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The Role of the Intrinsic Fibrinolytic System in the Prevention of Stasis Thrombosis in Small Veins

An Electron Microscopic Study

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THE INFUSION of mammalian serum into animals produces a transient state of hypercoagulability during which blood trapped in static areas in large vessels coagulates, forming grossly visible red thrombi.[1] These closely resemble the red tail of a natural thrombus, which consists primarily of fibrin and erythrocytes; platelet masses, although present, are much less numerous than in other parts of the thrombus. In a previously reported experiment,[2] Wessler *et al.* noted that an infusion of active serum factors capable of producing a complete thrombus in an isolated segment of the jugular vein of the hamster had no apparent thrombogenic effect in the cheek pouch when the latter was simultaneously clamped; the blood in the small vessels, which are less than 50 μ in diameter, remained fluid when viewed with a light microscope using transillumination. These findings were in keeping with the common observation that experimental traumatic injury to the vessels of the microcirculation regularly causes platelet accumulation but rarely produces coagulation thrombi even when the platelet mass completely obstructs the vessel.[3] The relative immunity of the very small veins to thrombosis in man is also borne out by the infre-

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quency with which these vessels are involved in patients with extensive phlebothrombosis or after trauma.

Although Virchow [4] observed that capillary blood is always fluid and incoagulable, the suggestion that this might be due to the fibrinolytic activity of the vessel wall was first demonstrated by Mole.[5] He reported an inverse relation between the vessel size and the fibrinolytic activity of the contained blood of cadavers. Astrup [6] identified this activity with tissue plasminogen activator which Todd,[7] using histochemical fibrin plate techniques, localized in the endothelium. Further work done by Kwann [8] and Warren [9] has confirmed this finding.

With this in mind, and using the electron microscope as a sensitive detector of fibrin, a study was undertaken to determine the effect of the intrinsic fibrinolytic system in preventing stasis thrombosis in the small veins. The results of these experiments confirm that the development of a serum-induced venous stasis thrombus is directly related to the size of the vessel involved. They also indicate that the relative absence of thrombi in the smaller vessels is in large measure attributable to a relative or absolute increase in fibrinolytic activity, since epsilon amino caproic acid (EACA), an inhibitor of plasminogen activator, results in a marked increase in the amount of thrombus formed under these conditions.

Methods and Materials

Male Sprague-Dawley rats, each weighing 250–500 gm. and fed ad libitum, were anesthetized with intraperitoneal sodium pentobarbital. The thoracic inferior vena cava was exposed, and loose ties were placed in position around the abdominal portion of the vena cava, the hepatic artery, and the portal vein. Meticulous care was taken not to touch or otherwise injure that portion of the vena cava to be studied. In 3 rats, an intravenous injection of a standard dose (0.75 ml./100 gm. of body weight) of fresh homologous serum was followed within 30 sec. by ligation of the loosely tied vessels and the placement of a neurosurgical clip at the junction of the vena cava and right atrium, thus insuring complete stasis in the thoracic caval segment. After 10 min., during which the body cavity was temporarily closed in order to prevent drying, fixative was poured into the chest cavity and allowed to remain in contact with the vessel for 1–2 hr. before the latter was removed en bloc for sectioning.

In a second group of experiments, the mesenteric vessels were exposed and loose ties were placed in position around the mesenteric arterial and venous arcades, the marginal veins, and the portal vein. Great care was again taken not to touch or otherwise injure that portion of the mesentery to be investigated. In 6 animals, an intravenous injection of the standard dose of serum was followed within 30 sec. by tying of the preplaced ligatures, thus insuring complete stasis in the mesentery of the terminal ileum. After 10 min., during which time the abdomen was again temporarily closed, a segment of mesentery and attached bowel were quickly removed and fixed en bloc. Three additional rats were similarly treated except for the injection of EACA (Amicar, 90 mg./kg.) immediately prior to the serum injection.

