HEAT-INDUCED REVERSIBLE HEXAGONAL PACKING OF SPINDLE MICROTUBULES

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ABSTRACT

Epithelial cells cultured from the lung of the Northwest rough-skinned newt (*Taricha granulosa granulosa*) were subjected to brief (10–15 min) elevated temperature shocks of 33°–36°C during metaphase. Electron microscope studies on these cells reveal that the spindle microtubules (Mts) are differentially stable to heat treatment. The great majority of nonkinetochore Mts are destroyed within the first few minutes of the shock while kinetochore and adjacent Mts rearrange to form hexagonal closely packed structures before disassembling, the latter occurring only after prolonged heat treatment.

The significance and theoretical implications of the formation of hexagonal closely packed Mt structures and of the differential stability of spindle Mts to heating are discussed. The data suggest the existence of one or more heat-sensitive structural component(s) which maintain the individual minimum spacing seen between spindle Mts. To our knowledge, this is the first reported instance of the experimental rearrangement of kinetochore Mts into reversible, hexagonal closely packed bundles.

KEY WORDS hexagonal packed microtubules · heat · mitosis

The controlled assembly-disassembly and spatial rearrangement of spindle microtubules (Mts) are basic features of every mitotic process. However, there is little information regarding the mechanism(s) regulating the rearrangement of spindle Mts.

A substantial amount of evidence shows that the polymerization of tubulin into Mts is a thermal-sensitive reaction and occurs at least partially through the hydrophobic bonding of dimer tubulin units with a concomitant release of bound water (5, 13, 28). The sensitivity of Mts to cold temperatures has been documented for Mts within the spindle (6, 11, 12, 15), cytoplasm (3), helizoa axopodium (44, 46), and nerve axons (24). Furthermore, the instability of the polymer at cold temperatures is being currently used in vitro to study the mechanics of Mt assembly-disassembly (5, 51).

Elevated temperatures have been used to study the stability of flagellar (19, 41), cytoplasmic (3), and axonal (10) Mts and the initiation and development of Mt patterns in the cytopharyngeal basket of the ciliate *Nassula* (48). Furthermore, polarized light studies (11, 12) show that elevated temperatures can also be used to reversibly abolish the birefringent component, i.e. Mts, of the spindle. We report here the fine structural effects of elevated temperatures on the rearrangement of spindle Mts in epithelial cells cultured from the lung of the newt, *Taricha granulosa granulosa*. We were primarily interested in determining how closely individual spindle Mts normally approach each other and whether this distance can be modified experimentally. The answers to these ques-

tions should provide information concerning the lateral spacing between individual Mts within the spindle.

MATERIALS AND METHODS

The method used to culture *Taricha granulosa granulosa* (Skilton) lung cells was essentially that of Seto and Rounds (32) modified by using 0.6 × Leibovitz medium (16) supplemented with 10% fetal calf serum, 5% whole egg ultrafiltrate, and antibiotics. Minced lung fragments (2 mm^2) were rinsed several times in 0.6 × Tyrode's salt solution and then transferred for 1 h to 5 ml of medium in a sterile petri dish. Fragments were then cultured in Rose chambers (25, 26) with cellophane strips and fed at weekly intervals. By the 8–10th day, divisions could be found in the epithelial monolayer. The initial mitotic index is about 2–3%, decreasing to less than 1% in cultures older than 2 wk.

A temperature control slide (TCS), described elsewhere (15, 23), was used for experimental temperature shocks. The slide permits continuous light microscope observations during experiments and fixation, thus allowing precise correlation between light and electron microscope data.

The temperature shifts were monitored by two thermistors (Yellow Springs Instrument Co., Yellow Springs, Ohio): one (YSI 423) was mounted directly in the inflow of the perfusing water-ethanol (1:1) mixture, the other (YSI 421) was in direct contact with the top of the fixation chamber. Readings from the two thermistors were either visually monitored on a calibrated galvanometer, or printed out on a calibrated Heath chart recorder. The detailed methodology for delivering temperature shocks and calibrating the TCS is described elsewhere (15, 23).

Unless otherwise stated, all cells were fixed during heat shock with 3.1% glutaraldehyde (pH 6.9, in 0.05 M Millonig's phosphate buffer) prewarmed to the same temperature as the cell. They were then postfixed in 1% OsO_4 at room temperature and embedded in Epon. Sections were cut on an LKB microtome (LKB Instruments, Inc., Rockville, Md.), placed on Formvar-coated 75-mesh grids, stained for 5 min in uranyl acetate followed by 5 min in lead citrate (22), and stabilized with evaporated carbon. Sections were then examined with a Philips 300 electron microscope operated at 60–80 kV.

