Influence of Taxol on the Response of Platelets to Chilling

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Platelets have a characteristic lenslike appearance in circulating blood. *In vitro* studies have shown that the discoid shape is supported by a circumferential band of microtubules. Early studies demonstrated that chilling caused platelets to lose their disklike form and become irregularly convoluted with multiple pseudopods. The cold-induced shape change was associated with disappearance of the annular band of microtubules. Rewarming caused reassembly of the circumferential bundle and recovery of platelet discoid form. The present study has examined the influence of taxol, a microtubule stabilizing agent, on the response of platelets to low temperature. Taxol-treatment protected platelet microtubules from disassembly in the cold and preserved the discoid shape of most platelets. Addition of taxol to platelets prechilled to remove microtubules and maintained in the cold resulted in assembly of tubular polymers at low temperature. Brief exposure to taxol in the cold did not prevent recovery of platelet discoid shape on rewarming to 37 C. However, the bundle of tubules was often located in the central axis, rather than in a circumferential band. Longer incubation with taxol at low temperature resulted in assembly of radiating bundles of tubules. On rewarming, these cells remained irregular in form and did not develop circumferential bands. (Am J Pathol 1982, 108:184–195)

A VARIETY of studies have demonstrated that blood platelets have a characteristic discoid form *in vivo* and *in vitro*.[1] A circumferential band of microtubules lying just inside the cell membrane supports the lentiform shape of the cell.[2-4] The importance of the circumferential band of microtubules as a cytoskeletal support system maintaining platelet discoid shape was demonstrated several years ago in studies of the effects of chilling.[5,6] Low temperature caused platelets to lose their lentiform appearance, assume globular configurations, and extend pseudopods. The shape change due to cold was shown to be associated with dissociation of the circumferential band of microtubules.

Taxol, a new chemical agent derived from plants, has recently been found to stabilize microtubules in a variety of cells and prevent their disassembly.[7-8] The present study has evaluated the influence of taxol on the response of platelets to low temperature. Results of the investigation demonstrate that taxol prevents dissociation of platelet microtubules on exposure to low temperature, promotes platelet microtubule assembly in the cold, and stimulates rapid formation of unusually located microtubules during rewarming.

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Materials and Methods

Blood for the present study was obtained from normal adult volunteers after informed consent. Immediately after venesection, the blood was mixed with citrate–citric acid dextrose, pH 6.5, in a ratio of 9 parts blood to 1 part anticoagulant.[10] Citrate–platelet-rich plasma (C-PRP) was separated from whole blood by centrifugation at 200*g* for 20 minutes at room temperature. Methods used to chill platelets to 4 C or lower in an ice-water bath and maintain the samples at low temperature for prolonged periods were described in earlier reports.[5,10] Taxol (Natural Products Branch, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, Md) was dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 10 mM. The stock solution was frozen at a −70 C between experiments. Samples of C-PRP were combined with taxol at final concentrations up to 10^{-4} M. The highest final concentration of taxol had no apparent deleterious influence on platelet structure. The final concentration of DMSO used to deliver taxol to PRP was equal to or less than 1.0%, an amount shown in previous studies to have no apparent physiologic or morphologic effect on platelets.[11] Samples of C-PRP combined with taxol or with the carrier, DMSO, in the same volume, but without taxol, were maintained at 37 C for 30, 60, and 90 minutes before fixation. Other portions of C-PRP were chilled to 2–4 C first for 15–30 minutes, then combined with taxol or carrier. Chilled samples were fixed in the cold at 5, 15, 30, and 60 minutes. Additional portions of prechilled C-PRP were combined with taxol or carrier, kept at 2–4 C for 5, 15, 30, or 60 minutes, then rewarmed to 37 C for 15 or 30 minutes before fixation. All samples were fixed according to the procedure established in this laboratory.[10] An equal volume of 0.1% glutaraldehyde was added to each platelet sample while the cells were in suspension. For samples at low temperature the glutaraldehyde was chilled and the platelets fixed in the cold. After 15 minutes the samples were sedimented at 800*g* for 15 minutes, and the supernatant was decanted and replaced with 3% glutaraldehyde in White's saline. After 30 minutes the pellets were washed with buffer and combined with 1% osmic acid in Zetterquist's buffer for 1 hour at 4 C. After dehydration in a series of alcohols the platelet samples were embedded in Epon 812. Thin sections stained with uranyl acetate and lead citrate to enhance contrast were prepared on an LKB ultramicrotome and examined in a Philips 301 transmission electron microscope. Samples of glutaraldehyde-fixed platelets were also frequently examined in the phase-contrast microscope to assess gross shape changes. Eight blood samples from 5 individuals were used in the present study.

Results

Normal Platelet Ultrastructure

The lentiform shape of platelets is well maintained by initial fixation at room temperature or 37 C in glutaraldehyde before exposure to osmic acid.[10] A circumferential bundle of microtubules lying just under the cell membrane supports the discoid form (Figure 1). Organelles, including granules of several kinds, dense bodies, and occasional mitochondria, are randomly dispersed interior to the annular bundle of microtubules in the hyaline matrix of the cytoplasm. Elements of two channel systems, the surface-connected, or open canalicular, system and the dense tubular system, are spread randomly in the cytoplasm.

Effects of Chilling

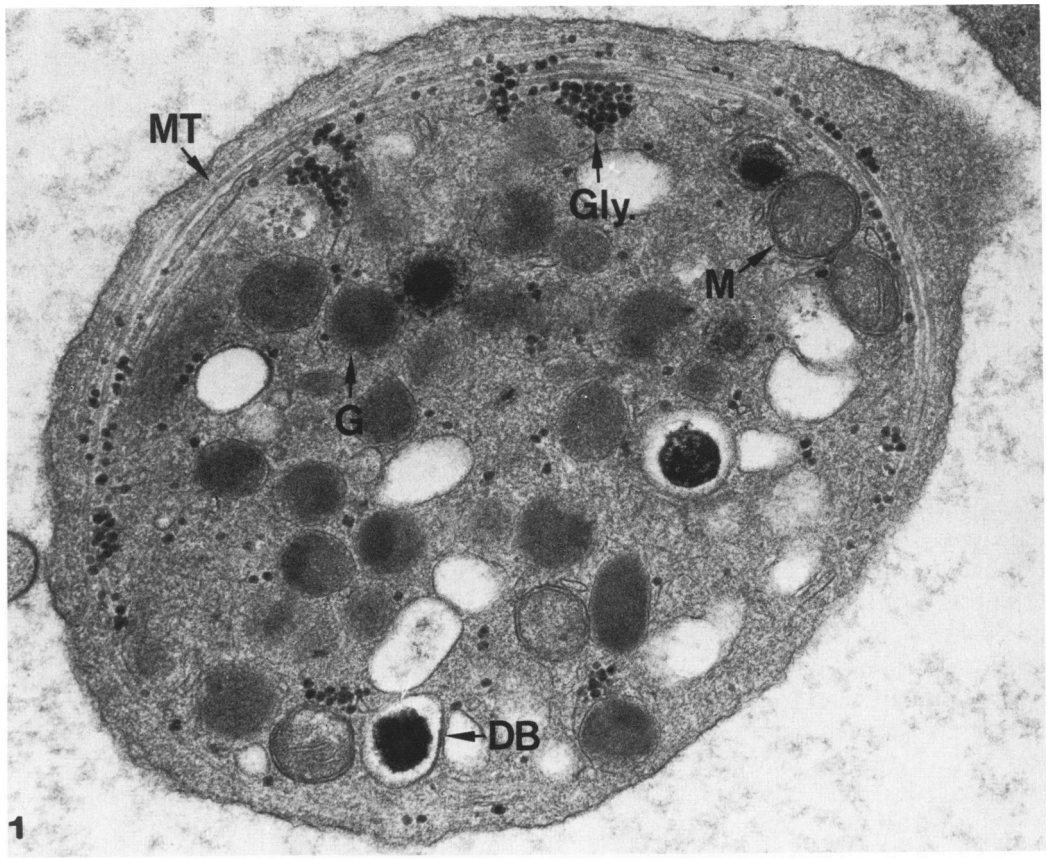
After exposure to temperatures below 13 C for periods as brief as 5–10 minutes, platelets lose their characteristic discoid configuration.[5] The cells become irregular and relatively spherical, and extend bulky and sharp pseudopods (Figure 2). Loss of discoid shape is associated with complete disappearance of microtubules in platelets chilled for 15 minutes at 2–4 C.

