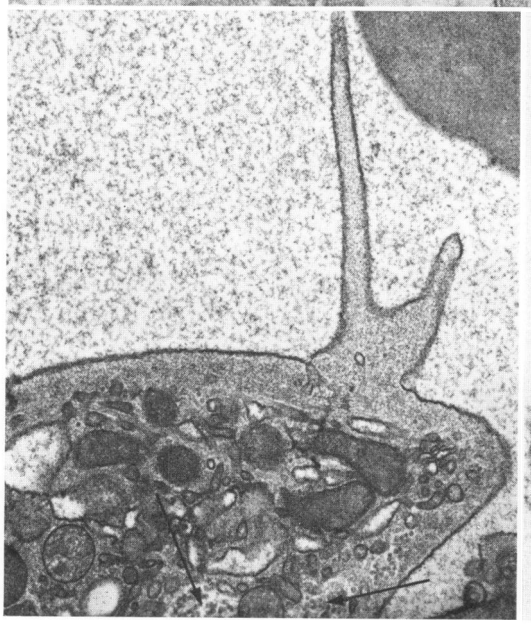

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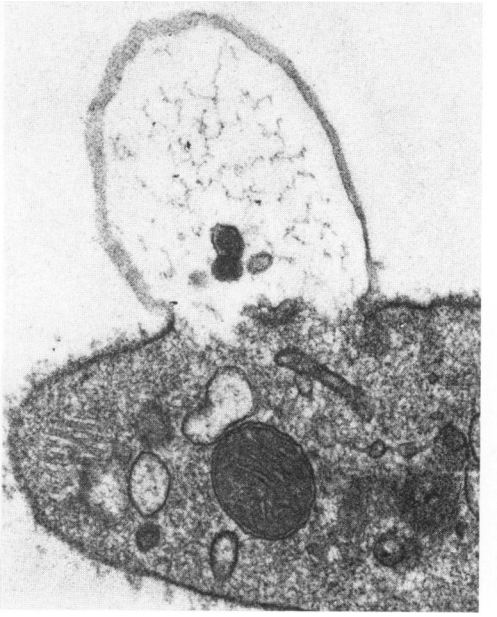

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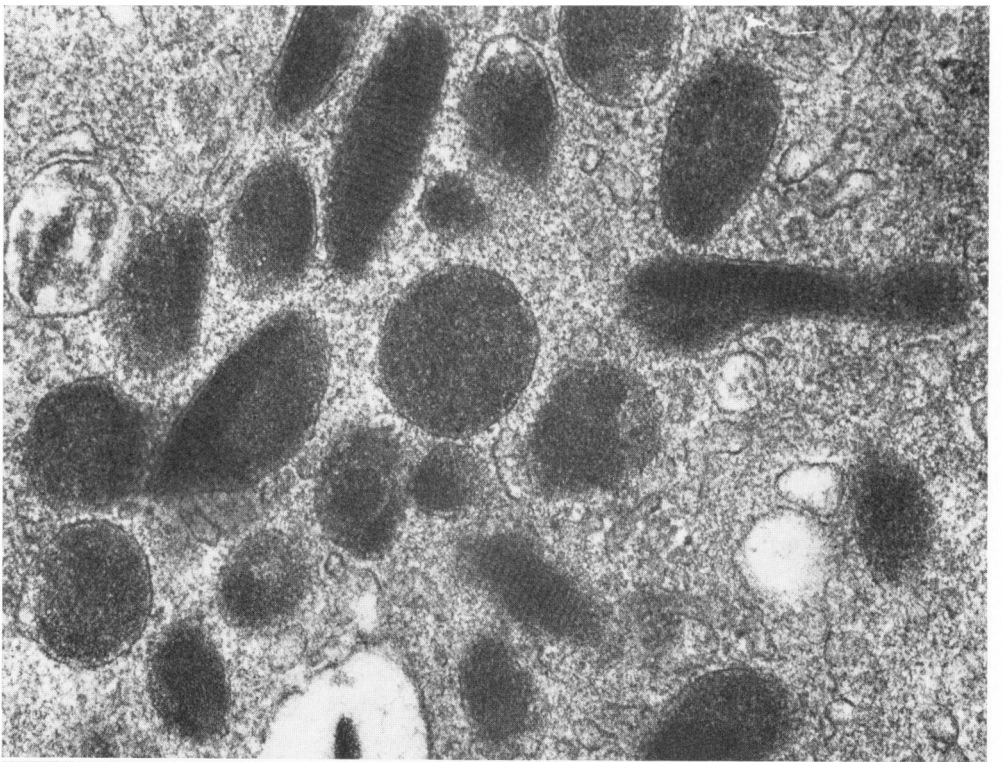
FIG. 13. Dense granules are evident in a platelet aggregate. The granules exhibit cross striation. Osmium fixation. × 60,000.

