MICROTUBULAR CRYSTALS IN MAMMALIAN CELLS

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ABSTRACT

Periwinkle alkaloids in very low concentrations cause an intracytoplasmic sequestration of microtubule protein in the form of symmetrical, microtubular bodies. These crystals, which may measure up to 8 μ in length, appear within 30 min in L-strain fibroblasts in vitro, but they increase in incidence and size with time of exposure to the alkaloids. Similarly, if exposed to these compounds, human leukocytes in vitro contain identical crystalline structures. Neither colchicine nor puromycin prevents the formation of these bodies; the latter compound, however, retards crystal growth.

Periwinkle (*Vinca*) alkaloids, helpful in the therapy of neoplastic diseases, are known to affect cell division through action similar to that of colchicine (1). Overdosage of these drugs damages primarily organs known to be rich in microtubules, such as neurons (2). Previous studies carried out by us showed a rapid (but, for vinblastine, reversible) decrease and disappearance of the birefringence of the mitotic spindle of living eggs of a marine annelid during perfusion with these compounds; electron microscopy confirmed the attrition and disappearance of microtubules by such treatment (3). The same alkaloids, when used in a study of microtubules of polymorphonuclear leukocytes, again made the microtubules disappear but produced in these cells intracellular bodies of singular regularity which fulfilled the criteria for definition of a crystal. This observation led to a systematic examination of the effects of the *Vinca* alkaloids vinblastine and vincristine sulfate on microtubules in mammalian cells. The results of these studies are reported here. Preliminary findings were reported briefly elsewhere (4).

MATERIALS AND METHODS

L-strain fibroblasts and human leukocytes were studied. The fibroblasts in tissue culture (5) were exposed for ½–24 hr to the periwinkle alkaloids which were added directly to the medium, resulting in final alkaloid concentrations ranging from 4×10^{-4} to 1×10^{-5} M. These cells, as well as control cultures, were subsequently fixed by addition of isotonic 2% phosphate-buffered glutaraldehyde solution (pH 5.9, temperature 24°–30°C) at a ratio of 1:1, followed within 5–15 min by centrifugal separation of the cells from the incubation medium-fixative mixture (6). Fibroblasts, but not leukocytes, were fixed also at 4°C and pH 7.4. The pellet was then overlaid with the above described fixative. Osmification (7) was carried out 1–240 hr later, after a 12 hr rinse of the cell pellet in isotonic phosphate buffer. The tissue embedded in epoxy resin (Maraglas) (8) was examined in an Elmiskop 1. Similarly, suspensions of human leukocytes (9) were incubated at 37°C for 2–3 hr in a phosphate-buffered Krebs-Ringer medium with 12% autologous serum, with or without the alkaloids (vinblastine 2.5×10^{-5} M, vincristine 1×10^{-4} M).

In other series of experiments, colchicine (final concentration $1–4 \times 10^{-5}$ M) was added to the fibro-

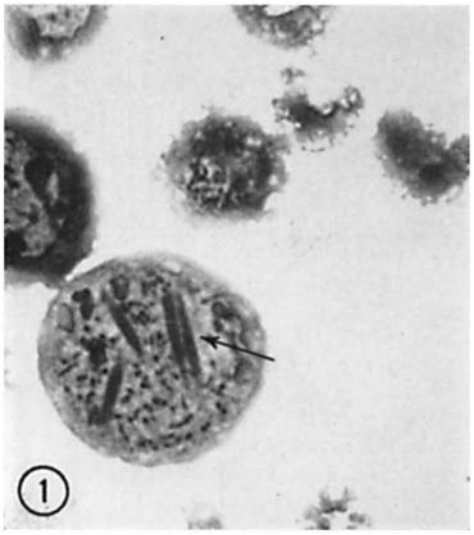
blast cultures 2 and 24 hr before addition of the periwinkle alkaloids. Similarly, suspensions of human polymorphonuclear leukocytes were treated with colchicine 5×10^{-5} M 1 hr before vinblastine 2.5×10^{-5} M or with colchicine 2×10^{-4} M 1 hr before vincristine 1×10^{-4} M.

In addition, leukocytes were exposed to 2×10^{-5} M puromycin (10 μ/ml) for 20 min before addition of vinblastine (2×10^{-5} M) or vincristine (1×10^{-4} M). The exposure of the leukocytes to the *Vinca* alkaloids in all of these experiments was 3 hr.

RESULTS

The results of the experiments in which only the periwinkle alkaloids were used were essentially identical, with little difference in effect between the two periwinkle compounds or the different cell types. Abnormal elongated intracellular crystalline bodies measuring up to 8 μ were identifiable in the 1 μ thick sections of the plastic-embedded material (Fig. 1).

Crystals, although encountered in all parts of a cell, were more abundant in the concave recess of the nucleus of the fibroblasts, normally an area of pronounced cytoplasmic activity, usually containing the Golgi apparatus, lysosomes, and the centrioles (Fig. 2). The latter were sometimes partly surrounded by crystals, a feature which could also be found in the leukocytes (Fig. 3). After ½–1 hr exposure to the alkaloids, most of the crystals were observed in this juxtacentriolar location. Prolonged exposure was associated with dispersal of the cell organelles throughout a cell's cytoplasm.



All illustrations depict fibroblasts, except for Figs. 3 and 4. *Abbreviations:* vinblastine sulfate, VB; vincristine sulfate, VC. Concentrations are given in moles per liter.

FIGURE 1 Four large crystals (arrow) in metaphase-arrested cell. 1 μ thick, plastic-embedded material observed under phase microscope. 10^{-5} VB; 8 hr. × 2000.

