# Cytochemical Electron Microscopic Studies of the Action of Phorbol Myristate Acetate on Platelets

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**Phorbol myristate acetate (PMA), the active principle of croton oil, is a potent platelet aggregating agent. A previous electron microscopic study indicated that PMA caused selective labilization of platelet granules. The present investigation has employed cytochemical procedures to clarify problems concerning the action of PMA on platelets. Results of this study confirm the suggestion that vacuoles in PMA-treated platelets derive primarily from granules, and demonstrate that the swollen vacuoles are continuous with channels of the open canalicular system (OCS) and surrounding plasma. Despite loss of the barrier separating vacuolated granules from the OCS, the contents of the organelles were not extruded from the PMA aggregated platelets. Stabilization of platelet surface membranes did not inhibit the action of PMA on platelet granules, and examination of replicas of freeze-fractured PMA platelets failed to reveal any specific injury produced by the agent. The findings have elucidated some of the effects of PMA on platelet structure, but have not solved the basic mechanism of drug action (Am J Pathol 74:453–466, 1974).**

Zucker, Kim, Belman and Troll were the first workers to study the effects of phorbol myristate acetate (PMA), the active ingredient of croton oil, on blood platelets.[1] They demonstrated that minute amounts of PMA could cause irreversible aggregation when added to stirred platelet-rich plasma. PMA-induced aggregation closely resembled the response of stirred platelets to adenosine diphosphate (ADP), and Zucker *et al* suggested that PMA and ADP might activate platelets in a similar manner. They also observed that PMA-stimulated platelets failed to secrete serotonin and adenine nucleotides until long after irreversible aggregation had occurred, and a granule-bound enzyme, β-glucuronidase, was incompletely released.

A recent report from this laboratory described the effects of PMA on platelet ultrastructure in stirred and unstirred systems.[2] In contrast to the known influence of ADP,[3] the primary action of PMA appeared to be on channels of the open canalicular system and storage granules rather than on platelet discoid shape. Granules inside platelets were

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converted to swollen vacuoles containing residual debris before the onset of aggregation, and the initial clumps developed from discoid cells. Shape change took place after platelet clumping, and was associated with irreversible aggregation.

The striking influence of PMA on the ultrastructure of platelet granules raised several questions about the mechanisms of drug action. Could the agent still affect granules if the platelet capacity to change shape was inhibited? Were the swollen granules in PE-treated platelets isolated vacuoles or were they continuous with channels of the open canalicular system and the plasma? Were the contents of the altered granules discharged or retained? In the present study several specialized ultrastructural techniques were used in an attempt to resolve these questions.

## Materials and Methods

The methods used in this laboratory to obtain blood from normal donors, mix the samples with 3.8% tri-sodium citrate or citrate-citric acid, pH 6.5, in a ratio of 9 parts blood to 1 part anticoagulant, separate citrate platelet rich plasma (C-PRP), expose samples to various drugs and experimental conditions *in vitro*, examine the response of platelets to aggregating agents by recording nephelometry at 37 C, and prepare control and experimental cell samples for study in the electron microscope were detailed in several recent publications.[3-7] The previous report in this series described the preparation and use of phorbol myristate acetate (12-O-tetradecanoyl-phorbol-13-acetate, PMA) to study platelet aggregation and morphologic changes following incubation with the agent.[2] Identical procedures, experimental systems and concentrations of PMA were employed in the present study.

Cytochemical electron microscopic methods discussed in previous publications were applied to the problem of platelet-PMA interaction. Thorium dioxide was diluted 1:10 or 1:100 in phosphate buffer.[4] A 0.1 ml of thorium dioxide solution was combined with 0.9 ml of C-PRP 5 minutes before addition of PMA in final concentrations of 10 ng to 10 μg on the platelet aggregometer. In some experiments the thorium dioxide was added to platelet samples which had been irreversibly aggregated by PMA. Other samples of C-PRP were combined with thorium dioxide 5 minutes before or after the start of incubation with graded concentrations of the drug. Additional platelet preparations incubated with or aggregated by PMA were fixed in the usual two-step procedure with glutaraldehyde, washed and then exposed to osmic acid containing lanthanum nitrate.[7] Polylysine was added to C-PRP at concentrations of 0.1 to 0.2 mg/ml just before graded concentrations of PMA on the aggregometer.[6] Other platelet samples were mixed with polylysine before addition of PMA and subsequently incubated without stirring for up to 60 minutes. In some experiments polylysine was added 5 to 15 minutes after the start of C-PRP incubation with PMA, and the incubation continued for intervals up to 1 hour. The method developed for the demonstration of endogenous platelet peroxidase activity was carried out on PMA-aggregated or incubated paltelets at intervals up to 60 minutes after exposure to the drug.[8] On the basis of previous studies demonstrating the stablizing influence of cytochalasin B on platelet discoid shape this agent was added to C-PRP at

final concentrations of 10 to 25 μg/ml for 15 minutes before introducing various concentrations of PMA.[9,10] The method used to prepare platelets for freeze-fracture in the Balzer's freeze-etch device was described previously.[11]

