MORPHOLOGIC ALTERATIONS OF HUMAN BLOOD PLATELETS DURING EARLY PHASES OF CLOTTING

Electron Microscopic Observations of Thin Sections

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Platelet aggregates develop *in vivo* during the formation of the hemostatic platelet plug and in the development of white platelet thrombi. Platelet aggregation also occurs during the clotting of blood *in vitro*. Since the earliest report of Wolpers and Ruska in 1939,[1] changes in individual platelets and the characteristics of platelet aggregates have been studied extensively by electron microscopic examination of whole platelets. Many studies of this type have been published, more recently by Braunsteiner and Pakesch,[2] de Robertis,[3] Haydon and Corey,[4] Hutter,[5] Köppel,[6] and Rebuck, Riddle, Johnson, Monto and Sturrock.[7] While many have used shadow casting methods, ultrathin section techniques have been utilized in relatively few electron microscopic studies of platelets. Rinehart in 1955[8] first observed the fine structure of individual platelets and described some of the changes which occur during the agglutination process. Kuhnke[9,10] extended this study. A detailed investigation of the alterations which occur in platelet ultrastructure during blood coagulation has not been reported.

This paper describes the progression of alterations in the ultrastructure of platelets during the clotting of blood plasma.

Material and Methods

The osmium tetroxide (OsO_4) fixative was freshly prepared on the day of the experiment by mixing 5 parts of 2 per cent OsO_4 in demineralized water with one part 0.28 M veronal-acetate. To this was added sufficient 0.2 N HCl to adjust the pH to 7.35, as measured with a glass electrode (Beckman Zeromatic). Water was added to bring the volume to 1.5 times that of the original.

All glassware used in handling the blood and plasma up to the point of fixation was silicone coated with Dri-Film No. SC-87 (General Electric). With the two-syringe technique and a 16-gauge untreated needle, blood was obtained from a

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cubital vein of human subjects whose prothrombin time and partial thromboplastin time [11] were found to be within normal limits. There was sufficient 0.11 M trisodium citrate in the second syringe so that the final ratio of blood to citrate solution was 8 to 1. The blood was centrifuged in a trunnion head at approximately 100 *g.* at room temperature, for 8 minutes. The supernatant platelet-rich plasma was immediately withdrawn and divided into 2 portions. To the first portion was added one-fifth volume of 0.154 M NaCl; simultaneously, one-fifth volume of 0.108 M $CaCl_2$ was added to the second portion. Aliquots of each mixture were delivered into test tubes in a water bath at 37° C. The water bath was placed on a Yankee rotator (Clay-Adams, Inc., New York City) which oscillated at 20 to 30 times per minute until clotting occurred, at which time the rotator was stopped. From the recalcified portion of platelet-rich plasma a separate tube was prepared. This was kept at room temperature, constantly but gently agitated, and observed with the aid of strong transmitted light in order to follow macroscopically the progress of platelet agglutination.[12] The method allowed correlation of macroscopically observed changes with microscopic appearances.

At successive time intervals platelets were fixed by adding 3 volumes of fixative to 1 volume of plasma. The final concentration of OsO_4 was 1 per cent. After 20 minutes' fixation at room temperature, the mixture was centrifuged for 10 minutes at approximately 625 *g.* The resultant button was dehydrated in solutions of increasing ethanol concentration, infiltrated with methacrylate monomer, and embedded in prepolymerized methacrylate. Polymerization of a mixture of 9 parts n-butyl methacrylate and 1 part methyl methacrylate was catalyzed under dry nitrogen at 53° C. with 2 per cent Luperco ® CDB (Lucidol Division, Wallace and Tiernan, Inc., Buffalo, New York). Clotted samples which did not require centrifugation were cut into appropriately sized blocks during dehydration but otherwise were handled similarly to unclotted samples.

Sections, cut at 300 to 600 Å with a Porter-Blum microtome equipped with a glass knife, were placed on supporting films of either formvar or carbon. Most sections were examined with no staining procedure other than OsO_4 fixation. Some were stained with saturated uranyl acetate solution for 1 hour. Sections were examined with an RCA EMU-3B electron microscope at magnifications ranging from 1,590 to 8,170 times. Micrographs were subsequently enlarged up to 32,700 times with an Omega D-2 enlarger.

Results

The results from 12 experiments performed in addition to the 2 experiments from which the illustrations were made corroborated the findings presented here. In the several experiments multiple samples were obtained at many different times from both nonrecalcified and recalcified plasma. The micrographs in the paper illustrate ultrastructure in platelets from citrated, nonrecalcified plasma and the sequential changes as they were observed at 3 specific points in time following recalcification. The first point, 10 minutes after recalcification, corresponded to the formation of minute platelet aggregates as seen with the electron microscope and to the first suggestion of agglutination which could be appreciated macroscopically. The second corresponded to complex platelet aggregates as they were observed in a filmy clot 13 minutes after recalcification. The third point corresponded to early platelet disintegration in

a partially retracted clot. This was 13 minutes after the formation of the filmy clot and 26 minutes after recalcification.