RESULTS

Anaphase chromosome movements in *Taricha* lung cells occur within a temperature range of 6°–32°C. The separation of centrosomes is disturbed and normal prometaphase chromosome movements are inhibited in prophase cells elevated to temperatures above 32°C. The resultant configuration, which resembles a C-metaphase, is only stable for 1–2 h, after which the chromosomes swell and form a restitution nucleus. A detailed ciné and electron microscope analysis of the effects of elevated temperatures on spindle Mts and the subsequent desynchronization of anaphase chromosome movements is presented elsewhere (23).

Metaphase cells shocked from room temperature (23°C) to 33°C for 10–15 min and then fixed before chromatid separation reveal kinetochore fibers under phase microscopy (cf. Fig. 1 to Figs. 5 and 6). Such fibers are not seen in room temperature controls fixed in room temperature glutaraldehyde or in room temperature cells fixed with 33°C glutaraldehyde.

Longitudinal sections through cells fixed after 10 min at 33°C show that the Mts of each kinetochore fiber are closely associated with one another for long distances (cf. Fig. 2 to Figs. 7 and 8). Individual Mts within these bundles can be followed for up to 10 μm. In cross sections of these cells, three features are immediately apparent: (*a*) there are very few nonkinetochore Mts in the heat-treated spindle; (*b*) the kinetochore fibers are no longer arranged around the periphery of the hollow spindle (see reference 52) as in controls, but are scattered at random throughout the spindle; and (*c*) the Mts of each kinetochore fiber are

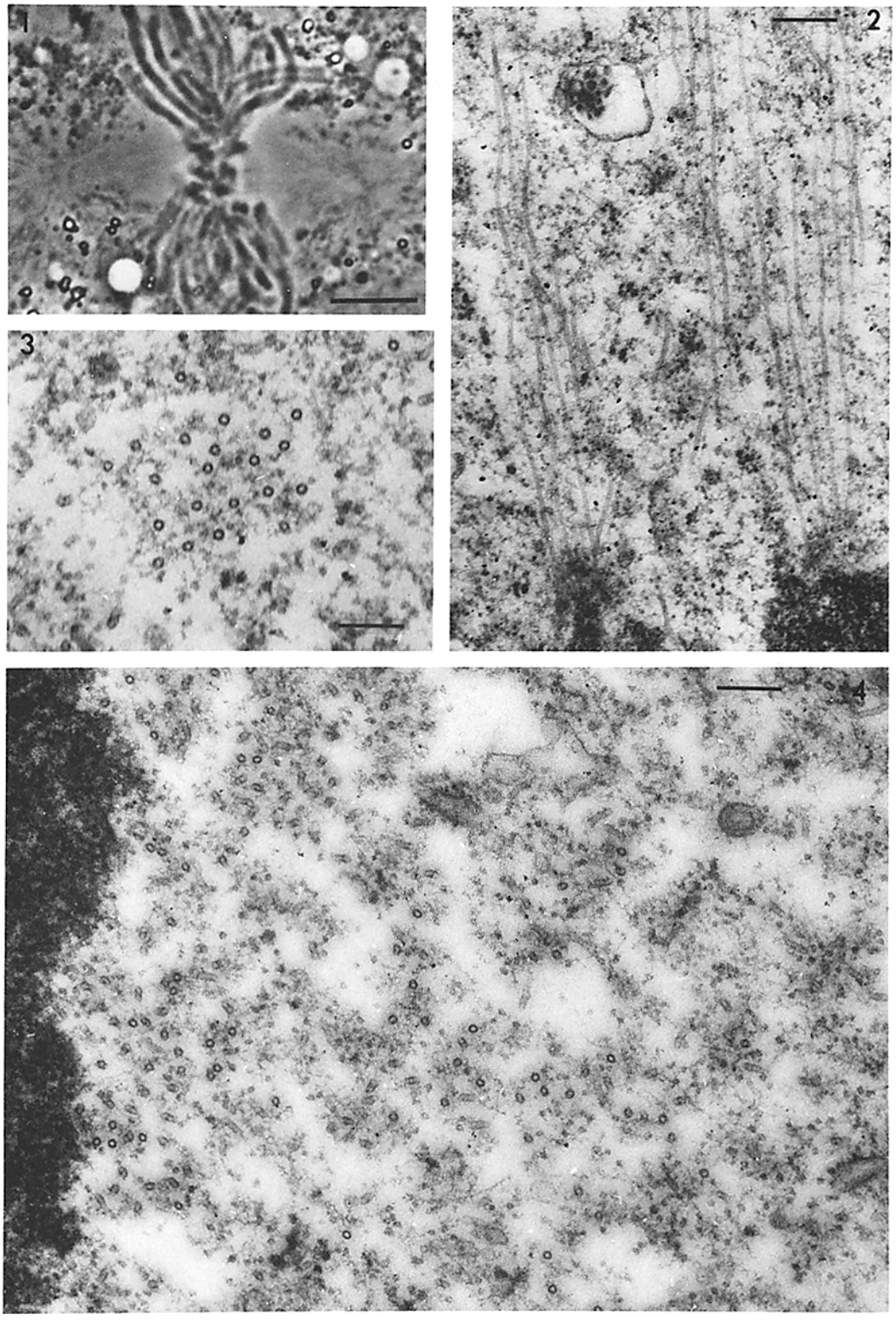
FIGURES 1–4 Figs. 1–4 are from room temperature cells.