In the final group of 6 rats, an inferior vena caval segment was isolated in the same fashion as in the first group, but only one tenth the standard dose (0.075 ml./100 gm. of body weight) of homologous serum was injected prior to the induction of stasis. Three of these animals received no EACA; the remainder received 90 mg./kg. immediately prior to the serum injection as in the previous group.

Initial en bloc fixation was accomplished with paraformaldehyde-glutaraldehyde in 0.1 M cacodylate buffer, pH7.4,[10] for 1–2 hr. After removal of the segments to be studied, they were appropriately sectioned and postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer at pH7.4 for 2 hr. Following dehydration in graded alcohols and propylene oxide, the tissues were embedded in Epon 812.[11] The sections for electron microscopic study were stained with lead [12] and with uranyl acetate and were examined with a Phillips 200 electron microscope. Adjacent sections, 1 μ in thickness, were stained with methylene blue [13] for light microscopy.

A considerable number of control animals subjected to stasis alone or to stasis preceded by EACA without serum were similarily studied. Multiple sections revealed no evidence of fibrin formed in small vessels by electron microscopy, and no further discussion of this group is therefore included.

Results

Observations Following a Standard Dose of Serum.

The Inferior Vena Cava. The introduction of a standard dose of serum followed by the induction of complete stasis in the inferior vena cava resulted in the formation of a red thrombus in every way similar to those previously produced in other large veins by this method.[14] Such thrombi tend to be grossly visible as complete or partial casts of the isolated segment and, on microscopic examination, are composed chiefly of erythrocytes trapped in a fibrin meshwork with groups of platelets scattered at random throughout. Under the electron microscope, these platelets are closely apposed and are intimately associated with a delicate fibrin reticulum which links the platelet aggregates and penetrates groups of adjacent red cells. The platelets also show such structural alterations as disappearance of granules and mitochondria, and loss of the normal discoid shape with pseudopod formation and peripheral budding of featureless dense zones (Fig. 1). As noted previously,[15] there is no visible association of the thrombus with, or attachment to, the adjacent vessel wall and the endothelium appears to be normal in every respect—a consistent finding throughout this study.

Mesenteric Veins of Small and Intermediate Size. With the exception of the major veins draining each mesenteric arcade, which were studied separately, the many small veins found in the mesenteric fat are less than 50 μ in diameter and show no detectable variation in response following a standard dose of serum and the induction of stasis. No evidence of thrombus formation was visible in any of these vessels by light microscopy. Under the electron microscope the large majority

of venules were also free of fibrin, but occasional venules contained minute thrombi ranging from wisps of fibrin seen independently of platelets (Fig. 2) to single or loosely aggregated groups of platelets intimately associated with sparse and delicate fibrin strands (Fig. 3). Platelet alteration could not be demonstrated even when fibrin strands were present. Except in those rare instances in which an erythrocyte or leukocyte was seen penetrating a cell junction, the endothelium appeared everywhere intact.

The major veins of the mesenteric arcades are the only veins visible grossly and are about 200–500 μ in diameter. Thrombus formation in these vessels following a standard dose of serum was more distinct than in the smaller vessels but far less complete than that seen in the vena cava. Thrombi could not be seen when the mesentery was studied with the dissecting microscope, but under the electron microscope they consisted of moderately dense masses of fibrin containing clumps of loosely aggregated platelets and virtually no red cells (Fig. 4).

When EACA was administered prior to the introduction of serum, the amount of fibrin seen in both the small veins and those of intermediate size was significantly increased. Fibrin strands were now seen in virtually all the small veins and ranged in amount from tiny wisps similar to those noted in the untreated group to well-developed thrombi almost filling the lumen and similar to those usually observed in the larger vessels (Fig. 5). In the presence of these larger amounts of fibrin, the small platelet aggregates often displayed detectable alterations, whereas those in contact with fibrin wisps still tended to remain unchanged. In the major veins draining the arcades, the thrombi became visible under the dissecting microscope as small white bodies. Electron microscopic examination now revealed many appreciably altered platelets and fibrin also tended to enmesh erythrocytes and to fill a larger portion of the lumen.

