

# ULTRASTRUCTURAL ANALYSIS OF MITOTIC SPINDLE ELONGATION IN MAMMALIAN CELLS IN VITRO

## Direct Microtubule Counts

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### ABSTRACT

The mitotic spindle of many mammalian cells undergoes an abrupt elongation at anaphase. In both cultured rat kangaroo (strain PtK<sub>1</sub>) and Chinese hamster (strain Don-C) fibroblasts, the distance from pole to pole at metaphase doubles during anaphase and telophase. In order to determine the organization and distribution of spindle microtubules during the elongation process, cells were fixed and flat embedded in Epon 812. Selected cells were photographed with the phase-contrast microscope and then serially sectioned perpendicular to the major spindle axis. Microtubule profiles were counted in selected sections, and the number was plotted with respect to position along the spindle axis. Interpretation of the distribution profiles indicated that not all interpolar microtubules extended from pole to pole. It is estimated that 55–70% of the interpolar microtubules are overlapped at the cell equator while 30–45% extend across the equator into both half spindles. This arrangement appeared to persist from early anaphase (before elongation) until telophase after the elongation process. Although sliding or shearing of microtubules may occur in the spindle, such appears not to be the mechanism by which the spindle elongates in anaphase. Instead, our data support the hypothesis that spindle elongation occurs by growth of prepositioned microtubules which “push” the poles apart.

### INTRODUCTION

The process of mitosis in all cells is characterized by a distinct series of subcellular movements involving chromosomes, mitotic centers, and cytoplasmic organelles and inclusions. Numerous investigations have been directed toward elucidating the mechanisms of mitotic movements. The earlier literature on this subject has been reviewed in detail by Schrader (1953) and Mazia (1961); more recent studies have been reviewed by Forer (1969), Nicklas (1970), and Bajer (1971). Although much conflict and discord over the molecular mechanism of mitotic movement is still prevalent, some important, basic generalities concerning the struc-

tural basis of movement have emerged in recent years. The mitotic spindle about which movement takes place is composed of oriented molecules organized into fibers which can be demonstrated in living cells (Inoué, 1964) and in fixed material (Inoué and Sato, 1967). A basic component of the spindle fiber is the microtubule which measures 200–240 Å in diameter. Jensen and Bajer (1969) have recently combined Nomarski differential-interference optics and electron microscopy on the same cell in both the living and fixed states to demonstrate that the smallest spindle fiber resolu-

ble with the light microscope is composed of at least three microtubules.

In general, most mitotic spindles are composed of two classes of microtubules: those which extend from the chromosome kinetochore (centromere) to the poles (chromosomal microtubules) and those which extend between the poles along the major spindle axis (interpolar or continuous microtubules).

The tendency to equate microtubules with mitotic movement has been widespread since their initial discovery in dividing cells by Roth and Daniels (1962) and Harris (1962). It should be pointed out, however, that definitive experiments to directly establish their true role in chromosome movement and mitosis have been conspicuously absent. Questions which are immediately encountered are: (a) Do spindle microtubules actively participate in the mechanics of subcellular movement, especially during mitosis, or do they simply provide a cytoskeleton for maintenance of cell form during the distribution of genetic material to opposite poles? (b) If they are active in movement, what is the mechanism in the production and/or transmission of forces, i.e., do they interact by a "sliding filament" mechanism analogous to thick and thin filaments of striated muscle or do they merely push by elongation from an anchored position in the cytoplasm, or perhaps a combination of these events?

In order to elucidate the above questions, the present study was designed to determine the quantitative distribution and organization of microtubules during spindle elongation in mammalian cells in culture. In many cell types and especially mammalian tissue culture cells, the spindle undergoes an abrupt elongation at the onset of anaphase. In effect, pole-to-pole distance increases as the chromosomes move to opposite poles. The phenomenon of anaphase elongation, therefore, lends itself to the following experiments concerning the behavior and distribution of spindle microtubules. If we assume that the interpolar microtubules are repositioned at metaphase, the existence and degree of sliding or repositioning of microtubules should be evident by directly counting microtubule profiles in ultrathin serial transverse sections at points along the spindle of cells in various stages of the elongation process. A similar investigation of mammalian mitotic cells has been independently carried out by McIntosh and coworkers (1969, 1971). Some features of the experiment, namely

counting of microtubule profiles to establish organization, have also been independently applied to studies of mitosis and meiosis in a marine diatom, *Lithodesmium undulatum* by Manton and coworkers (1969a, b).

## MATERIALS AND METHODS

Cultured Chinese hamster fibroblasts (strain Don-C) and rat kangaroo fibroblasts (PtK<sub>1</sub>) were grown as monolayers in modified McCoy's 5 a medium supplemented with 20% fetal calf serum. For each experiment, approximately  $1 \times 10^6$  cells were seeded into plastic 30-ml Petri dishes (Falcon Plastics, Division of B-D Laboratories, Inc., Los Angeles, Calif.) and incubated overnight at 37°C to produce a uniform monolayer of cells.

The cells were fixed *in situ* in 3% glutaraldehyde in Millonig's phosphate buffer for 1 hr and postfixed in 1% osmium tetroxide buffered in a similar manner for 1 hr. The cells were flat embedded in Epon 812 according to the procedure of Brinkley, Murphy, and Richardson (1967).

After polymerization at 60°C for 48 hr, the Epon layer containing the cell monolayer was "snapped" free of the Petri dish. Cells in various stages of mitosis from metaphase to late telophase and postmitotic stages were selected and photographed through the Epon plastic with a 100× phase oil-immersion-objective lens. The cells were then marked by scoring the Epon with a sharp needle under the 10× phase-objective lens. The immersion oil was carefully removed and a drop of Epon mixture was placed over the selected cell and allowed to polymerize at 60°C overnight. Thus the cell to be sectioned was "sandwiched" into two Epon layers.

The area containing the selected cell was cut with a cork borer and positioned in a clamp chuck. The block face was trimmed in an LKB Ultratome III with glass knives. During the trimming process, it was necessary to examine the block face frequently with a phase-contrast microscope using the 10× objective lens to monitor the position of the cell. When trimming was completed, the cell to be sectioned was contained in the center of a block face which measured approximately  $0.5 \times 0.25$  mm. Serial sections of the entire cell were cut with a diamond knife and picked up on collodion-coated, slotted grids. A total of 50 grids, each containing 10–15 silver sections (approximately 600 Å thick), were collected for each cell. The grids were kept in proper sequence by storing them in LKB grid boxes.