## Results

### Morphology of Platelets Following Exposure to PMA

The effects of PMA on platelet fine structure were presented in detail in a previous report.[2] PMA in small amounts (1 to 100 ng) acted primarily on channels of the OCS and intracellular organelles. The granules were converted to swollen vacuoles containing variable amounts of residual matrix material. Most platelets remained discoid until after aggregation. Larger amounts of PMA (100 ng to 10 μg) caused various degrees of shape change prior to aggregation, and alterations in discoid form regularly developed when EDTA was added to the system to inhibit aggregation (Figure 1). Changes in discoid form appeared related to the development of distended vacuoles inside the cells. The degree of alteration observed in PMA-treated platelets was roughly proportional to the amount of drug added to the system and the duration of platelet exposure to the agent.

### Electron-Dense Tracers

The possibility that swollen vacuoles in PMA-treated platelets were either open to the surrounding plasma or sealed from it was explored with electron-dense tracers. Thorium dioxide added to C-PRP before or after exposure of the platelets to PMA was observed on cell surfaces, within channels of the open canalicular system, and inside the dilated vacuoles derived from platelet granules (Figure 2). Platelets treated with PMA, fixed in glutaraldehyde, washed and exposed to osmic acid containing lanthanum nitrate were also examined. Electron-dense stain coated the exterior of the platelets, filled channels of the open canalicular system and entered the distended vacuoles in PMA-treated cells (Figure 3). On the basis of these observations PMA appears to produce a direct communication between dilated granules, elements of the open canalicular system and the surrounding plasma.

### Polylysine

Previous investigations demonstrated that polylysine and other cationic polypeptides polymerize matrix material in platelet granules and stabilize them during extrusion from activated platelets.[6] In the present study polylysine was used to determine whether matrix material remained in the dilated vacuoles of PMA-treated platelets or was extruded

via the canalicular system to the exterior. Examination of platelets exposed to polylysine before addition of PMA on the aggregometer and of PMA-treated platelets incubated subsequently with polylysine revealed no evidence of granule extrusion (Figures 4 and 5). Polylysine was taken up by the PMA-treated platelets and appeared in the swollen vacuoles. The polyelectrolyte produced the characteristic polymerization of residual matrix material identified previously in the unaltered granules of platelets before and after aggregation. This observation suggests that the reduced opacity of swollen vacuoles derived from granules after exposure of platelets to PMA is not due to extrusion of all granule substance. A substantial amount must remain in order for polylysine to polymerize the typical lattice structure.

#### Cytochalasin B and PMA

To determine if PMA could induce changes in the granules of platelets stabilized in the discoid configuration samples of C-PRP were incubated with cytochalasin B (CB) prior to the addition of PMA. Examination of these preparations revealed nearly complete conversion of granules to distended vacuoles without loss of platelet discoid form or pseudopod formation (Figure 6).

#### Endogenous Peroxidase

To be sure that swollen vacuoles were derived from granules and not from channels of the dense tubular system, samples of C-PRP exposed to PMA were fixed and incubated for the cytochemical demonstration of endogenous peroxidase. Enzyme reaction product was localized specifically to the dense tubular system in PMA-treated platelets and never appeared in the distended vacuoles (Figure 7).

#### Freeze-Fracture

The possibility that PMA induced a change in platelet membrane structure was investigated by the technic of freeze-fracture and etching.[11] Replicas of freeze-fractured, PE-treated platelets revealed no significant difference in the appearance of exposed membrane surfaces or the number and distribution of intercalated particles (Figure 8).

## Discussion

Results obtained in the present study have clarified some of the problems posed by earlier investigations into the action of phorbol myristate acetate (PMA) on blood platelets.[1,2] Initial examination of platelets after aggregation or incubation with PMA indicated that the agent

caused vacuole formation in the cytoplasm without causing the cells to lose their discoid configuration. The origin of the intracellular vacuoles was uncertain, but careful study of many samples suggested that PMA acted primarily on channels of the open canalicular system (OCS) and on the storage organelles. Granules were certainly involved because they disappeared as vacuoles increased in number. Since swollen vacuoles were occasionally continuous with platelet surfaces at least some channels of the OCS must have taken part in vacuole formation. Mitochondria were not involved in vacuole development because they remained intact in PMA-treated platelets. Only channels of the dense tubular system (DTS) remained as a reasonable alternative to granules and the OCS in the evolution of vacuoles, and that possibility has been ruled out in the present investigation. When PMA-treated platelets were incubated for endogenous peroxidase activity the enzyme reaction product was confined to the narrow channels of the DTS and did not appear in swollen vacuoles. The distribution of peroxidase in PMA cells was identical to that observed in normal platelets.[8] Therefore, elements of the DTS are not involved in the formation of vacuoles found in platelets following exposure to PMA.

