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Taxol-induced anaphase reversal: Evidence that elongating microtubules can exert a pushing force in living cells

(mitotic spindle/microtubule lateral association/chromosome movement)

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ABSTRACT **The effects of taxol on mitosis in *Haemanthus* endosperm were studied. Immuno-gold staining was used to visualize microtubules; observations on microtubule arrangements were correlated with studies *in vivo*. Mitosis is slowed down, but not arrested, by taxol over a wide range of concentrations. Taxol promotes the formation of abundant new microtubules and lateral association within and between microtubule arrays (spindle fibers). This leads to a pronounced reorganization of the spindle, especially at the polar regions. Chromosome arms may be pushed toward the equator in metaphase. Anaphase chromosomes, with their kinetochores still pointing to the poles, move backward before resuming their poleward migration. During anaphase, the interzone is depleted of microtubules and trailing chromosome arms are stretched and often torn apart by rapidly elongating polar microtubules. Fragments are transported away from the poles, apparently "riding" on the tips of microtubules. This provides evidence of "pushing" by elongating microtubules. The desynchronization of anaphase, often observed as one of the first effects of taxol, indicates that the anchorage of different kinetochore fibers varies. The data draw attention to modifications of spindle structure due to increased microtubule lateral associations and to the role of this process in spindle integrity and chromosome movement.**

Taxol, a low molecular weight alkaloid from the eastern yew (*Taxus brevifolia*) promotes the assembly of microtubules *in vitro* by lowering the critical concentration of tubulin required for polymerization (1–3). Experiments with taxol carried out on living systems (4–8) are in general agreement with studies *in vitro*. In a variety of cells, taxol induces the formation of microtubules that are resistant to disassembly. Most studies have been done with animal cells and describe the long-term effects of this drug. It has been reported that taxol blocks mitosis either in the G_2 or the M phase. The data on immediate short-term effects on mitosis are either fragmentary (9) or do not include direct observations *in vivo* (10).

We have studied the effects of taxol on mitosis in living cells of a higher plant, *Haemanthus* endosperm, and correlated these experiments with immuno-gold staining (IGS) (11, 12). The IGS technique allows us to examine spindle microtubule organization at high resolution in the light microscope on a large number of cells under precisely the same experimental conditions. This overcomes the limited sampling inherent in studies *in vivo* or with an electron microscope. It also makes the variation between cells, which normally increases the difficulty of interpretation of small samples, an advantage in identifying the essential features of the effects studied because extensive comparison of average and extreme cases can be made.

The absence of centriolar asters in *Haemanthus* is a further advantage for this type of study. Centrioles (centrosomes) induce microtubule nucleation, a process that is enhanced by taxol. This introduces additional complexities in microtubule organization and therefore in the interpretation of the effects of taxol on an astral spindle.

We report here the initial effects of taxol on chromosome movement in living cells of *Haemanthus* endosperm in different stages of mitosis, particularly metaphase and anaphase. Over a wide range of concentrations, taxol does not block mitoses already in progress. The effects are practically immediate, and the large spindle of *Haemanthus* shows clearly the details of the microtubule rearrangements that occur. Extensive previous studies on *Haemanthus* (13) provide a broad background for interpretation. Taxol can reverse midanaphase and chromosomes move backward before resuming their polar migration. Chromosomes in later stages are often fragmented. We present evidence that this is the result of the rapid elongation of microtubules combined with their lateral interaction (association). This is a clear example of such properties of microtubules in plant cells and has profound implications for understanding the mitotic mechanism.

MATERIAL AND METHODS

Endosperm of *Haemanthus katerinae Bak* was used as material. The technique for studies *in vivo* has been described (14, 15).

Taxol, obtained from the National Cancer Institute, was dissolved at 10 mM in 100% dimethyl sulfoxide and stored at −20°C. This served as a stock solution. Working solutions, used no longer than 3 days, were stored at 4°C. In most experiments reported here, 0.1–10 μM solutions were used. The concentration of dimethyl sulfoxide in the medium was 0.1–0.01%. The course of mitosis in medium containing 0.5% dimethyl sulfoxide alone is indistinguishable from controls.