The sections were stained in ethanolic uranyl acetate followed by lead citrate, and examined with a Hitachi HU-11C electron microscope operated at 75 kv. By comparing the electron image to the phase image of the same cell, it was possible to estimate the position along the spindle.

### Microtubule Counts

Microtubule counts were made from Fotorite prints with a final magnification of 30,000–50,000. The “counters” were trained to recognize microtubule profiles in both transverse and longitudinal sections, and they scored each profile by shading it with a black felt-tip pen. The counts were tabulated in groups of 10 microtubules.

Each micrograph was scored by two and sometimes three separate counters. The counting efficiency of the counters and the reproducibility of the counts in one set of experiments are shown in Table I. When these data were subjected to a two-way analysis of variance test, it was shown that the counts for individual micrographs as well as counts of various positions along the

spindle at different stages of mitosis were significantly reproducible at the 95% confidence level.

### RESULTS

#### *Kinetics of Spindle Elongation*

The transition of cultured rat kangaroo fibroblasts from metaphase to telophase requires approximately 10–15 min. As illustrated in Fig. 1, time-lapse cinematography records indicate that the daughter chromosome moves a distance of approximately 10–12  $\mu$  from the metaphase plate during anaphase. During the same period, the distance from pole to pole is increased from 10  $\mu$  at

TABLE I  
*Microtubule Counting Efficiency*

Mitotic stage	Spindle position	Counter			Total	Average	<i>F</i> ratio	
		I	II	III				
Early anaphase	Pole	418	280	404	1102	367.3	0.681	
		438	284	331	1053	351		
		405	487	370	1262	420.6		
		Total	1261	1051	1105	3417		1138.9
		Average	420.3	350.3	368.3	1139		379.6
		Half spindle	472	403	449	1324		441
			608	586	490	1684		561.3
			Total	1080	989	939		3008
		Average	540	494.5	469.5	1504		501.1
		Interzone	324	356	270	950		316
			487	333	445	1265		421.6
			407	680	742	1829		609.6
	Total	1218	1369	1457	4044	1347.2		
	Average	406	456.3	485.6	1348	449.1		
Late anaphase	Pole	280	261	265	806	268.6	4.016	
		330	219	271	820	273.3		
		Total	610	480	536	1626		541.9
		Average	305	240	268	813		271
Telophase	Midbody	775	787	734	2296	765	1.065	
		739	780	782	2301	767		
		894	842	735	2471	823.6		
		Total	2408	2409	2251	7068		2355.6
		Average	802.6	803	750.3	2356		785.2

This table illustrates the counting efficiency of three counters (I, II, and III) with respect to the same micrographs. The micrographs were selected to represent (a) different stages of mitosis and (b) different positions along the spindle. The values were subjected to a two-way analysis of variance test. The *F* ratios indicate that the degree of error between individual counters was insignificant at the 95% confidence level.

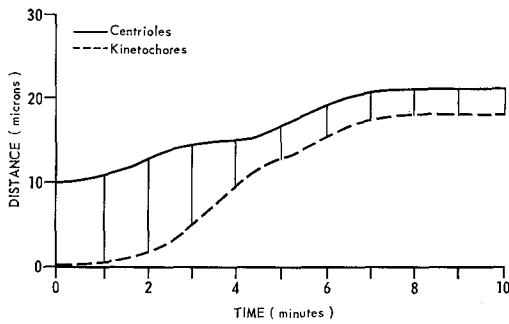


FIGURE 1 Kinetics of centriole migration (spindle elongation) and kinetochore movement from metaphase to telophase in rat kangaroo (PtK<sub>1</sub>) cells in vitro. The movement of only one daughter kinetochore is depicted on the graph.

metaphase to approximately 20  $\mu$  at telophase. Similar kinetics were noted in the shorter, smaller spindle of Chinese hamster fibroblasts. After the chromosomes moved to within approximately 3  $\mu$  of the poles, they maintained a constant separation from the poles and continued to move at approximately the same rate as the poles during the final stages of elongation. A second, slight burst of movement sometimes was seen which coincided cytologically with the onset of cytokinesis (Brinkley and Stubblefield, 1969). Figs. 2 *a-d* are phase-contrast photomicrographs taken of rat kangaroo cells embedded in Epon before sectioning. These depict the essential cytological stages of anaphase movement and spindle elongation. In this study, spindle elongation was divided into five stages: (*a*) *meta-*

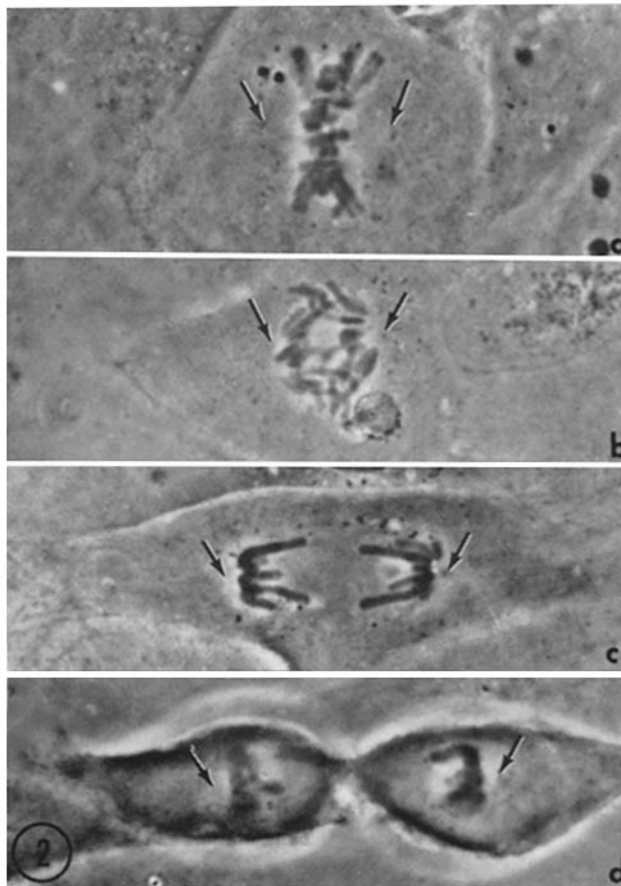


FIGURE 2 Phase-contrast photomicrographs of Epon-embedded PtK<sub>1</sub> cells showing representative stages of anaphase movement and spindle elongation (arrows point to centrioles). (*a*) metaphase, (*b*) anaphase, (*c*) telophase, and (*d*) telophase with midbody. These same cells were later sectioned and analyzed for microtubule counts.  $\times 1000$ .

phase (Fig. 2 a), (b) anaphase (Fig. 2 b), (c) telophase (Fig. 2 c), and (d) telophase midbody (Fig. 2 d). A fifth stage,  $G_1$  midbody (not shown), where the nuclei assume an interphase appearance but the midbody is still evident, was also studied.