The selective influence of PMA on storage granules in discoid platelets noted in our previous study led to the conclusion that some effect of the drug on the OCS altered the permeability barrier separating granule contents from channels, thereby leading to osmotic swelling of the organelles. It was also possible that the drug gained access to the intracellular organelles by traversing the cytoplasm, though the absence of any damage to other cell structures made this unlikely. The relationship of the PMA-induced vacuoles to channels of the OCS and the platelet surface was explored in this study with electron-dense tracers.[4,5] Thorium dioxide used as a vital stain and lanthanum nitrate as a passive electron-dense tracer coated the surfaces of platelets aggregated by or incubated with PMA, filled channels of the OCS and entered the large vacuoles. Neither stain was observed in the cytoplasmic matrix of PMA-altered platelets. The findings indicate that PMA not only alters a permeability barrier but breaks the wall separating granule contents and open canaliculi. Absence of tracer particles from the cytoplasm supports the suggestion that the site of communication between channels and granules is more susceptible to drug action than the rest of the platelet surface.

The possibility that membranes of granules and the OCS were more sensitive than the cell wall to the action of PMA stemmed from the first study which revealed conversion of granules to vacuoles without loss

of platelet discoid shape.[2] To examine this hypothesis, platelet samples in the present investigation were incubated with an agent which stabilizes the cell wall and inhibits shape change before exposure to PMA. Cytochalasin B has been used in previous studies to protect the discoid shape of platelets from the influence of aggregating agents and low temperature.[9,10] Platelets combined with cytochalasin B before treatment with PMA retained their discoid form but developed multiple cytoplasmic vacuoles. Stabilization of the cell wall does not appear to influence the barrier separating granule contents from channels of the OCS.

The morphologic features of the platelet response to agents which stimulate the release reaction and irreversible aggregation have been characterized in previous publications.[3,5,12] All of the agents caused platelets to lose their discoid form, become irregular with multiple pseudopods and undergo internal reorganization before, during or after the initial phase of aggregation. Organelles were moved to cell centers where they were embedded in contractile gel and encircled by tight fitting bands of microtubules. The external and internal alterations developed before the contents of storage organelles were extruded and were essential for irreversible aggregation. Vacuoles were seldom observed in platelets during the early phases of clumping associated with the release reaction, but developed to some extent during late phases of irreversible aggregation. The force of internal contraction leading to extrusion of products from storage organelles into channels of the OCS and out of the platelet does not appear to be associated with significant vacuolization.

The marked vacuole formation in platelets shortly after addition of PMA to C-PRP differed significantly from the response of the cells to other aggregating agents. Even though the granules in PMA-treated cells were labilized and permitted entry of electron-dense tracers, it was possible that they failed to extrude their stored contents. Zucker *et al*[1] noted that the secretion of adenine nucleotides, serotonin and β-glucuronidase was very delayed and reduced in quantity after platelets were aggregated by PMA. Polylysine was employed in this study to determine if the granule products were extruded during conversion to vacuoles or remained in the platelets after exposure to PMA. The cationic polypeptide was chosen on the basis of previous work showing that the agent is taken up by platelets and deposited in storage orgnelles.[6] The matrix of granules developed a characteristic lattice structure after contact with polylysine which remained intact during extrusion from the platelets. As a result it has been possible with the aid

of polylysine and other cationic polyelectrolytes to identify the substance stored in granules before or after discharge, and to follow sequentially the platelet release reaction in the electron microscope.[13] When platelets were combined with polylysine before or after exposure to PMA there was no evidence that products of the granules were extruded. All of the vacuoles in PMA-treated cells developed the characteristic lattice structure identified previously in the intact or discharged granules of normal platelets.[6] The findings suggest that the matrix substance of granules is diluted during conversion to vacuoles, but to a large extent remains in the distended organelles even though they communicate directly with channels of the OCS.

Results of investigations into the effects of PMA on blood platelet structure and function emphasize the importance of shape change and internal transformation to the platelet release reaction. Labilization of platelet storage organelles and subsequent swelling does not necessarily mean that stored products will be delivered to the surrounding plasma even though the distended vacuoles are connected by channels of the OCS to platelet surfaces. Vacuole development appears to inhibit rather than to facilitate extrusion of products destined for release.