The larger crystals predominated in dividing cells treated with the lower concentrations of vinblastine for 4–8 hr. These structures occupied, as confirmed by electron microscopy, a considerable portion of a cell. Sections of these crystalline bodies parallel to their long axis revealed their fine structure to be reflected in highly regular arrays of electron-opaque lines which ran parallel to the long axis of these crystals. The predominant periodicity of these lines was 280 A and was less frequently about 240 A (Fig. 4). However, a 200 A periodicity was also observed repeatedly, particularly on examination of thinner sections; in this instance, the parallel lines consisted of rows of electron-opaque isodiametric (80 A) dots which were spaced regularly every 240 A (Fig. 5). Dots forming a line were interconnected with one another by thin strands of electron-opaque material. The counterpart of a dot on a neighboring row was not present at the smallest possible distance, i.e. 200 A, but at a certain greater distance (230–240 A), thus giving rise to a second type of periodicity caused by rows of dots which formed a 22° angle with the short axis of a crystal (Fig. 6). Sections perpendicular to the long axis of these crystalline bodies showed what appeared to be (at lower magnification) a regular pattern of circles conveying the impression of cross-sectioned stacks of microtubules (Fig. 7). Closer examination at high magnification revealed each of these crystalline structures to consist of a hexagonal electron-lucent center surrounded by an electron-opaque rim of even width. This rim, which was shared with six surrounding tubules, measured approximately 80 A in width. The outer diameter of each tubule was that of the predominant periodicity of the longitudinally sectioned crystals, namely 270–280 A, the inner diameter thus being about 200 A (Fig. 7, insert, and Fig. 8).

Relatively infrequently, collections of separate microtubules among a relatively dense population of ribosomes (Fig. 9) were observed in fibroblasts and mononuclear leukocytes but not in poly-

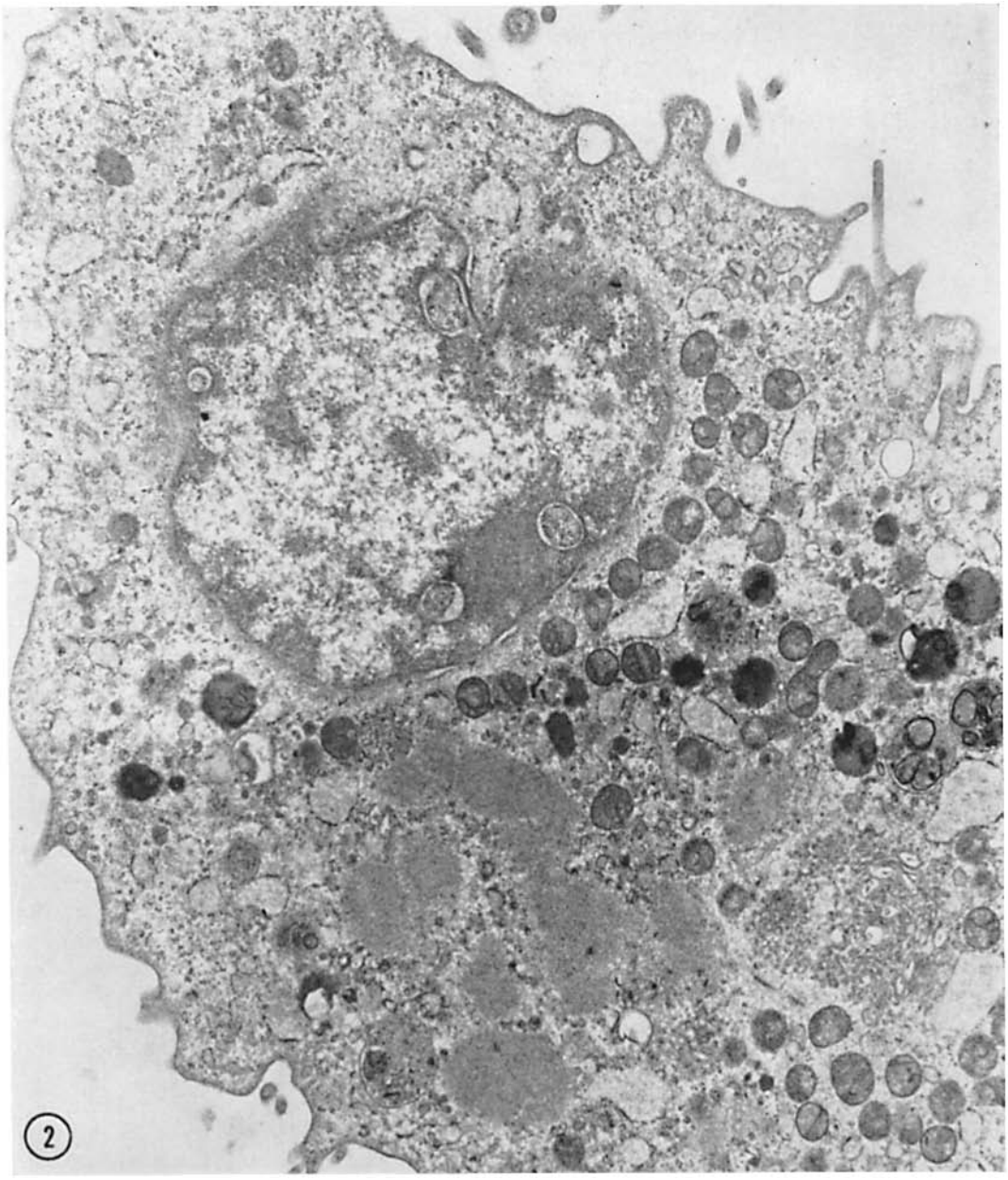


FIGURE 2 The replacement of cytoplasm by crystals, particularly after prolonged incubation in the presence of alkaloids, is very extensive. 2×10^{-4} VC; 4 hr. × 16,000.

morphonuclear leukocytes. The diameter of each of these tubules was 270–280 A, while the diameter of the tubules in control cells averaged 240 A. Longitudinal sections through these collections of microtubules confirmed the increased numbers of ribosomes in these areas if compared with the adjacent cytoplasm (Fig. 10).