Morphologic Influence of Taxol on Platelets

Platelets were incubated for 30, 60, and 90 minutes with taxol in final concentrations of 10^{-6}, 10^{-5}, and 10^{-4} M or exposed to the carrier alone. The discoid shape and other morphologic features of platelets appeared unaffected by taxol or the carrier (Figures 3 and 4).

Influence of Taxol on the Platelet Response to Chilling

Platelets combined with taxol at final concentrations of 10^{-4}–10^{-6} M or exposed to the carrier alone were maintained at 37 C for 15 minutes. Subsequently the samples were chilled to 2–4 C and maintained at that temperature for intervals up to 60 minutes. Over half the platelets exposed to low temperature after treatment with taxol retained their discoid shape throughout the period of observation (Figure 5). Circumferential bands of microtubules remained intact in discoid and irregular platelets fixed in the



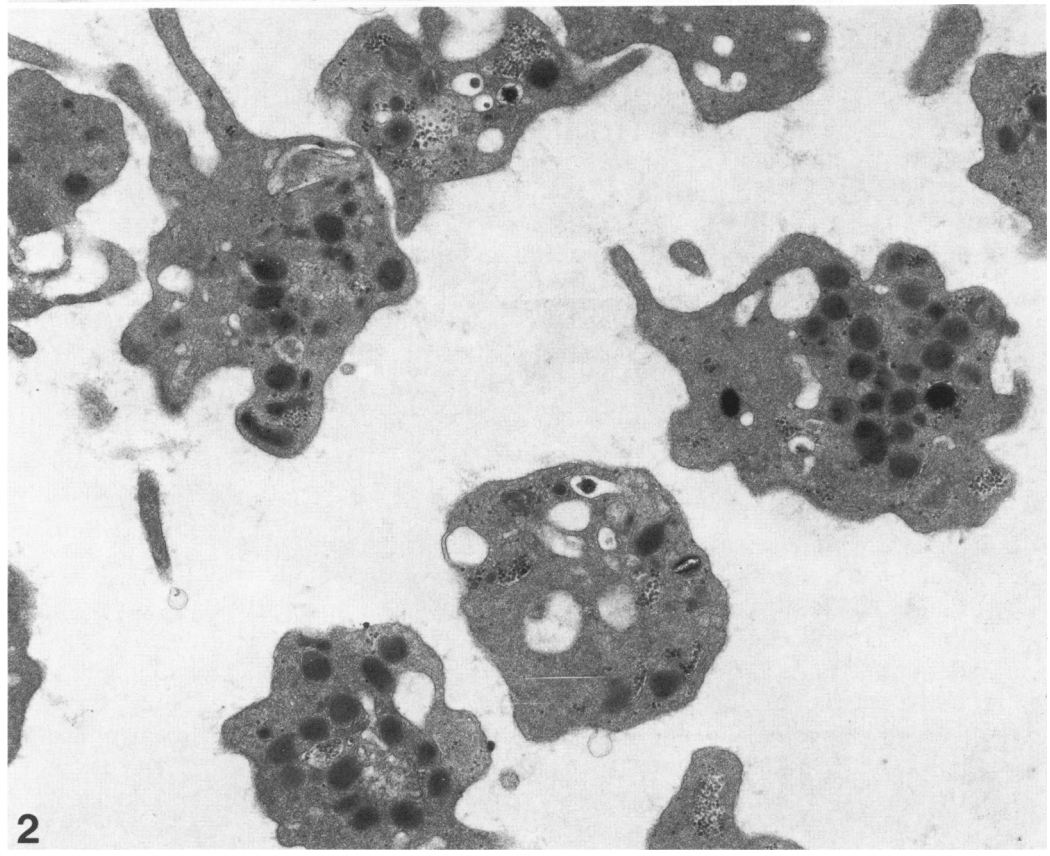
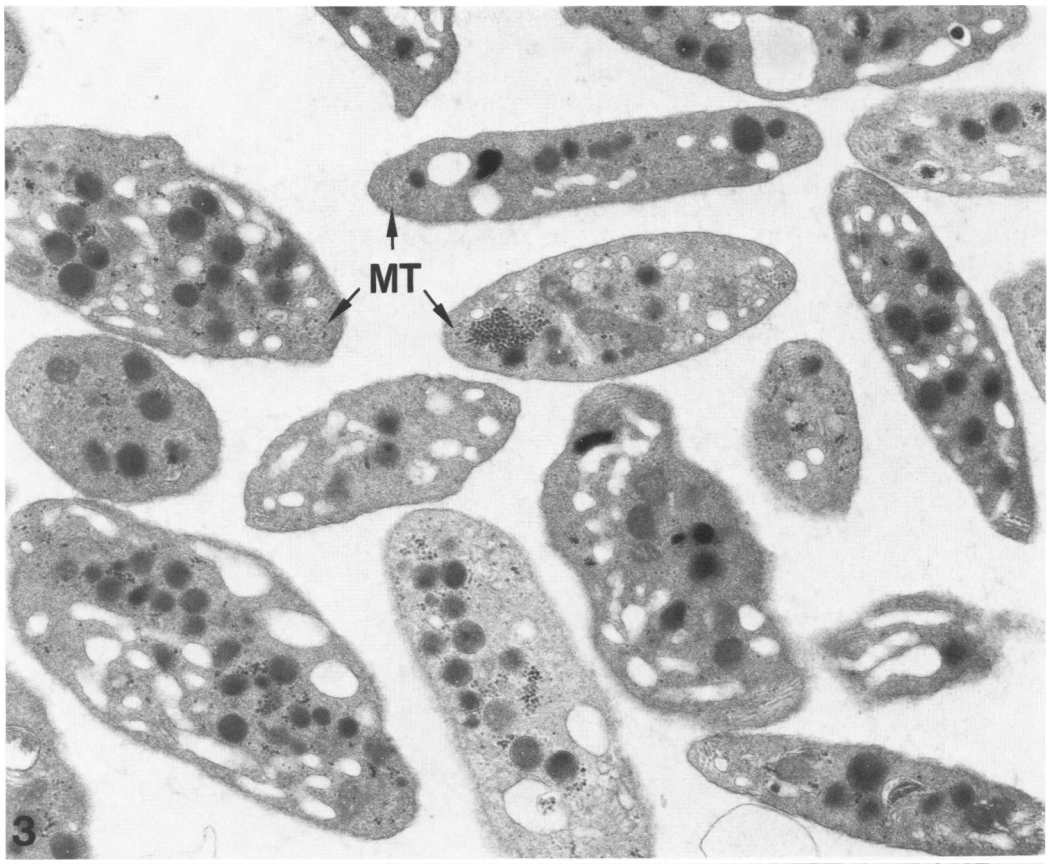