A serious problem remains to be solved. If PMA does not stimulate the release reaction, how does it cause irreversible platelet aggregation? The process of irreversible aggregation follows from the platelet release reaction and appears to be dependent upon it.[14] Close cell contact is a sufficient stimulus to produce the platelet release reaction.[15] The aggregation induced in stirred C-PRP following addition of PMA may provide the propinquity necessary for the secretion of enough stored substances to cause irreversible aggregation. In a subsequent report we will demonstrate that PMA does cause the secretion of small but significant quantities of serotonin and adenine nucleotides, and that the modified release is important for irreversible aggregation induced by this agent.[16,17]

The mechanism by which PMA selectively labilizes platelet granules has not been ascertained. Other workers have shown that PMA can damage cell membranes, mitochondria and lysosomes, but the concentrations used to demonstrate lytic effects were far higher than the amounts needed to aggregate platelets.[18] However, because PMA is a membrane-active agent its influence was studied in replicas of freeze-fractured platelets treated with the drug.[11] Inspection of the replicas failed to indicate any significant change in the exposed faces of the split lipid bilayer or in the number and location of intercalated particles. Further investigations will be required to clarify the specific action of

PMA, and such efforts may reveal basic mechanisms involved in membrane secretory phenomena.

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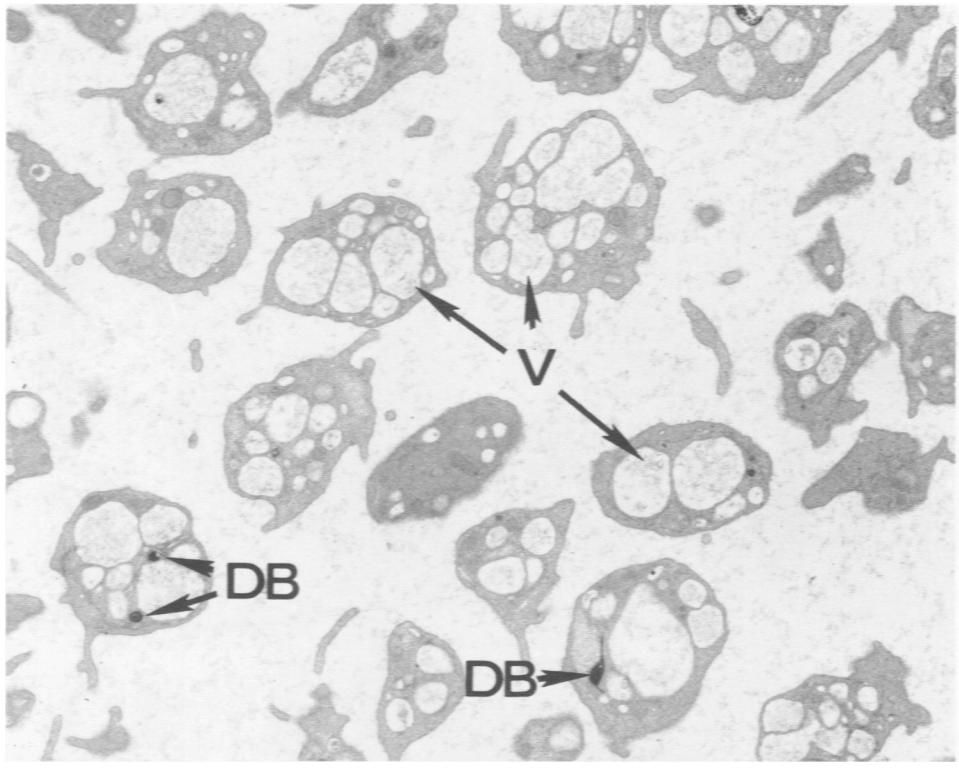
[*Illustrations follow*]

## Legends for Figures

**Fig 1**—Platelets from sample of citrate platelet-rich plasma (C-PRP) combined with EDTA (10 mg/ml) and exposed to phorbol myristate acetate (PMA) for 5 minutes at a final concentration of 100 ng/ml. Most of the granules in the cells have been replaced by swollen vacuoles (*V*). Dense bodies (*DB*) are less affected than granules and often survive exposure to high concentrations of the agent. The platelets have undergone slight degrees of shape change and pseudopod formation, but internal transformation is minimal. Alterations evident in platelet surface configuration appear to be due to the chelating agent as well as vacuole swelling in samples containing EDTA (× 8200).

**Fig 2**—Platelet from C-PRP combined with thorium dioxide before addition of PMA (100 ng/ml) to the sample on the aggregometer. The sample was fixed 2 minutes after exposure to PMA. Mitochondria (*M*), microtubules (*MT*), channels of the dense tubular system (*DTS*) and the cytoplasmic matrix appear undamaged. One granule (G) remains in the cell, but most have been converted to vacuoles (*V*). Particles of thorium dioxide coat the surface of the platelet, fill channels of the open canalicular system (*OCS*) and are present in all the vacuoles (× 40,000).

