PRESSURE-INDUCED DEPOLYMERIZATION OF SPINDLE MICROTUBULES

III. Differential Stability in HeLa Cells

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ABSTRACT

Evidence from light microscopy (principally polarization microscopy) has demonstrated that hydrostatic pressure can reversibly inhibit mitosis by rapidly depolymerizing the spindle fiber microtubules. We have confirmed this finding in ultrastructural studies of mitotic HeLa cells incubated at 37°C and pressurized at 680 atm (10,000 psi). Although there are many spindle microtubules in the cells at atmospheric pressure, electron micrographs of cells pressurized for 10 min (and fixed while under pressure in a Landau-Thibodeau chamber) show few microtubules. Pressure has a differential effect on the various types of spindle microtubules. Astral and interpolar MTs appear to be completely depolymerized in pressurized cells, but occasional groups of kinetochore fiber microtubules are seen. Surprisingly, the length and density of microtubules of the stem bodies and midbody of telophase cells appear unchanged by pressurization. In cells fixed 10 min after pressure was released, microtubules were again abundant, the density often appearing to be higher than in control cells. Reorganization seems incomplete, however, since many of the microtubules are randomly oriented. Unexpectedly, kinetochores appeared diffuse and were difficult to identify in sections of pressurized cells. Even after 10 min of recovery at atmospheric pressure, their structure was less distinct than in unpressurized cells.

The effects of pressure on mitotic spindle microtubules, primarily in *Chaetopterus* oocytes, were reported and discussed in the previous papers of this series (27–29). The state of spindle fiber microtubule (MT) assembly was judged in these studies from measurements of the spindle's birefringence retardation (BR). The changes in spindle BR induced by pressure were similar to changes induced by lowering temperature (11) or by adding the drug colchicine (10). Increased pressure caused a rapid, but reversible decline in spindle BR; the rate and extent of BR decay was directly related to the magnitude of pressure. Thermodynamically, the characteristics of spindle MT assembly-disassembly in vivo resembled those of purified brain MTs in vitro (8, 12, 21, 28, 30). In general, the spindle fiber MTs appeared to behave as if they were in a labile equilibrium with a cellular pool of tubulin subunits, with an appropriate distribution of nucleating centers (13). The MT polymerization both in vivo and in vitro is characterized by a large increase in volume, approximately 90 ml/mol of polymerizing subunit, based on a nucleated condensation mechanism of polymerization (28, 30).

Although the polymerization-depolymerization characteristics of spindle microtubules in vivo are

similar to those of the assembly of purified labile brain MTs in vitro, unlike the purified brain tubulin, there is some evidence that the assembled spindle MTs are not a homogeneous population. The kinetics of spindle BR decay in response to sudden increases in pressure are biphasic (an initial rapid decrease in BR, followed by a second phase of slower BR decay) reflecting the persistence of kinetochore fiber BR over that of the interpolar MTs (27). A similar differential effect on the visibility of spindle fiber BR also occurs in response to cooling and colchicine (10). These studies and others (3, 5, 26) have suggested that there are several types of MTs within the spindle in terms of stability to depolymerizing agents. This differential stability may be important when considering both the mechanism of spindle MT assembly and the role of spindle fiber MT assembly and disassembly in the production of chromosome movement.

The present study was designed to verify at the ultrastructural level the depolymerization of spindle MTs by pressure and their repolymerization after pressure release. Critics have suggested that the loss of BR observed in the earlier experiments could have resulted from fragmentation or disorientation of the spindle MTs rather than from their depolymerization. We also hoped to obtain additional evidence concerning the differential stabilities of spindle MTs.

HeLa cells were chosen for these experiments because they have been well characterized ultrastructurally (20, 22, 25), and large numbers of mitotic cells can be obtained by selective detachment from monolayer cultures. We decided to use 680 atm (10,000 psi) as the experimental pressure on the basis of previous experiments with brain MTs in vitro (30). At 37°C, the physiological temperature for HeLa cells and the temperature at which our experiments were performed, most, but not all microtubules were depolymerized by 680 atm at a tubulin concentration of 1–3 mg/ml. Assuming that the spindle MTs of HeLa cells would behave similarly, we expected to observe substantial depolymerization of MTs with 680 atm and hoped that any remaining MTs would provide information about possible MT stability differences. We were also interested in extending our knowledge of the effects of pressure on meiotic and mitotic cells to include mammalian cells, since they have not previously been studied in this respect.

MATERIALS AND METHODS

HeLa cells were maintained at 37°C as monolayer cultures with minimal essential medium (Earle's salts) plus 10% fetal calf serum, L-glutamine, and antibiotics. Before an experiment, mitotic cells were selectively harvested by shaking from the monolayer in calcium-magnesium-free Earle's balanced salt solution. After rapid cooling to 10°C, the cells were centrifuged at 1,000 *g* for 2 min and resuspended in fresh normal culture medium. The cell suspension was divided into three equal samples: two experimental samples and a control sample.

The pressure apparatus, similar to one designed by Landau and Thibodeau (16), consists of two stainless steel chambers. The outer chamber is the basic pressure bomb. The inner chamber, when assembled, consists of two 7-ml compartments separated by a thin cover glass. The ends of the two compartments are covered with rubber diaphragms which allow the pressure to be transmitted equally to the specimen and fixative compartments. The lower compartment contains a stainless steel ball. At the time of fixation, the pressure bomb is inverted and shaken vigorously, causing the steel ball to break the cover slip and mix the initially double-strength fixative with the cell suspension. A Blackhawk P76 hydraulic pump system was used to generate 680 atm within 3 s (31). Decompression by opening a needle valve occurred within a similar time period.