Figure 1 — Platelet from a sample of C-PRP maintained at 37 C and fixed in glutaraldehyde at the same temperature before exposure to osmic acid. A circumferential bundle of microtubules (*MT*) lying just inside the surface membrane supports the discoid form of the cell. A large number of granules (*G*), a few dense bodies (*DB*) and occasional mitochondria (*M*) are randomly dispersed in the cytoplasm. Glycogen (*Gly*) particles occur singly or in clumps. (× 43,000) **Figure 2** — Platelets from a sample of C-PRP chilled in ice water to 2–4 C for 15 minutes before fixation in cold glutaraldehyde. The chilled platelets lose their discoid form, become irregular, and extend bulky, long, thin pseudopods. The shape change is associated with complete disappearance of microtubules.[5,6] (× 20,000)



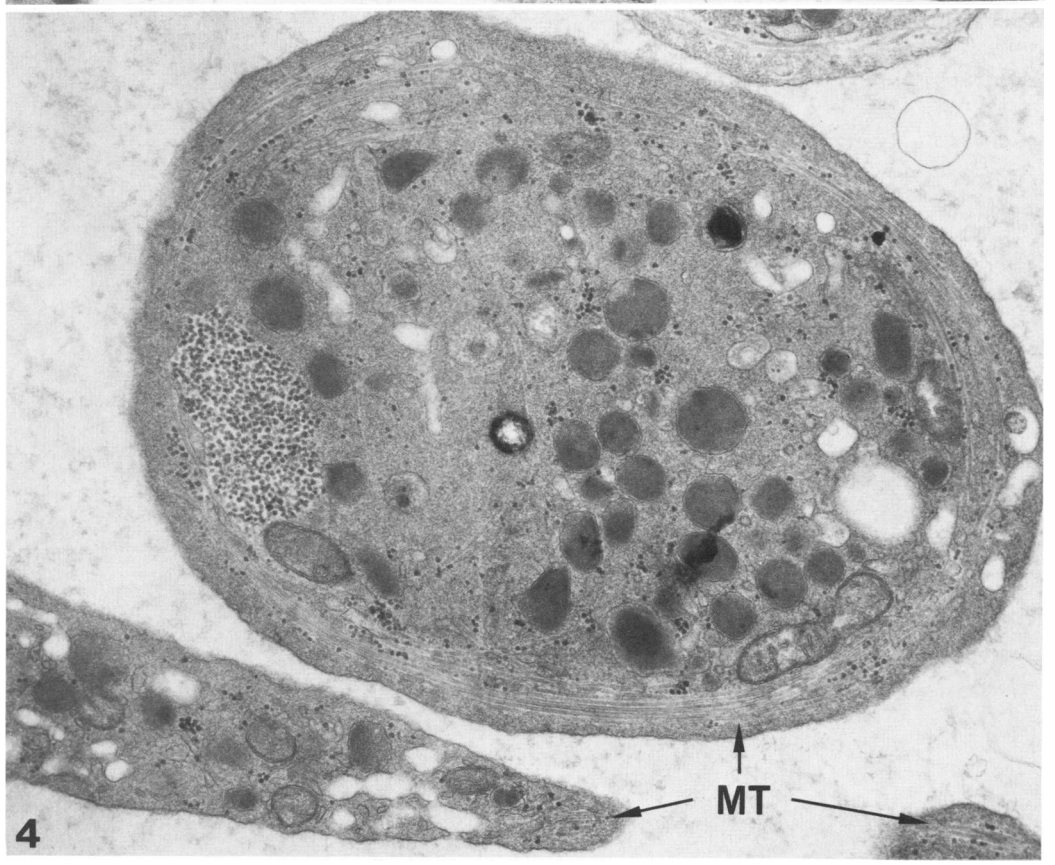
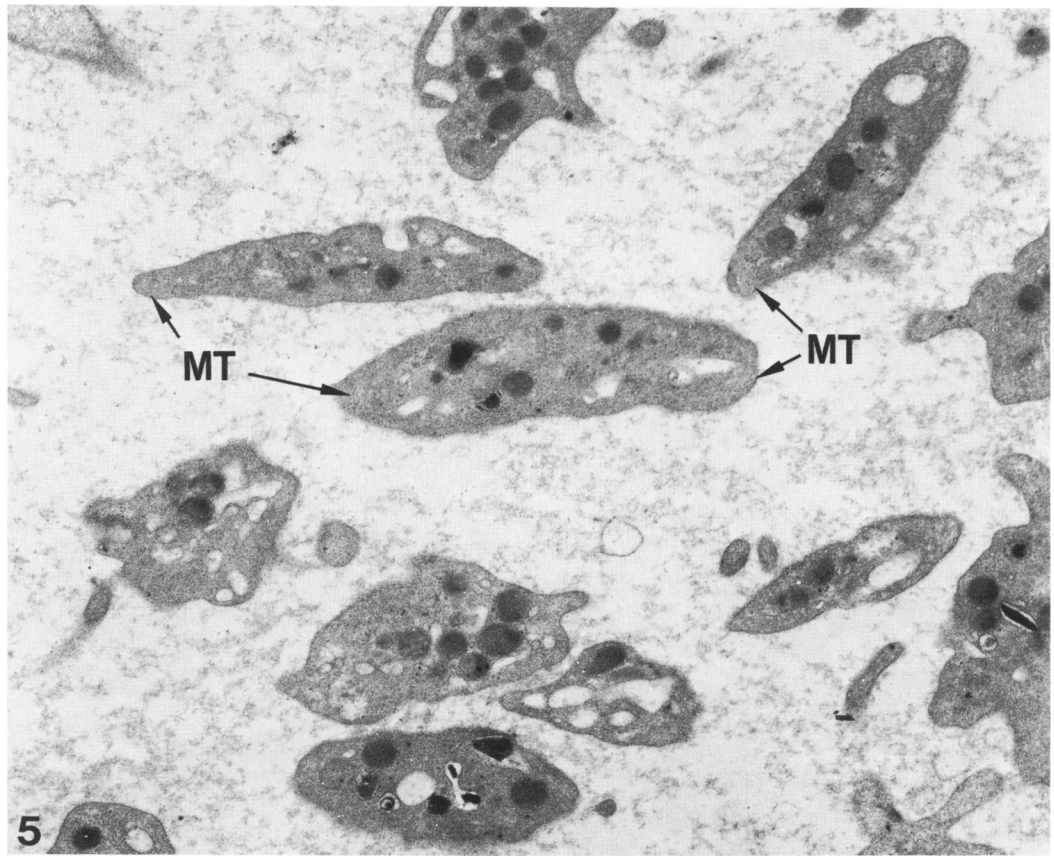


Figure 3 – Platelets from a sample of C-PRP combined with taxol at a concentration of 10^{-4} M for 60 minutes at 37 C. Circumferential bands of microtubules (*MT*) support the discoid form of the cells, which are well preserved in the presence of taxol. Other morphologic features of platelets, appear unaffected by prolonged exposure to the drug. (× 20,000) **Figure 4** – Platelet from a sample of C-PRP combined with 10^{-4} M taxol and incubated for 90 minutes at 37 C. The cell is oval in shape but has a complete circumferential band of tubules (*MT*). Adjacent cells also have annular bundles of tubular elements. (× 28,000)