FIG. 14. Cross striation is evident in an elongated dense granule. This platelet is separated from the endothelium (E) and another platelet (P) by spaces (arrows) containing abundant amorphous material. Osmium fixation. × 90,000.



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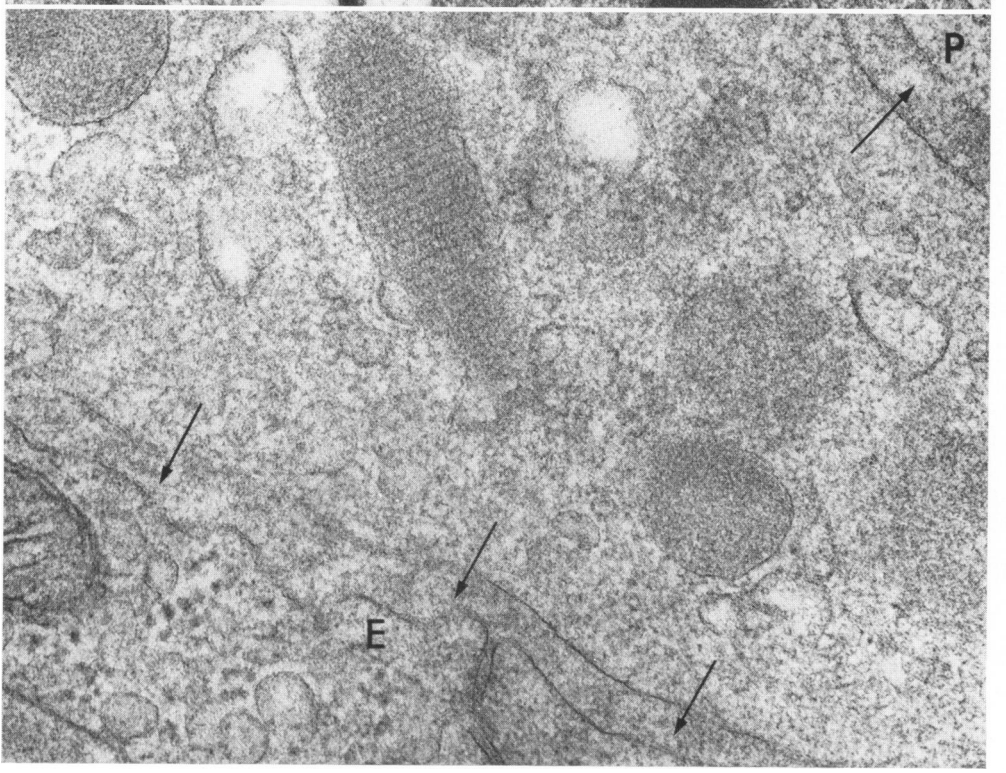