Preincubation of fibroblasts in media containing as much as 4×10^{-4} M colchicine for 24 hr followed by addition of vinblastine to the incuba-

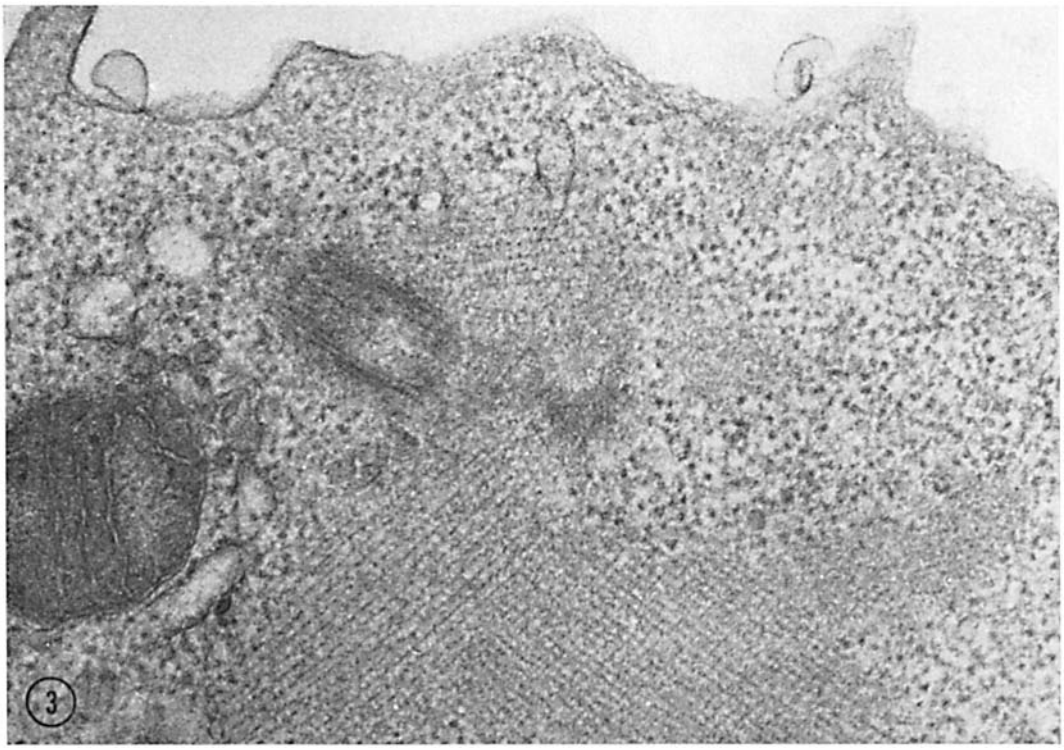


FIGURE 3 Part of a leukocyte with tangentially sectioned centrioles partly surrounded by crystals. The presence of crystals in the centrosphere was a recurrent feature in the different cell types. 2 × 10^{-5} VB; 2 hr. × 58,000.

tion fluid did not prevent the formation of microtubular crystals. The latter were identical with those found in cells exposed to vinblastine alone. Crystals were also present in leukocytes exposed to colchicine and vinblastine or vincristine; their incidence, intracellular distribution, and size were identical with that found in leukocytes exposed to the periwinkle alkaloids alone. Puromycin did not prevent the formation of crystals which, however, appeared smaller and fewer in their numbers.

DISCUSSION

There can be little doubt that the crystals formed after exposure of mammalian cells to periwinkle alkaloids consist mainly of microtubules; the structural resemblance between the tubular subunits of the crystals and normal microtubules is obvious. In addition, there is a complete absence of normal microtubules in the treated cells, except for occasional collections of tubules with a diameter identical with that found in the crystals. Moreover, colchicine, which is thought to occupy binding sites on its cellular target protein that are different from those held by periwinkle alkaloids (vinblastine) (10), does not prevent formation of these crystalline bodies. Periwinkle alkaloids, however, block sites essential for the formation of normal tubular structures. Thus, vinblastine and vincristine bring about not only the formation of larger than normal microtubules and microtubule crystals but also a stabilization of these structures; the rigid requirements for the preservation of the microtubular structure by fixation at high temperature (37°) and low pH also are not necessary after alkaloid treatment. Yet, as shown in the experiments with *Pectinaria* eggs (3), the process of spindle dissolution is fully reversible by diluting the concentration of vinblastine (but not vincristine) in the suspension medium. The normal precarious equilibrium between the microtubular monomer and polymer leading to formation of tubules is also emphasized by the short period of time necessary for crystal

