Effects of Colchicine and Vinca Alkaloids on Human Platelets

II. Changes in the Dense Tubular System and Formation of an Unusual Inclusion in Incubated Cells

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A PREVIOUS INVESTIGATION from this laboratory demonstrated that colchicine and the vinca alkaloids, vincoleukoblastine (Velban) and vincristine, removed circumferential band and hyaloplasmic microtubules from blood platelets without impairing the capacity of the cells to retract clots.[1] The results indicated that intact microtubules were not essential for platelet contractile function but did not rule out the possibility that a subcomponent of disassembled microtubules might play a role in the contractile mechanism. During the course of this study, additional effects of alkaloids on platelets were noted. The agents caused alterations in a system of channels associated with the circumferential band of microtubules and promoted formation of an unusual inclusion in the platelet hyaloplasm which has not been previously described.

Materials and Methods

The methods used in this laboratory for collection of blood in 3.8% trisodium citrate, separation of citrated platelet-rich plasma (C-PRP), fixation of platelets for electron microscopy in glutaraldehyde-osmium, and embedment in Epon 812 were described in recent reports.[2,3] Material for the present study was obtained from the previous investigation on the influence of alkaloids on platelet microtubules and clot retraction.[1] Colchicine, vincristine, and Velban were dissolved in buffered saline to obtain concentrations of 100, 10, and 1 mg./ml., and 100 and 10 μg./ml. A 0.1-ml. volume of the dilution of one of the agents was combined with 0.9 ml. of C-PRP and returned to the 37° C. water bath for 30 min. At the end of incubation, one sample was fixed for electron microscopy, and the other recalcified and observed for clot retraction. The present study will be concerned with the samples incubated with alkaloids for 30 min. and then prepared for ultrastructural evaluation. Thin sections of plastic-embedded platelets were stained

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with uranyl acetate and lead citrate, and were examined in the Philips 200 electron microscope.

The platelet shown in Fig. 3A is from a sample of C-PRP, 0.9 ml. in volume, combined with 0.1 ml. of a solution containing 25 mg. of acetylsalicylic acid (aspirin) per milliliter. Addition of this amount yielded a final concentration of 2.5 mg. of aspirin per milliliter of C-PRP. The sample was incubated at 37° C. for 30 min., then prepared for electron microscopy in the same manner as the cells combined with alkaloids. Experiments to define the effects of aspirin on platelets in vitro over a wide range of concentrations have been completed and will be described in a subsequent report.[4]

Results

Normal Platelet Ultrastructure

The fine structural morphology of platelets obtained from blood collected in citrate anticoagulant and fixed initially in glutaraldehyde at 37° C. has been described in recent reports.[2,3] Platelets prepared in this manner are shaped like flattened discs. The lentiform shape is supported by a circumferential band of microtubules lying just under the cell wall along its greatest circumference (Fig. 1A). A system of fenestrated clear channels is irregularly distributed in the hyaloplasm, and its peripheral elements are continuous with the cell surface. Granules, mitochondria, dense bodies, and masses of glycogen are spread randomly in the matrix of the cell.

A system of channels closely interwoven with the circumferential bundle of microtubules is also present in discoid platelets (Fig. 1A and 1B). The canaliculi are irregular in shape and therefore appear discontinuous in thin section. An amorphous material, similar in density to the hyaloplasmic matrix, is evident in the channels, and fine 50 Å filaments are also noted occasionally. The close association of the channels with the circumferential microtubules and their content of amorphous substance distinguish them from elements of the canalicular system. As a result, Behnke[5] suggested that the channels associated with the marginal band be referred to as the dense tubular system (DTS).

Effects of Colchicine, Vincristine, and Velban DTS of Platelets. In the previous report,[1] the gross morphologic effects of alkaloids on blood platelets were equated with the functional capacity of the cells to retract clots. Low concentrations of the drugs removed platelet microtubules and caused the cells to lose their typical discoid shape. Disappearance of intact microtubules and loss of disc shape did not impair the ability of the cells to retract clots. The effects of the alkaloids on platelet structure, however, were not limited to the circumferential band and hyaloplasmic microtubules.