FIGURE 1 Phase-contrast photomicrograph of a metaphase newt lung cell. Bar, 10 μm. × 1,350.

FIGURE 2 Longitudinal section through two kinetochore fibers showing the organization of kinetochore Mts. Bar, 0.3 μm. × 3,000.

FIGURE 3 Cross section through a kinetochore fiber close to the metaphase plate. Notice the spacing between neighboring Mts (cf. Figs. 9–11). Bar, 0.2 μm. × 52,000.

FIGURE 4 Cross section through a half-spindle showing the distribution of spindle Mts. Kinetochore and nonkinetochore Mts intermingle and are indistinguishable from one another. Bar, 20 μm. × 50,000.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arranged parallel to one another and are closely packed in a hexagonal array (cf. Figs. 2–4 with Figs. 7 and 9–11). When compared to controls, the overall number of nonkinetochore Mts on a cross section is reduced. The number of Mts present depends on the duration and temperature of the shock and on the location of the section within the spindle, e.g., a section cut near the kinetochores in one of the cells studied after 10 min at 33°C revealed that 80% or more of the remaining Mts were hexagonally packed and could be identified as kinetochore fiber Mts while 20% were in the process of packing or remained individual (see Fig. 11).

Nonkinetochore Mts which run parallel to and are closely associated with the Mts of the kinetochore fiber are occasionally incorporated into these closely packed structures (Figs. 7 and 8). Hexagonally packed kinetochore and associated Mts are of the same diameter as Mts in control cells, i.e., 24 nm, with a wall-to-wall separation distance of 4.5–5.5 nm, a center-to-center spacing of 28.5–29.5 nm (see Fig. 9), and are connected by occasional weakly staining cross bridges. Furthermore, when only 5 Mts are present, they still assume a hexagonal close-packing symmetry and leave one space open (see Fig. 10). Although the heat-treated Mts seem to absorb more stain than Mts fixed at room temperature, there is no reason to believe that they represent the transformed tubulin structures discussed extensively by Tilney (43). Sections of kinetochore fibers close to the metaphase plate show bundles containing up to 25–30 Mts per fiber while those closer to the poles show fewer Mts per bundle. However, the number varies from cell to cell. As a result, it is not clear whether there is in fact a reduction of kinetochore fiber Mts, or, if there is such a reduction, how and where along the fiber it takes place.

Only occasional single Mts can be detected in the spindle of cells shocked to 35°–36°C for 15–20 min. The closely packed kinetochore fiber Mts, which are seen in phase-contrast microscopy in cells fixed during the first few minutes of a 35°–36°C shock, are missing entirely after 15–20 min. The centrosomal area appears heavily vacuolated, and most of the astral Mts are also missing.

Metaphase cells, elevated to temperatures between 33°–36°C for 10 min and then allowed to cool slowly to room temperature over a 10-min interval, subsequently start and complete anaphase. These cells may show heat-induced chromosome damage, i.e., sticky chromosomes, bridges, and/or akinetic fragments, but the movement of the chromosomes to the poles and the elongation of the spindle are similar in all respects to those of room temperature controls. The characteristic fine structural features of such meta-

FIGURE 5 A metaphase spindle before the initiation of a 33°C heat shock. Bar, 10 μm. × 1,200.