The IGS method has been described (12). The following primary rabbit antitubulin antibodies, gifts from J. De Mey, K. Fujiwara, and J. B. Olmsted were used: (*i*) antibody against dog brain tubulin (12), (*ii*) antibody induced against vinblastine paracrystals isolated from sea urchin eggs (16), and (*iii*) antibody against cilliary tubulin from *Tetrahymena* (17). The IGS technique did not allow us to follow the same cells *in vivo* and after IGS processing because cells were lost during the procedure. The number of cells remaining in our preparations varied between 100 and 900.

Taxol was either perfused during recording *in vivo*, or cells were observed after incubation in taxol solutions. About 50 cells were observed by differential interference contrast microscopy, and data were recorded with a Dage 67 Newvicon video camera and a Sony TVO 9000 video cassette recorder.

OBSERVATIONS

Controls. The IGS technique revealed various features of spindle microtubule organization in control *Haemanthus* cells

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Abbreviation: IGS, immuno-gold staining.

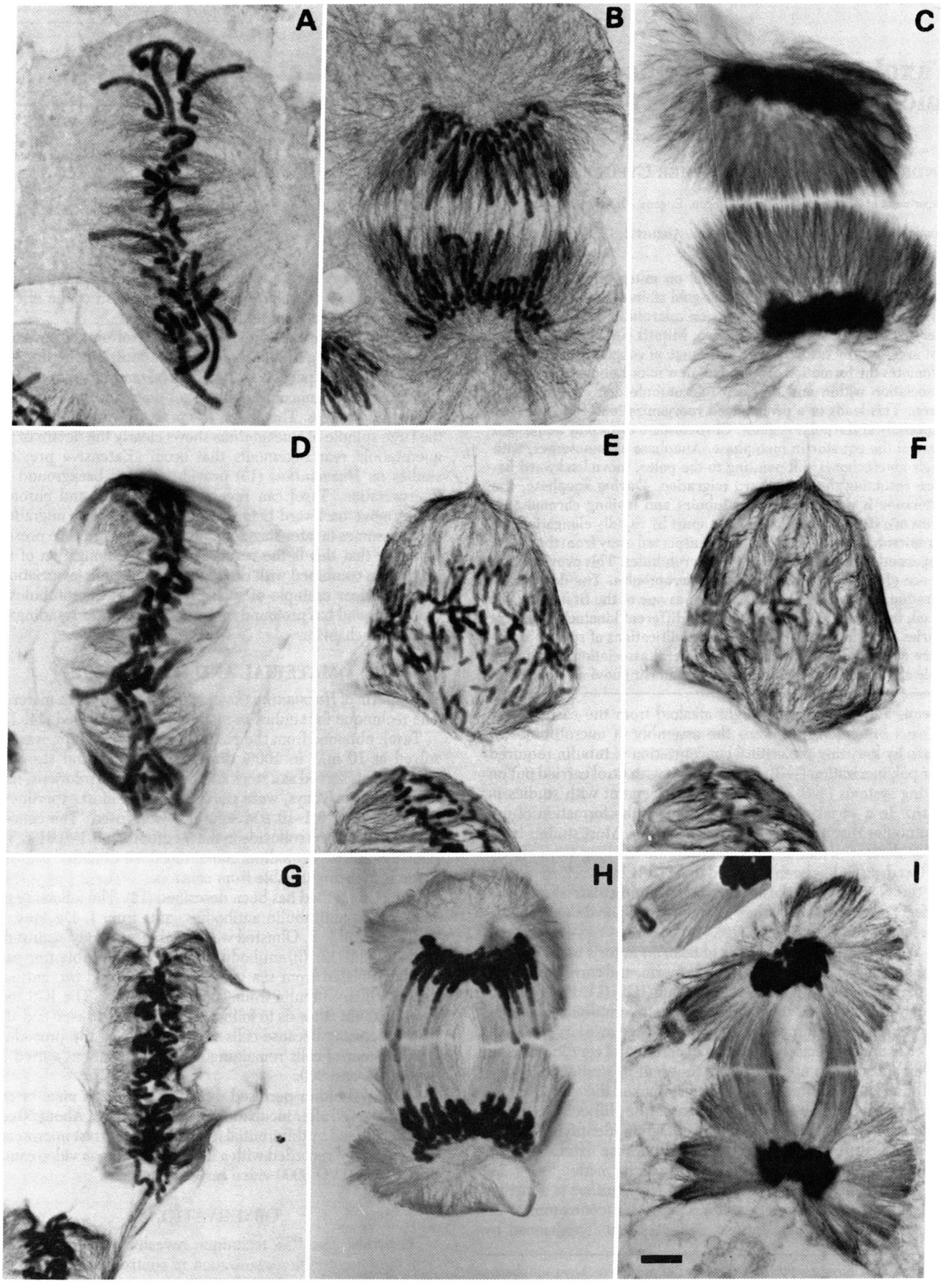

FIG. 1. *(Legend appears at the bottom of the next page.)*

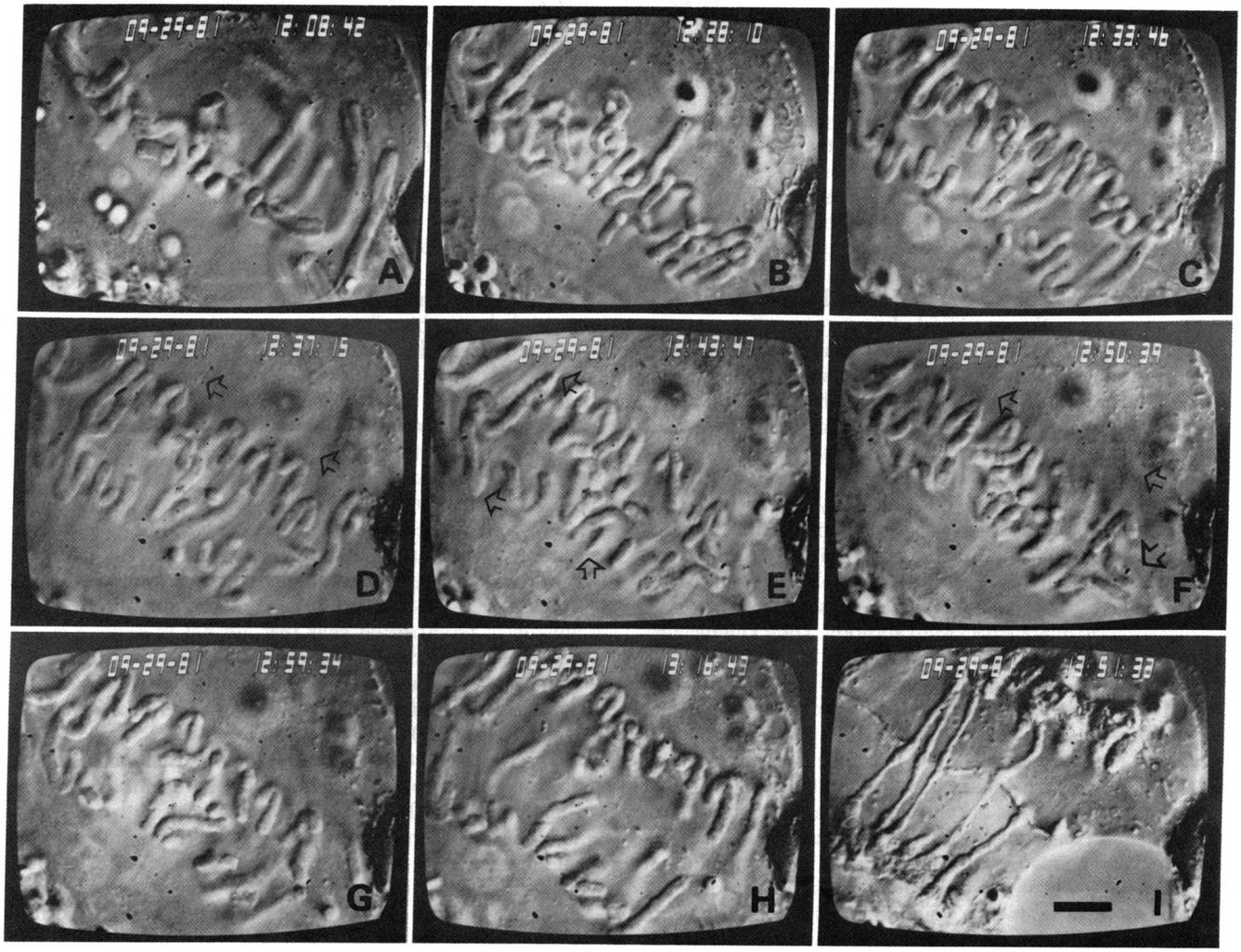

FIG. 2. An endosperm cell was perfused with 1 μM taxol at 12:34 and with medium without taxol at 12:52. The arrows in *E* mark stretched kinetochore regions. The arrows in *D* and *F* mark kinetochore fibers. The distal portion of some of the chromosome arms are parallel to the equatorial plane (thick arrow in *F*). Time (hr, min, and sec) is given on each print. The reversal of anaphase is clearly seen. Bar = 10 μm.