Platelets from Citrated Plasma

These platelets, fixed 15 to 20 minutes after venipuncture, were rounded to oval with occasional short, stubby projections (Fig. 1). Most of the platelets, as seen in sections, measured 2.5 to 3 μ in greatest dimension, but some measured up to 5 μ. The outer membrane was single and moderately dense. It was segmentally interrupted with a "staccato" appearance and was 100 to 150 Å thick. The granulomere structures in most platelets had a random distribution while a few platelets contained a narrow peripheral zone free of granulomeres. No correlation was observed between platelet shape and granulomere distribution. Several types of granulomere structure, similar to those described by Schulz, Jürgens and Hiepler,[13,14] were observed. The alpha granulomere had a dense border and homogeneous ground substance. The beta granulomere was the mitochondrion. The gamma granulomere consisted of microvesicles. The delta granulomere had a dense border and an electron-lucent center.

Platelets fixed 20 to 25 minutes after venipuncture (Fig. 2) differed in that they had more numerous and more elongated projections than shown in Figure 1. They remained rounded to oval and were similar in size, in character of the limiting membrane, and in structural content and distribution.

Earliest of the Sequential Changes in Platelets from Recalcified Plasma

The earliest changes observed in platelets from recalcified plasma (Fig. 3), at 10 minutes after recalcification, were a striking alteration in platelet shape, a much more sharply defined limiting membrane with absence of the "staccato" appearance, and a concentration of the granulomere structures toward the center of the platelet. Bizarre shapes were uniformly noted in single platelets. In these individual platelets the several different types of granulomere were still identifiable. At the same time, as demonstrated in adjacent fields of the same section, an occasional platelet (Fig. 4) had multiple protrusions of the limiting membrane (pseudopods) which contained cytoplasm and which varied markedly in size and shape. In addition, in a rare minute area (Fig. 5), a few fibrin strands had appeared, and platelets had begun to fit together as the pieces of a jigsaw puzzle. Platelets in these small aggregates had intact membranes and were separated by a space which varied from 200 to 400 Å in width. Fibrin had appeared between most of the platelets;

it was closely apposed to the surface of some and was also observed in areas devoid of platelets.

Transition Phase to Large Platelet Aggregates

During the time from earliest agglutination to the formation of a macroscopically visible filmy clot, platelet aggregates became larger and much more intricately constructed. At the same time the areas in which platelet aggregates were found became increasingly larger, and fibrin appeared in greater and greater amounts. The numbers of single platelets decreased until, at the time of formation of the filmy clot, only a rare single platelet was observed.

Large Platelet Aggregates

Two different types of platelet aggregates were observed in the filmy clot 13 minutes after recalcification. In the first type (Figs. 6 to 8), which was seen more frequently, limiting membranes were easily identified throughout the aggregates although a rare individual membrane (Fig. 8) was either partially degenerated or cut tangentially. The aggregates had varied patterns and were extremely complex. Alpha, beta, gamma and delta granulomeres were identified as specific structures with less certainty than formerly. In these aggregates fibrin was occasionally absent (Fig. 6) although in most areas fibrin was abundant adjacent to platelets and in loci remote from platelets (Figs. 7 and 8).

The second type of platelet aggregate (Fig. 9), much less commonly encountered, had two distinctly different zones. Centrally there was poorly defined granular material and absence of identifiable membranes. Peripherally there were sac-like protrusions with partial limiting membranes, which were well defined where they presented to the surrounding fluid medium and were frequently absent where the protrusions attached to the body of the aggregate. These sac-like protrusions contained finely granular material with occasional microvesicles and other ultrastructures, some of which were suggestive of mitochondria. Fibrin lay within and adjacent to all of the masses observed at this time.

Disintegration of Individual Platelets

In the partially retracted clot (Fig. 10), 26 minutes after recalcification, there were many agglutinated masses resembling those found at the stage of the filmy clot (Fig. 9). Centrally the masses comprising the partially retracted clot were irregularly granular with no specifically identifiable structures. Peripherally there were numerous sac-like protrusions of varying shapes. Many of the protrusions were rounded, some had irregular shapes, and some had a teardrop configuration. Many such

structures appeared to be attached at the periphery of a mass while a few, seemingly detached, were short distances from it. As in the similar aggregates seen earlier, fibrin was observed within and adjacent to the mass; it was also present in spaces separating the masses.

Discussion

The experimental results, as presented above, describe platelet ultrastructure as seen in specimens obtained from citrated plasma and then alterations in ultrastructure as they were observed successively at several specific points in time after recalcification. The transition of intact discrete platelets to platelet aggregates appeared to be a graded process. That this graded process occurred at different times in different platelets was demonstrated at 10 minutes after recalcification (Figs. 3 to 5) when a few minute foci of platelet aggregation and fibrin were found, while most of the platelets were single and had altered shapes with concentration of the granulomeres toward the center. Also, at the time of large, very complex platelet aggregates, corresponding with the macroscopic appearance of a filmy clot, there were two strikingly different patterns of platelet aggregation.