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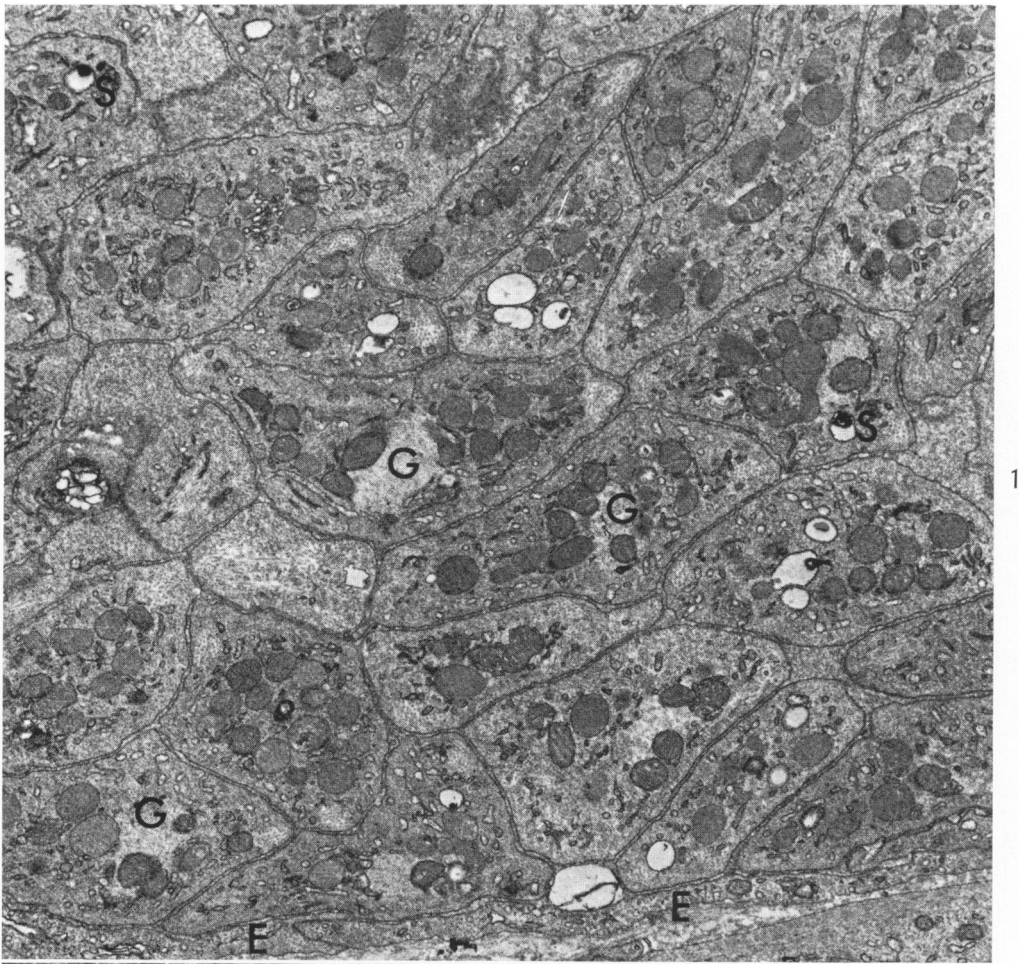
FIG. 15. Platelets are arranged in a closely packed mosaic. Cytoplasmic organelles are shown. Zones of glycogen granules (G). The granules are pale staining possibly due to utilization. Endothelium (E); siderosomes (S). Glutaraldehyde fixation. × 15,000.

FIG. 16. Platelets in a thrombus. Pale-staining glycogen granules (G) are encircled by dense granules and mitochondria. Arrows indicate vesicular structures containing small densities. Glutaraldehyde fixation. × 30,000.