Channels of the DTS, usually associated with the circumferential band of microtubules, were redistributed in alkaloid-treated cells (Fig. 2A and 2B). The alteration was evident in the few cells affected by the lowest concentrations of alkaloids and prominent in platelets exposed to higher concentrations. Prominence of the DTS and irregular distribution of its channels in the hyaloplasm were associated with disappearance of the circumferential band of microtubules. The redistribution of the DTS in alkaloid-treated cells, however, was not strictly a specific effect of the drugs tested in this study. Similar changes in the DTS occurred in platelets after exposure to high concentrations of acetylsalicylic acid in vitro (Fig. 3A).[4]

Formation of a Crystalloid in Platelets. In addition to changes in external contour, disappearance of intact microtubules, and random dispersal of the DTS, another significant alteration developed in platelet structure following incubation with the alkaloids. An inclusion was apparent in a few platelets exposed to the lowest drug concentrations and in most cells that had been exposed to large amounts of the three agents. The appearance of the inclusion varied with the plane of section and the degree of cell swelling. Most commonly, the lesion consisted of groups of parallel lines (Fig. 3B, 4A and 5A). Each line was approximately 50 Å in diameter and was separated from adjacent heavy lines by intervals of about 300 Å. Finer lines could occasionally be distinguished between the heavy filaments. In other platelets, or in different parts of the same cell, the inclusion consisted of closely packed circles (Fig. 3B, 4A, and 4B). Individual circles were approximately 350 Å in diameter, and many contained central filaments 50–75 Å thick. Between the laminar arrangements and compact groups of circles were many variations. In some cases the inclusion resembled a grid-like pattern of small squares (Fig. 4A) or appeared as diamond shapes similar to a picket fence (Fig. 5B). The unusual structure was still evident in platelets even at concentrations of the drug which severely damaged most cells and destroyed clot-retracting capacity.

Discussion

Colchicine, vincristine, and Velban influence a variety of functions in different cells by selectively depolymerizing microtubules.[6-9] The three agents do not appear to produce irrevocable damage to cells, since removal of alkaloid from the incubation media allows cells to reform tubules and recover functional capacity.[6,8] Utilization of these agents by many investigators has permitted new insights into mechanisms of cell division, saltatory particle movement, pseudopod formation, and other motile processes.

Many of the cell functions affected by the alkaloids are believed to be contractile in nature.[10,11] Therefore, the agents were chosen for the present study to assess the role of intact microtubules in platelet contractile activity.[1] High concentrations of colchicine, vincristine, and Velban destroyed platelets, but smaller amounts removed intact microtubules without impairing the ability of the cells to retract clots. Since clot retraction is an energy-dependent contractile process requiring metabolically active platelets,[12] it is doubtful that the cells were seriously damaged by exposure to concentrations as high as 1 mg./ml. These findings indicated that intact microtubules are not essential for the clot-retracting capacity of platelets but did not rule out the possibility that a component derived from microtubules during disassembly might still be involved. Therefore, a careful study of platelets after exposure to various concentrations of alkaloid has been made to assess other effects of the drugs and to determine the fate of the microtubules.

Behnke [5] previously pointed out the existence of two unconnected systems of channels in platelets, and demonstrated that they were morphologically and cytochemically different. The canalicular system (CS), composed of irregular, clear channels continuous peripherally with the cell surface, permits plasma and plasma-borne elements to bathe the interior of the platelet, and may serve as a conduit for the extrusion of hyaloplasmic components during the release reaction.[13] Channels of the DTS do not appear to share the transport function of the CS, and their role in platelet physiology has remained obscure.

Results of this study have not clarified the function of the DTS. However, the relationship between channels of the DTS and the circumferential band of microtubules appears more intimate than previously suspected. Prominence and irregular distribution of DTS elements occurred in platelets rendered devoid of intact marginal bands of tubules by exposure to alkaloids. Platelets unaffected by exposure to small amounts of the three agents did not develop changes in the DTS. These findings suggest that channels of the DTS are in some way structurally related to circumferential microtubules. What the relationship has to do with the function of the DTS or formation of circumferential tubules, however, remains to be determined.

The prominence of channels of the DTS in alkaloid-treated platelets may be due to an increase in their number. Proliferation of new channels secondary to the action of alkaloids cannot be ruled out, but the possibility implies an influence of the drugs on structures other than microtubules. The highly selective action of alkaloids on tubules and the avidity of the agents for microtubule protein are well known.[9] An

effect on structures other than microtubules in platelets by low concentrations of the drugs would be unlikely. Therefore, changes in the DTS are probably due to the direct action of the agents on microtubules and not to drug effects on the platelet DTS channels per se. Furthermore, other agents which can affect the state of polymerization of microtubules produce changes in the DTS similar to those caused by the alkaloids.[4] High concentrations of acetylsalycilic acid also destroy the marginal band and leave the DTS clearly defined (Fig. 3A). Changes in the DTS, therefore, are not drug specific, but are intimately related to alterations in the circumferential microtubules.