(12, 15). A major finding was the formation of abundant polar microtubules arranged in double "dome-like" structures, one around each half-spindle, in the polar regions during anaphase (Fig. 1 *B* and *C*). These two dome-like microtubule arrays form outside the spindle. They grow and fuse at the equator, forming a ring-shaped phragmoplast and cell plate that does not bisect the cell (12, 15). The formation of these structures explains the increase in microtubule number in anaphase (18) and shows the site of their assembly (19). It also provides evidence that the continuous fibers of the anaphase spindle contribute negligibly, if at all, to the formation of the phragmoplast (20).

Taxol Treatment. The effects of taxol on metaphase to late anaphase are detected with IGS in about 25% of the cells within 3 min and in more than 90% in 5–10 min. These primary changes of microtubule organization are followed by slower "secondary" effects that partially reverse the primary effects and lead to a more regular spindle structure (unpublished data). The data presented here concern mostly the primary effects. The large number of cells studied in different mechanical conditions (flattening) and mitotic stages allowed us to examine and evaluate the progressive phases of the action of taxol. Observations on living cells confirmed and extended the immunocytochemical data.

Late prometaphase/metaphase. The first detectable effects of taxol are pronounced changes of microtubule arrangement in the polar regions of the spindle. The microtubules appear more closely bundled together (laterally associated) than in controls (cf. Fig. 1 *D* and *G* with *A*) and there is an increase in the