FIGURE 6 Same cell as in Fig. 5, 10 min after the initiation of a 33°C heat shock. The cell is fixed in glutaraldehyde. Notice the thin fibers (open arrows) and centrosomes within the spindle (cf. Figs. 1 and 5). Bar, 10 μm. × 1,200.

FIGURE 7 Longitudinal section through a kinetochore fiber treated as in Figs. 5 and 6. Notice the close association of kinetochore Mts and the incorporation of some nonkinetochore Mts within the closely packed structure (arrow). Note the slight counter-clockwise twist of the kinetochore fiber. Bar, 0.3 μm. × 35,000.

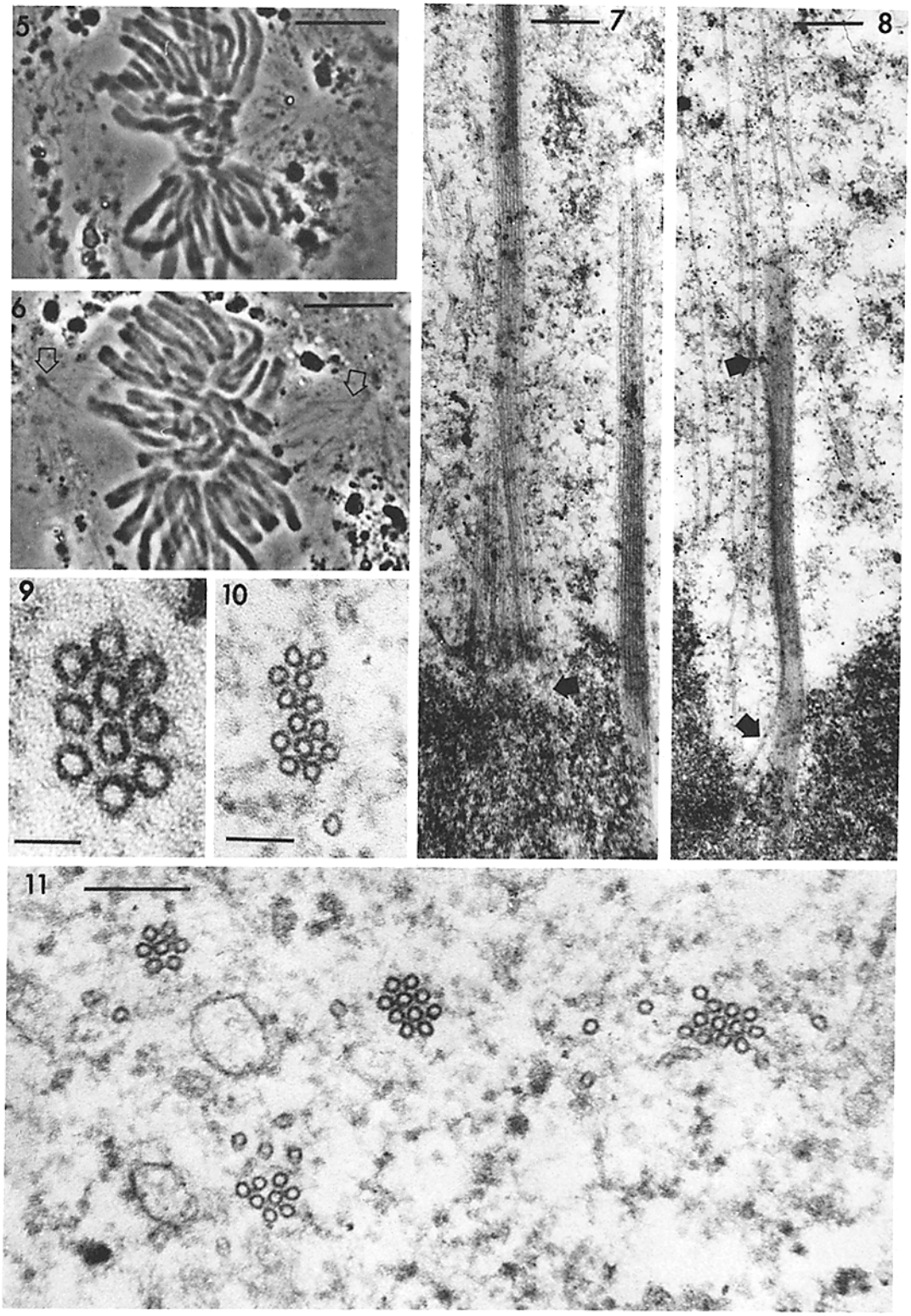
FIGURE 8 Similar to Fig. 7. Notice the bending of some Mts incorporated into the bundle. Bar, 0.4 μm. × 27,000.