The first experimental sample was placed in the pressure-fixation chamber, the second in a glass vial sealed with Parafilm. We carefully excluded all trapped air. These two experimental samples were then placed in the pressure bomb and maintained at 37°C by a water bath, while the control sample was incubated in the water bath outside of the bomb. Within 12 min of harvesting the cells, we applied 680 atm of pressure. After 10 min of pressurization the cells in the first sample were fixed under pressure by breaking the thin glass cover slip separating equal volumes of cell suspension and 5% glutaraldehyde in Millonig's phosphate buffer, pH 7.0. The pressure bomb was repeatedly inverted for the next 2 min to guarantee good mixing of the fixative and cell suspension as the stainless steel ball travelled back and forth. Then the pressure was released to atmospheric.

At the time the pressurized cells were fixed, the control sample was fixed by mixing the cell suspension with an equal volume of fixative. 10 min after pressure release, the second experimental (recovery) sample was fixed in a similar manner. 20 min later, the cells in each sample were pelleted and fixed overnight in fresh 2.5% glutaraldehyde in Millonig's phosphate buffer, pH 7.0, at 4°C.

After postfixation for 0.5 h in 1% osmium tetroxide in the same buffer, the pelleted cells were dehydrated in ethanol, then propylene oxide, and embedded in Epon 812. Thin sections were cut with a diamond knife, picked up on uncoated grids, stained with uranyl acetate followed by lead citrate, and examined in a Hitachi HU-12 electron microscope.

Small samples of cells were observed with phase-contrast microscopy to follow recovery and division after pressure treatments. A repetition of the above-described experiment gave the same results.

RESULTS

At 37°C, 10 min at 680 atm (10,000 psi) produces almost complete disappearance of spindle MTs in HeLa cells (Figs. 1, 3, 6, and 8). In cross-sections through the metaphase plate region of control cells, there are typically in excess of 1,000 MTs (Fig. 2). Similar sections through mitotic cells fixed at 680 atm contain few, if any, visible MTs (Fig. 3). In the pole regions of control cells, MTs are abundant (Fig. 5); in pressurized cells, spindle MTs are scarce or absent (Fig. 6). On the other hand, the centriole triplet MTs, and the electron-dense amorphous material surrounding them, and the internal structures such as the triplet base and associated "foot" (33) appear to have been unaffected by 680 atm.

Astral and interpolar MTs appear completely depolymerized in pressurized cells, but occasionally individual kinetochore fiber MTs are seen in groups of two to six (Figs. 1, 3, and 8). In longitudinal sections most MTs attached to the kinetochores appear to extend poleward for at least 1 μm before leaving the plane of the section. As seen in Fig. 1, only some kinetochores have MTs attached and, in general, it is otherwise difficult to identify kinetochores.

Spindle MTs appear to be in a pressure-sensitive equilibrium with a small tubulin oligomer (the tubulin dimer?). At 680 atm no identifiable MT fragments are observed. Ring and spiral structures of tubulin dimers have been reported in conspicuous numbers in solutions of purified brain MTs depolymerized in vitro by cooling (7, 14, 21). These structures, apparently formed from 50–60-Å diam tubulin protofilaments, appear as 340-Å diam rings or stacked disks, or 480-Å diam double rings or spirals, depending on the preparation conditions. We have seen near the poles in pressure-treated cells a very few beaded, protofilament-like structures measuring about 50 Å in diameter (Fig. 6). Even if these structures are, in fact, composed of tubulin, their abundance in the pressurized cells does not seem sufficient to account for all the tubulin that would have been assembled into a normal spindle. These structures are most likely to be cross-sections of vesicles.

In contrast to its effect on the spindle MTs of prometaphase, metaphase, and anaphase spindles, 680 atm does not produce any apparent change in the MTs of the telophase stem bodies and midbody (Figs. 11, 12, and 13). The cross-section through the midbody of a pressurized telophase cell in Fig. 13 contains 1,200–1,500 MTs, about the same number of MTs as contained in that region of a normal HeLa cell midbody (20). The length and appearance of the midbody MTs also appear unchanged in the 3-μm regions on either side of the midbody center in Fig. 12 where the MTs are clearly visible.

In addition to affecting the spindle MTs, pressure causes changes in the normal appearance of the kinetochore's tripartite structure (4). The normally dense central or axial element (Fig. 7) is difficult to distinguish and appears to be absent in many kinetochores (Figs. 1 and 8). The chromosomes often fuse into large aggregates in cells pressurized during metaphase and early anaphase, but not generally in cells pressurized during prometaphase. Continuous bands of chromatin are frequently seen in pressurized metaphase cells (Fig. 1) with the chromosome arms no longer distinct.

Rapid repolymerization occurs after release of pressure. In mitotic cells fixed after 10 min of recovery, MTs are again abundant (Figs. 4, 9, and 10). Numerous MTs extending from kinetochores are visible as well as astral MTs and a mixture of interpolar and kinetochore MTs around the chromosomes. As seen in Fig. 10, the normal poleward orientation of the MTs within the central spindle is fairly well established, but occasional MT cross-sections in the equatorial region suggest that reorganization is not complete. Numerous MTs appear to pierce and pass through the chromosomes (Fig. 10). Although we did not frequently observe this phenomenon in control cells, it can occur in normal cells (20). Unlike the MTs which repolymerize quickly, the kinetochores and chromosomes still appear somewhat abnormal after the 10-min recovery period. By this time the tripartite structure is again discernible in some kinetochores, but others remain more diffuse than controls (Figs. 9 and 10). The chromosomes regain some of their identity, but chromosome fusion still persists in many metaphase cells (Fig. 10).