### *Structure of the Spindle in Longitudinal Profile*

The ultrastructural aspects of mitosis have been studied in a variety of cells, and the events of metaphase, anaphase, telophase, and daughter cell formation have been specifically dealt with in studies of HeLa cells by Robbins and Jentzsch (1969), of rat L cells by Krishan and Buck (1965), of chick embryonic cells by Allenspach and Roth (1967), and of insect cells by Roth et al. (1966). Since the general ultrastructural features of the mitotic apparatus of the cell types in the present study showed little departure from those reported for other mammalian cells, a detailed discussion of mitotic fine structure would be redundant. For the sake of orientation, however, a brief reiteration of some of the essential features will be presented. For a detailed, light microscopic description of mitosis in cultured rat kangaroo cells, readers are referred to a recent paper by Heneen (1969). At metaphase, all components of the mitotic apparatus were fully formed (Fig. 3). A pair of parent and daughter centrioles along with electron-opaque pericentriolar materials accentuated each pole. Numerous microtubules converged to the poles, generally ending in the amorphous pericentriolar matrix surrounding the centriole. The kinetochores were fully differentiated and were clearly visible on the chromosomes. The detailed structure of the mammalian kinetochore has been described earlier (Brinkley and Stubblefield, 1967, 1969; Jokelainen, 1967). Chromosomal microtubules extended from the kinetochore to the poles, a distance of from 4.5 to 5.5  $\mu$  in rat kangaroo fibroblasts and from 2.5 to 3  $\mu$  in Chinese hamster cells. Evidence for inter-polar microtubules at metaphase was seen when serial sections cut parallel to the spindle were viewed. A few microtubules radiated out from the poles to form the asters. In Chinese hamster cells the aster was less well developed. The mean distance from pole to pole in the rat kangaroo metaphase spindle was 10  $\mu$ , while the Chinese hamster spindle measured 5  $\mu$ .

Observation of serial sections cut parallel to the spindle axis in anaphase and telophase revealed

several important features which may have some bearing on microtubular function in mitosis. At metaphase and early anaphase, microtubules can be seen extending from the electron-opaque axial element of the kinetochore directly to the poles in almost parallel orientation. In addition, microtubules assumed to be of the interpolar variety can be seen in close proximity to kinetochore microtubules (Fig. 4). In some cases these actually penetrate the chromosome (Brinkley and Stubblefield, 1969). Direct counts of microtubules arising from the axial elements of 10 kinetochores showed the number to range from 21 to 30, with a median value of 25 microtubules per kinetochore in rat kangaroo chromosomes. The kinetochores which were counted were chosen at random, and no effort was made to equate the number of microtubules with the size of the chromosome or its position on the spindle.

By late anaphase, when the chromosomes had completed their migration to the poles, the number of microtubules in the half spindle became greatly reduced and disoriented. Spindle microtubules in the interzone, however, were maintained in parallel array throughout anaphase and telophase. By telophase, conspicuous electron-opaque material surrounding microtubules was evident in the region of the cell equator. This material was clearly identifiable as the early stem bodies, and these bodies were identical to structures described by Buck and Tisdale (1962) and Robbins and Gonatas (1964). Careful examination of serial sections taken through the electron-opaque masses revealed 10–20 microtubules in each stem body, some of which appeared to overlap the others for a distance of up to 1.7  $\mu$  (Figs. 5 a–c).

During cytokinesis, the stem bodies were forced closer together as the cleavage furrow formed. Finally, the two daughter cells were held together by a cytoplasmic bridge or midbody which contains many stem bodies clustered together forming a singular large bundle of microtubules. The electron-opaque fibrous matrix around the microtubules formed an irregular band in the center of the midbody. Overlapping of microtubules in the vicinity of the electron-opaque band was again clearly indicated when serial sections of the midbody were examined (see also Paweletz, 1967).