Keeping in mind the variations in individual platelets at any specific time, it is of interest to reconstruct what might be the expected events in several human platelets in juxtaposition in platelet-rich plasma, in a silicone-treated vessel, being gently swirled, and maintained at 37° C. while clotting occurs. Several minutes after recalcification the granulomere structures of these platelets gather toward the center. Pseudopods of various sizes and shapes appear, giving the platelet a bizarre configuration. Some of these platelets then fit together as do pieces in a jigsaw puzzle. The limiting membrane of each platelet remains intact, although its character is altered. The limiting membrane of each platelet is separated from that of its neighbor by a small space. Fibrin may or may not be present between or adjacent to the platelets. Several additional platelets then join the small mass, producing enlargement of the aggregate. Platelet limiting membranes in the central part of the mass disintegrate, as do granulomere structures, producing an area of irregular granular material. At the periphery of the mass, there are sac-like remains of platelets partly covered by intact limiting membranes. Some of these sac-like remains appear to break away from the mass. Fibrin is present in the mass, centrally and peripherally, and in the spaces between masses. It is logical to assume that a chain of events similar to those described may occur at the site of injury to a blood vessel. The resulting aggregation of platelets would be an effective hemostatic plug.

Summary

Alterations in the ultrastructure of human blood platelets during coagulation were described. Single platelets, obtained from citrated platelet-rich plasma, were rounded to oval with well-defined granulomere structures. Following recalcification the platelets assumed bizarre shapes with multiple pseudopods and a gathering of the granulomeres toward the center. Aggregates formed, and fibrin appeared in and about most of the aggregates. The aggregates enlarged in a variable and complex pattern. Later, in a partially retracted clot, degeneration was noted in the central part of the platelet aggregate. The variations in pattern observed at specific times indicated that the alterations occurred at different times in different platelets.

References

1. Wolpers, C., and Ruska, H. Strukturuntersuchungen zur Blutgerinnung. *Klin.Wchnschr.*, 1939, **18**, 1077–1081.
2. Braunsteiner, H., and Pakesch, F. Thrombocytoasthenia and thrombocytopathia—old names and new diseases. *Blood*, 1956, **11**, 965–976.
3. De Robertis, E. Electron microscope observations of the platelet-fibrin relationship in blood clotting. *Blood*, 1955, **10**, 528–533.
4. Haydon, G. B., and Corey, D. L. Platelets in the thromboplastin generation test. Electron microscopic studies. *Arch. Path.*, 1961, **71**, 615–620.
5. Hutter, R. V. P. Electron microscopic observations on platelets from human blood. *Am. J. Clin. Path.*, 1957, **28**, 447–460.
6. Köppel, G. Elektronenmikroskopische Untersuchungen zur Funktionsmorphologie der Thrombozyten und zum Gerinnungsablauf im normalen menschlichen Nativblut. I. Frühe Veränderungen der Thrombozyten. *Ztschr. Zellforsch.*, 1958, **47**, 401–439.
7. Rebuck, J. W.; Riddle, J. M.; Johnson, S.A.; Monto, R. W., and Sturrock, R. M. Contributions of electron microscopy to the study of platelets. *Henry Ford Hosp. M. Bull.*, 1960, **8**, 273–292.
8. Rinehart, J. F. Electron microscopic studies of sectioned white blood cells and platelets; with observations on the derivation of specific granules from mitochondria. *Am. J. Clin. Path.*, 1955, **25**, 605–619.
9. Kuhnke, E. Elektronenoptische Untersuchungen über die Veränderung der Thrombocyten und des Fibringerinnsels im Verlaufe der Gerinnung unter besonderer Berücksichtigung der Retraktion. *Pflügers Arch. ges. Physiol.*, 1958, **268**, 87–104.
10. Kuhnke, E. Elektronenmikroskopischer Nachweis von Strukturveränderungen des Thrombocyten während der Gerinnung. In: Fourth International Conference on Electron Microscopy, Berlin, Sept. 10–17, 1958. Springer-Verlag, Berlin, 1960, vol. 2, pp. 263–266.
11. Rodman, N. F., Jr.; Barrow, E. M., and Graham, J. B. Diagnosis and control of the hemophilioid states with the partial thromboplastin time (PTT) test. *Am. J. Clin. Path.*, 1958, **29**, 525–538.
12. LeRoy, E. C.; Mason, R. G., and Brinkhous, K. M. Species differences in

platelet agglutination in man and in the dog, swine and rabbit. *Am. J. Physiol.*, 1960, **199**, 183–186.