Within 2 h after pressure release, most treated cells that are allowed to recover without fixation have completed anaphase and normal cytokinesis as seen at the level of resolution of the phase-contrast microscope.

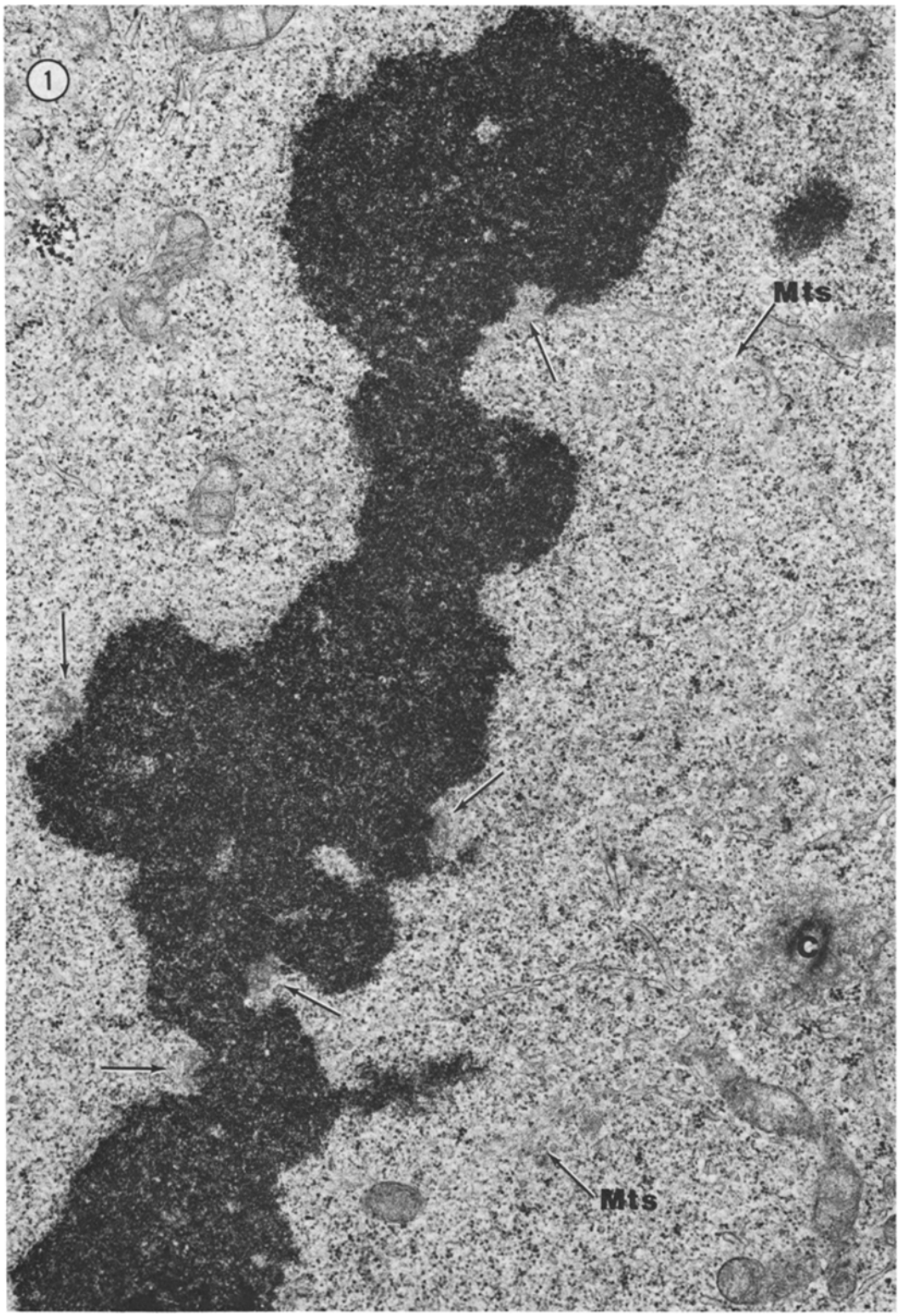
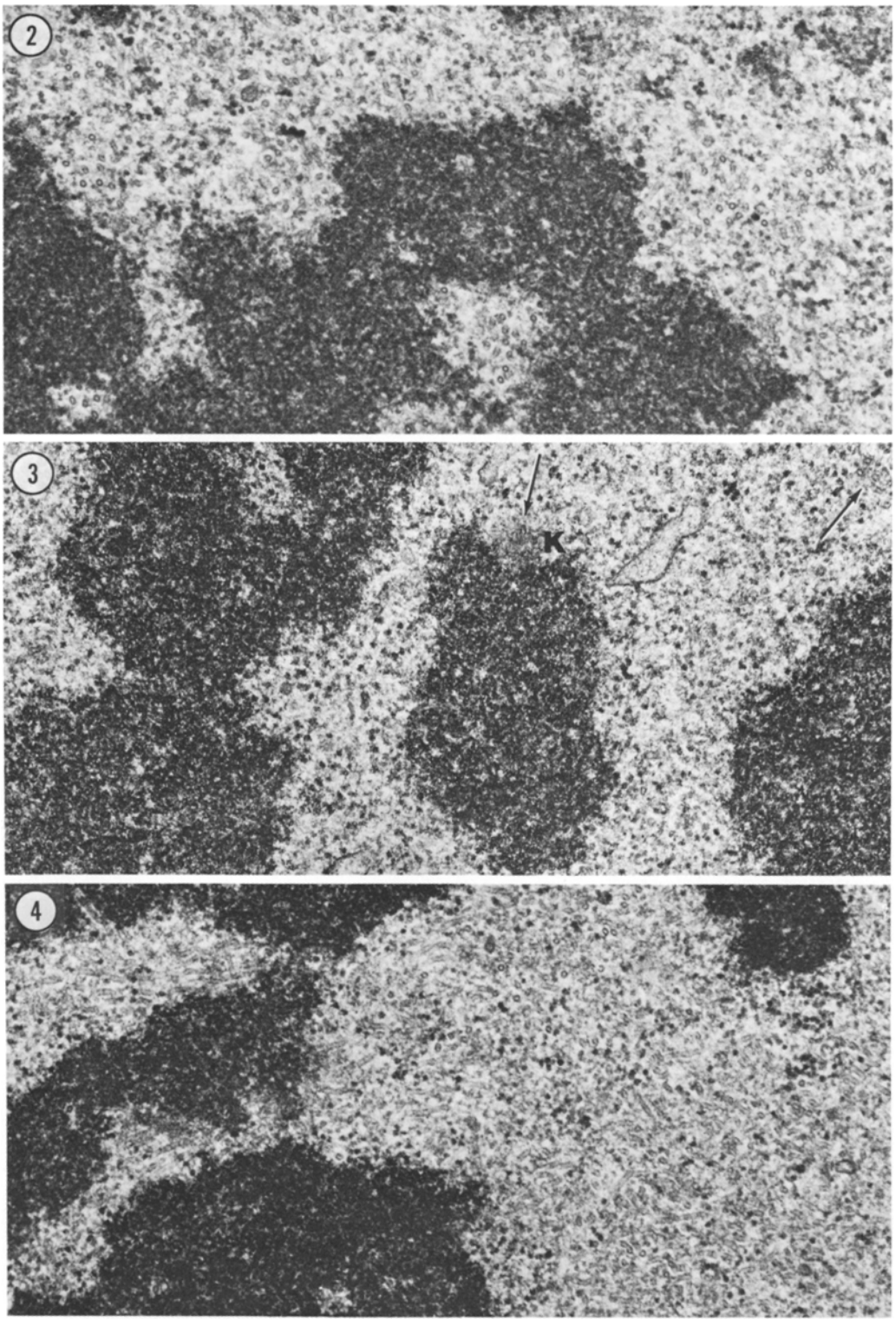