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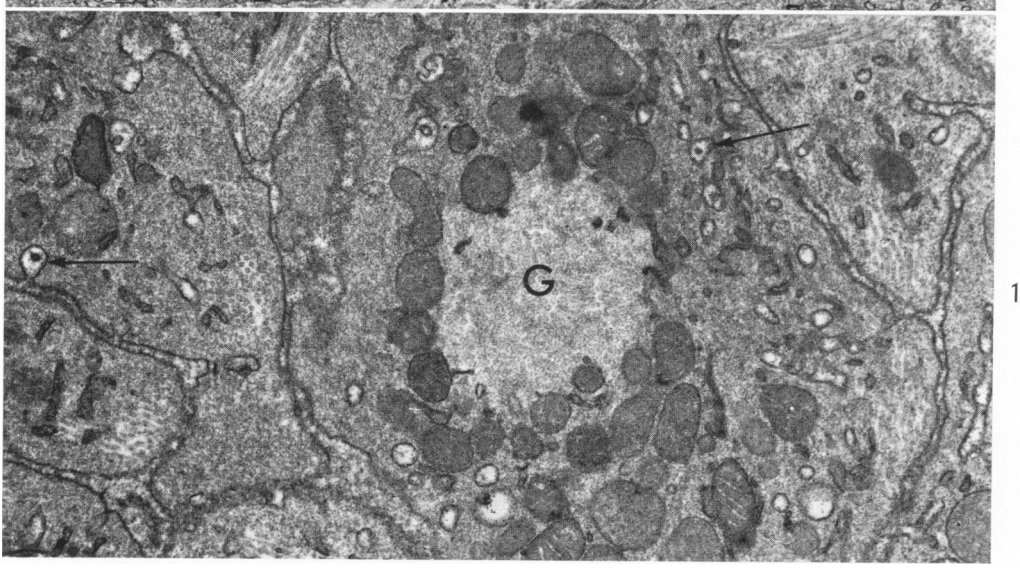

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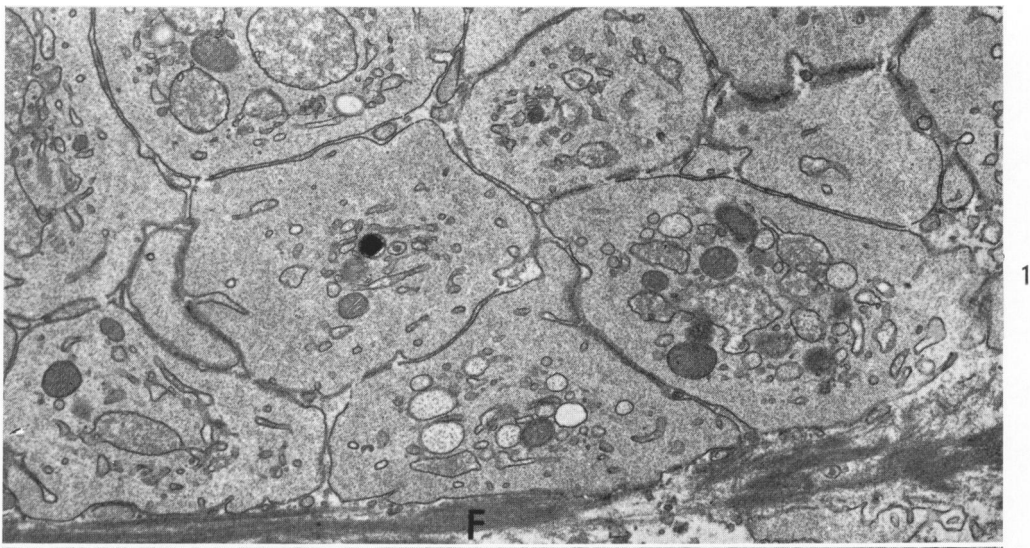
FIG. 17. Platelets in a more advanced thrombus are associated with dense strands of incompletely polymerized fibrin (F). Within the platelets the large vacuolar spaces contain irregularly distributed amorphous material. The contours of the platelets are more irregular than those shown in Figure 15. Glutaraldehyde fixation. × 30,000.