FIG. 1 (*on preceding page*). Flattened control cells (*A–C*) and cells treated with 1–100 μM taxol of *Haemanthus* endosperm were processed with the IGS technique. (*A*) Metaphase. Distinct chromosomal fibers are seen, some of which are loosely associated. (*B*) Anaphase. The chromosomes have moved to the polar regions and the "bushy" appearance of the half-spindles is becoming clear. (*C*) Telophase. Two focal planes are shown, illustrating the arrangement of phragmoplast microtubules and the uneven splayed microtubules in the dome-like microtubule array. (*D*) Metaphase. Kinetochore fibers intermingle with each other, especially distally where they fuse together, tapering centrally. Such associations are so strong that groups of fibers split apart forming subpoles (5 μM taxol, 20 min). (*E* and *F*) Anaphase. Chromosome movement is desynchronized. In this unflattened cell, strong microtubule–microtubule associations in the polar regions result in sharply focused poles (10 μM taxol, 5 min); a view of the surface of the spindle with abundant curved microtubules is shown in *F*. (*G*) Midanaphase. A flattened cell shows the splitting of the half-spindles at the polar regions, suggesting strong microtubule–microtubule associations (10 μM taxol, 5 min). (*H*) Telophase. Unlike bridges seen occasionally in control cells, which are thinnest at the equator, these are thicker (5 μM taxol, 20 min). (*I*) Telophase. The polar microtubules are uniform in length and strongly associated at their distal ends (100 μM taxol, 35 min). Bar = 10 μm.

density of fiber staining (see *Discussion*). The microtubule arrays in the polar regions are tilted and bent together. The overall staining density of the spindle is occasionally so increased that identification of single chromosomal fibers is not possible.