FIGURE 1 Pressurized cell at metaphase. Cell was fixed after 10 min at 680 atm and 37°C. The tangential section passes through a centriole (*C*) at one pole of the spindle; the other pole is not visible. Few microtubules (*Mts*) can be seen (compare with Fig. 10). The chromatin appears fused and the kinetochores do not show the typical tripartite structure. The amorphous, densely stained material around the centriole, however, appears normal. Mitochondria and other cytoplasmic vesicles have invaded the spindle region. Unlabeled arrows indicate probable kinetochores. × 8,800.



FIGURES 2, 3, and 4 Transverse sections through the equatorial region of metaphase cells. × 30,000. Fig. 2, At atmospheric pressure there are numerous microtubules. Fig. 3, After 10 min at 680 atm only occasional bundles of microtubules are visible (arrows), some clearly associated with a kinetochore (*K*). Fig. 4, 10 min after pressure was released there is, again, a high density of microtubules.

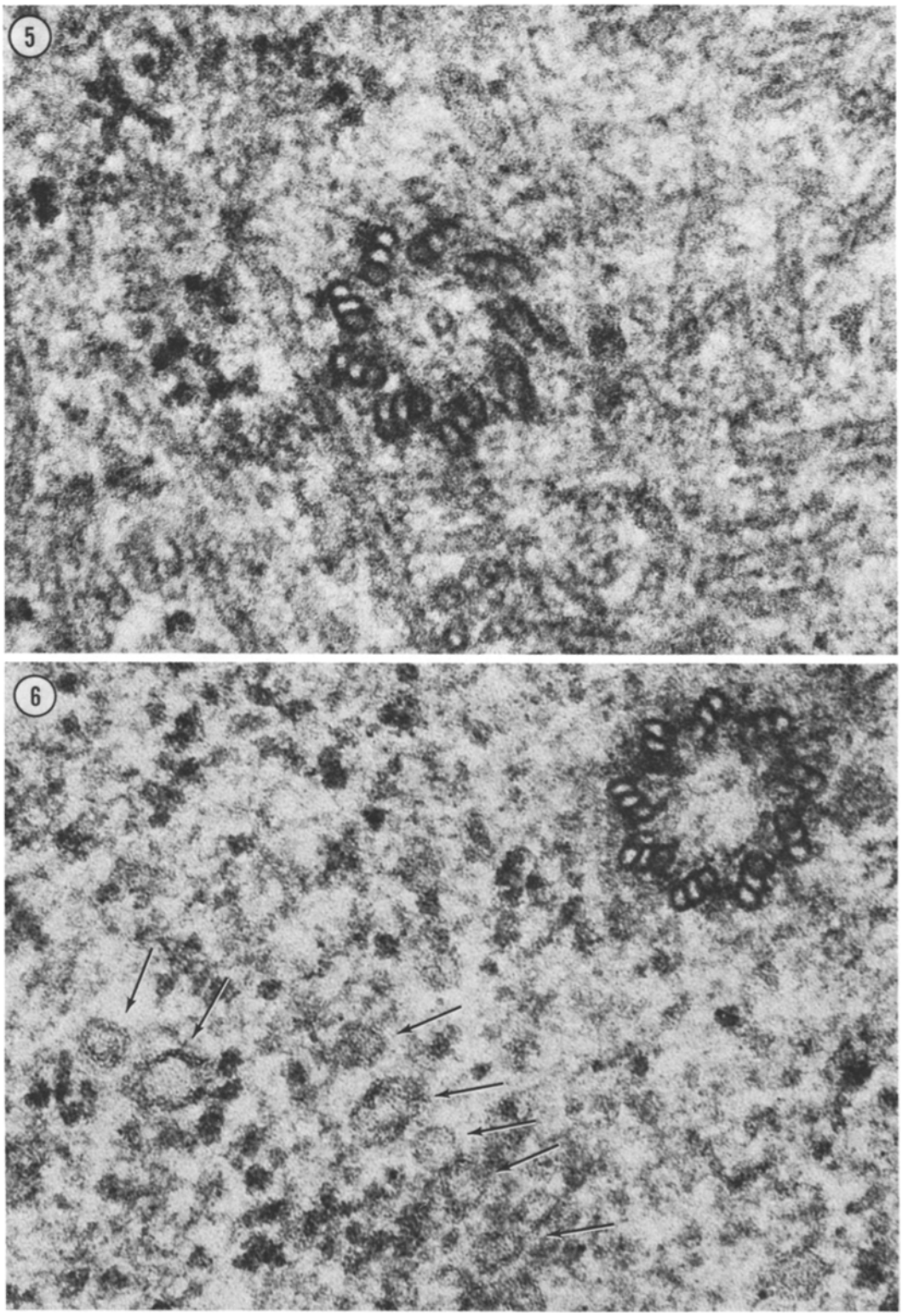


FIGURE 5 Section through the pole region of a metaphase cell at atmospheric pressure. Many spindle microtubules and the triplet microtubule structure of the centrioles are clearly visible. × 160,500.

FIGURE 6 Section through a pole region in a cell fixed after 10 min at 680 atm. Polar microtubules are absent, but the structure of the centriole and its microtubule triplets is apparently normal. Arrows indicate spiral structures discussed in the text. × 160,000.