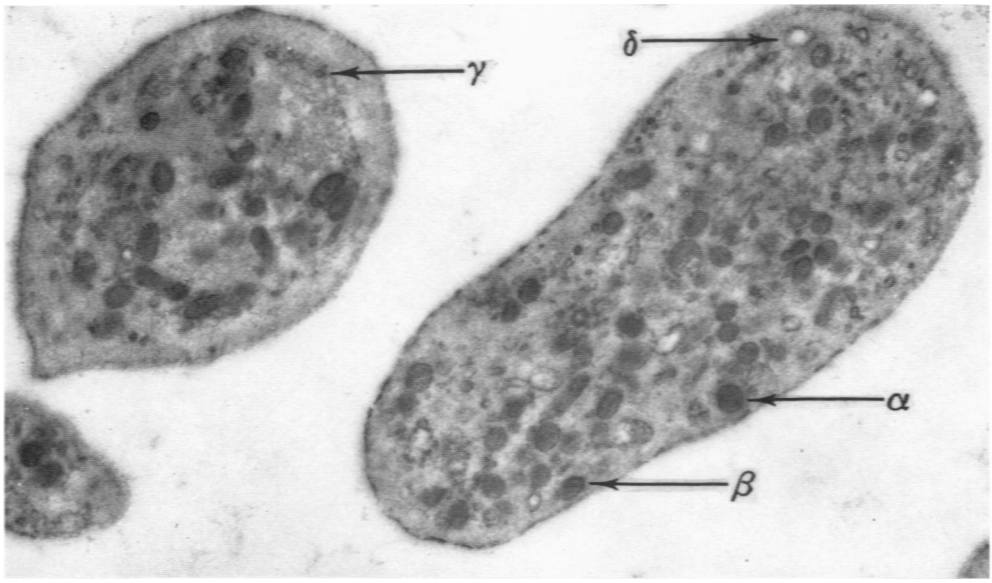
13. SCHULZ, H.; JÜRGENS, R., and HIEPLER, E. Die Ultrastruktur der Thrombozyten bei der konstitutionellen Thrombopathie (v. Willebrand-Jürgens) mit einem Beitrag zur submikroskopischen Orthologie der Thrombozyten. *Thromb. Diath. Haem.*, 1958, **2**, 300–323.
14. SCHULZ, H., and HIEPLER, E. Über die Lokalisierung von gerinnungsphysiologischen Aktivitäten in submikroskopischen Strukturen der Thrombocyten. *Klin. Wchnschr.*, 1959, **37**, 273–285.

[*Illustrations follow*]

Legends for Figures

FIG. 1. Platelets from citrated plasma 15 to 20 minutes after venipuncture or 5 minutes after addition of saline. α = alpha granulomere; β = beta granulomere; γ = gamma granulomere; δ = delta granulomere. Note the distribution of the granulomeres. The limiting membrane is segmentally interrupted with a "staccato" appearance. × 20,400.

FIG. 2. Platelets from citrated plasma 20 to 25 minutes after venipuncture or 10 minutes after addition of saline. These are unchanged from those seen in Figure 1 except that their shapes are more variable. The small oval to round structures separate from the bodies of platelets probably represent projections of platelets in other planes. × 16,800.



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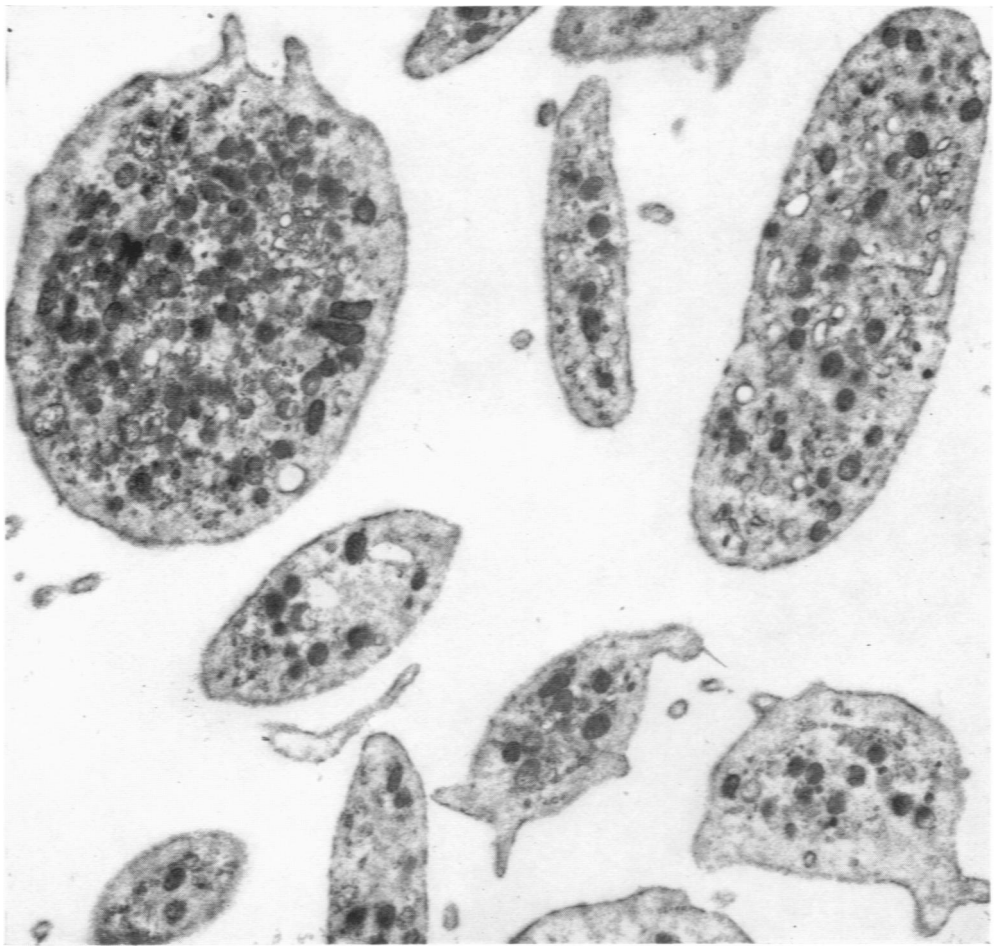