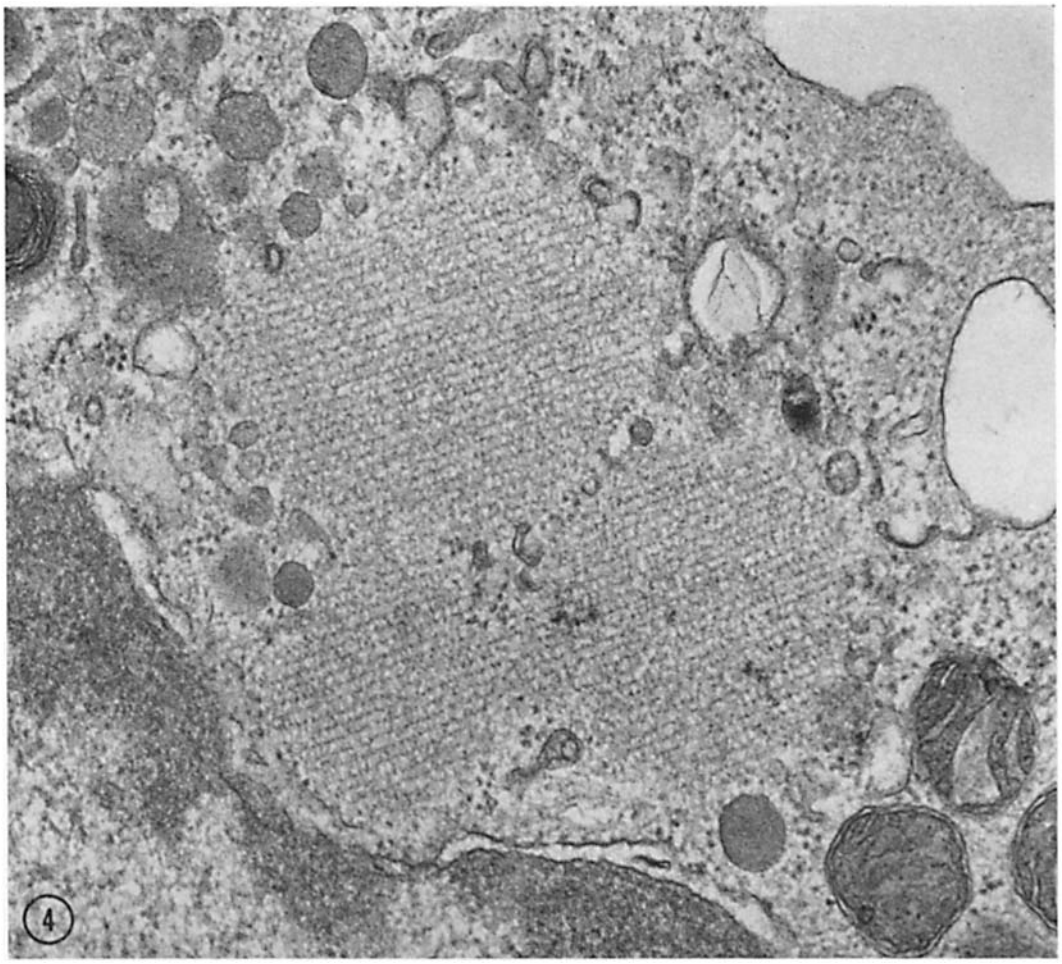


FIGURE 4 Tangentially sectioned crystals in a leukocyte. The line distance is approximately 270 A. 2×10^{-5} VB; 2 hr. × 63,000.

formation. These crystalline bodies may occupy a relatively huge volume estimated conservatively as 10% or more of the total cytoplasm of a cell; the short time interval between exposure and appearance of crystals in fibroblasts as well as the presence of voluminous crystals in the polymorphonuclear leukocytes, a cell type usually considered to carry out very little protein synthesis after its release into the bloodstream, indicates that normally most of the microtubular protein must be present in a disoriented state and must not be newly formed after exposure to the alkaloids. The presence of crystals in the experiments in which the leukocytes were exposed to puromycin supports this notion. However, prolonged exposure to the drug, with the concomitant sequestration of the microtubules in the form of crystals, may accelerate microtubular protein synthesis, as is borne out by the observed increase in number and size of the crystalline structures with time as well as the appearance of stacks of the single (abnormal in respect to their diameter) tubules between rows of ribosomes. Another striking feature is the proximity of crystals to centrioles in interphase cells, a finding which supports the assumption that these organelles may very well be a point of origin in the assembly of microtubules (11).

The observed change in the normal cytoplasmic distribution of cell organelles, i.e. their dispersal after prolonged alkaloid exposure (12–24 hr), was in contrast particularly with the confinement of