Observations on the Inferior Vena Cava Following a Reduced Dose of Serum

Since it was impractical to determine whether EACA had any effect on the advanced thrombus formation induced in the inferior vena cava following the standard serum dose, these experiments were repeated using one-tenth the amount of serum. As demonstrated by Wessler, Reimer, and Sheps,[16] the degree of thrombus formation is directly related to the amount of serum infused, and use of 0.075 ml./100 gm. of body weight resulted in the formation of microthrombi similar to those seen in the veins of intermediate size following a standard dose. Single or small loosely associated aggregates of platelets were bound by a

delicate fibrin meshwork which failed to penetrate between adjacent erythrocytes and did not always appear to bind adjacent platelet aggregates (Fig. 6). Some of these platelets were extensively altered but most were only partially changed and retained some of the features of normal platelets. Many platelet aggregates interspersed between these microthrombi showed neither change nor association with fibrin.

When EACA was administered prior to the infusion of the reduced serum dose, the amount of fibrin was markedly increased and the result obtained was indistinguishable from that observed following a standard dose of serum (Fig. 7).

Discussion

The results of these experiments confirm previous observations that production of a serum-stasis thrombus is dependent not only on such factors as the amount of serum or active factors infused and the degree and duration of stasis but also on the size of the vessel involved.[17] A standard dose of homologous serum capable of producing a grossly visible thrombus in a static portion of a large vein such as the inferior vena cava produces microscopic thrombi in veins of intermediate size, whereas only submicroscopic fibrin formation is found in veins smaller than 50 μ in diameter. The presence of traces of fibrin demonstrable by electron microscopy in these small vessels indicates, however, that thrombin has been released and that very slight changes have, in fact, taken place. When plasminogen-activator activity has been blocked by EACA, the amount of fibrin seen is markedly increased; the thrombi in the small veins come to resemble those observed in much larger vessels, and those noted in the veins of intermediate size are indistinguishable from those in the inferior vena cava. It is apparent, therefore, that the fibrinolytic activity in these vessels must be playing a significant role in limiting the amount of visible fibrin in these static segments when prothrombin is activated via the intrinsic pathway by the injected serum. The striking effect of fibrinolysin inhibition in the inferior vena cava can also be demonstrated when a submaximal thrombogenic dose of serum is used.

There is now considerable evidence that plasminogen-activator activity is present in endothelium,[5-8] but whether the differences observed represent quantitative differences in vessels of varying size or merely reflect a greater surface-to-volume ratio in smaller vessels has not been determined. The latter seems a likely possibility since considerable fibrinolytic activity can be demonstrated adjacent to the endothelium of even the larger veins when the fibrin plate technique is employed.[8]

The considerable fibrinolytic activity demonstrable in the inferior vena cava in those experiments in which the thrombogenic stimulus was reduced also suggests that this may be so although some of the latter effect may also be due to the inhibition of circulating plasminogen activator by the EACA.

Of particular interest in these studies was the observation that small amounts of fibrin were frequently seen in association with individual platelets and even small platelet clumps which showed no evidence of degranulation or other recognizable alteration. The presence of fibrin presupposes the presence of thrombin, and platelets are known to respond by clumping and alteration to minute concentrations of this enzyme.[18] The absence of changes under these conditions, even allowing for the problems inherent in drawing three-dimensional conclusions from electron microscopic sections, suggests a reduced response to thrombin. Since platelets were often discernibly altered in the larger thrombi noted in these same vessels after EACA was used, and since the amount of thrombin released is presumably not altered by this procedure, the possibility exists that this finding may represent a morphologic expression of the depression of platelet function that has been attributed to fibrin breakdown products.[19] Local inhibition of thrombin by such products or by the products of fibrinogen denaturation or breakdown is another possibility.