The tilting of microtubules in the polar regions is usually accomplished after 10 min in 1 μM taxol. By that time, spindles have assumed an oval or circular shape. After longer times, the polar regions are flatter, with microtubules bent in irregular wavy arrays often arranged about 90° to the spindle axis. The changes result in a shorter flat barrel-shaped spindle, often with one or more protruding pointed poles (Fig. 1 *D* and *G*). Long chromosome arms, which in controls usually point poleward, are often parallel to the equatorial plane.

Midanaphase. This is the stage when long trailing chromosome arms still intermingle. The primary effects of taxol are basically the same as in metaphase and result in reorganization of the spindle pole. Another effect is a depletion of microtubules in the interzone, especially pronounced in late stages when the spindles are elongated.

In vivo, a characteristic feature of early/midanaphase is a partial reversal of anaphase occurring within 10 min after perfusion of taxol. Often, the first detectable sign is a "limpness" of trailing chromosome arms, which in controls are straight. This is clearly seen in both living and fixed cells. Often, the chromosomes start to migrate backward abruptly. Their kinetochore regions are often stretched (arrows in Fig. 2*E*) and still point toward the poles. Depending on the stage of anaphase, the distance between separate sister kinetochores decreases by a few to 10–15 μm—i.e., about one-fourth of the spindle length. The velocity of backward chromosome movements varies from 0.2 to 1.2 μm/min; i.e., the maximal velocity is identical to normal poleward migration rates in controls. This initial action of taxol may result in considerable desynchronization of anaphase in some cells and points to variations in the anchorage of different chromosomal fibers at the polar regions (Fig. 1 *E* and *F*).

Anaphase reversal *in vivo* can be stopped by perfusion with medium without taxol (Fig. 2). In cells continuously immersed in taxol (1 μM), movements backward stop after about 10 min and polar migration is resumed. The distance of maximum separation is less than in controls and the velocities of resumed poleward migration range from 0.1 to 0.3 μm/min—i.e., about 10–30% of those of controls. The movements are often speeded up in mid- to late anaphase, which corresponds to anaphase B—i.e., spindle elongation (21). Comparison with fixed cells indicates that often there are few microtubules in the interzone between two half-spindles, which appear in this stage as two independent units.

Late anaphase. The stretched trailing chromosome arms are clearly seen in late stages of anaphase when only short remnants of half-spindles are present. *In vivo* observations indicate that the distance between separated chromosome groups usually does not decrease and chromosomes seldom move backward. Trailing chromosome arms, which no longer interdigitate, are stretched in the direction of the equator, where their distal parts are often aligned parallel to and within the cell plate (Fig. 1*H*). Chromosome bridges and fragments are often seen. Some fragments are caught in the cell plate, but those located at the periphery of the developing phragmoplast are located away from the pole (Fig. 1*I*, in which no bridging is seen).

DISCUSSION

An advantage of the IGS method applied to endosperm is that we could compare cells observed *in vivo* with a large number of nearly identical cells fixed in both control and experimental conditions. This allowed us to correlate the microtubule organization of the spindle with chromosome behavior.

Taxol slows down but does not arrest mitosis in endosperm cells of the higher plant *Haemanthus*. Taxol does modify the microtubule organization of the spindle. Our results indicate that this is due to an abrupt increase in tubulin polymerization combined with increased lateral associations between microtubules. Both effects are well documented *in vitro* (2, 4, 22).

The present observations point to an abundant formation of new microtubules in taxol-treated cells, predominantly in the polar regions of the spindle (cf. Fig. 1 *C* and *I*). It should be stressed, however, that we interpret our results with caution since the IGS technique does not allow precise quantitation of microtubule numbers.*

In taxol-treated metaphase cells, we observed closer associations between microtubules (bundling) and an increase in microtubule fiber staining density. Using the IGS technique, we cannot tell whether the increase in density is due solely to the bundling of existing microtubules or to bundling and an increase in the total number of microtubules present. An increase of microtubule lateral association may obscure microtubule assembly. We also cannot distinguish between the elongation of existing microtubules and the nucleation of new microtubules. However, because there is an increase in staining density and we observe the formation of arrays of microtubules in the cytoplasm of taxol-treated cells that are not present in control cells, we believe that taxol does promote the formation of new microtubules in these cells *in vivo*. This conclusion is also supported by the *in vitro* data on taxol-induced microtubule polymerization (1, 2).