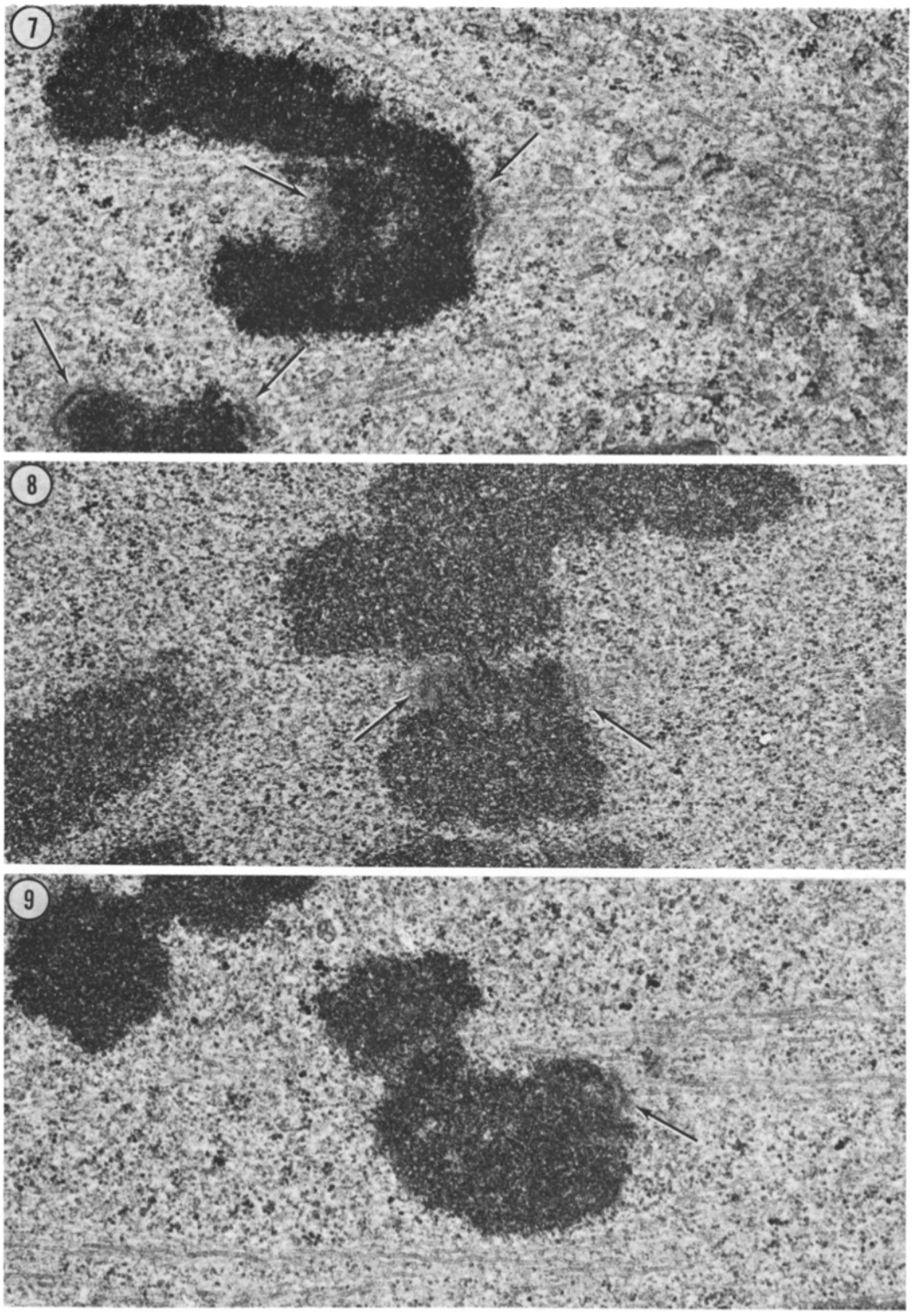


FIGURE 7 Longitudinal section through prometaphase cell. At atmospheric pressure there are numerous kinetochore microtubules and interpolar microtubules. The kinetochores (arrows) have a distinct, tripartite structure. × 32,000.

FIGURE 8 Longitudinal section through prometaphase cell. After 10 min at 680 atm, few kinetochore microtubules, and no interpolar or polar