Formation of the unusual inclusion in alkaloid-treated platelets was also closely related to alterations in the circumferential bundle of microtubules. The arrangement of parallel filaments with a period of 350 Å, the compact groups of 350 Å circles, and the grid-like patterns occurred only in platelets rendered devoid of intact tubular elements following incubation with the three agents. Inclusions may have resulted from the influence of alkaloids on structural components of platelets other than microtubules. Platelet hyaloplasm contains masses of 50 Å microfilaments which often become associated in parallel bundles during pseudopod formation.[3] Lysis of platelet granules could provide tubular elements and masses of parallel filaments, since both configurations have been identified in the substructure of intact particles.[14] However, the inclusions developed in platelets exposed to concentrations of alkaloid which did not damage granules or other hyaloplasmic components, and the inclusion was not prominent in pseudopods. Therefore, it appears probable that the inclusion results from the direct action of the agents on tubules, rather than on other platelet structures. In fact, careful evaluation of unaltered platelet microtubules by several techniques and analysis of the various arrangements assumed by the alkaloid-induced inclusion observed in this study have indicated that inclusions are formed from disassembled microtubules of the marginal band. In order to comprehend the effect of the three alkaloids, it is essential to examine the structure of unaltered platelet microtubules.

Each microtubule examined in negative-stain, whole-mount preparations of normal platelets is composed of 11–15 parallel subfilaments.[15,16] Individual subfilaments are made up of stacks of helically wound, 40 × 50 Å globular subunits. Adjacent subfilaments of spread tubules have a center-to-center period of 60 Å. A diagonal pattern of lines across the width of the spread tubules indicates that the entire complex is a helical association. Thin sections of unaltered plastic-embedded platelets, on the other hand, reveal intact marginal microtubules as parallel 250 Å

fibers, or hollow circular profiles when cut in cross section.[2,3,17] Each tubule appears hollow, except for an occasional central filament, and is separated from adjacent tubules by a structureless matrix.

After exposure to alkaloids, this arrangement of fibers disappears, and the inclusions develop. The compact groups of 350 Å circular profiles with central filaments are clearly similar to microtubules in cross section except for their size and packing. Lamellar inclusions composed of 50 Å filaments separated by 300 Å intervals would resemble horizontally sectioned tubules if the tubules were spread and pressed together. All other arrangements of the inclusion can also be explained by reference to a model in which individual tubules dilate and pack together with similarly dilated neighbors (Fig. 4A).

The inclusion observed in platelets exposed to alkaloids, therefore, appears to result from dilatation of individual tubules making up the circumferential band. Increase in diameter of microtubules may be explained by the spreading apart of individual subfilaments, uncoiling of the entire helical complex of subfilaments, or both. The precise chemical basis for dilatation is not known, but one critical point emerges which is of value to this discussion. The subfilaments which make up each microtubule remain intact even after exposure to 10 mg. of alkaloid per milliliter, an amount sufficient to destroy the integrity of the cell and its function. Only the bonds between adjacent subfilaments appear to be affected. Therefore, the action of colchicine, vincristine, and Velban does not appear to result in complete destruction of platelet microtubules down to the level of 40 × 50 Å globular subunits of which each subfilament is composed. Rather, the agents appear to split each subfilament from its neighbor, resulting in dilatation of the whole tubule. At points where the inclusions become disorganized, the filaments appear to remain intact. No masses of globular subunits from destroyed microtubules were noted in alkaloid-treated platelets at any drug concentration. The tendency for subfilaments of inclusions to remain intact when no longer associated with the crystalloid is in agreement with previous observations on the integrity of subfilaments spraying out of broken microtubules.[16] Subfilaments escaping from inclusions cannot be differentiated from hyaloplasmic microfilaments, nor can subfilaments be distinguished from microfilaments in whole-mount preparations.