Summary

Fresh mammalian serum was infused into rats and 10-min. stasis thrombi of large and small veins were studied by electron microscopy. A standard dose of serum (0.75 ml./100 gm.) produced well-developed thrombi in the vena cava while only occasional fibrin wisps were found in the small veins of the ileal mesentery. Pretreatment with epsilon amino caproic acid (EACA, 90 mg./kg.) resulted in well-developed small-vessel thrombi. This enhancement of thrombosis by EACA could also be demonstrated in the vena cava when serum doses were reduced to one-tenth of the standard dose.

The results of these experiments confirm that the development of a serum-induced venous stasis thrombus is directly related to the size of the vessel involved and indicate that the relative absence of thrombi in the smaller vessels is in large measure attributable to a relative or absolute increase in fibrinolytic activity.

Platelets associated with the wisps of fibrin found in small vessels showed no recognizable alteration. Since platelets in small vessels often show distinct change after EACA administration, this failure to alter

may represent a morphologic expression of the depressed platelet function that has been attributed to fibrin breakdown products.

References

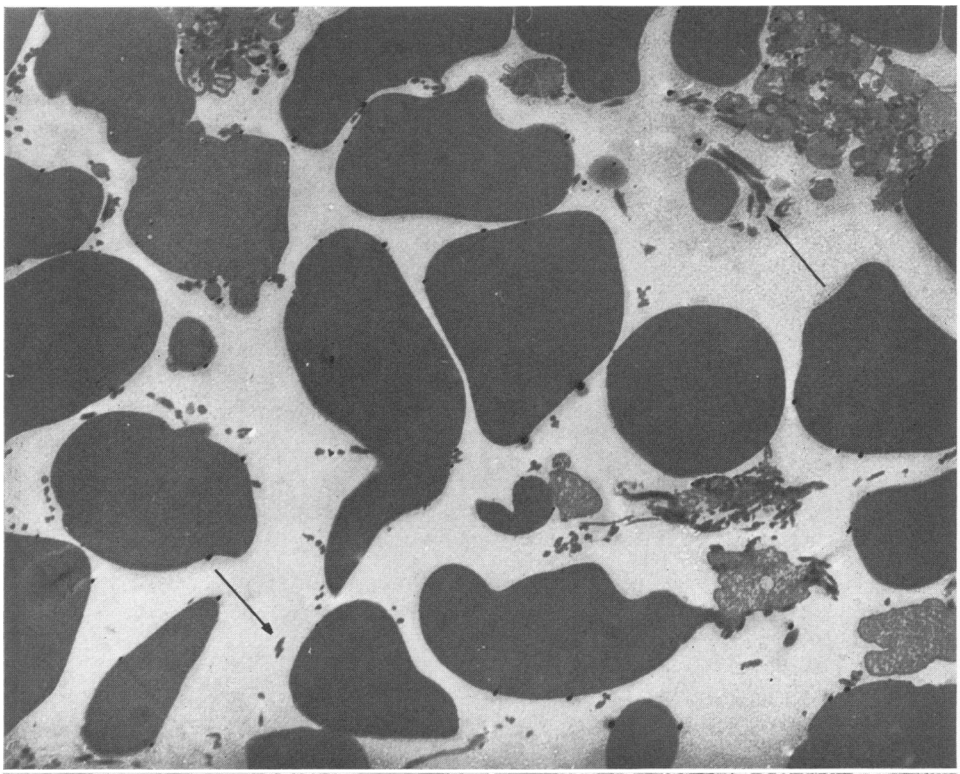
1. WESSLER, S. Studies in intravascular coagulation. III. The pathogenesis of serum-induced venous thrombosis. *J Clin Invest 34*:647–651, 1955.
2. WESSLER, S., FREIMAN, D. G., SUYEMOTO, J., and REIMER, S. M. Serum thrombotic accelerator (STA) activity: A possible key to massive venous thromboembolism in man. *Trans Ass Amer Physicians 74*:111–117, 1961.
3. BERMAN, H. G., and FULTON, G. P. Platelets in the peripheral circulation. *Henry Ford Symposium: Blood Platelets*, Johnson, S.A., Ed. Little, Boston, 1961, pp. 7–20.
4. VIRCHOW, R. *Die cellular Pathologie* (ed. 4). Hirschwald, Berlin, 1871, p. 194.