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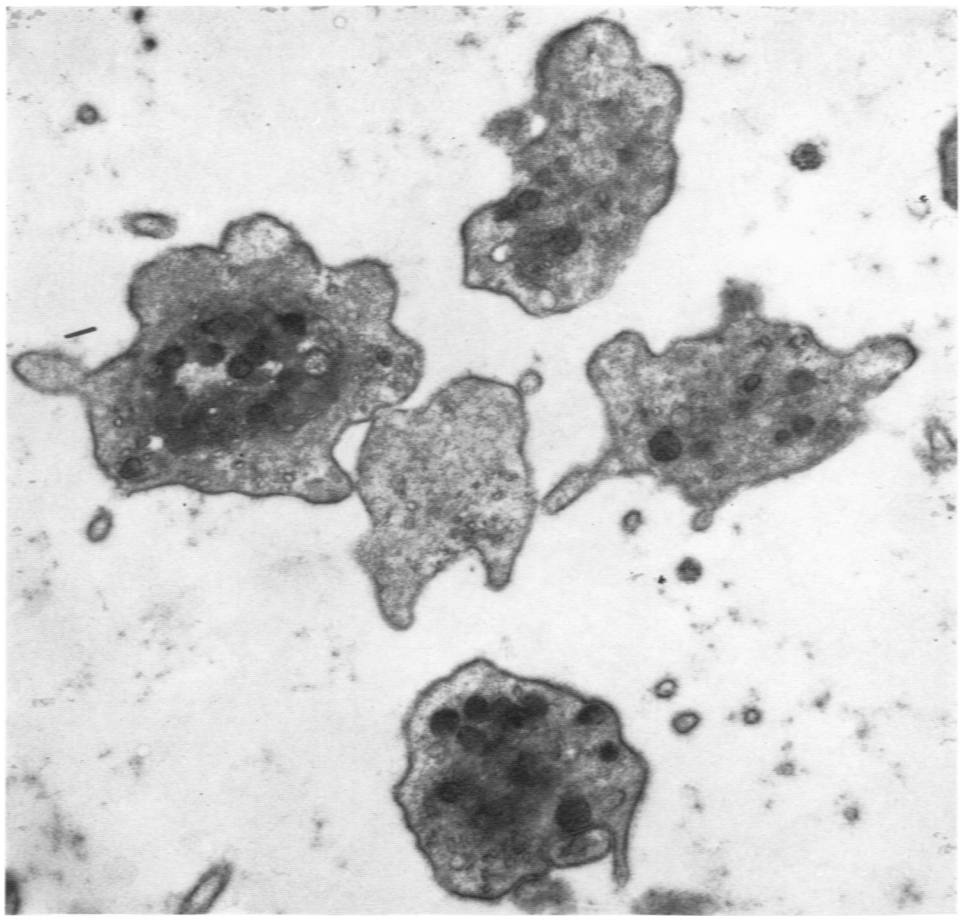
FIG. 3. Individual platelets from plasma 10 minutes after recalcification. Note the alterations in the limiting membrane, shapes of the platelets, and the concentration of the granulomere toward the center. Compare with platelets in Figures 1 and 2. × 16,800.

FIG. 4. A platelet with multiple pseudopods, from the sample shown in Figure 3. Although this is interpreted as a single platelet, an indistinct line at "A" suggests the possibility of two aggregated platelets. At "B" a rounded structure appears to be continuous with the limiting membrane. × 16,800.

FIG. 5. A minute platelet aggregate from the sample shown in Figures 3 and 4. The platelets have intact limiting membranes, and fibrin has appeared. × 16,800.