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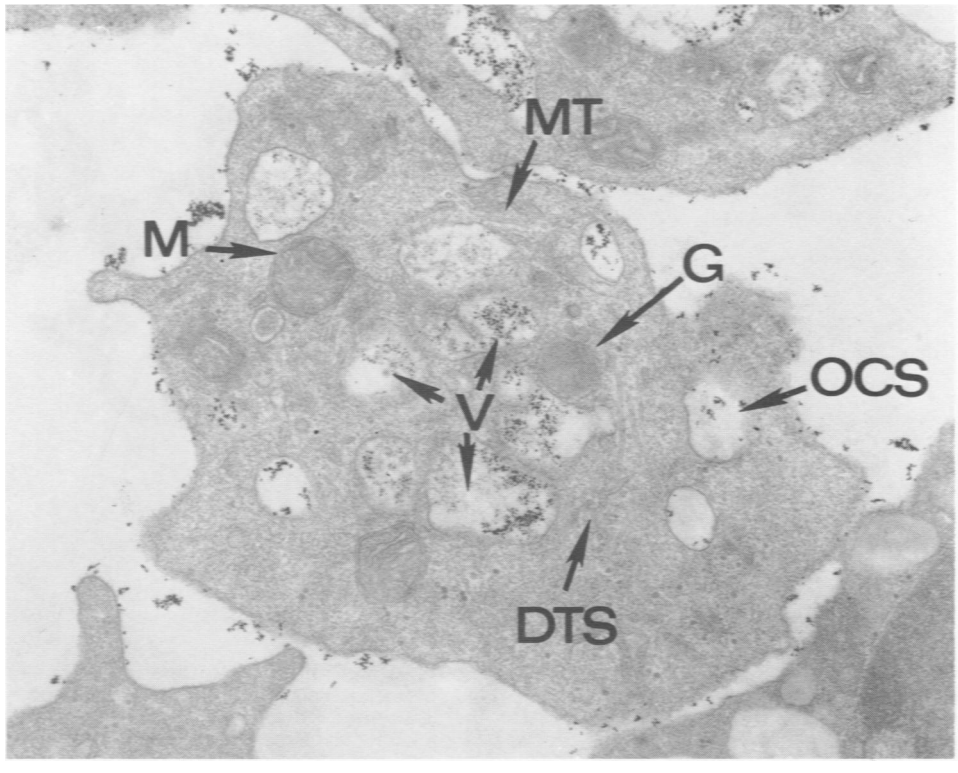


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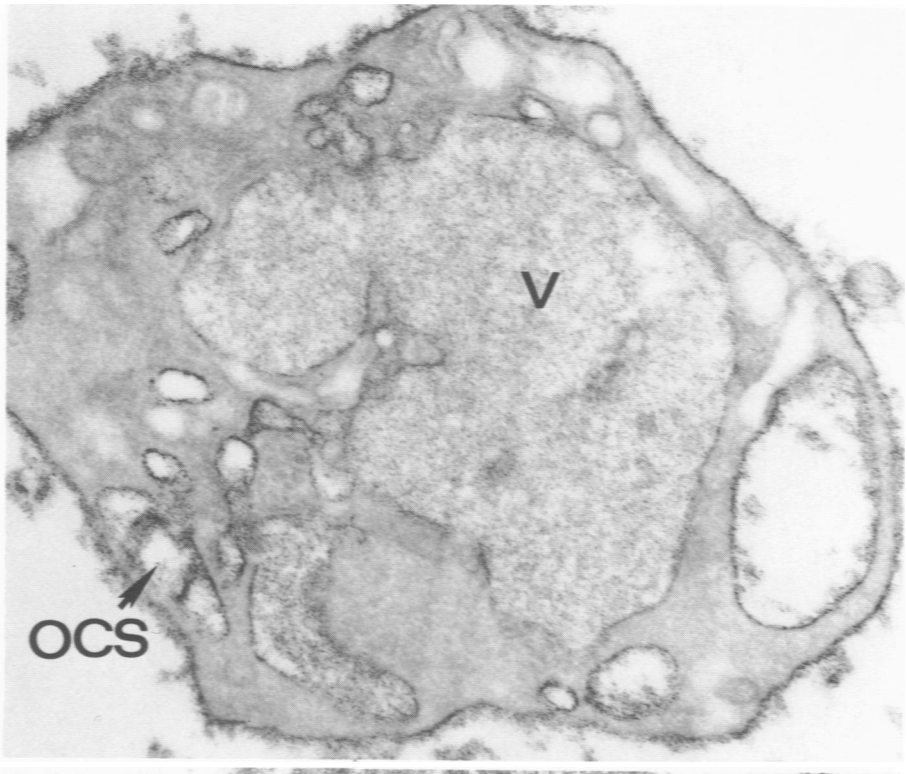