### *Transverse Section Analysis*

**METAPHASE:** Several new features became apparent when metaphase cells were sectioned per-

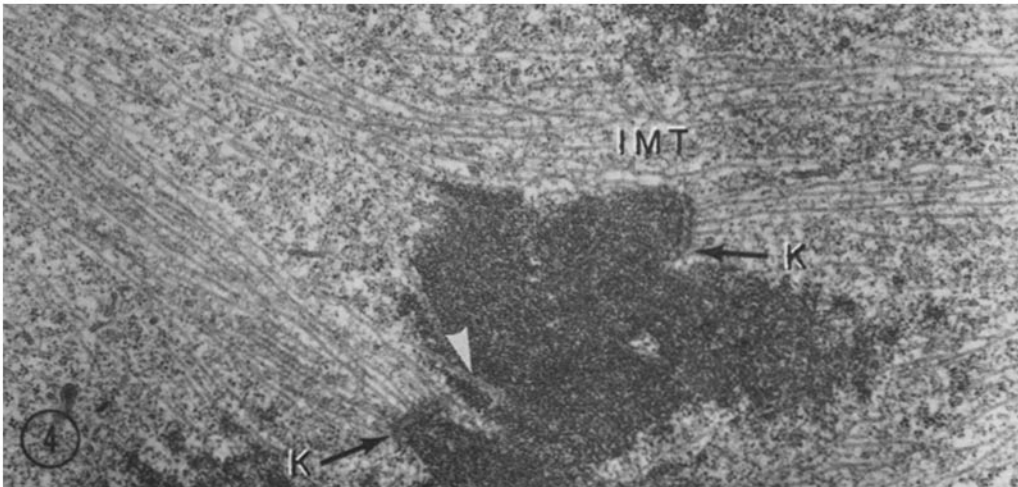
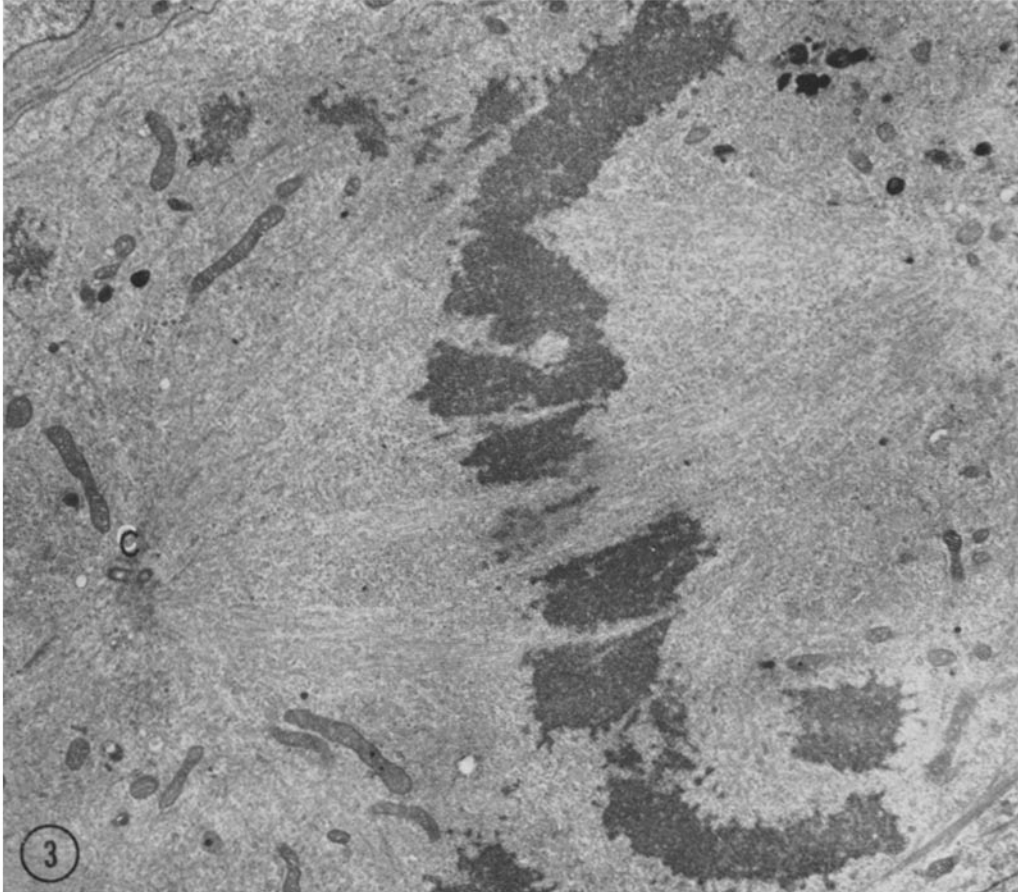


FIGURE 3 Typical longitudinal profile of metaphase spindle of PtK<sub>1</sub> cell showing the spindle tubules and centriole (C) positions.  $\times 8600$ .

FIGURE 4 Higher magnification of PtK<sub>1</sub> metaphase cell showing kinetochores (K), interzonal microtubules (IMT), and microtubules which penetrate the chromosome near the kinetochore (white arrow).  $\times 21,000$ .

pendicular to the spindle axis. Microtubules were frequently connected by fine cross bridges which measured approximately 50 Å in diameter and 250–600 Å in length (Figs. 6 *b–g*). In some sections, single microtubules exhibited arms which were not attached to other tubules (Fig. 6 *a*). In a few cases, the crossarms appeared to attach microtubules to membranes. The intermicrotubular connections were similar to those described in manchette microtubules of developing spermatids (McIntosh and Porter, 1967) and mitotic spindle tubules of algae (Wilson, 1969).

A second feature of spindle microtubules viewed in horizontal profile was their tendency to maintain a fairly constant spacing even when cross bridges were not evident. Measurements of center-to-center spacing of 100 pairs of microtubules in each stage of spindle elongation are shown in Fig. 7. For this analysis, we measured the distance from the center of a given microtubule to that of its "nearest neighbor." Although a rather wide range was evident in some extreme cases, the majority of the pairs had a center-to-center spacing of approximately 540 Å. The average distance between nearest neighbor microtubules appeared to decrease slightly during telophase. During this stage, microtubules were frequently grouped into hexagonal bundles with an average center-to-center spacing of 427 Å.

Aside from the apparent uniform spacing between adjacent pairs, microtubules of the metaphase spindle exhibited no unique organizational pattern. Moreover, no class distinction, in the form of interpolar or chromosomal microtubules, was evident from morphological appearances.