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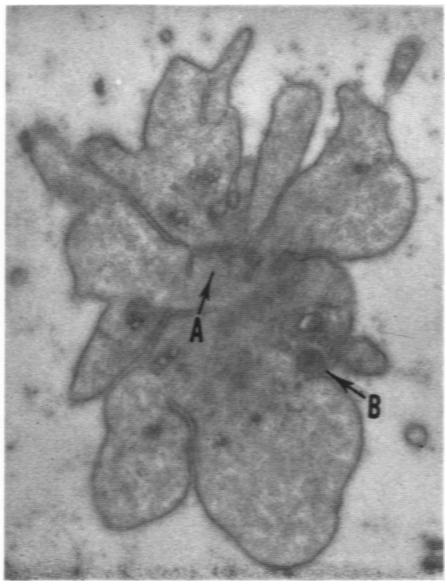

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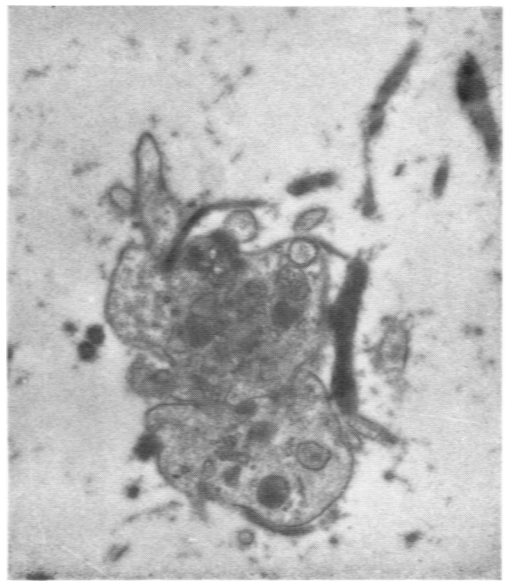

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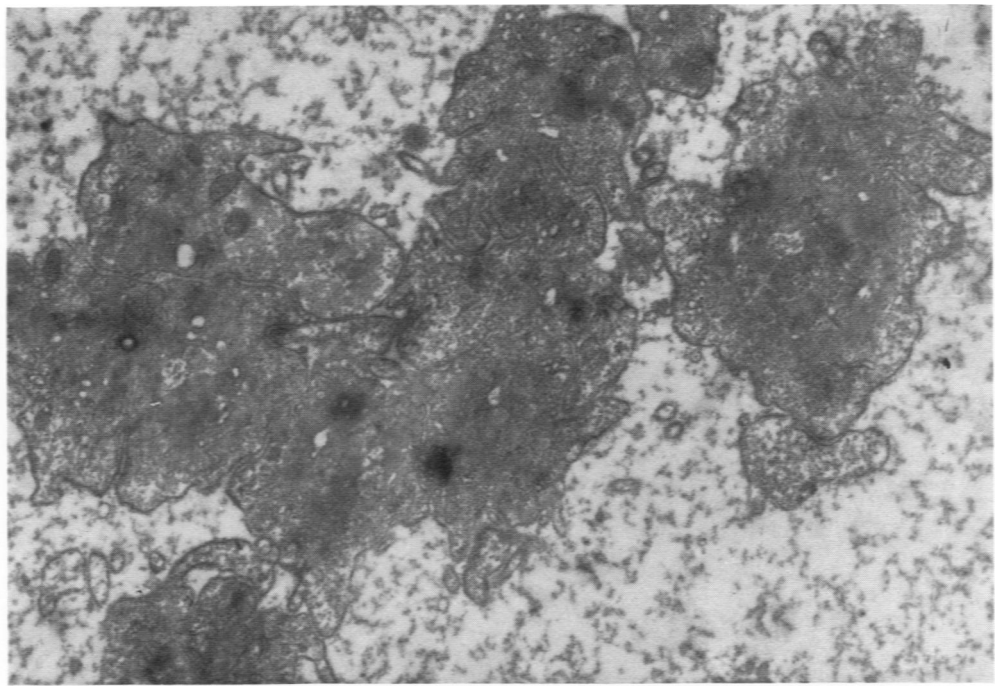