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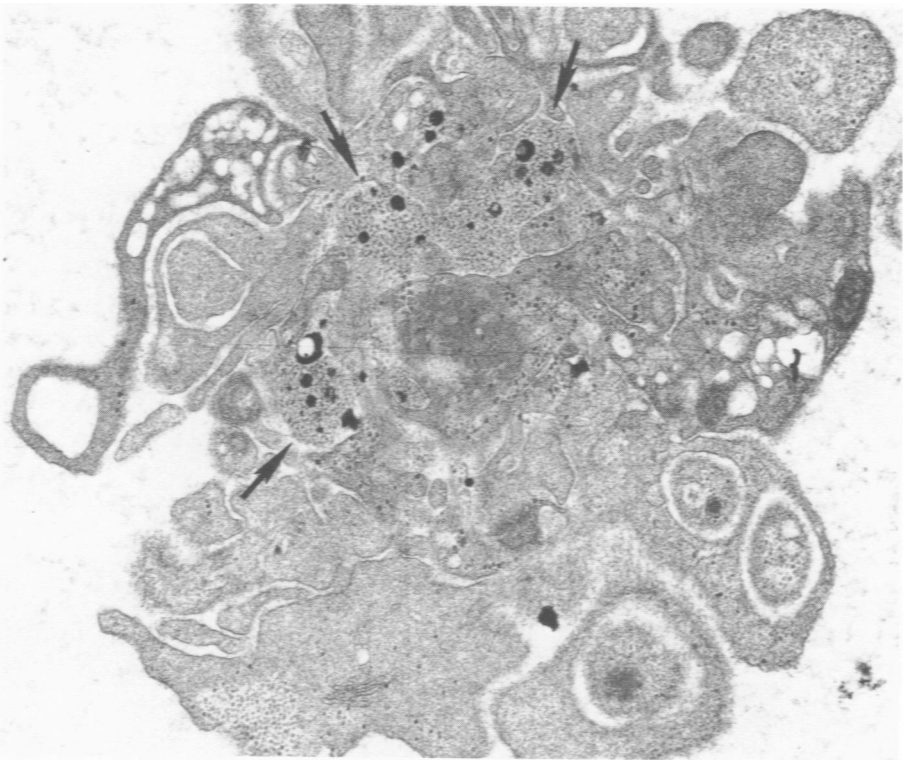
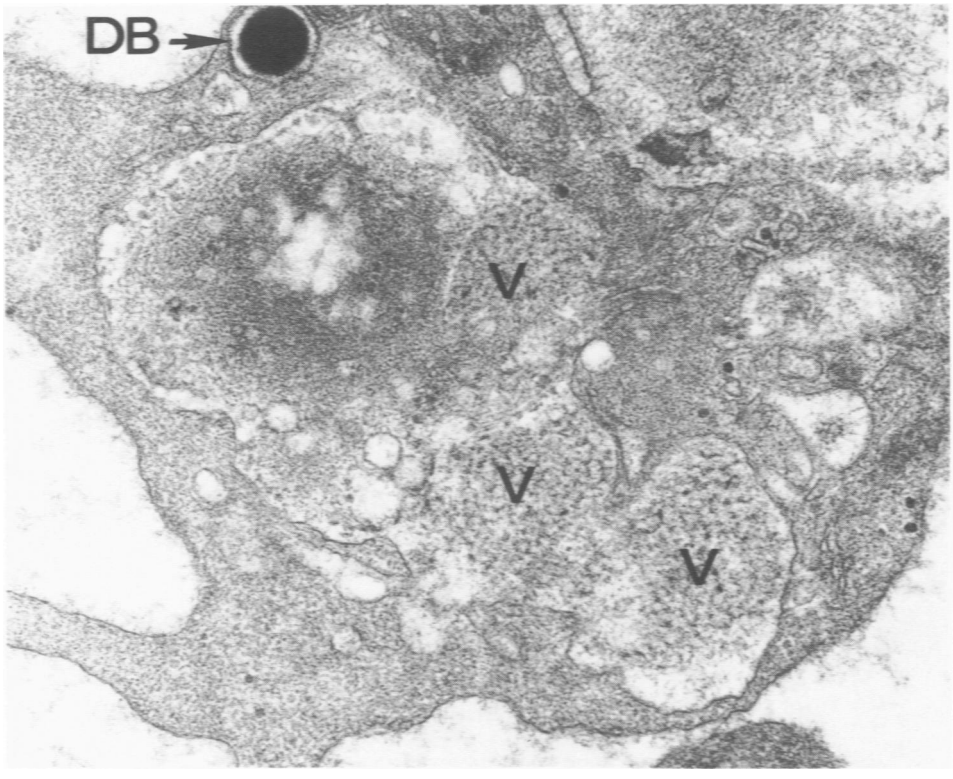