5. MOLE, R. H. Fibrinolysin and the fluidity of the blood post mortem. *J Path Bact 60*:413–427, 1948.
6. ASTRUP, T. Tissue activators of plasminogen. *Fed Proc 25*:42–51, 1966.
7. TODD, A. S. Fibrinolysis autographs. *Nature (London) 181*:495–496, 1958.
8. KWAAN, H. C. Tissue fibrinolytic activity studied by a histochemical method. *Fed Proc 25*:52–56, 1966.
9. WARREN, B. A. Fibrinolytic activity of vascular endothelium. *Brit Med Bull 25*:52–57, 1966.
10. KARNOVSKY, M. J. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. (abstr.) *J Cell Biol 27*:137A–138A, 1965.
11. LUFT, J. H. Improvements in epoxy resin embedding methods. *J Biophys Biochem Cytol 9*:409–414, 1961.
12. MILLONIG, G. A modified procedure for lead staining of thin sections. *J Biophys Biochem Cytol 11*:736–739, 1961.
13. RICHARDSON, K. C., JARETT, L., and FINKE, E. H. Embedding in epoxy resins for ultrathin sectioning in electron microscopy. *Stain Techn 35*:313–323, 1960.
14. WESSLER, S., REINER, L., FREIMAN, D. G., REIMER, S. M., and LERTZMAN, M. Serum induced thrombosis. Studies of its induction and evolution under controlled conditions *"in vivo." Circulation 20*:864–874, 1959.
15. ASHFORD, T. P., and FREIMAN, D. G. The role of the endothelium in the initial phases of thrombosis. An electron microscopic study. *Amer J Path 50*: 257–273, 1967.
16. WESSLER, S., REIMER, S. M., and SHEPS, M. C. Biologic assay of a thrombosis-inducing activity in human serum. *J Appl Physiol 14*:943–946, 1959.
17. ASHFORD, T. P., and FREIMAN, D. G. Role of fibrinolysis in the prevention of stasis thrombus formation in small veins. (abstr.) *Fed Proc 26*:319, 1967.
18. HOVIG, T. The ultrastructure of rabbit blood platelet aggregates. *Thromb Diath Haemorrh 8*:455–471, 1962.
19. KOWALSKI, E., KOPEC, M., and WEGRZYNOWICZ, Z. Influence of fibrinogen degradation products (FDP) on platelet aggregation, adhesiveness and viscous metamorphosis. *Thromb Diath Haemorrh 10*:406–423, 1964.