microtubules, are visible. The kinetochores (arrows) appear diffuse. × 32,000.

FIGURE 9 Longitudinal section through prometaphase cell. 10 min after pressure release, many microtubules of all types are apparent. One kinetochore (arrow) shows the normal tripartite structure. Mitochondria have been excluded from the spindle region. × 32,000.

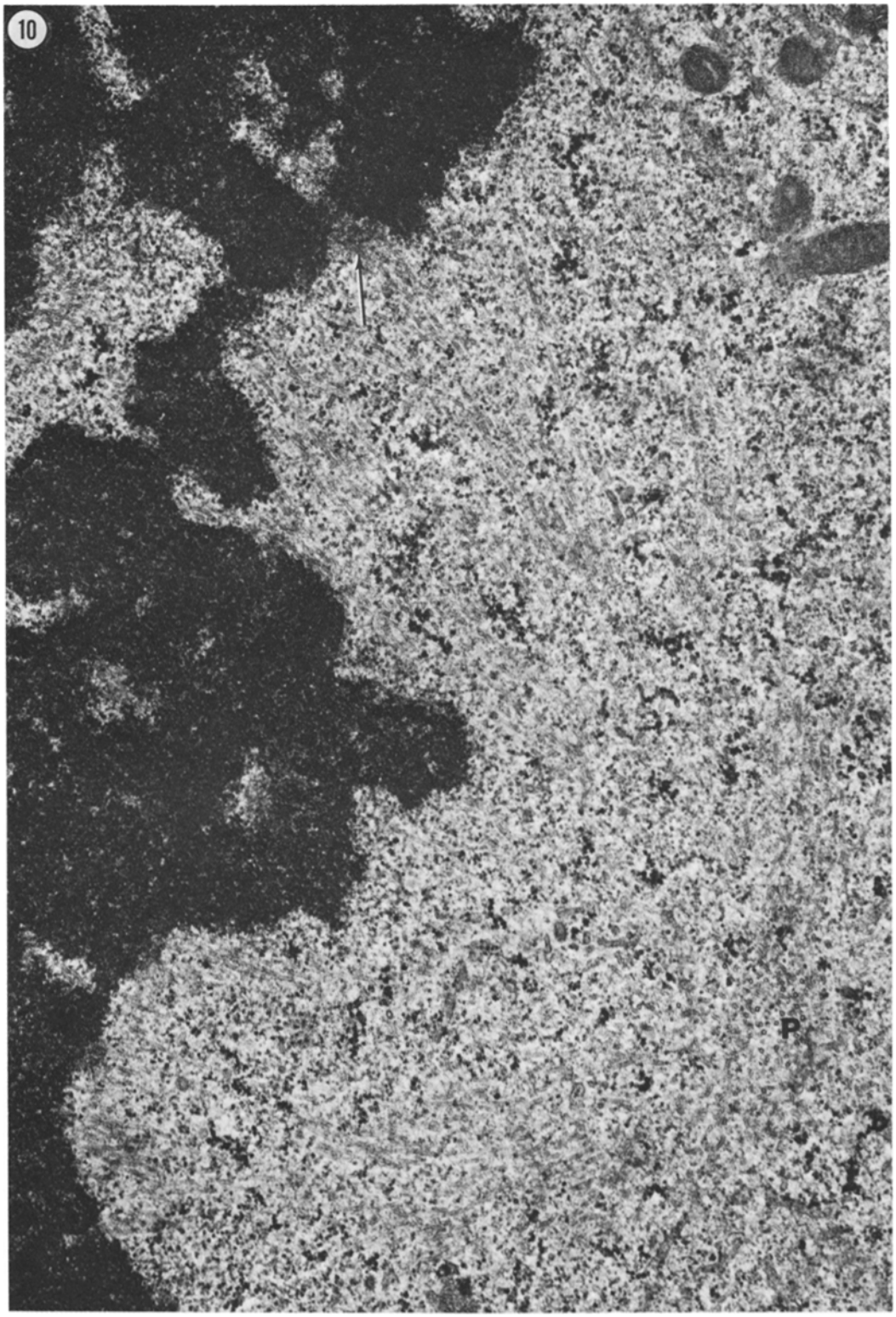
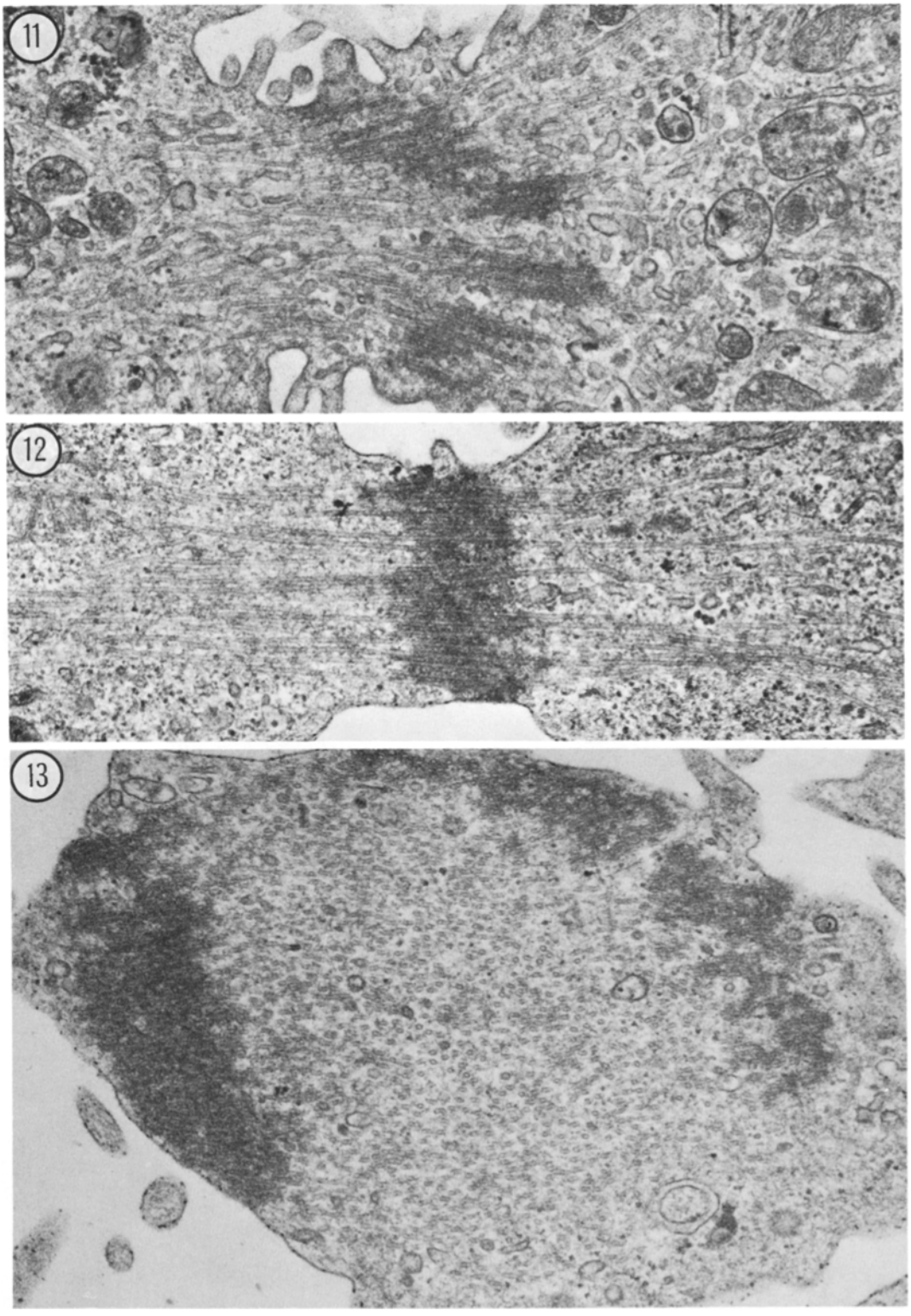