Though intact microtubules are virtually ablated in platelets exposed to colchicine and vinca alkaloids, results of this study indicate that the subfilaments of which the microtubules are composed remain in the cells. While many subfilaments make up the framework of inclusions, large numbers probably become part of the unorganized mass of microfilaments filling the hyaloplasm. These findings lend support to the

concept that microfilaments and subfilaments of microtubules are identical, or at least very similar fibers, differing only in their state of organization.[10] While intact microtubules are not necessary for the functional activity of platelets in clot retraction, it is evident that subfilaments derived from microtubules remain in alkaloid-treated cells. Elimination of the need for intact microtubules does not remove the possibility that subfilaments derived from the tubules may plan an active role in the platelet contractible mechanism.[3]

The dilatation of platelet microtubules following exposure to alkaloids has apparently not been observed in other investigations. However, the alteration is not without precedent. Tilney and Porter noted a transformation of 220 Å microtubules in axopodia of Heliozoa into 340 Å tubules following exposure to low temperature.[18] The only major difference between their observations and the findings of this report is that packing of dilated tubules did not develop in Heliozoa. Tilney and Porter's work is not strictly applicable to the present study. Microtubules in axopodia may differ from platelet microtubules, alkaloids may have a different mechanism of action than chilling, and previous studies have shown that platelet microtubules disappear completely when the cells are exposed to 0–4° C.[2] Despite these reservations, the fact that microtubules of any cell species can dilate by separation of component subfilaments under the influence of a condition or agent which also profoundly affects the stability of platelet microtubules requires consideration. It is probable that chilling causes platelet microtubules to shear into individual subfilaments, rather than to break down the subfilaments to their globular components. This possibility was not explored in the previous study, but is currently under investigation.

Summary

A previous investigation demonstrated that colchicine and the vinca alkaloids removed intact microtubules from platelets without destroying the capacity of the cells to retract clots. During the course of the investigation, it was noted that platelets exposed to the agents developed structural alterations in addition to disappearance of microtubules. Channels of a dense tubular system, usually interwoven with circumferential microtubules in unaltered platelets, became prominent and randomly dispersed in the hyaloplasm. An unusual inclusion also developed in cells rendered devoid of microtubules by exposure to alkaloids. Both alterations appear to be direct results of the action of the drugs on microtubules, rather than on other components of the cell. The nature of the two structural alterations is discussed.

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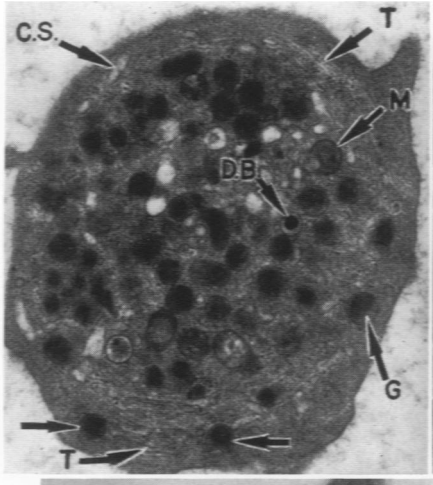
[*Illustrations follow*]

Legends for Figures

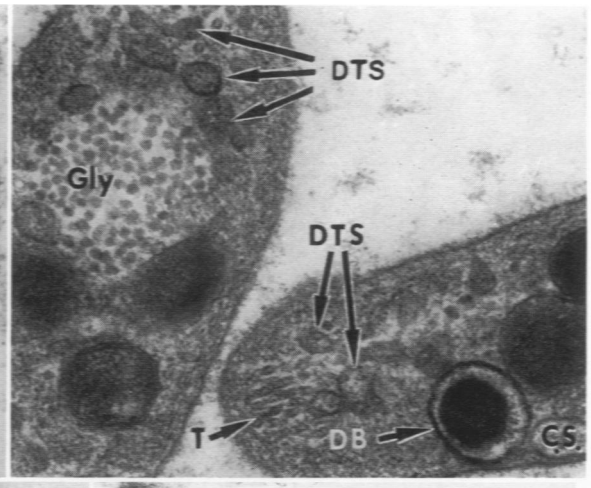
Fig. 1. A. Discoid blood platelet from control sample of C-PRP sectioned in a plane parallel to circumferential bundle of microtubules (*T*). Band of tubules lies just under cell wall along its greatest circumference. Most hyaloplasmic organelles are located interior to marginal band. Granules (*G*) with two zones of electron density dominate hyaloplasm. A few mitochondria (*M*), occasional dense bodies (*DB*), and a complex system of clear channels—the canalicular system (*CS*)—are spread randomly in platelet substance. Another system of channels, the DTS, is obscured by bundle of microtubules at cell periphery *(arrows)*. × 14,000. **B.** Cross sections of two platelets, revealing elements of DTS lying just inside circumferential bundle of microtubules (*T*). Material contained in these channels is similar in density to hyaloplasm. Some of the contained elements resemble filaments cut in cross section. A mass of glycogen (*Gly*) is present in one platelet; a dense body (*DB*) and channel of canalicular system (*CS*) are indicated in the other cell. × 57,700.