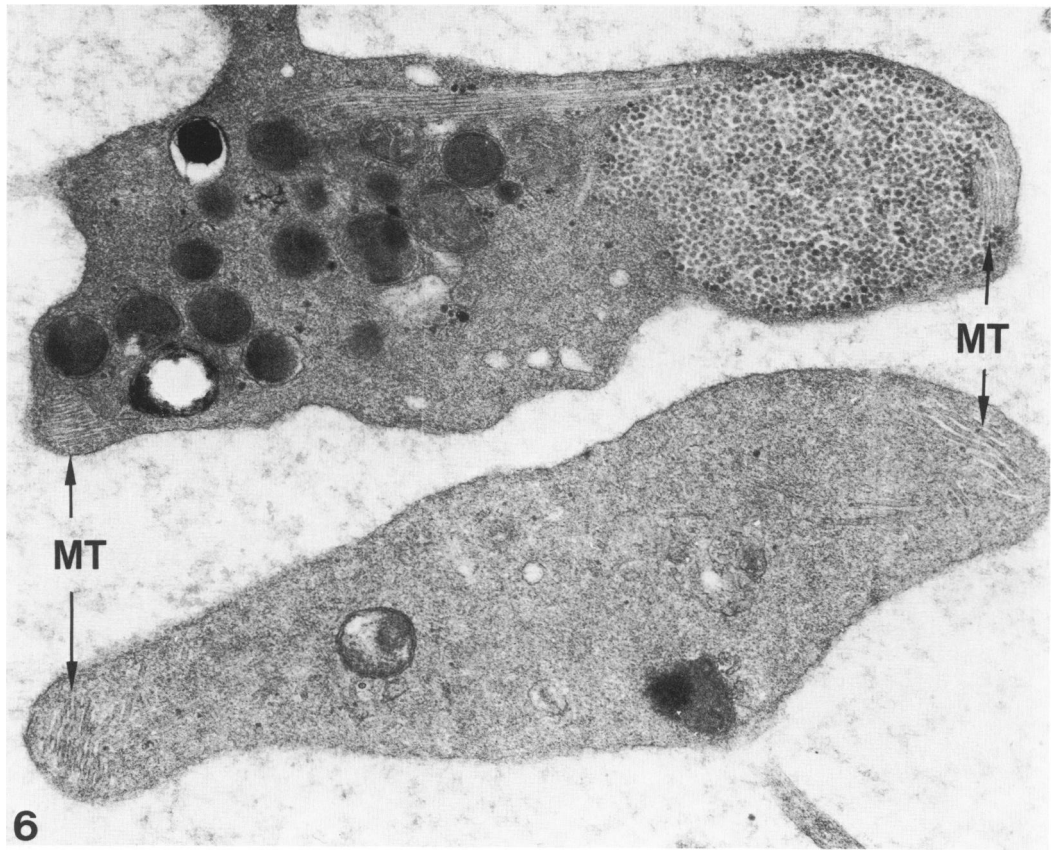
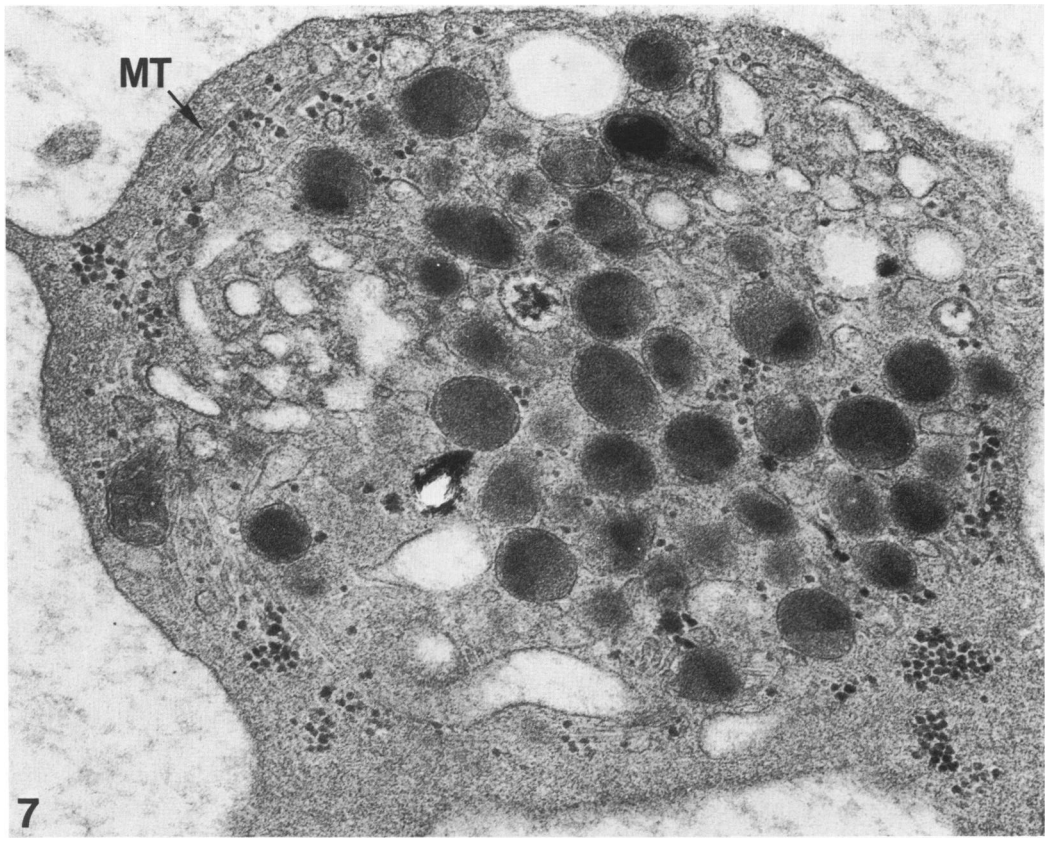


Figure 5 — Platelets from a sample of C-PRP combined with 10^{-5} M taxol at 37 C for 15 minutes, and then chilled to 2-4 C for 30 minutes. Some of the cells have become irregular, but most retain a relatively discoid shape. Clearly the cells are susceptible to some degree of shape change despite retention of circumferential bundles of microtubules (*MT*). (× 18,000) **Figure 6** — Platelet from a sample of C-PRP incubated with 10^{-4} M taxol for 30 minutes and then chilled to 2-4 C for 60 minutes. One of the cells is irregular, but both retain circumferential bands of microtubules (*MT*). (× 38,000)