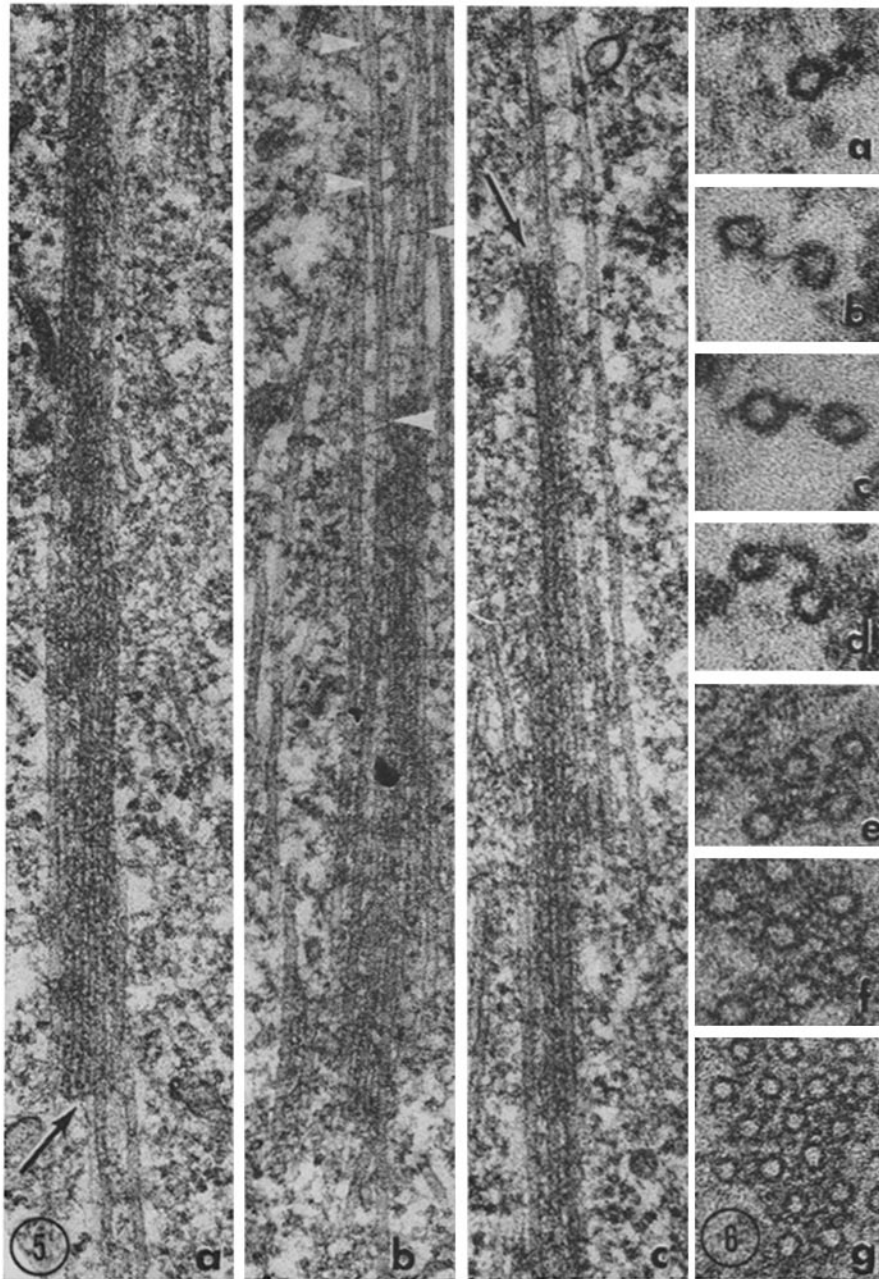
When counts were made from serial sections along the spindle and were plotted with respect to approximate position on the spindle axis, a characteristic microtubule distribution profile was evident as shown in Figs. 8 and 9. Although the actual number of microtubules differed in the two spindles, it should be noted that the distribution profiles of microtubules for both rat kangaroo fibroblasts and Chinese hamster cells appeared greatly similar. Moreover, as expected from birefringence measurements (Swann, 1951; Inoué, 1964), the distribution profile of microtubules in each half spindle was a mirror image of its opposite counterpart. The greatest number of microtubules was consistently observed in the half spindle immediately adjacent to the kinetochores. In rat kangaroo cells, the maximum number was approximately

1500, while in the Chinese hamster spindle it was about 500. The numbers decreased progressively in the vicinity of the poles.

A second characteristic feature of microtubule distribution in the metaphase spindle was the consistent decrease in number in the vicinity of the metaphase plate, resulting in a dip or valley between the "peaks" on each half spindle. An almost identical profile has been obtained from birefringence measurements of sea urchin eggs (Swann, 1951). The reduction in the number of microtubules in this region is apparently due to the existence of primarily interpolar microtubules extending across the metaphase plate between opposite sister kinetochores. Since metaphase chromosomes were never perfectly aligned such that sections would be taken exactly between all of the daughter chromosomes, some contamination from kinetochore microtubules was invariably experienced. Thus, a concise estimate of the number of interpolar microtubules in the metaphase spindle was not possible. Nevertheless, it can be safely estimated that 50–60% of the microtubules in the metaphase half spindle were of the interpolar variety. Since both chromosomal and interpolar microtubules were so closely interspersed at metaphase, the arrangement and organization of microtubules in each class was impossible to distinguish by transverse section analysis.

**ANAPHASE:** By examining cells in which daughter chromosomes had begun their movement to opposite poles, it was possible to analyze the distribution of microtubules in the interzone between the separating chromosomes. In so doing, we could essentially rule out chromosomal microtubules and focus on the distribution of interpolar microtubules in the vicinity of the cell equator. The distribution profiles for early anaphase are shown in Figs. 8 and 9. The pattern was consistent in both cell types. The maximum numbers of microtubules were consistently observed at the cell equator, the numbers being reduced by approximately one-third at a distance of 1  $\mu$  on either side of the equator. The maximum number of microtubule profiles in the interzone of rat kangaroo cells ranged from 250 to 500, while the number observed in a single Chinese hamster cell was approximately 200. It should be pointed out that spindle elongation had not been completed in these early anaphase cells. In fact, the spindle length had increased less than 2  $\mu$  in each case.

As in metaphase, the largest number of micro-



FIGURES 5 *a-c* Stembodies from late anaphase cell. Overlapping of microtubules is clearly indicated. Black and white arrows point to apparent free ends of microtubules. White arrows point to structures which may represent crossarms.  $\times 68,500$ .

FIGURES 6 *a-g* Transverse profiles of microtubules showing (*a*) single microtubule with a short arm and (*b-d*) crossarms between pairs of microtubules. In late anaphase and telophase, pairs of microtubules become grouped into hexagonal bundles (*e-g*). (*a-f*)  $\times 245,000$ ; (*g*)  $\times 143,000$ .



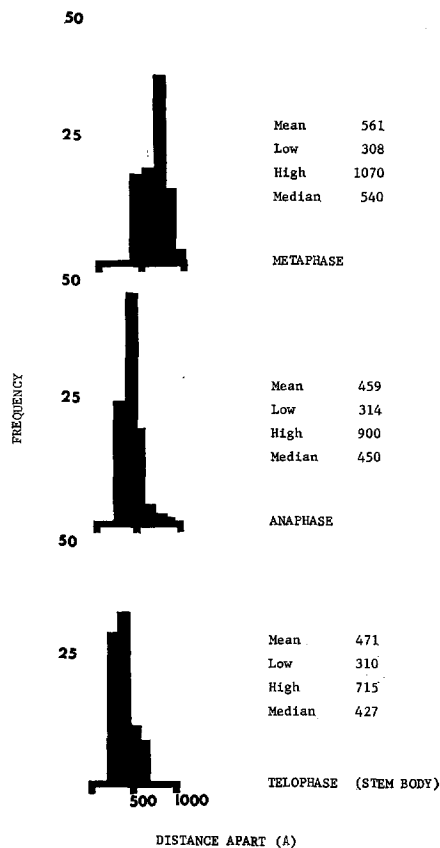


FIGURE 7 Histogram showing the center-to-center spacing between adjacent microtubules in the spindle. Cross bridges such as shown in Figs. 5 and 6 may help maintain the near-uniform spacing.

tubules was observed in the region immediately adjacent to the kinetochores in the half spindles. Even in early anaphase, however, the maximum number in the half spindle was only 50–60% of the number in metaphase, indicating a loss of chromosomal microtubules. The apparent reduction in the number of microtubules in the half spindle at anaphase occurred before an increase in microtubule number in the interzone as discussed below.