Fig. 2. A. Platelet from C-PRP incubated for 30 min. with a final vincristine concentration of 1 μg./ml. Cell is devoid of intact microtubules. Two types of channels are evident. Clear channels of canalicular system (*CS*) are somewhat dilated after exposure to alkaloid. Elements of DTS, freed of their relationship with the microtubules, appear randomly spread in hyaloplasm (*arrows*). Other platelet organelles are undisturbed by exposure to drugs at this concentration. Granules (*G*); glycogen (*Gly*); dense body (*DB*). × 30,300. **B.** Platelet exposed to a final colchicine concentration of 10 μg./ml. Narrow ribbons of the DTS *(arrows)* are spread irregularly in cell substance. Angular shape and content of opaque material distinguish elements of this system from channels of canalicular system (CS). × 27,600.

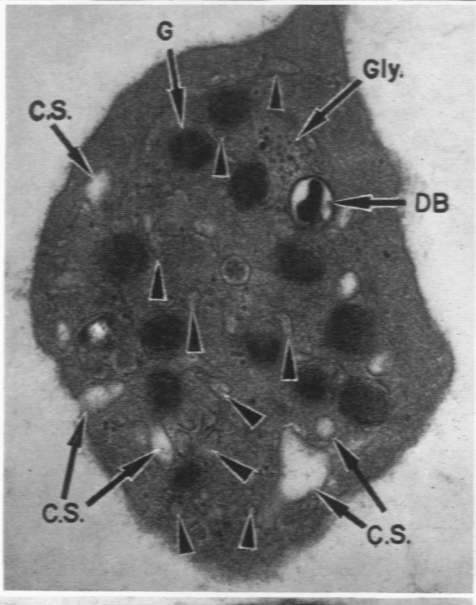
Fig. 3. A. Exposure of platelets to high concentrations of acetylsalicylic acid results in damage to circumferential bundle of microtubules. This cell is from C-PRP combined with aspirin, 2.5 mg./ml. for 30 min. Channels of *DTS* and canalicular system (*CS*) are clearly demarcated. Filaments sectioned in various planes are evident in channels of DTS. × 32,900. **B.** Platelet from sample combined with 100 μg. Velban per milliliter. A series of fine 50 Å parallel lines separated by intervals of 300 Å are evident at right (*I*), and a group of compact 350 Å circles at left (*I*). Inclusions of this type have not been seen in normal platelets or in cells exposed to agents other than alkaloids. × 29,400.



1A



1B



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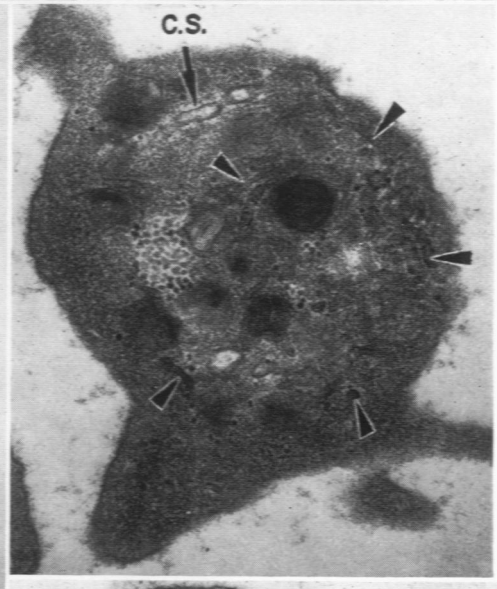