**Fig 3**—Platelet from sample of C-PRP and exposed in the third step of fixation to osmic acid containing lanthanum nitrate. Electron-dense stain covers the cell surface, lines channels of the OCS and fills the large vacuole in the cell center (× 49,500). **Fig 4**—Platelet aggregration from a sample of C-PRP combined with polylysine before addition of PMA on the platelet aggregometer. Vacuoles (*arrow*) in the aggregated cells are filled with granule matrix material polymerized into a lattice. Dark particles in the polymerized granule matrix are nucleoid fragments and dense bodies. The presence of these products in swollen vacuoles indicates their origin from granules and suggests that the material is not entirely extruded as a result of the changes induced by PMA (× 16,000).

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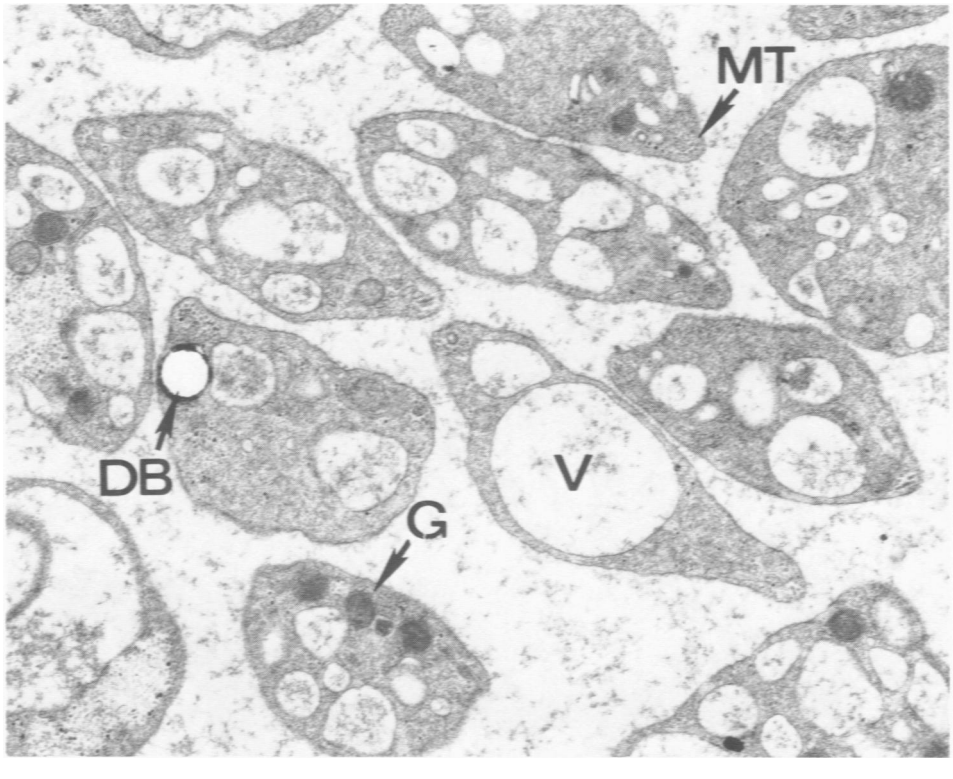