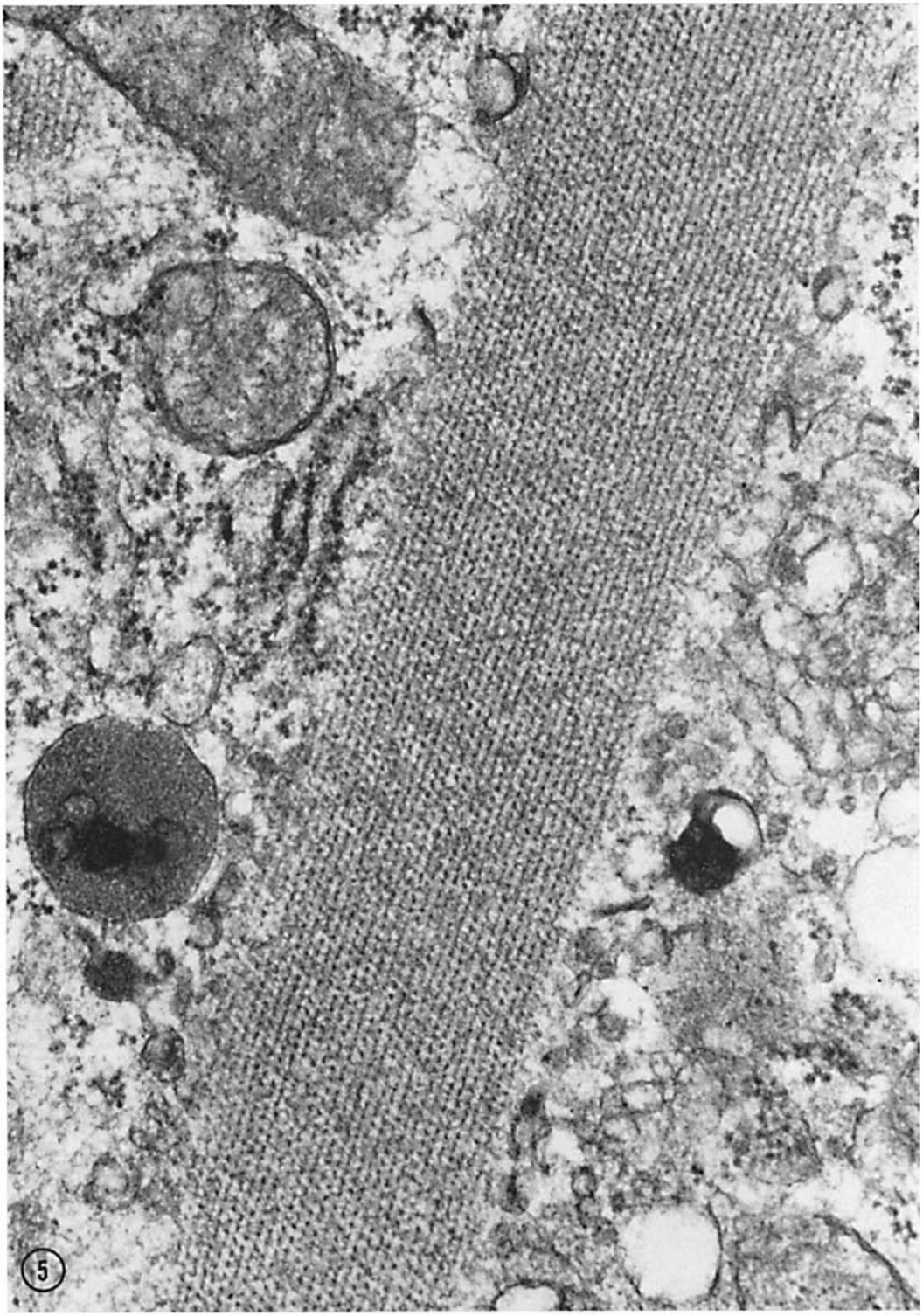


FIGURE 5 A longitudinally sectioned crystal shows clearly rows of dots with fine interconnections between the latter. 10^{-5} VB; 4 hr. × 81,000.

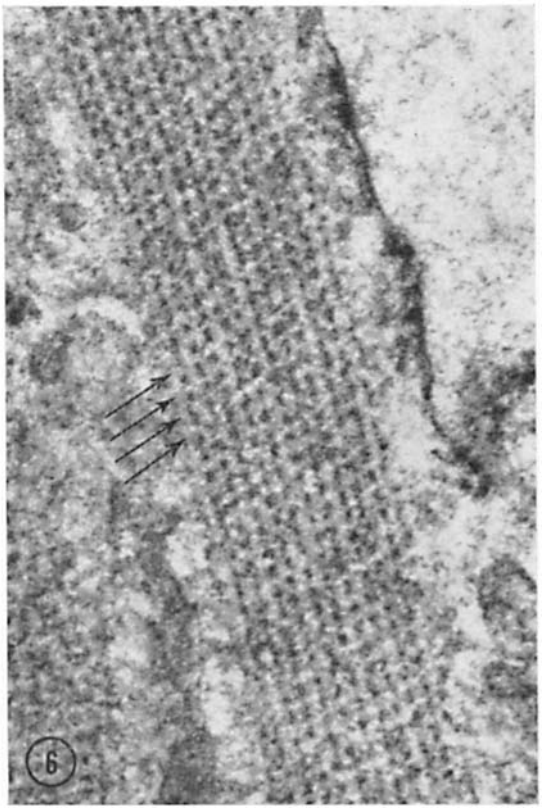


FIGURE 6 Neighboring dots on adjacent rows are out of register but are regularly spaced. Thus, they give rise to an oblique periodicity (arrows). 2×10^{-5} VB; 4 hr. × 86,000.

lysosomes to a small part of an untreated cell; this finding may reflect on one of the functions of microtubules, namely direction of the intracellular saltatory movements of cell particles (12). This function may be one of the requirements for the efficient merger of lysosomes with phagosomes (13). Indeed, we have evidence that the periwinkle alkaloids do not affect phagocytosis but retard intracellular digestion (14; Bensch, K. Unpublished data).

Examination of the crystals per se suggests that the periwinkle alkaloids, like colchicine (15), permit the polymerization of the tubule protein to protofibrils. However, in the case of colchicine we found, as others have, the presence of ubiquitous fibrillary aggregates; in cells treated with *Vinca* alkaloids, such aggregates were relatively rare. Instead, the assembly of protofibrils under the influence of vincristine and vinblastine apparently may proceed to formation of single cylindrical microtubules with a diameter 40 A greater than that seen in untreated human leukocytes or L-strain fibroblasts. These single microtubules, however, are relatively rare when compared with the crystalline aggregates in these cells. The most surprising aspect of the latter is the fact that the wall of a tubule is shared with the six surrounding units. (Noteworthy of mention here is the partial common wall of the outer doublets of microtubules [fibers] of cilia [and spermatozoa] of species ranging from protozoa to mammals [16–18]. A partial common wall is also normally present in the nine sets of triplets of a centriole.) Closer analysis of cross-sections of these crystals, as well as theoretical considerations, led to the conclusion that these tubules are hexagonal on cross-section and have a greater diameter of approximately 280 A and a lesser diameter of 240 A (Fig. 11). Theoretically, electron microscopic study of sections of infinite thinness parallel to the long axis of a crystal should thus give line distances ranging from 140 to 280 A; however, the greatest and by far most frequently encountered periodicity was 280 A, although smaller line distances were found, predominantly 240 and 200 A. Obviously the sec-