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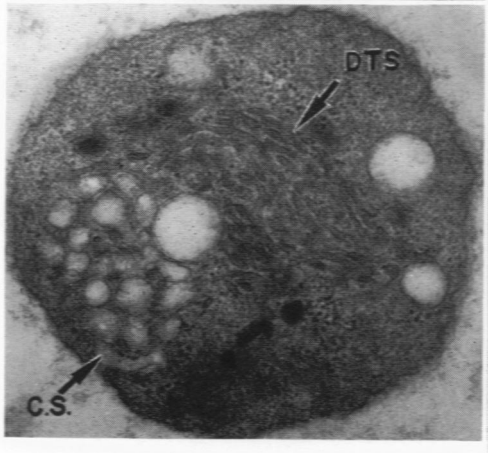
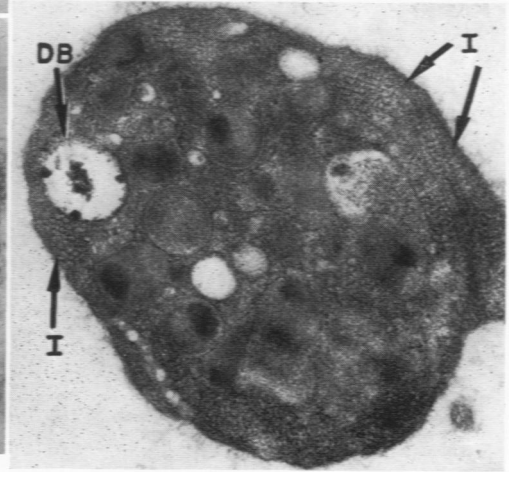
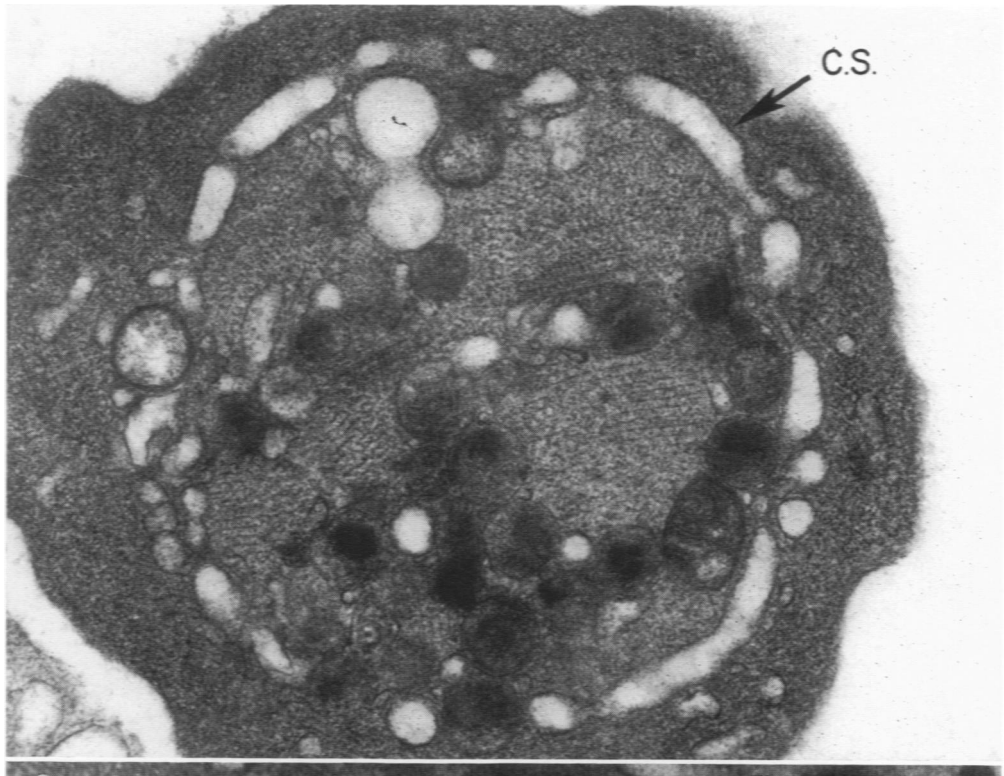