The interzonal microtubule distribution profile remained essentially the same throughout anaphase and telophase. In every case, the maximum number of microtubule profiles was found at the cell equator and decreased by approximately one-third on each side of the equator. In both rat kangaroo and Chinese hamster cells, the total number of microtubules in the interzone region appeared to increase at late anaphase and throughout

telophase. At telophase, when spindle elongation was completed and chromosomes were beginning to coalesce into a daughter nucleus at each pole, the number of interzonal microtubules increased significantly in both cell types and was maintained throughout telophase until the midbody was initially formed.

In mammalian cells, the behavior of the midbody (Flemming body) has been well documented (Byers and Abramson, 1968). Fig. 10 *d* shows a Chinese hamster cell with a forming midbody. Microtubule profiles at three positions along the midbody are shown in Figs. 10 *a-c*. After cleavage, the two daughter cells remain attached by the midbody. In subsequent stages, the daughter cells enlarge and their nuclei undergo a morphological transition to a more interphase appearance. During this period, which may require 2 hr or more, the midbody continues to remain associated with the two cells but undergoes an abrupt elongation. The distribution of microtubules in the G<sub>1</sub> midbody of rat kangaroo cells after elongation is shown in Fig. 8. Although the number of microtubules was greatly reduced, their density was greatest in the exact center of the midbody. Complete serial sections in two different cells showed, however, that the number of microtubules was reduced to 50% of this value at a distance of less than 1  $\mu$  to the right and left of center. It was only in this late midbody stage that a near 2:1 distribution ratio was observed, indicating complete interdigitation of microtubules from each half of the midbody as proposed by Paweletz (1967).

## DISCUSSION

Quantitation of microtubules in transverse sections taken at various positions along the spindle should elucidate several important features about the mitotic apparatus. First, a consistent distribution profile with a small intercellular variance would support the observations from living cells that a high degree of order is maintained in the fibers of the mitotic spindle. Consistency in the actual numbers of microtubule profiles at precise positions in the spindle would attest to the completeness of fixation as well as confirm the presence of precise numbers of assembly sites for microtubules in the mitotic cell. The over-all shape of the profile distribution would predict the lengths of various microtubules with respect to the major spindle axis. Finally, a predictable shift in microtubule

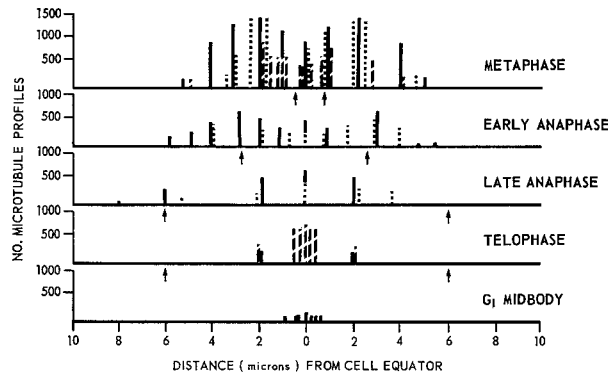


FIGURE 8 Microtubule counts for rat kangaroo (PtK<sub>1</sub>) cells presented as histograms with respect to position along the spindle at metaphase, early anaphase, late anaphase, telophase, and G<sub>1</sub> midbody. The arrows indicate approximate positions of the kinetochores at each stage as indicated. Each vertical bar pattern represents counts from a different cell.

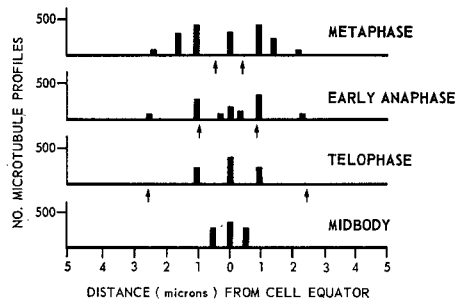


FIGURE 9 Distribution profile of four Chinese hamster (Don-C) cells analyzed at metaphase, early anaphase, telophase, and telophase midbody. Although the total number of microtubules is fewer than in PtK<sub>1</sub> cells, the distribution profiles for both types of cells are almost identical.

distribution during the metaphase-to-telophase transition would support the argument for repositioning or sliding of microtubules during spindle elongation. Although the absence of a shift in profile distribution during spindle elongation would not necessarily rule out sliding or "shearing" as a mechanism for spindle movement, it would give greater credence to the hypothesis that the interpolar microtubules were repositioned into a rigid framework which accompanied elongation by the assembly of subunits at some point along the tubules.

As shown in Fig. 8, considerable variation in actual number of microtubules was noted between cells cultured and fixed at different times. Analysis of cells cultured and fixed at different times re-

vealed a variation of 40–50%. A variation of less than 10% was noted between cells of a single culture which were, of course, fixed at the same time. Although our sample is much too small to apply statistical analysis, it is likely that culturing conditions and/or fixation affected microtubule number. Indeed, Inoué and Sato (1967) have shown that birefringence levels observed in living cells are not preserved after fixation unless special additives are included in the fixation vehicle.

Although actual numbers of microtubules varied considerably from cell to cell, the distribution profiles were amazingly uniform. Thus, the loss of microtubules due to culture condition and/or fixation appeared to be uniform throughout the spindle.