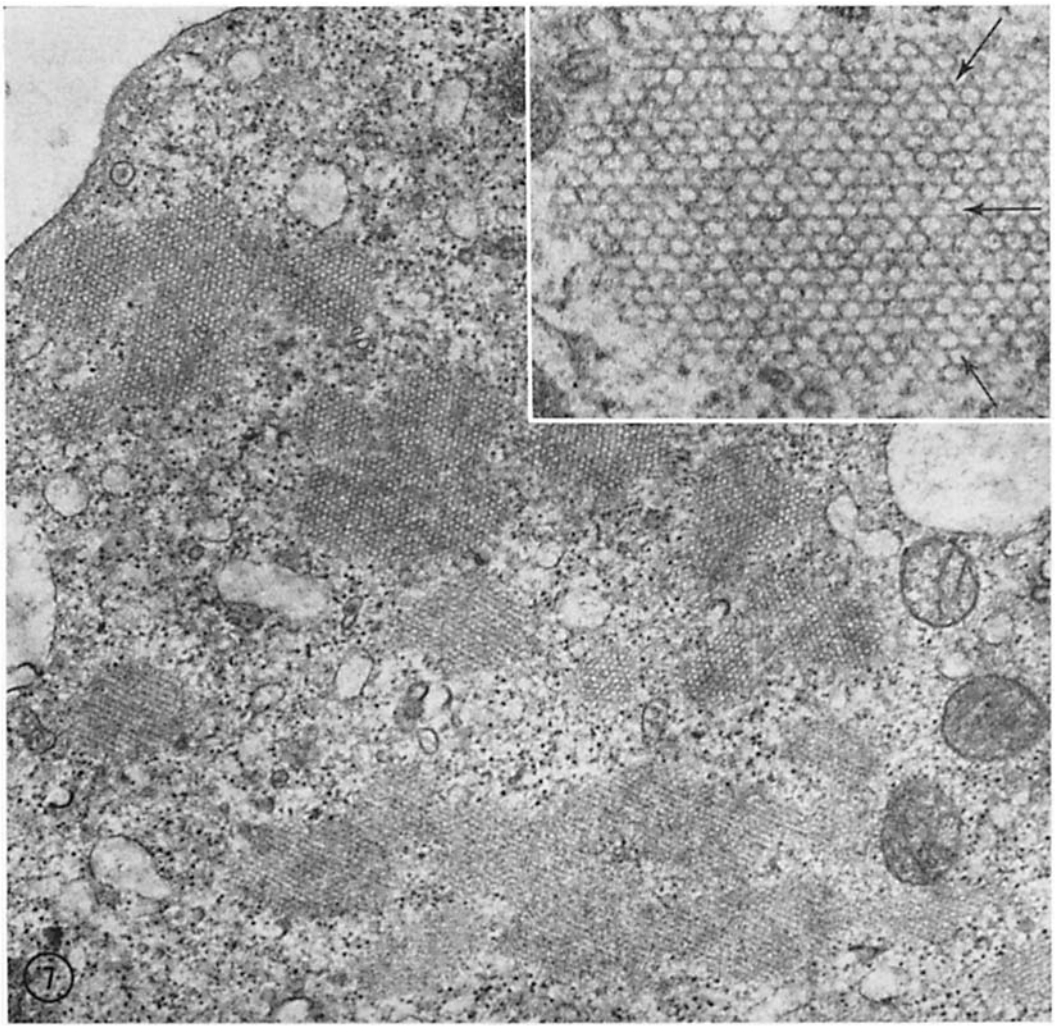


FIGURE 7 Several cross-sectioned small crystals are present in the upper half of the photograph; tangentially cut crystals are present in the lower half. 2 × 10^{-5} VB; 24 hr. × 35,000. The insert depicts part of an almost perfectly cross-sectioned crystal at higher magnification. Note the rows of tubules which intersect at 60° (arrows). 2 × 10^{-5} VB; 1 hr. × 100,000.

tions studied showed only an occasional crystal cut perfectly parallel to its long axis, and more important the sections were usually at least 500 A thick; these factors should give rise to a variety of patterns. Thus, for instance, the 200 A periodicity, as illustrated in Fig. 11, may be produced by a superimposition of the zigzag of tubular walls if cut and looked at from a certain angle (beneath the dots of Fig. 11). But a grazing cut through the surface of a crystal would probably produce an identical pattern. Remarkable about the 200 A periodicity is also the fact that the lines consist of regularly spaced dots which in turn, in conjunction with dots on neighboring lines, give rise to an oblique periodicity of 240 A (Figs. 6 and 11). The lines of the latter form an angle of 22° with the transverse diameter of a crystal. Is this indicative of a helix with a pitch angle of 68°? Of course, this coiling of the microtubule wall would actually be due to a spiral arrangement of its subunits which are assumed to consist of protofibrils (19–22). According to

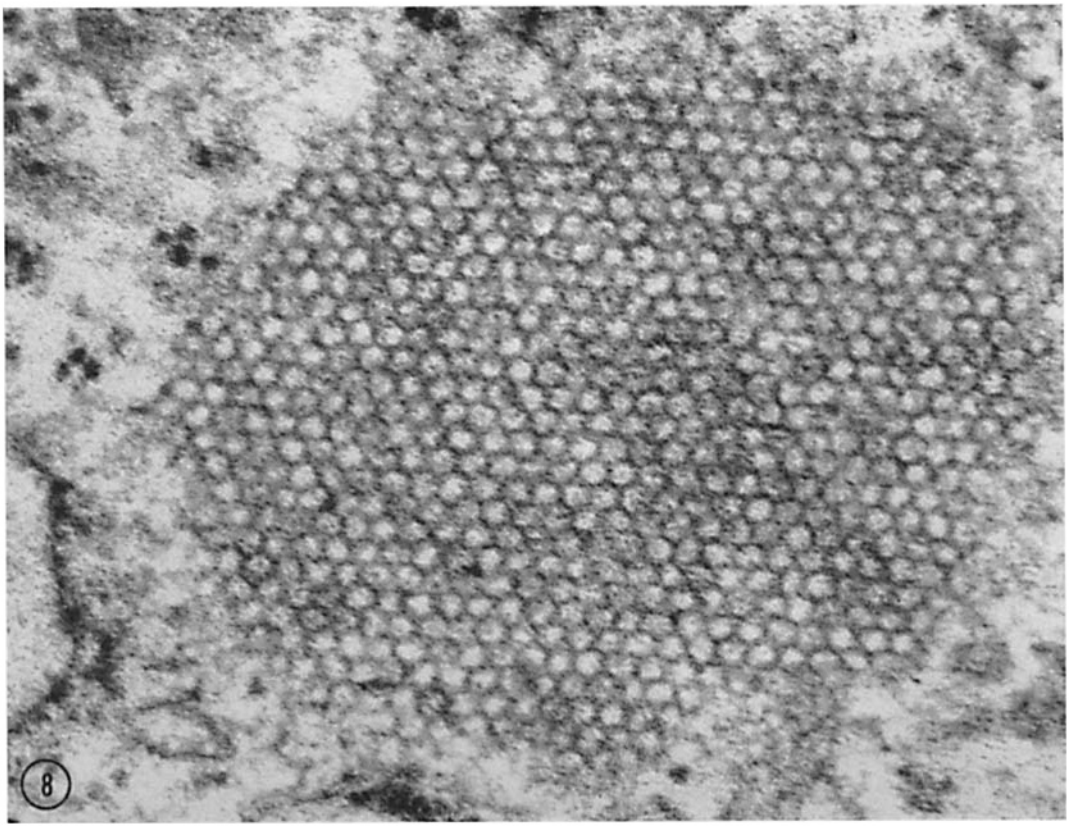


FIGURE 8 Cross-sectioned crystal showing the tubular nature of its subunits. 10^{-5} VB; 8 hr. × 140,000.