FIGURE 10 Metaphase cell fixed 10 min after release of 680 atm. Microtubules are abundant, often passing through the chromosomes. Most microtubules curve toward the pole (*P*) in a normal manner from the equatorial region and many astral microtubules are evident. Mitochondria are excluded from the spindle region. The tripartite structure of the kinetochores is still not so distinct as in control cells (arrow). The chromosomes still appear to be fused. × 30,000.



FIGURES 11, 12, and 13 Stem body and midbody microtubules in telophase cells all fixed under 680 atm. Fig. 11, The cleavage furrow has forced the stem bodies together. × 40,000. Fig. 12, Midbody formed from clustered stem bodies. Apparently normal microtubules extend at least 3 μm into both daughter cells from the dark band of electron-opaque matrix material. × 40,120. Fig. 13, Oblique section through another midbody shows the large number and high density of microtubules despite pressurization. × 40,050.

DISCUSSION

The results presented here demonstrate that MTs of the mitotic spindle are depolymerized by hydrostatic pressure and that the effects of pressure on the MTs are reversible, thus confirming conclusions of earlier studies in which changes in the assembly of the mitotic spindle of marine oocytes were monitored by measuring spindle BR (27, 28, 29). Pressure caused MTs to dissociate into tubulin aggregates which, with some possible exceptions, are too small to be resolved in thin sections by the electron microscope. Consequently, the decrease in BR of pressurized spindles does not result from fragmentation or disorientation of the MTs, but from their depolymerization. Similarly, Tilney et al. (36) found no identifiable tubule remnants after depolymerizing MTs of *Echinosphaerium* axopodia with pressures greater than 544 atm (8,000 psi).

Within the HeLa cell mitotic spindle there are distinct differences among MTs in their stability to pressure. These differences coincide with differences in stability to depolymerization produced by either lowered temperature (3, 26), colchicine (5), or a reduction in the exogenous tubulin concentration of partially isolated HeLa cell spindles (18). Although a few kinetochore MTs remain at 680 atm, there is no evidence of polar MTs nor of free MTs. (According to a categorization by McIntosh et al. [18], "kinetochore MTs" attach to a kinetochore and extend towards a pole, "polar MTs" begin in a pole and extend radially to form the aster and interpolar spindle, and "free MTs" are unattached at both ends but are part of the interpolar spindle.)