In rat kangaroo cells the maximum number of microtubule profiles counted in the metaphase spindle was approximately 1500. Since this count was taken in the half spindle immediately adjacent to the kinetochores, we can assume that it represents both chromosomal and interpolar microtubules. The consistent slope in the profile near the poles can be taken to indicate that not all of the spindle microtubules extend to the poles. The characteristic dip in the number of microtubules near the cell equator at metaphase indicates that some microtubules end at the chromosome (chromosomal microtubules) while others extend past the chromosomes into both half spindles (interpolar microtubules). The actual number of microtubules in these sections is, however, an unreliable index to the true number and organization of interpolar microtubules, since lack of precise

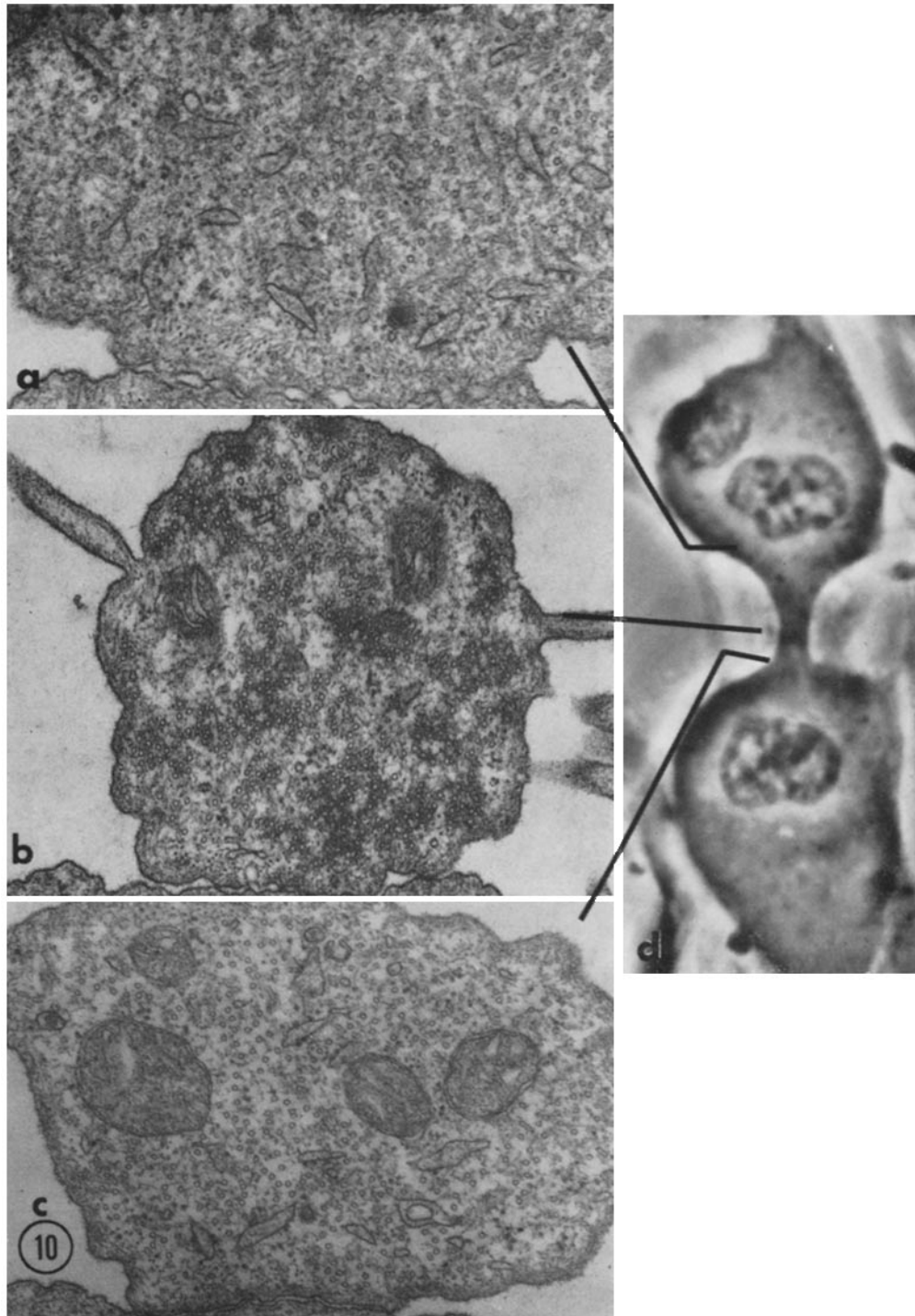


FIGURE 10 Transverse sections at three positions along the midbody of a Chinese hamster (Don-C) cell. The black lines indicate the approximate position where the sections were cut along the midbody. (a) Few microtubules are evident in this section taken well into the body of one daughter cell. (b) Electron-opaque matrix surrounding the microtubules depicts the stembodies also seen as a dense band in the phase-contrast photomicrograph (Fig. 10 d). (c) Numerous microtubules are evident but no dense material is seen in this section cut near one end of the midbody. (a-c)  $\times 37,000$ . (d)  $\times 2160$ .

alignment of chromosomes on the metaphase plate leads to "contamination" of the cell plate with chromosomal microtubules.

The distribution of inter-polar microtubules was more accurately determined when we looked at the interzone between separating chromosomes at early anaphase. The maximum number counted in the half spindle of rat kangaroo cells was 750 microtubules. Since the spindle had hardly begun to elongate, we can assume that counts in the half spindle (between kinetochore and poles) represent both chromosomal and inter-polar microtubules as in metaphase. In the interzone the maximum number of microtubules was 550. Here we can rule out contamination from chromosomal microtubules. The numbers were not consistent throughout the interzone. Instead, the number of microtubules was reduced by approximately 30-40% on each side of the equator. Thus, in the cell with 550 microtubules at the equator, only 350-400 microtubules were observed at a distance of 1  $\mu$  on each side of the equator (Fig. 8). A similar distribution was observed in Chinese hamster cells at approximately the same stage (Fig. 9). Moreover, the ratio of microtubules at the equator to those on each side of the equator remained fairly constant throughout spindle elongation in both cell types studied.