3A

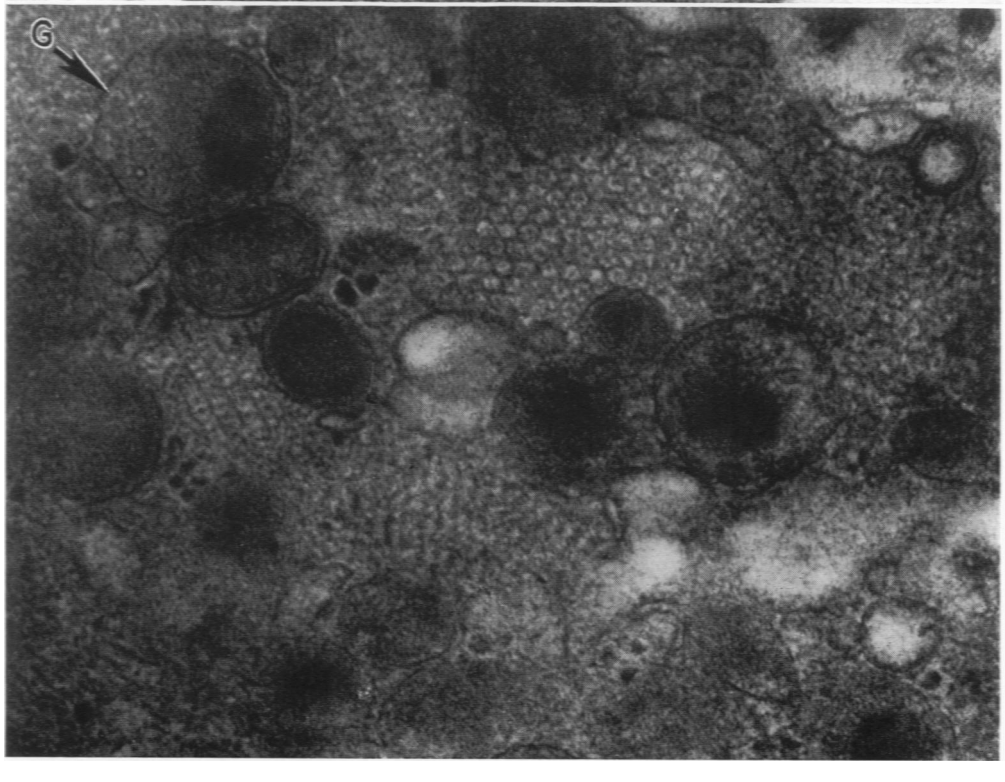


3B

Fig. 4. A. Platelet incubated with vincristine for 30 min. at final concentration of 1 mg./ml. Central area of cell reveals grid-like pattern composed of fine parallel lines and compact circular profiles. Canalicular system *(CS)*. × 36,800. **B.** Central area of platelet after exposure to colchicine, 1 mg./ml. Compact groups of circular profiles and other arrangements of inclusion are apparent. Many circular elements contain central filaments. An adjacent granule (G) contains a tubular element in its matrix. Profiles are frequently encountered in granules of unaltered platelets. × 81,000.