[*Illustrations follow*]

Legends for Figures

Fig. 1. Electron micrograph of standard serum thrombus in vena cava. Delicate fibrin strands *(arrows)* connect and are intimately associated with small clumps of altered platelets scattered at random. × 5000.

Fig. 2. Electron micrograph of standard serum thrombus in small vessel of mesentery. Gross sections of a few delicate wisps of fibrin (*F*) are seen in lumen. Endothelium is intact. × 17,000.



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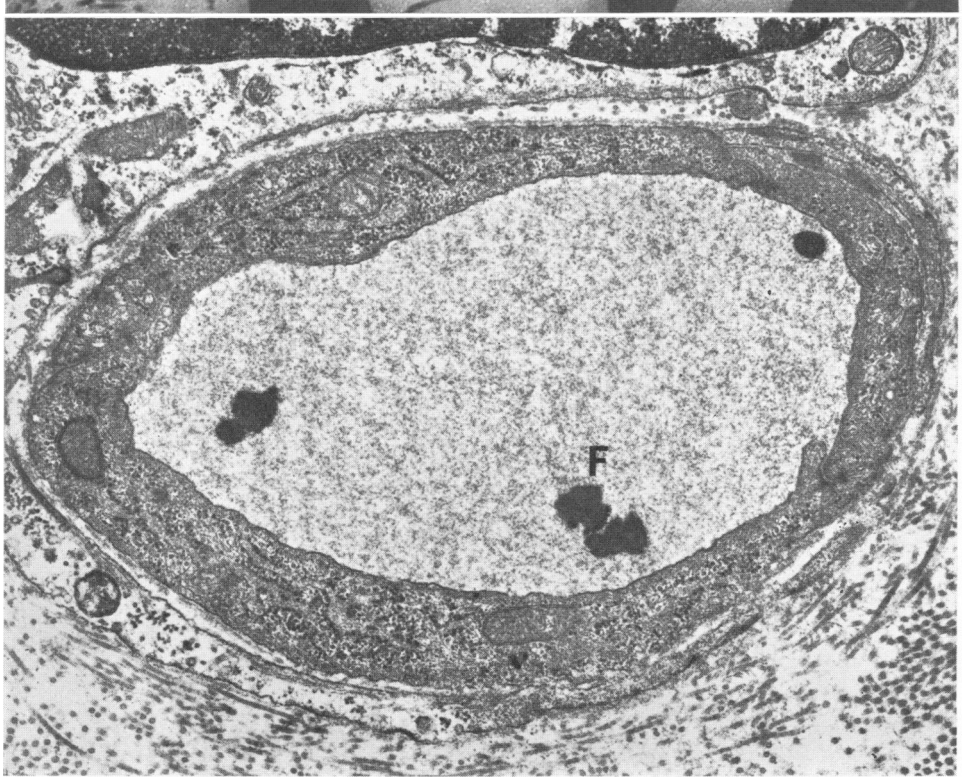

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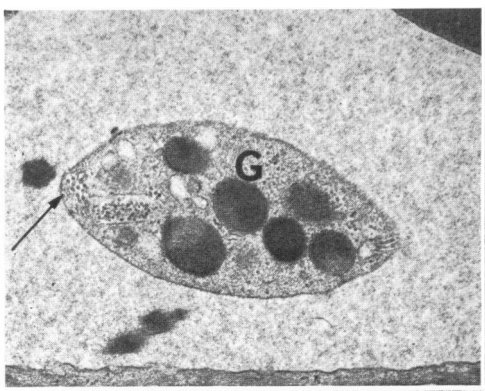
Fig. 3. Intact platelet intimately associated with fibrin in serum thrombus of small vein. Platelet retains discoid shape and granules (G). Microtubules are seen in cross section at periphery (*arrow*). × 17,000.

Fig. 4. Platelet seen in standard serum thrombus in intermediate size vessel. Shape is altered although granules (G) and mitochondria (*M*) are seen. Some fibrin strands *(arrow)* surrounding platelet display characteristic 220 A periodicity. × 23,000.

Fig. 5. Standard serum thrombus of small vessel after EACA administration. Dense fibrin connects partially altered platelets *(P)*. Endothelium *(E)* appears intact. × 20,000.

Fig. 6. Electron micrograph of platelets from serum thrombus of vena cava resulting from 1/10 dose of serum. Although platelets have altered shape, granules and mitochondria remain. × 13,000.

Fig. 7. Electron micrograph of higher magnification demonstrating alteration of clumped platelets found in standard serum thrombus of vena cava. *F*, fibrin; *E*, erythrocyte. × 9000.