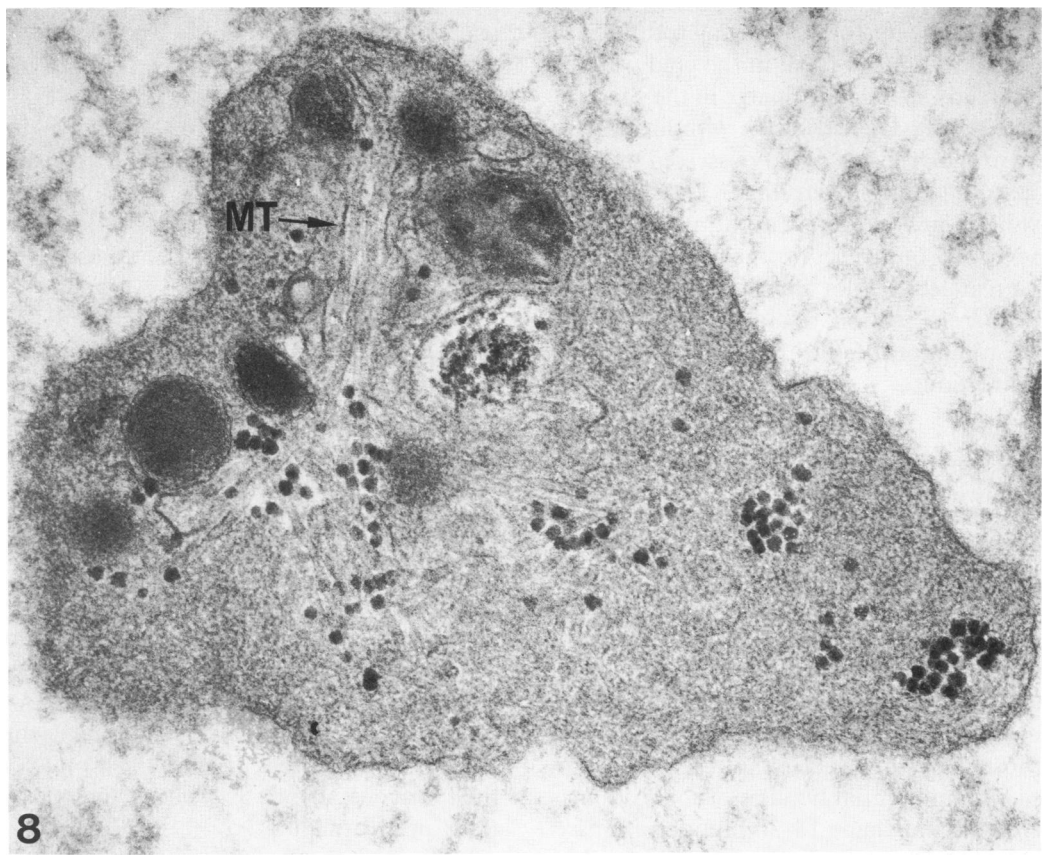


Figure 7—Platelet from a sample of C-PRP combined with 10^{-4} M taxol for 15 minutes before chilling to 2–4 C for 1 hour. General morphologic features and the circumferential bundle of microtubules (*MT*) are well maintained. (×38,500) **Figure 8**—Platelet from C-PRP chilled to 2–4 C for 15 minutes to remove all microtubules, then combined with 10^{-5} taxol for 15 minutes at low temperature and fixed in the cold. Clusters of microtubules (*MT*) are radiating in several directions from a centrally located nucleation site. (×60,000)

cold at 5, 15, 30, and 60 minutes after exposure to low temperature (Figures 6 and 7).

Influence of Taxol on Chilled Platelets

Samples of C-PRP were chilled to 2–4 C for 15 minutes, then combined with taxol at final concentrations of 10^{-4}–10^{-6} M, or to DMSO alone. The chilled samples were kept at 2–4 C and fixed in the cold at 5, 15, 30, and 60 minutes. Control platelets and samples combined with taxol for 5 minutes were irregular in form and devoid of microtubules. Fifteen minutes after combining with taxol, microtubules were apparent in a few platelets (Figure 8). At 30 minutes, platelets containing microtubules were commonly found; and at 60 minutes, nearly every platelet contained numerous microtubules (Figure 9). The location of microtubules in the taxol-treated, chilled cells differed strikingly from control platelets fixed at room temperature or 37 C. Circumferential bands of microtubules supporting the discoid shape of control platelets were rarely seen in platelets exposed to taxol in the cold. Instead, single tubules and bundles of microtubules were present throughout the cytoplasm of the cells. Often the tubules appeared to radiate in all directions from a central axis, resembling sheaves of wheat (Figure 10). Restoration of microtubules in chilled platelets exposed to taxol in the cold was not associated with any significant change in the shape of the deformed cells. Chilled cells without microtubules were irregular in form and possessed bulbous or sharp pseudopodial extensions. The irregular shape did not improve with restoration of microtubules under the influence of taxol. Conversely, taxol-treated platelets did not appear to become more deformed with reformation of microtubules in the cold, despite radiation of the relatively rigid structures in all directions.

Influence of Taxol Treatment in the Cold Recovery of Platelets Rewarmed to 37 C

As indicated, taxol did not cause reassembly of large numbers of microtubules in most chilled platelets until the cells had been incubated with the agent for at least 30 minutes at low temperature. However, taxol did appear to have an effect on the distribution of microtubules in rewarmed platelets, even when added to chilled platelets just 5 minutes before rewarming. Most chilled platelets in the absence of taxol reassembled circumferential bundles of microtubules and recovered discoid shape 15–30 minutes after exposure to 37 C.[5,6] Platelets combined with taxol in the cold and rewarmed 5 minutes later also recovered discoid shape. Some of these cells also developed circumferential bundles of microtubules (Figure 11), but in many discoid appearing platelets bundles of microtubules appeared to lie in the central axis of the cells (Figure 12). The appearance was even more striking in samples combined with taxol for 10–15 minutes in the cold before warming for 15–30 minutes. Single microtubules as well as bundles were often arranged in parallel in the long axis of discoid platelets.

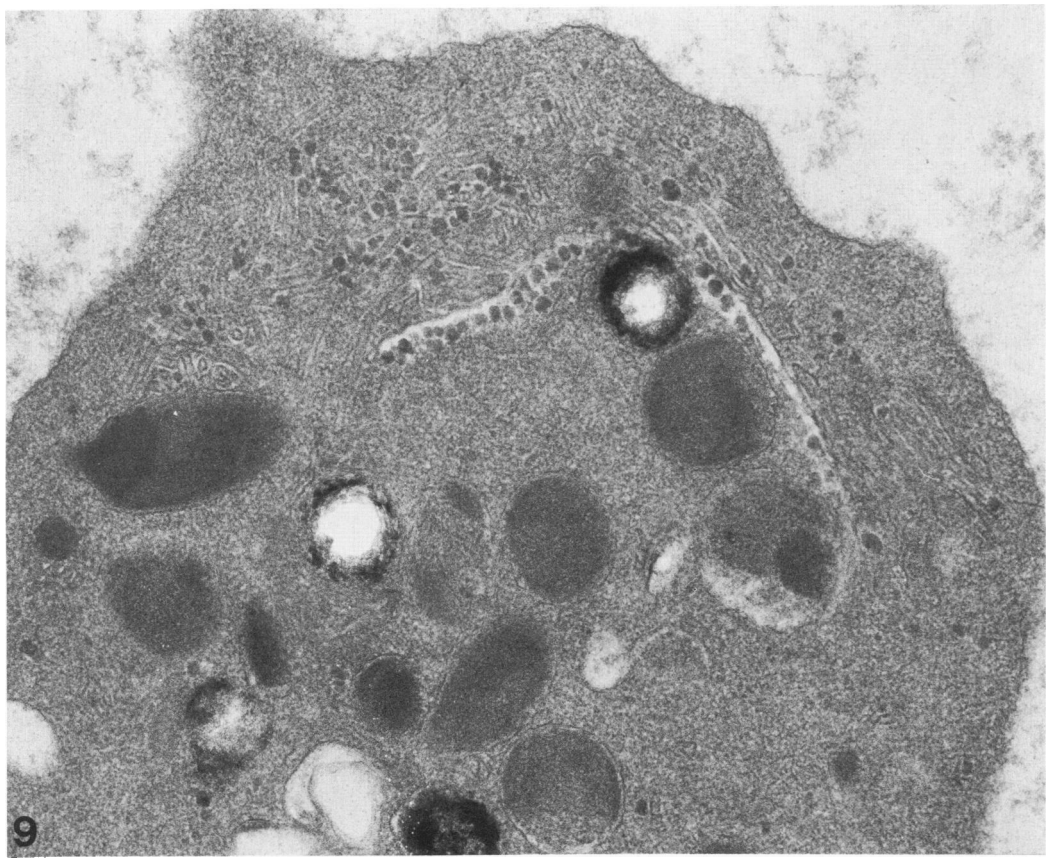
Samples treated with taxol for 30–60 minutes at 2–4 C before warming gave a different appearance. Most of the cells were round or oval in form, and clear-cut discoid cells, though present, were uncommon. Masses or bundles of microtubules were present in these platelets but were seldom organized into a circumferential band (Figures 13 and 14).