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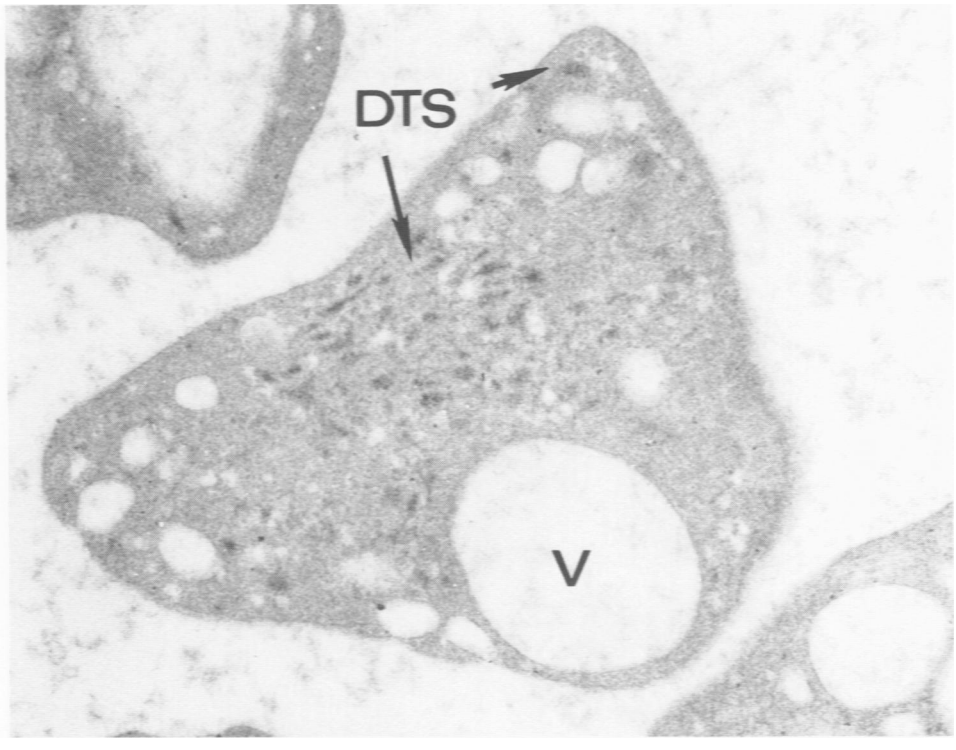
**Fig 5**—Platelet from sample of C-PRP aggregated by PMA and then incubated with polylysine. The appearance is identical to that observed in the previous illustration. Vacuoles (*V*) are filled with the polymerized material derived from the interaction of granule matrix and polylysine. An intact dense body (*DB*) remains in this cell (× 45,000). **Fig 6**—Platelets from sample of C-PRP combined with cytochalasin B (25 μg/ml) before exposure to PMA. Most of the cells have retained their typical discoid form with bands of microtubules (*MT*) located at the polar ends of each platelet. A few granules (*G*) and dense bodies (*DB*) remain in the cells, but most of the storage organelles have been converted to large vacuoles (*V*) by the action of PMA (× 18,000).

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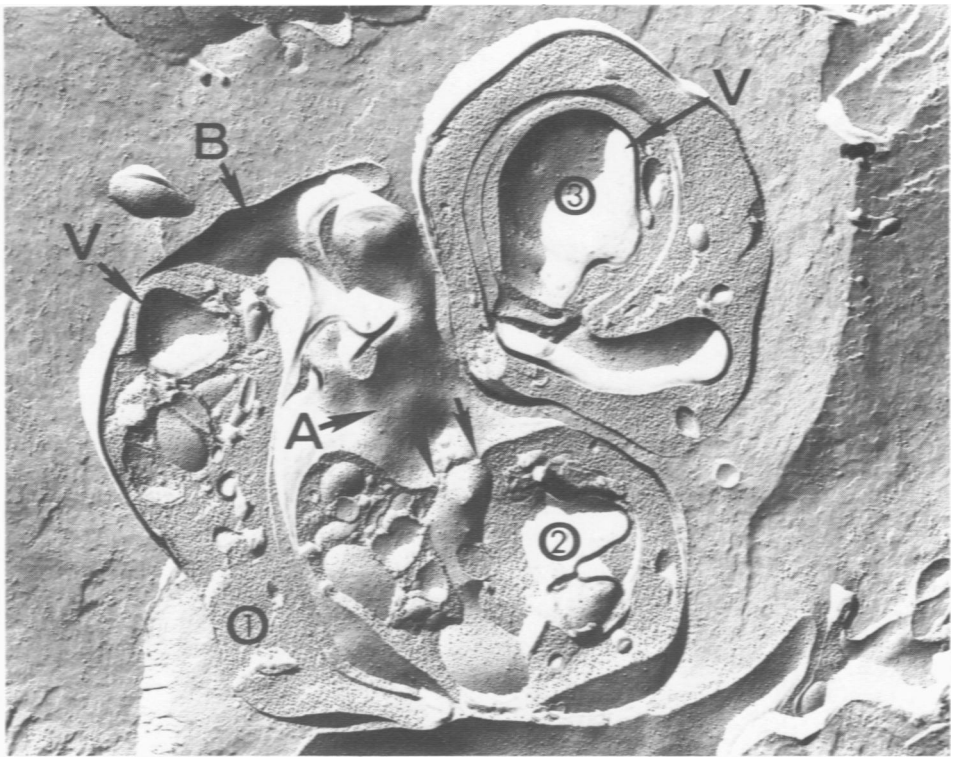


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**Fig 7**—Platelet from sample of C-PRP aggregated with PMA, fixed and incubated for endogenous peroxidase activity. Reaction product is evident in undilated channels of the DTS but not in the swollen vacuoles (× 42,900). **Fig 8**—Replica of freeze-fractured platelets aggregated by PMA. Three cells are present in this clump. Dilated vacuoles (*V*) are evident in cells *1* and *3*. Two faces of the split lipid bilayer (*A* and *B*) are evident in the replica. The number and distribution of 60 to 80A intercalated particles on both faces do not differ from those observed on replicas of normal cells. *Arrows* on cell 2 indicate sites of communication between channels of the open canalicular system and the cell wall (× 23,000).