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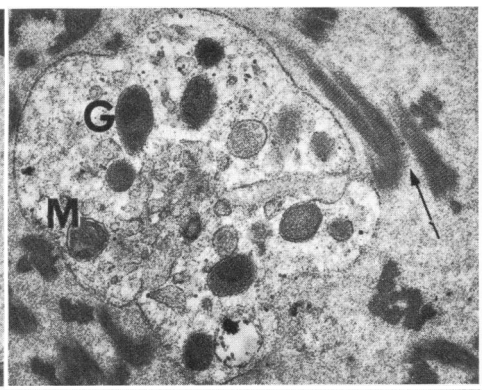

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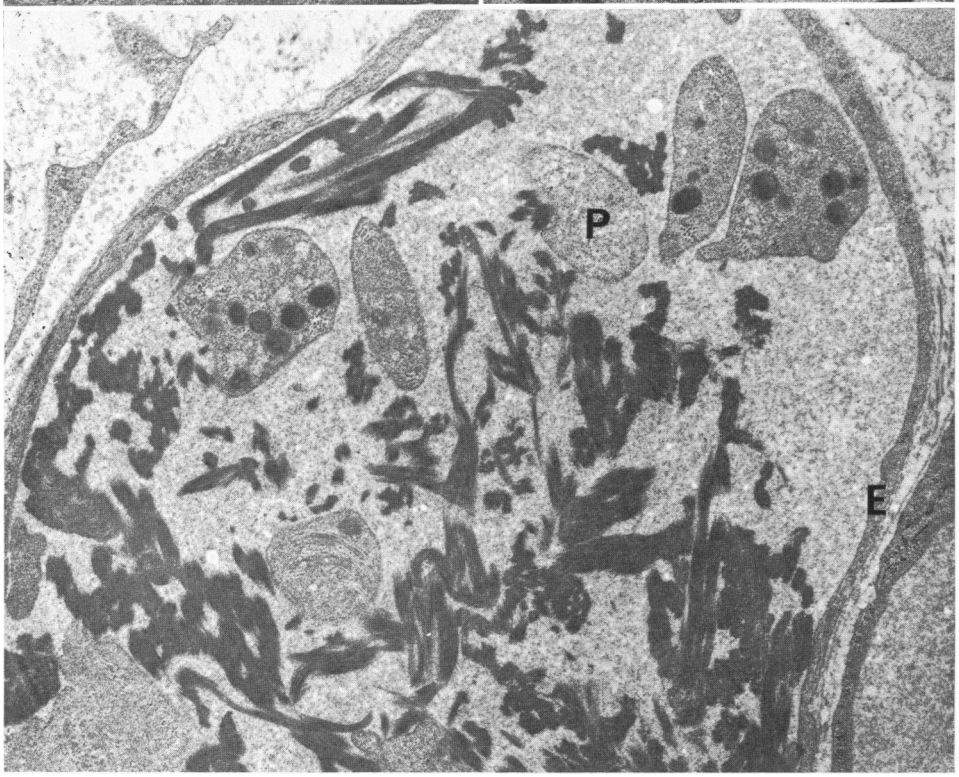

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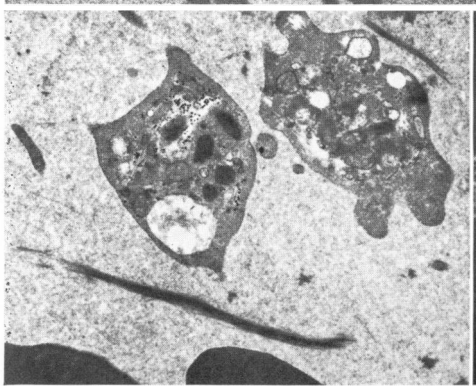

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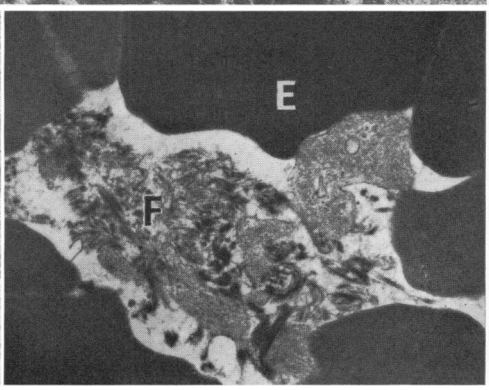

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