Discussion

The present study has shown that the microtubule-stabilizing agent, taxol, prevents disassembly of the circumferential band of microtubules when discoid platelets are exposed to low temperature and promotes formation of microtubules in prechilled platelets maintained in the cold. Taxol was derived from the plant *Taxus brevifolia* and originally studied for its potential as an antitumor agent.[7] Subsequently, taxol was found to enhance assembly of tubulin into microtubules *in vitro*, promote formation of microtubules in intact fibroblasts, and inhibit their dissociation in the cold.[8] Recently it has been reported that taxol causes assembly of microtubule-myosin arrays in postmitotic fibroblasts.[9] Although the precise mechanism through which taxol stabilizes microtubules is not known, it is clear that the agent may be useful for evaluating the role of labile microtubules in cell function.

The normal form of platelets was well preserved after incubation with taxol at room temperature or 37 C. Most platelets were discoid even after exposure to the agent for 2 hours. Circumferential bands of microtubules were well preserved, and there was no apparent injury to organelles, channel systems or other cytoplasmic structures. Taxol treatment prevented disassembly of circumferential bands of microtubules in platelets exposed to low temperatures sufficient to remove all tubular elements from control cells.[5,6] Most of the chilled platelets retained their discoid shape, as well as microtubules, after taxol pretreatment.

The relationship of circumferential microtubules to lentiform platelets was suggested in early studies



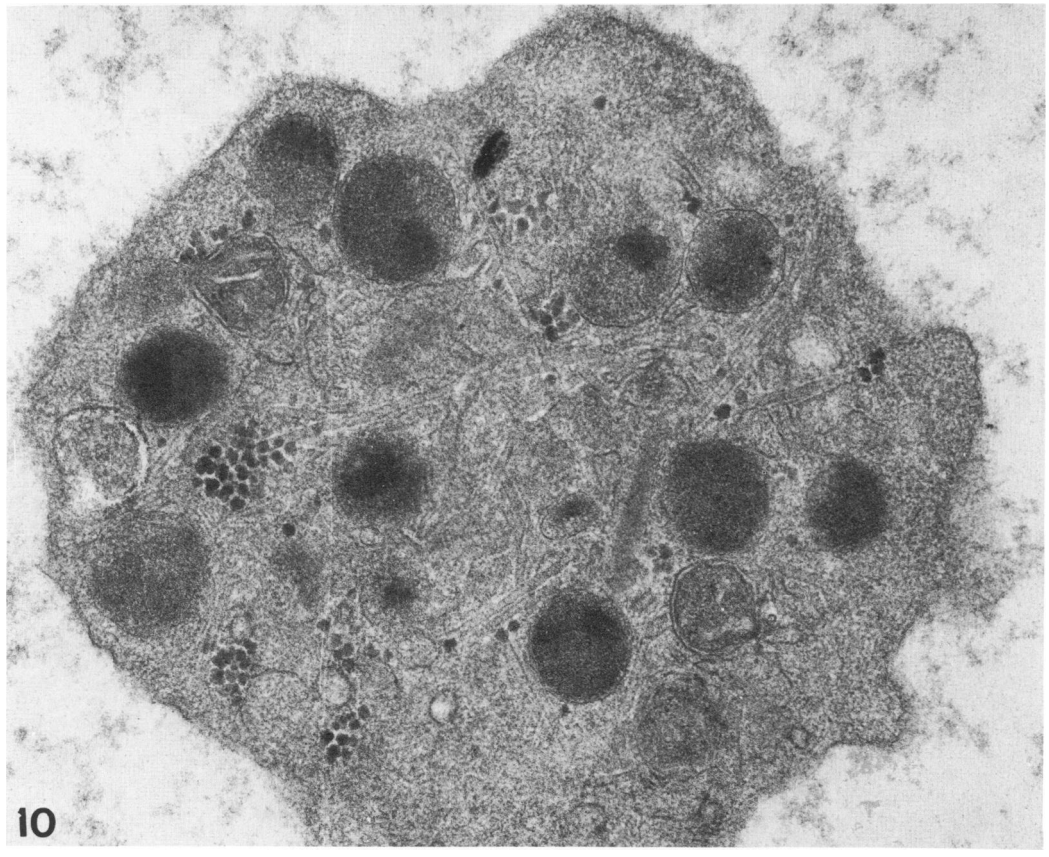
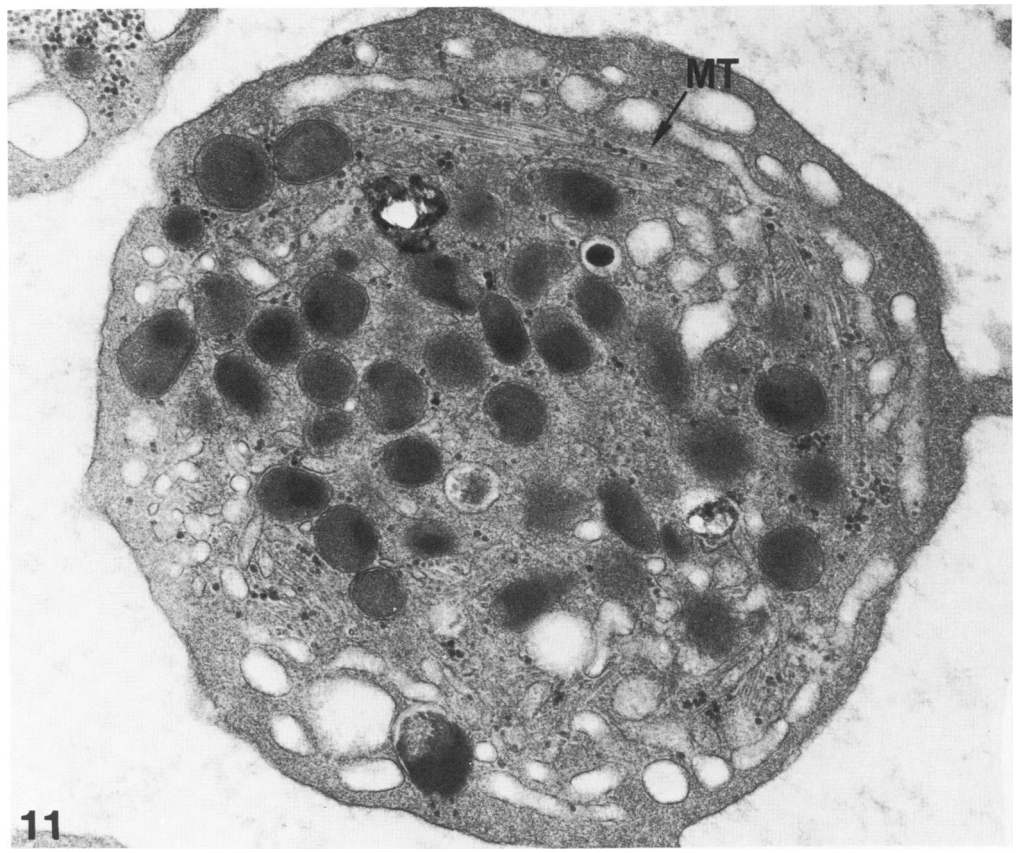


Figure 9 — Platelet from C-PRP chilled first to remove microtubules and combined with 10^{-4} M taxol for 60 minutes. The area of cytoplasm between the cell membrane and storage organelles is filled with a mass of microtubules radiating in all directions. (× 60,000) **Figure 10** — Platelet from a sample of C-PRP chilled to eliminate microtubules, mixed with taxol at a final concentration of 10^{-5} M, and incubated at low temperature for 60 minutes before fixation in the cold. Microtubules fill the cell cytoplasm and radiate in all directions. (× 60,000)