FIG. 18. A lamellated structure, possibly of lipid nature, lies between two platelets. Microtubules (T); pale-staining glycogen (G). Glutaraldehyde fixation. × 60,000.

FIG. 19. An extravascular platelet mass is accompanied by partially polymerized fibrin (F). The irregularity of platelet outlines may be compared with those shown in Figure 15. Many platelet profiles contain only cytoplasmic matrix. The large lacunae (L) contain irregularly distributed amorphous material. Glutaraldehyde fixation. × 15,000.



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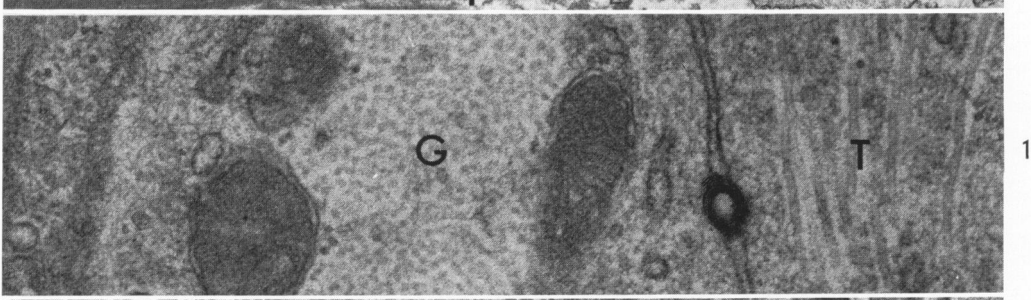

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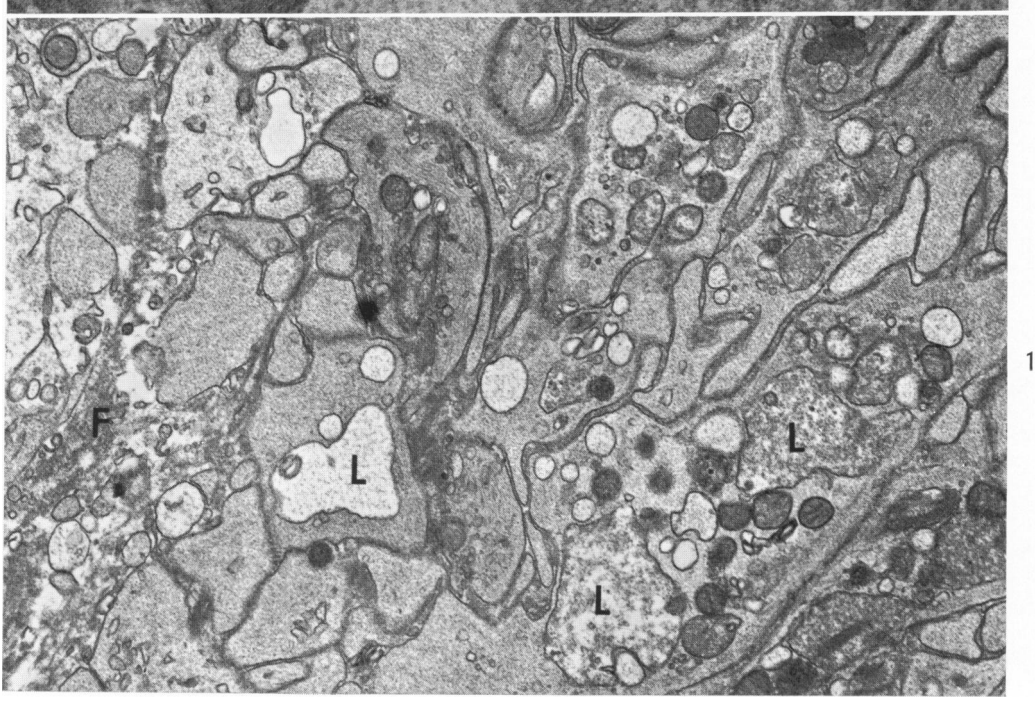

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