FIGURES 9–11 Figs. 9–11 are cross sections through the spindle pictured in Fig. 6.

FIGURE 9 Section through a kinetochore fiber illustrating the hexagonal packing arrangement of Mts. Notice the close wall-to-wall spacing, and note that some Mts seem to be connected by indistinct bridges. Bar, 0.05 μm. × 200,000.

FIGURE 10 Notice the maintenance of hexagonal symmetry when only 5 Mts are present. Bar, 0.1 μm. × 105,000.

FIGURE 11 Typical morphology of kinetochore fiber Mts in the polar area after heat shock (cf. Fig. 4). Bar, 0.2 μm. × 82,000.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phase and anaphase spindles are: (*a*) the kinetochore fiber Mts are no longer hexagonally packed, and their morphology closely resembles that of room temperature and 32°C control cells; (*b*) nonkinetochore Mts can be detected between separating anaphase chromosome groups; and (*c*) occasional short regions of hexagonally packed Mts may still be present close to the polar areas.

A control experiment was performed to determine whether the close packing of kinetochore Mts might be induced by fixing the experimental cells with heated fixative. Normal room temperature metaphase cells were fixed with 34°C glutaraldehyde. The fine structure of these cells resembles that of the room temperature and 32°C controls. Kinetochore fibers were normally arranged, contained 25–30 Mts per fiber, and nonkinetochore Mts could be easily detected. All three types of metaphase controls (room temperature, 32°C, and room temperature fixed in 34°C glutaraldehyde) contain kinetochore Mts which show a minimum center-to-center separation distance of 40–50 nm (see Fig. 3). Hexagonal closely packed Mt structures were never encountered in these cells.

DISCUSSION

Heat shocks have been previously used to study the structural stability and polymerization of flagellar Mts (3, 19, 41), the regrowth of axonal Mts (10), and the development of the Mt arrangement in the cytopharyngeal basket of the ciliate *Nassula* (48). Very little is known, however, about the in vivo thermal sensitivity of spindle Mts to elevated temperatures. Behnke and Forer (3) studied the effects of a 15-min 50°C heat shock in the crane fly and found that spindle Mts were destroyed while flagellar Mts were differentially stable.

The data presented here are the first to indicate that spindle Mts are differentially stable to elevated temperatures. The great majority of nonkinetochore Mts are destroyed within the first few minutes of the shock while kinetochore and adjacent Mts form hexagonal closely packed structures before depolymerizing. The data also confirm the finding of Inoué (11, 12) which indicates that the effects of short duration heat shocks on the spindle are reversible.

The differential stability of Mts to various physical (cold, heat, and pressure) and chemical agents is well documented. One or several interrelated factors may be involved in confirming a greater stability to heat-induced hexagonally packed Mts. This differential stability has been hypothesized to be the result of such factors as: (*a*) the number of free ends and/or the presence or absence of tension (28–30); (*b*) linkage formation and/or the interaction between Mts (1, 3, 15, 27, 44, 45, 48); (*c*) physical-chemical differences in tubulin subunits (6, 41, 42) which may result in differing equilibrium constants between different Mts (6, 28, 29); (*d*) the presence or absence of a dynamic equilibrium between different Mts of the same cell (31); or (*e*) differing cellular responses to the experimental treatment (2, 3). The presence or absence of an outer component (2, 40, 45) and/or a Mt-associated protein (MAPS, 33, 34), or a combination of these factor(s), may be responsible for the observed differential stability between kinetochore and nonkinetochore Mts reported in this study.

The rearrangement and subsequent hexagonal close-packing of kinetochore Mts may confer a greater stability to those Mts and possibly represents their lowest possible energy state under the experimental conditions. Further heating of these structures results in energy levels which are too high to maintain the polymer, and the structures disassemble. That the regular parallel arrangement of Mts may confer a greater stability to spindle Mts is also suggested by experiments with pressure (30) and cold (6, 15). Under these conditions, the most persistent Mts are those of the kinetochore fiber which undergo spatial rearrangement and form bundles of associated Mts. It may be, however, that these Mts are differentially stable to the experimental treatment before their rearrangement and consequent packing.

At present, this study does not allow us to single out any of the above reason(s) why kinetochore Mts show a greater stability than nonkinetochore Mts to heat shock. The results do indicate, however, that the differential stability of the kinetochore Mts can be correlated with a change of kinetochore Mt arrangement from one of divergence to one of hexagonal close-packing.