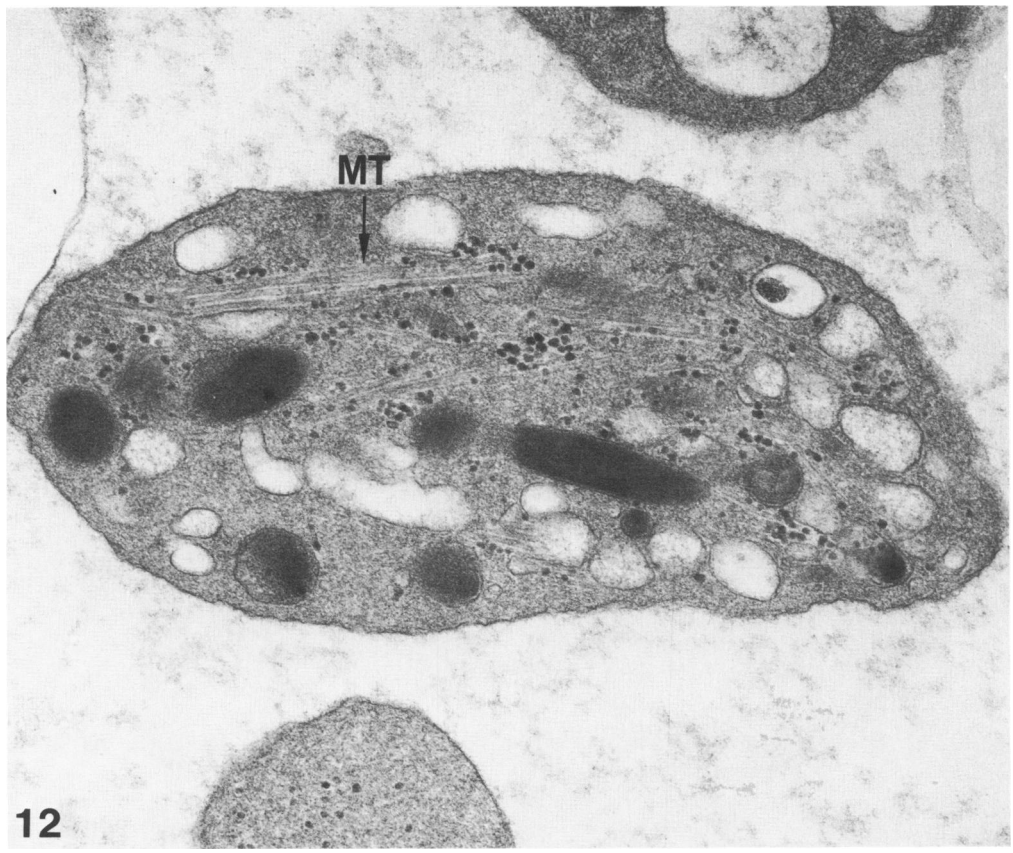
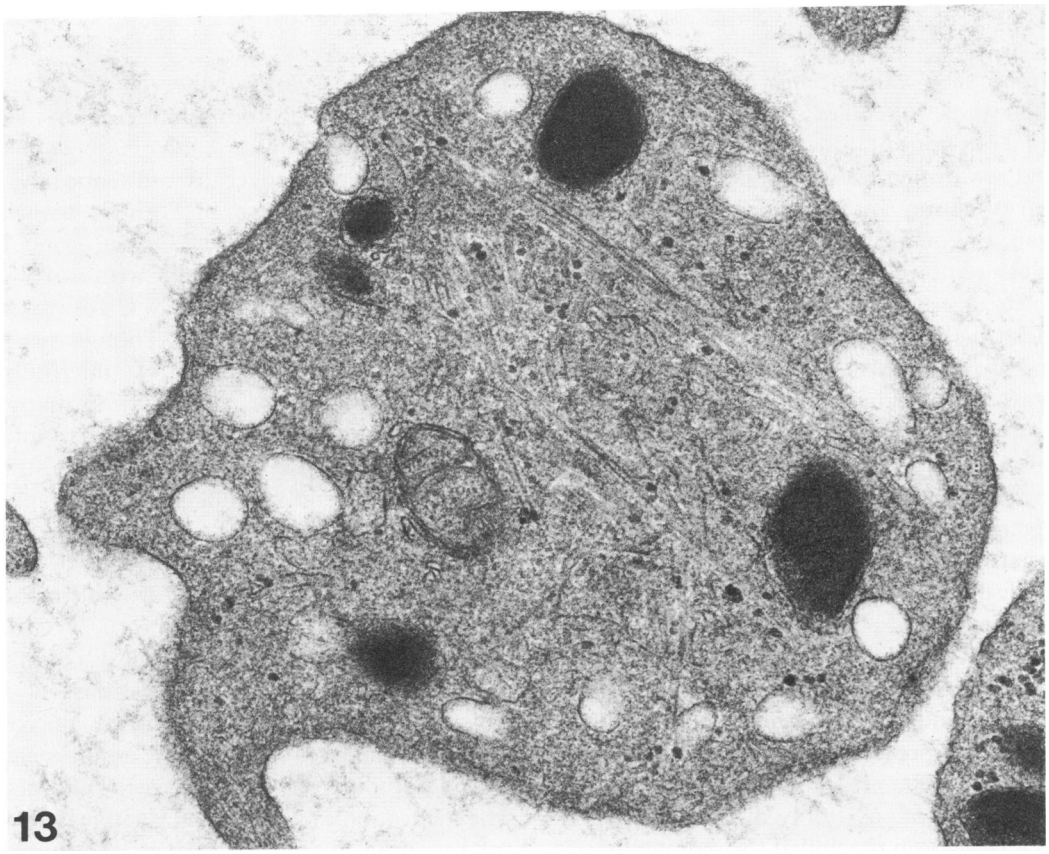


Figure 11 – Platelet from C-PRP chilled to remove tubules, combined with 10^{-5} M taxol in the cold for 5 minutes and then rewarmed to 37 C for 15 minutes. A circumferential bundle of microtubules (*MT*) has reformed in this cell. (× 27,500) Figure 12 – Platelet from a sample of C-PRP treated in the same manner as the cell in the previous illustration. The cell appears to have recovered its discoid shape, but microtubules lie in the central axis, rather than at its poles. (× 38,500)