the literature but not well demonstrated in our study, 10–13 filamentous subunits (protofibrils) are thought to be bundled together symmetrically to form a microtubule cylinder with a 50–60 A spacing of the subunits (21, 23). The hexagonal pattern observed in our experiments suggests that the walls of the microtubule crystals may consist of protofibrils arranged in multiples of six. Thus 12 longitudinal filaments per microtubule would be spaced at 70 A center-to-center, 18 (four per side of the hexagon) spaced at approximately 45 A. Neither of these calculated spacing distances agrees with those found in normal plant or animal microtubules. The hexagonal form of the cross-sectioned tubules is also of interest in light of recent studies of Moor (22). This investigator found occasional hexagonal mitotic spindle fibers in yeast cells recovering from exposure to an anaerobic medium; the diameter of these microtubules was different from that found randomly in the cytoplasm or nuclei.

Assuming that there are 12 protofibrils per microtubule, the ratio of the spacing of the subunits in our model to published spacing measurements would be 70/60 A, exactly the same ratio found for the diameter of our abnormal single microtubule if compared with a control, i.e. 280/240 A. This difference in dimensions may be due to swelling in volume of the subunits brought about by a change in the ionic environment (24). Or more likely, it may be caused by a rearrangement of the binding sites in the tubule protein molecules by the alkaloid. Analogous changes in the property of self-assembling systems are known for the secondary and tertiary folding of proteins and virus polymerizations.

The modification of mitotic behavior induced by the alkaloids under study is remarkably similar to that of other metaphase arresters of which the prototype is colchicine. A comparison of the chemical formulae of the *Vinca* alkaloids with those of the *Colchicum autumnale* alkaloids and

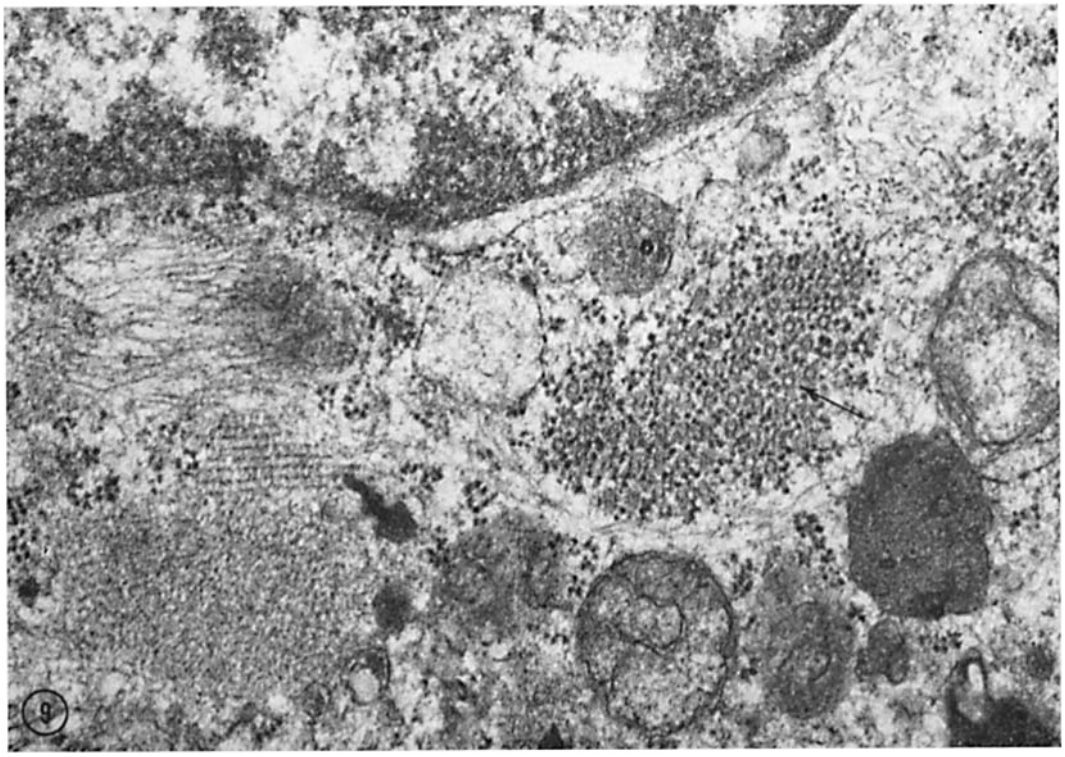


FIGURE 9 A bundle of cross-sectioned microtubules (arrow) of 280 A diameter lies among an aggregate of ribosomes. 5×10^{-5} VC; 24 hr. × 50,000.