Stem bodies and the midbody, on the other hand, appear to be completely resistant to depolymerization by 680 atm. Cooling and colchicine also have little effect on the appearance of midbody MTs (3, 22). On the basis of this high stability, which indicates strong subunit bonding forces and little subunit turnover, MTs of the midbody fit better in the class of stable MTs that includes cilia, flagella, basal bodies, centrioles, and axostyles, all of which are resistant to disruption by cold, colchicine, or pressure, rather than into the class of labile MTs to which the spindle MTs belong (2, 6, 34, 35).

The origin of the spindle MT stability differences is difficult to ascertain. As proposed in a previous paper (27), the differential stability of spindle MTs may be directly related to the number of attached ends. Tubulin subunits may be capable of entering or leaving an MT all along its length (13), but it is assumed that subunits are more easily exchanged and have lower bonding strengths at "free ends," i.e. ends that are not attached on a kinetochore or in the pole region. According to this hypothesis, free MTs (two free ends) should be less stable than polar MTs (one free end) which, in turn, should be less stable than kinetochore MTs (many having no free ends, at least at metaphase before treatment). This is, in fact, the observed hierarchy of stabilities. The stability of stem body and midbody MTs, however, cannot easily be accounted for by the proposed hypothesis, since they are much more stable than the spindle MTs and little is known about the ends of these MTs, or about their origins.

Although a strong argument has been made for the formation of the midbody from the sliding or repositioning of pre-existing interzone MTs (18, 19, 20, 22), the high stability of midbody MTs compared to the spindle MTs causes us to question this conclusion. As pointed out by Brinkley and Cartwright (3), if the midbody MTs are the same MTs that make up the metaphase and anaphase interpolar spindle, then some event must occur that changes these MTs from being very sensitive to depolymerization during metaphase and early anaphase to being very resistant to depolymerization during telophase. On the other hand, it may be that the MTs of the stem bodies and midbody are newly assembled during late anaphase and early telophase such as occurs during the formation of phragmoplasts in plant cells (1, 15). These newly polymerized MTs could be composed of a type of tubulin different from that of the metaphase-anaphase spindle MTs. Alternatively, they could be assembled from the same tubulin, but be associated with other molecules that result in more stable microtubules. Ciliary MTs and purified neuronal MTs, which differ in their stability to depolymerization, also have specific peptide differences in the tubulin from which they are made (17, 32). Similarly, the A and B tubules of ciliary axonemes differ in stability characteristics as well as tubulin composition (32). Revealing information concerning the origin of the midbody should come from a biochemical comparison of midbody MTs and MTs of the spindle.

The fusion of chromosomes in pressurized cells has been noted previously (23, 24, 37). In *Tradescantia* pollen mother cells fixed under pressure during anaphase (24), there were noticeable

bridges between separating chromosomes at 200 atm (3,000 psi). The bridging became more restrictive and the chromosomes more rounded as pressure was increased until the chromosomes were fused into a single mass at 1,000 atm. These effects were not completely reversed by releasing pressure; fusion bridges between chromosomes remained, resulting in abnormal chromosome segregation. In the HeLa cells, 680 atm induces rounding and aggregation of the chromosomes, but with less severity, it seems, than that reported by Pease. Fused metaphase chromosomes persist for at least 10 min in recovered cells (Fig. 10). Unfortunately, we have no sections of recovered cells that entered anaphase after pressure release, so we do not know how long fusion bridges persist after pressure release. The cells seem to separate (or break) fused chromosomes, since most recovered cells eventually go through anaphase and cytokinesis which appear normal by phase-contrast microscopy.

Like the changes induced by pressure in the chromosomes, changes in the kinetochores appeared only partially reversed 10 min after pressure was released. We wonder if the apparent change in the structure of the kinetochore may be causally related to its ability to function as a MT organizing center (11, 13). Perhaps in *Chaetopterus* oocytes subjected to pressures higher than 300 atm (4,500 psi) the delay preceding BR recovery may be related to necessary reorganization of the kinetochores before kinetochore MTs can be repolymerized (27). A similar delay, however, is seen before spindle MTs begin reformation after complete depolymerization by low temperature treatment, and the delay can be explained in other ways (9). Certainly, the major effect of pressure on spindle MTs cannot be attributed to changes in the kinetochores, since low temperature and colchicine also induce reversible depolymerization of MTs but have no apparent effect on the structure of kinetochores (3, 4, 5).

In summary, we have confirmed at the ultrastructural level that increased hydrostatic pressure, like lowered temperature and colchicine, causes mitotic spindle MTs to depolymerize, and thus we have substantiated conclusions of earlier investigations performed with light-microscopy techniques on the effects of pressure. The rapid depolymerization of spindle MTs to subunits not readily identifiable in thin sections and their reassembly after pressure release supports the concept that spindle fiber MTs are tubulin polymers in equilibrium with a pool of subunits. Also, like lowered temperature and colchicine, pressure has a differential effect on the various types of MTs in the mitotic spindle. It depolymerizes free and polar MTs more rapidly and completely than it depolymerizes kinetochore MTs. In contrast, pressure (680 atm) has no effect on the MTs of the stem bodies or midbody. Pressure also induces prolonged changes in the structure of chromosomes and kinetochores, but the significance of these changes has yet to be determined.

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