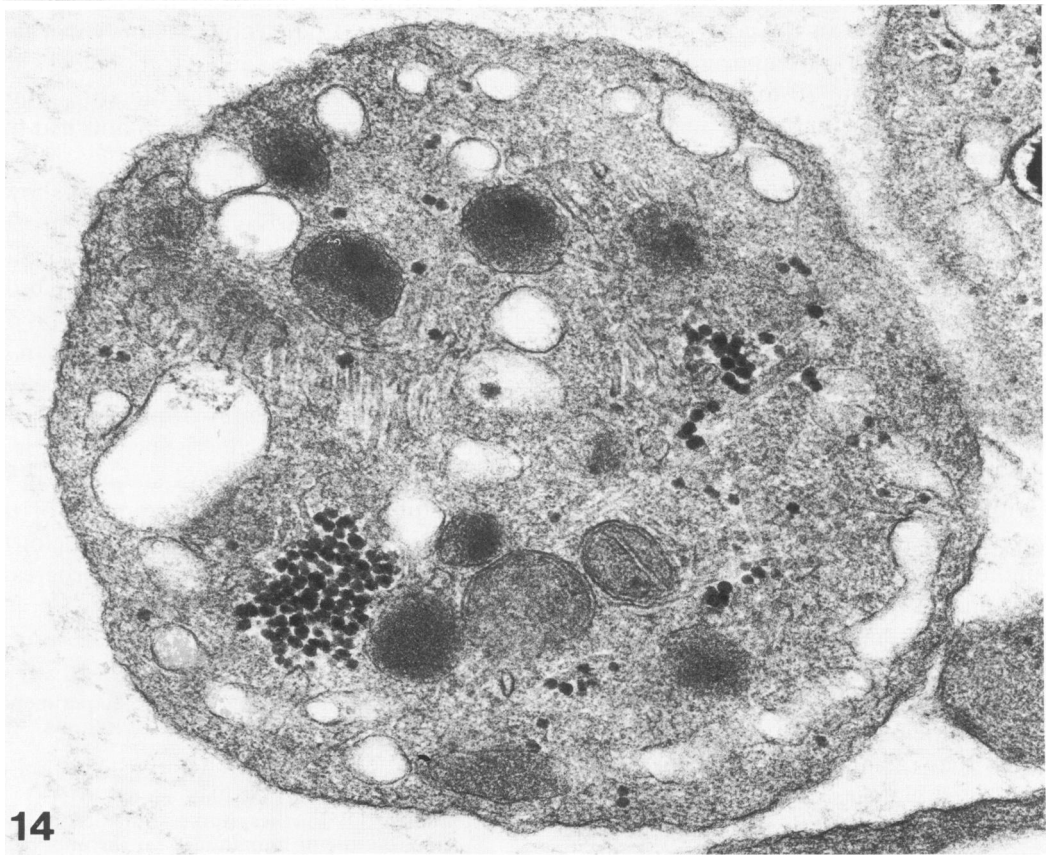


Figure 13 – Platelet from C-PRP chilled to remove microtubules, combined with 10^{-4} M taxol for 60 minutes in the cold, then rewarmed to 37 C for 30 minutes. Microtubules radiate in all directions in the cytoplasm. (×50,500) **Figure 14** – Platelet from a sample of C-PRP treated in the same manner as the cell in the previous illustration. Microtubules, singly and in bundles, radiate in many directions throughout the cytoplasm of the platelet. (×50,500)

that described their existence in platelets and was established in studies of chilled cells.[5,6] Chilling caused the simultaneous loss of disklike shape and microtubules, and rewarming was associated with recovery of both. Investigations into the effects of antimitotic agents, such as colchicine and vincristine, showed that chemical removal of microtubules would also cause platelets to lose their disklike form.[12] Thus, the direct association between annular bundles of microtubules and disklike form established in the early studies seems supported in the present work by the ability of taxol to protect both shape and microtubules in most cells from the influence of low temperature. However, treatment of platelets with chelating agents, such as EDTA, can convert the cells to spiny spheres without apparent changes in annular bands of microtubules.[12] The present study demonstrates that exposure to low temperature can also cause some taxol-treated platelets to lose their disklike shape and become irregular despite retention of circumferential bundles of tubular elements.

In addition to preventing cold-induced microtubule disassembly, the present study has shown that taxol will also promote assembly of microtubules in platelets that have been prechilled for removal of microtubules and maintained in the cold. The process of assembly at low temperature appears to be quite slow; most platelets require 30 minutes for the development of substantial numbers of microtubules. Although microtubules formed under the influence of taxol in chilled platelets appear identical to those in untreated cells fixed at 37 C, the organization was quite different. Circumferential bundles of microtubules were rarely seen in chilled platelets combined with taxol. Instead, the microtubules appeared to be assembled singly or in masses or bundles in central and peripheral areas of the cytoplasm. Also, the bundles, masses, and single tubules appeared to radiate in all directions.

This unusual organization of microtubules was not associated with restoration of discoid shape in chilled cells. However, the irregular, chilled cells with taxol-induced bundles of radiating microtubules did not appear to become more irregular. Radiating bundles of microtubules might have been expected to distort the platelet surface into multiple pseudopods, producing the appearance of "spiny spheres."[14] This alteration was anticipated because some workers have suggested that radiating, newly formed microtubules in activated platelets cause pseudopod extrusion during shape change,[15] but the expected transformation was not observed in the present study.

Examination of rewarmed platelets exposed to taxol in the cold for various intervals suggested that the drug has a more profound influence on the organization of microtubules than endogenous factors. Chilled platelets recover discoid shape on warming to 37 C, and restoration of form is associated with reassembly of the circumferential band of microtubules. Platelets incubated with taxol for 5–10 minutes before warming to 37 C for 30 minutes recovered their discoid form. Though reassembly of the circumferential band of microtubules was associated with shape restoration in many platelets, the majority revealed bundles of microtubules oriented in the long axis. This finding suggests that reassembly of an annular bundle of microtubules may not be absolutely essential for recovery of discoid shape.

Incubation of platelets with taxol in the cold for 30–60 minutes before warming to 37 C limited the ability of the cells to recover their discoid form. Most of the platelets were relatively spherical or oval in thin sections, and phase-contrast microscopy revealed a similar appearance. Circumferential bands of microtubules were rare in such platelets. Instead, the microtubules were organized in masses or bundles of radiating microtubules, just as they were at low temperature. Endogenous factors favoring assembly of microtubules in annular bundles appeared incapable of reorganizing the radiating framework or central mass of microtubules established through the action of taxol in chilled cells.

In summary, the present study has shown that the microtubule-stabilizing agent taxol prevents disassembly of platelet microtubules due to chilling, promotes reformation of disassembled microtubules in chilled platelets, and causes organization of microtubules into radiating bundles, rather than circumferential bands. Preliminary studies indicate that taxol also prevents disassembly of platelet microtubules by the antimitotic agent vincristine. It will be of interest to determine whether taxol stabilization of platelet microtubules influences the response of the cells to aggregating agents or the process of clot retraction. Such experiments are currently in progress.

References

1. Eberth JL, Schimmelbusch C: Experimentelle Untersuchungen uber Thrombose. Virchows Arch [Pathol Anat] 1886, 103:39–87
2. Haydon GB, Taylor AB: Microtubules in hamster platelets. J Cell Biol 1965, 26:673–675
3. Behnke O: Further studies on microtubules: A marginal bundle in human and rat thrombocytes. J Ultrastruct Res 1965, 13:469–477
4. Bessis M, Breton-Gorius J: Les microtubules et les fib-

rilles dan les plaquettes etalies. Nouv Rev Franc Hematol 1965, 5:657-662
5. White JG, Krivit W: An ultrastructural basis for the shape changes induced in platelets by chilling. Blood 1967, 30:625-535
6. Behnke O: Incomplete microtubules observed in mammalian blood platelets during microtubule polymerization. J Cell Biol 1966, 34:697-701
7. Wani MC, Taylor HL, Wall M, Cozzan P, McPhail AT: Plant antitumor agents: VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from Taxus brevifolia. J Am Chem Soc 1971, 93: 2325-2327
8. Schiff PB, Horwitz SB: Taxol stabilizes microtubules in mouse fibroblast cells. Proc Natl Acad Sci 1980, 77: 1561-1565
9. Antin PB, Forry-Schaudies S, Friedman TM, Tapscott SJ, Holtzer H: Taxol induces postmitotic myoblasts to assemble interdigitating microtubule-myosin arrays that exclude actin filaments. J Cell Biol 1981, 90:300-308
10. White JG: Fine structural alterations induced in platelets by adenosine diphosphate. Blood 1968, 31:604-622
11. White JG, Krumwiede M: Influence of cytochalasin B on the shape change induced in platelets by cold. Blood 1973, 41:823-832
12. White JG: Effects of colchicine and vinca alkaloids on human platelets: I. Influence on platelet microtubules and contractile function. Am J Pathol 1968, 53:281-291
13. White JG: Effects of ethylenediamine tetracetic acid (EDTA) on platelet structure. Scand J Haematol 1968, 5:241-254
14. Zucker MB, Borelli J: Reversible alterations in platelet morphology produced by anticoagulants and by cold. Blood 1954, 9:602-608
15. Crawford N: Platelet microfilaments and microtubules, Platelets in Biology and Pathology. Edited by J Gordon. Amsterdam, North-Holland, 1976, pp 121-155