Apart from the hexagonal packing of Mt paracrystals induced by various drugs (37–39) and those Mt-like structures reported in insect nuclei (36) and prokaryotes (4, 14), reports of Mt hexagonal packing are generally confined to systems which show a high degree of inherent Mt ordering. Some of these systems are stabilized by cross bridges (18, 35, 46) while others seem to be formed and maintained by space limitations (21; see references 43 and 44).

Reports concerning the close packing of spindle

Mts are scarce. Lambert and Bajer (15) report a 55–65 nm center-to-center spacing of closely packed Mts in *Haemanthus* endosperm shocked to 4°C or below. Tippit et al. (47) report the close rectangular packing of central spindle Mts in *Melosira* which is also a characteristic feature of many other diatoms (17). In this case, each Mt is surrounded by four neighbors which maintain a separation distance of at least 1 Mt diameter, i.e., 20–25 nm.

The data presented here represent the closest (4.5 nm wall to wall) individual hexagonal packing yet induced from Mts of the spindle. Some possible implications of such an arrangement are discussed below.

A substantial body of evidence suggests that the clear zone, found around some Mts (2, 40), may contain MAPS. Weingarten et al. (50) and others (5, 7, 9) have shown that accessory proteins are needed for Mt initiation and elongation in vitro. It is unknown whether these proteins constitute clear zone components. However, Mts assembled in the presence of some of these proteins can be decorated with other protein(s) which co-purify with Mts isolated and purified by in vitro assembly-disassembly. These proteins (MAPS) are distinct from the initiation proteins, have a high molecular weight and are not needed for the in vitro assembly of Mts. Mts assembled in the presence of MAPS show trypsin-sensitive (49) filamentous decorations and do not pack so tightly during centrifugation as do those assembled in the absence of MAPS (8).

Mts can also be decorated with a variety of cationic substances (2, 40). The electron-transparent halo seen in thin sections contains structural elements which can be visualized by histochemical means. The composition of these element(s) is not known with certainty, but glycoproteins are thought to be at least partly involved (2, 31, 40). Furthermore, such structural components may be found only on some, or some parts of, Mts composing a particular Mt system.

The hexagonally packed Mts induced by heat in our experiments show an approximate 10-fold decrease in their wall-to-wall packing distance when compared with the minimum separation distance in controls. This suggests that there may be an associated heat-labile structural element(s) on these spindle Mts. It is irrelevant in this connection how these structural element(s) are distributed along the Mt. It is clear, however, that they keep Mts a certain minimum distance apart. Our data do not allow us to distinguish whether this structural element(s) is reversibly inactivated, disassembled, or undergoes a conformational change producing a contractive force (20). Tilney (44) suggests that Mts are brought together either by physical and/or mechanical forces, or by external compression applied to the system (as in the tip of the frog cilia [21]). The shape of the bundles in the present experiments (see also reference 15) is consistent with the former but not the latter explanation. The ordering of Mts around a sixfold axis of symmetry, even when only 5 Mts are present, suggests that attractive forces between Mts play a role in forming and maintaining the closely packed structure. Regardless of their nature, the forces causing this heat-induced hexagonal packing are strong enough to bend Mts.

The structural factor(s) under consideration here are reversibly affected by heating the cell. During the recovery from the shock, existing closely packed Mts must either: (*a*) separate and splay out into a kinetochore fiber of normal morphology; (*b*) depolymerize and regrow from the kinetochore; or (*c*) move poleward and be replaced by Mts of normal divergence growing from the kinetochore. Since our electron microscope observations are consistent with all of these interpretations, none can be ruled out, and all are possible courses of kinetochore fiber recovery. However, the presence of hexagonally packed Mt bundles in the polar area of recovering cells suggests that recovery proceeds from the kinetochore towards the pole.

Lambert and Bajer (15) have shown that spindle Mts in normal and cold-shocked *Haemanthus* endosperm maintain an individual Mt spacing of 55–65 nm. In cells which have been exposed to sub 4°C temperatures, some nonkinetochore Mts, especially those which pass through the chromatin, may bend sharply and cluster together with the packed kinetochore Mts. This "bending" of Mts can also be seen in room temperature control cells. The same phenomenon was observed in this study (Fig. 8), suggesting that the force(s) which bring Mts together in heat-shocked cells are quantitatively similar to those at work in normal and cold-shocked cells. However, the possibility exists that elevated temperatures induce or release a new force within the spindle.

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