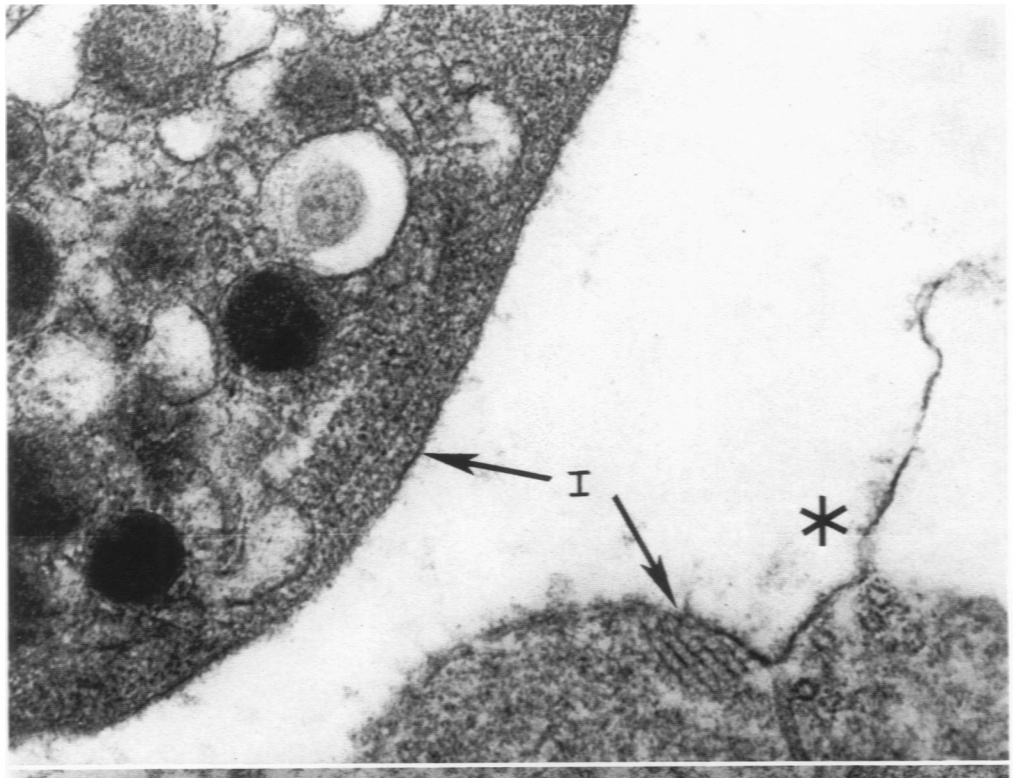


4A

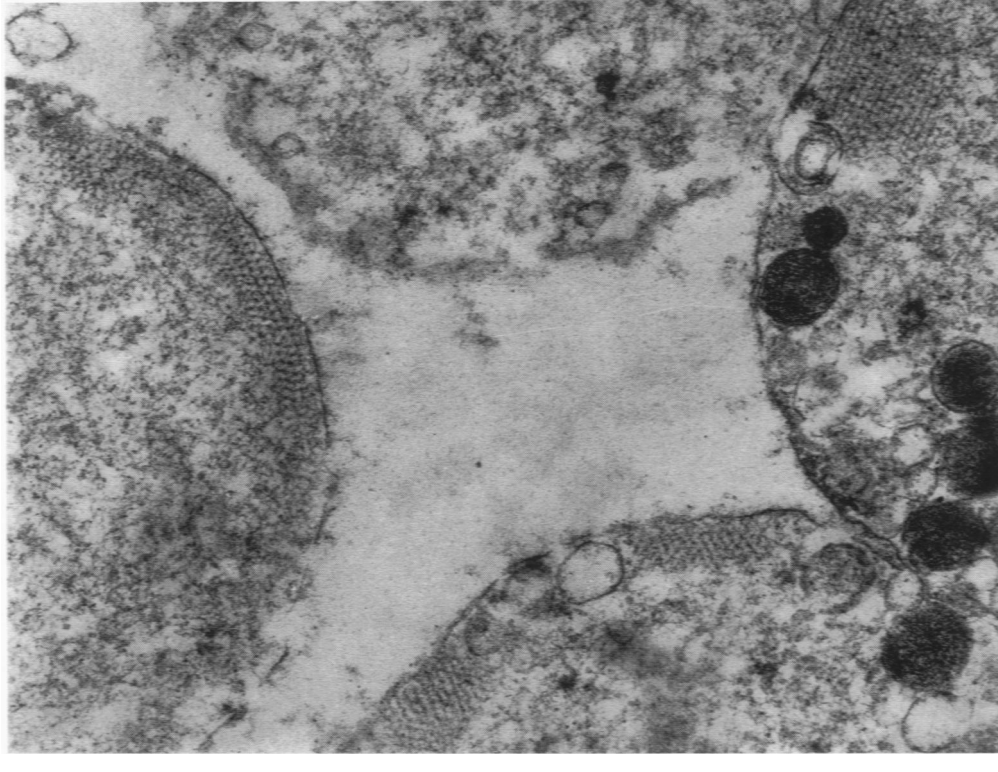


4B

Fig. 5. A. Platelets from C-PRP combined with Velban, 1 mg./ml. Inclusions composed of fine parallel lines and compact groups of circles are evident in adjacent cells (*I*). Area under *asterisk* is of interest. Dilated microtubules in cross section reveal subfilaments of which each tubule is composed. One tubule is against the side of another, cut in a plane parallel to its long axis. Alterations evident in individual tubules suggest that alkaloids cause parallel subfilaments to separate, resulting in dilatation. The inclusions, therefore, are formed directly from altered microtubules. × 55,200. **B.** Platelets after exposure to colchicine, 10 mg./ml. High concentrations of the drugs cause severe platelet damage. Inclusions survive despite the injury to cell structure and function. Picket-fence arrangements and grid-like patterns composed of small squares are apparent. The differing arrangements depend upon the various planes in which the inclusion is sectioned. × 42,300.



5A



5B