The experiments reported here draw attention to the importance of microtubule–microtubule lateral interactions for spindle integrity. It has been proposed (23, 24) that lateral associations between microtubules may be responsible for chromosome transport and that this property of microtubules may play a significant role in mitosis (25). Our data show a more extensive association of the portions of microtubules distal to the nucleus or chromosome group in taxol-treated cells as compared with controls. The variations in the anchorage of individual kinetochore fibers seen during the desynchronization of anaphase can be explained by differences in lateral association between neighboring microtubule arrays (spindle fibers). Pronounced reorganization of the spindle pole, which is the first detectable effect of taxol, can also be explained by the assumption that taxol promotes lateral associations between microtubules and microtubule arrays. This process is partly responsible for the differentiation of the spindle into regions of high and low microtubule density—i.e., the breaking or splitting apart of the spindle. The nature of the increased lateral association, seen here as denser microtubule arrays, is not known. Electron microscopy of taxol-treated cells (unpublished data) does not provide clear evidence of increased cross-bridging or a change in the minimum microtubule–microtubule distance.

Short backward movements of chromosomes during anaphase were observed during low temperature shocks in *Lilium* pollen mother cells (26) and in the fern *Hymenophyllum* (27) and were related to rapid microtubule disassembly in *Haemanthus* (28). The backward movements reported here, however, are three to five times longer and may involve a desynchronization of anaphase. During the latter process, some, but not all, trail-

* The IGS technique, like all immunochemical techniques, is sensitive to the density of antigen but the label may not be able to penetrate into areas of highly concentrated antigen. A discussion of this problem, which has not been evident in immunofluorescent methods, must be considered for precise quantitation of antigen and is beyond the scope of this paper.

ing chromosome arms are stretched away from the pole while their kinetochores are still "pulled" toward the pole. The observed stretching of kinetochore regions, occurring simultaneously with the stretching of the arms, demonstrates that the pulling does not cease during backward movements. There are several reasons why we believe that new nonkinetochore microtubules grow from the poles toward the equator during both metaphase and anaphase. (*i*) Observations *in vivo* show that, during metaphase, chromosome arms that extend perpendicular to the metaphase plate tilt toward and become parallel to the plate during taxol treatment. (*ii*) In late anaphase, even short chromosome arms are stretched toward the plate. (*iii*) Chromosome arms are often broken and fragments are transported away from the poles.

The movement backward is little affected by shortening of the spindle because wavy microtubules are found only on the surface of the spindle while kinetochore microtubules remain straight. These data show that the anchorage of different kinetochore fibers is not identical. Only those that are no longer "anchored" are pushed toward the equator. The classical centrifugation work of Shimamura (29) demonstrated the same point. The movement backward induced by taxol, however, is so great that it is possible that it results from a temporary reversal of the anaphase transport mechanism. Speculation concerning the molecular mechanism of anaphase is beyond the scope of the present report. Concerning microtubule force production, however, it must be noted that the backward movement of the chromosomes and the transport of fragments toward the equatorial plane occur during periods in which there is extensive growth of microtubules in the direction of these movements. These motions, therefore, supply evidence that such microtubule elongation can exert a pushing force in living cells, as has been suggested (30, 31).

The comparison of cells *in vivo* at different stages of anaphase with the IGS preparations indicates that the speeding up of chromosome movement in late anaphase in taxol-treated cells corresponds to a stage when the number of interzonal microtubules drastically decreases and the two half-spindles are not connected by microtubules. As the chromosomes are still moving and the distance between the poles increases, we consider this evidence that each half-spindle in *Haemanthus* is an autonomous motile unit like those in the astral mitosis of the newt (15, 32). Astral mitosis is complicated, however, by the unexplained autonomous motility of asters (15, 32, 33). The anastral spindle of *Haemanthus* endosperm deserves special attention as an object for further studies of mitosis due to its size, optical clarity, and lack of cellulose cell walls permitting extensive immunocytochemistry.

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