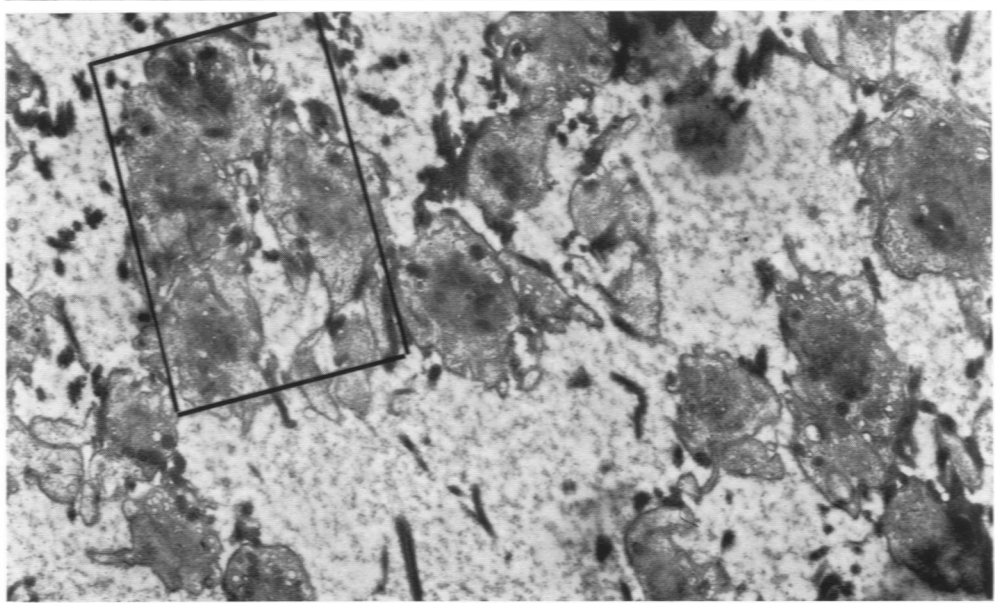

FIG. 6. A complex platelet aggregate in the filmy clot 13 minutes after recalcification. Fibrin is not identified in this field. Platelet limiting membranes are intact throughout. Specific granulomere structures are identified with less certainty than formerly, although there are numbers of vacuoles and microvesicles. × 15,100.

FIG. 7. A complex platelet aggregate from the sample shown in Figure 6 and similar except for platelet distribution and the presence of abundant fibrin. Fibrin is round to elongated and lies between, adjacent to, and seemingly away from platelets. Stained with saturated uranyl acetate solution for 1 hour. × 7,480.

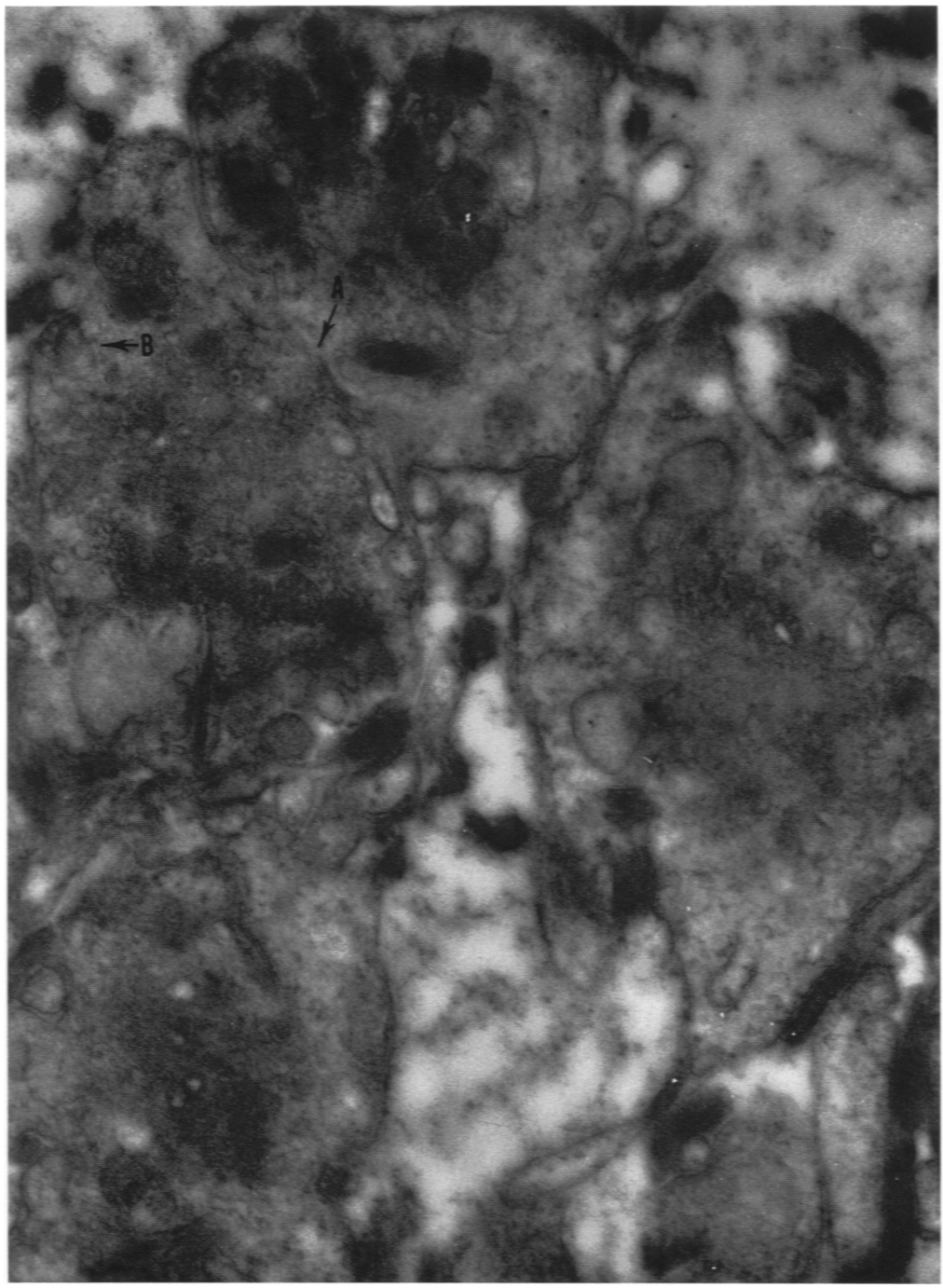

FIG. 8. Insert in Figure 7. Limiting membranes are clear and intact except for a possible membrane at "A" which may be either partially degenerated or cut tangentially. The structure at "B" is probably a mitochondrion. Note the relation of fibrin to the platelets, and the presence of vacuoles and microvesicles. Stained for 1 hour with saturated uranyl acetate solution. × 32,700.