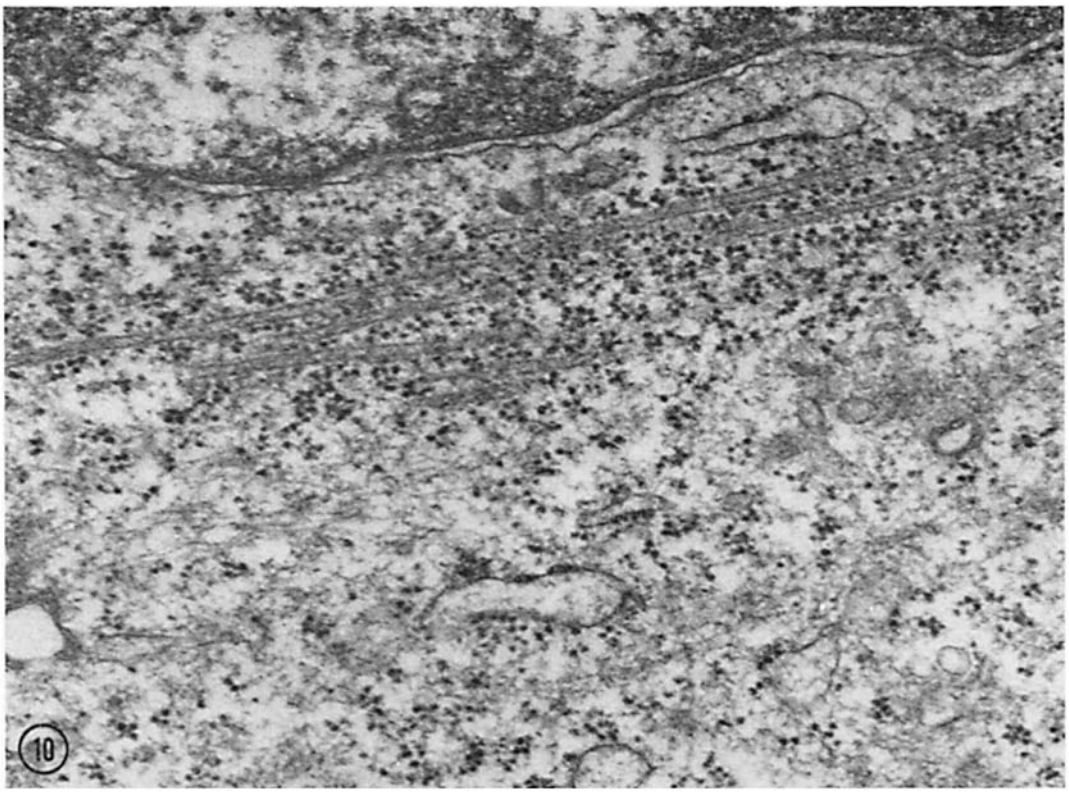


FIGURE 10 Longitudinally sectioned group of abnormally large microtubules of the type shown in the previous illustration. Note the numerous polysomes in this area. 10^{-5} VB; 4 hr. × 50,000.

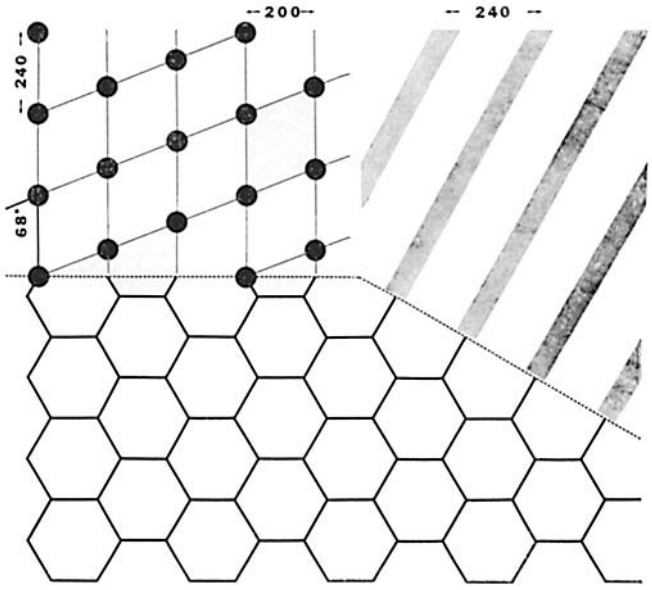


FIGURE 11 Schematic representation of pattern of three different planes of section through a crystal, perpendicular (lower half of drawing), and parallel to long axis. Distances are given in Ängstroms.

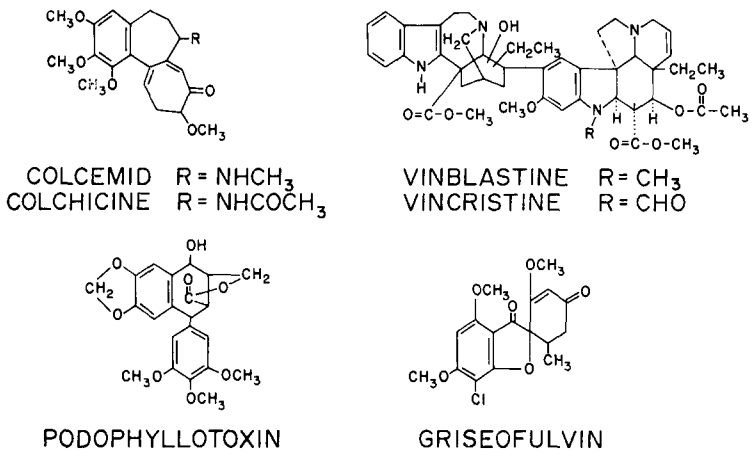


FIGURE 12 Chemical formulae of the best known metaphase arresters that are effective in very low concentrations.

other mitotic poisons shows their common denominators to be their hydrophobic cyclic carbon rings and unusual richness in methoxyl side chains (Fig. 12). Levan and Oestergren (25) and Oestergren (26), in studying the metaphase arresting abilities of benzene and naphthalene derivatives, found a direct correlation between the effect on mitosis and lipid solubility. These observations are also supported by our finding on the action of the highly lipophilic metaphase arrester griseofulvin (3). Does this all suggest the presence of a hydrophobic moiety in (one of) the microtubule protein(s), which controls the formation, size, and sharing of the walls of these cell organelles?

The fact that microtubule protein may form crystals should allow application of polarization and diffraction methods, possibly on isolated crystals, to the elucidation of this protein's molecular configuration and the mechanics of formation of the various forms of this cell organelle.

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