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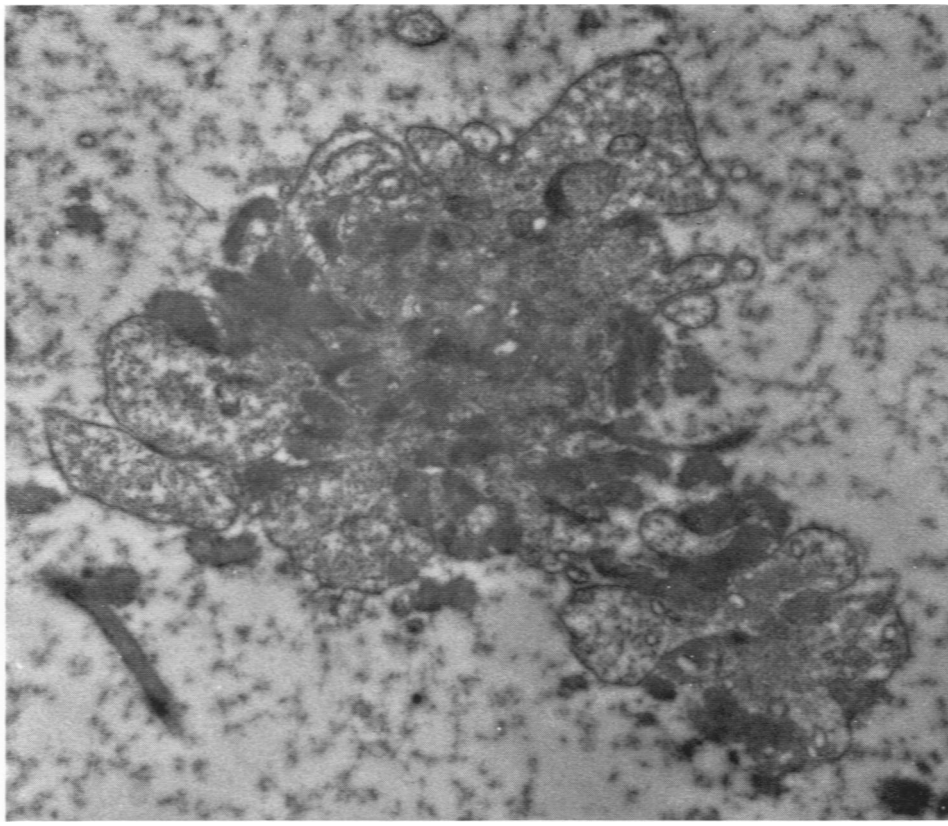

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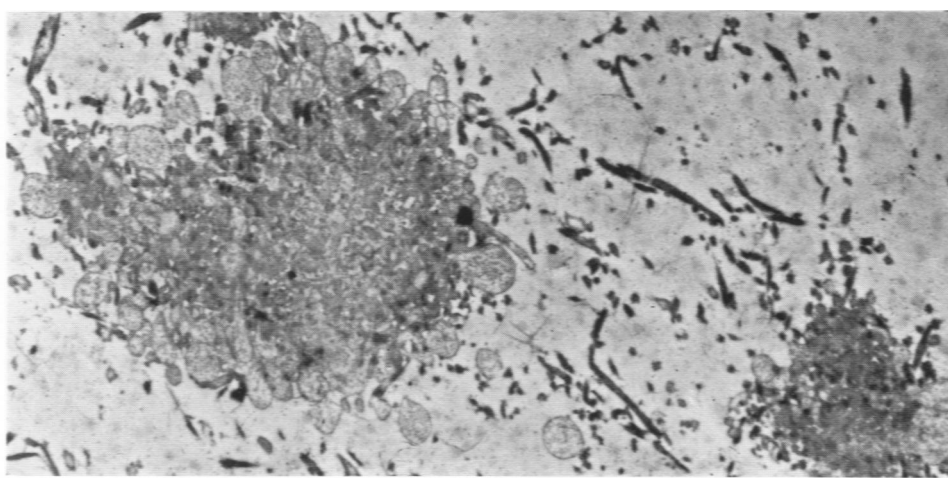

FIG. 9. The second type of complex platelet aggregate with beginning disintegration of platelet structures centrally and sac-like protrusions peripherally. × 25,000.

FIG. 10. Masses of platelet remains in a partially retracted clot, 26 minutes after recalcification. Centrally the masses contain irregular, granular material with no specifically identifiable structures. Peripheral sac-like protrusions have intact limiting membranes. Fibrin lies within and between the masses. Stained with uranyl acetate solution for 1 hour. × 4,930.