The most plausible interpretation of these consistent observations is that some inter-polar microtubules overlap at the equator while others extend from pole to pole. From our studies of both mammalian cell types, we would predict that 55-70% of the inter-polar microtubules were overlapped at the equator while 30-45% extended from one half-spindle into the other. This interpretation can be supported by a general mathematic expression. First, we define the region at the cell equator as  $a$ , the region between the equator and the separating daughter kinetochores as  $b$ , and the region between the kinetochores and the poles as  $c$ . Then, let  $x$  be the number of inter-polar microtubules in one half-spindle which overlap at the equator,  $y$  be the number of microtubules which extend from pole to pole, and  $z$  be the number of chromosomal microtubules in one half-spindle. Using the values given for the rat kangaroo (PtK<sub>1</sub> cell discussed above, we can solve for  $x$ ,  $y$ , and  $z$  as follows:

$$\begin{aligned} a &= 2x + y = 550 \text{ microtubules} \\ b &= x + y = 400 \text{ microtubules} \\ c &= x + y + z = 750 \text{ microtubules.} \end{aligned}$$

Thus,  $x = 150$ ,  $y = 250$ , and  $z = 350$ . From these values, the per cent of the inter-polar microtubules which overlap at the cell equator can be calculated as follows:

$$\frac{2x}{2x + y} \times 100 = \frac{300}{550} \times 100 = 55\%.$$

Microtubule counts obtained in transverse sections are in close agreement with counts made in longitudinal sections. For example, in serial longitudinal sections through kinetochores, we observed an average of 25 microtubules per kinetochore (see Results). If we divide the value for  $z$  in the above equation by the average number of chromosomes in this cell line ( $2N = 11$ ), a value of 32 microtubules per kinetochore is obtained. This value is in close agreement with the actual counts reported in Results.

While it is essential to understand the organization of microtubules in the spindle of higher organisms before we can predict mechanisms of spindle movement, mammalian cells are difficult in this respect for several reasons. First, the fact that both chromosomal and continuous (inter-polar) microtubules form at approximately the same time during prometaphase makes it essentially impossible to observe each class independently. Secondly, the large number of microtubules in the spindle along with the close apposition of both chromosomal and inter-polar microtubules adds to the confusion.

Manton and coworkers (1969 *a, b*) have been able to overcome the above difficulties by selecting an organism which is far more suitable for transverse microtubule analysis. In the marine diatom, *Lithodesmium*, the formation of inter-polar microtubules is completely independent of that of chromosomal microtubules. By a similar analysis of spindle cross-sections, these investigators have shown that most of the inter-polar microtubules overlap at the cell equator. It was established that no more than 10% of the microtubules passed from one pole to the other.

Little is known about the formation of inter-polar microtubules in cells of higher organisms. They first appear during the migration of centrioles or mitotic centers to opposite poles before or at the time of nuclear envelope disruption (see Mazia, 1961). Well-known microtubule inhibitors such as Colcemid (Brinkley et al., 1967) and chloral hydrate (Molè-Bajer, 1967, 1969) have been shown

to differentially inhibit interpolar microtubules and thus prevent the formation of a bipolar spindle.

The microtubules which comprise the interpolar apparatus of mammalian cells are thought to be generated, in part, by the centrioles (Stubblefield and Brinkley, 1967) or by the associated pericentriolar material (Murray, et al., 1965). Thus, microtubules generated at one centriole pair would be of opposite polarity with respect to those formed by the sister pair (Stubblefield and Brinkley, 1967; McIntosh et al., 1969). Centriole migration at the onset of spindle formation may be brought about by an interaction of microtubules of opposite polarity (McIntosh et al., 1969; McIntosh and Cleland, 1969). McIntosh and coworkers have proposed a sliding filament model to account for all forms of microtubule-associated spindle movement. According to their theory, microtubules with opposite polarity are unstable and tend to slide past each other by the action of forces generated at the active mechanochemical cross bridges. According to this attractive scheme, centriole migration, chromosome movement, and spindle elongation can all be explained on the basis of sliding or shearing between antiparallel microtubules. Metaphase, by this mechanism, is a least energy state in which all opposing forces are equal and a stable equilibrium between chromosomal and interpolar microtubules is achieved. Anaphase movement is characterized by a shift in equilibrium resulting in the sliding of chromosomal microtubules past interpolar microtubules with opposite polarity. Thus, daughter chromosomes are driven to the poles while interpolar microtubules are pushed to the cell equator during spindle elongation. The attributes and shortcomings of their model will not be discussed since they have been recently reviewed by Nicklas (1970).

One important feature of the hypothesis of McIntosh et al. (1969) is testable at the ultrastructural level. As mentioned earlier, sliding or repositioning of interpolar microtubules can be detected by a predictable shift in microtubule profiles. Thus, before spindle elongation, the microtubules would be completely overlapped from pole to pole, and the resulting ratio of microtubules at the cell equator to those on each side of the spindle would be 1:1. After elongation, a shift in microtubule profile distribution to a ratio of 2:1 (or even a 3:2 ratio) would be compelling evidence for sliding of all or part, in the

case of the 3:2 ratio, of the microtubules in the interpolar apparatus.

As is evident from the data in Figs. 8 and 9, a 2:1 ratio was not detected during spindle elongation in rat kangaroo or Chinese hamster cells. Moreover, analysis of microtubules in the interzone of early anaphase reveals that some microtubules appeared to be overlapped before spindle elongation while others extended from pole to pole. Instead of a 2:1 ratio at the end of elongation, a near 3:2 ratio is observed at early anaphase before spindle elongation and is maintained throughout anaphase and telophase.

These observations certainly do not negate the hypothesis of sliding or shearing of microtubules as a mechanism of movement, especially chromosome movement. Indeed, some form of shearing between chromosomal and interpolar microtubules can best explain observations on living mitotic cells (see Nicklas, 1970). A sliding filament mechanism is also a likely possibility in ciliary and flagellar motion (Satir, 1968) as well as during nuclear elongation in avian spermiogenesis (McIntosh and Porter, 1967). These data do, however, offer little support for the hypothesis that sliding or repositioning of interpolar microtubules accounts for total spindle elongation either by complete sliding of all interpolar microtubules as diagrammed in Fig. 11 or by partial sliding of only a few of the microtubules (Fig. 12). Both models require an initial 1:1 distribution of microtubules along the interpolar apparatus before elongation and a shift to a 1:2 (complete sliding) or 3:2 (incomplete sliding) ratio at the end of elongation.

The most direct interpretation of our data on the basis of spindle elongation is diagrammed in Fig. 13. Two types of microtubules are formed in the interpolar apparatus: those which arise from the poles and overlap at the cell equator and those which extend from pole to pole. Such an arrangement would give the 3:2 distribution ratio observed before elongation begins. Thus, at metaphase, a rigid interpolar apparatus is formed and is, perhaps, partially stabilized by the crossarms between adjacent microtubules. As elongation begins in anaphase, the interpolar microtubules increase in length by the addition of subunits at their polar ends. This interpretation supports the hypothesis of Ris (1949) that spindle elongation is brought about by a lengthening of interpolar fibers which push the poles apart (see also Ris,

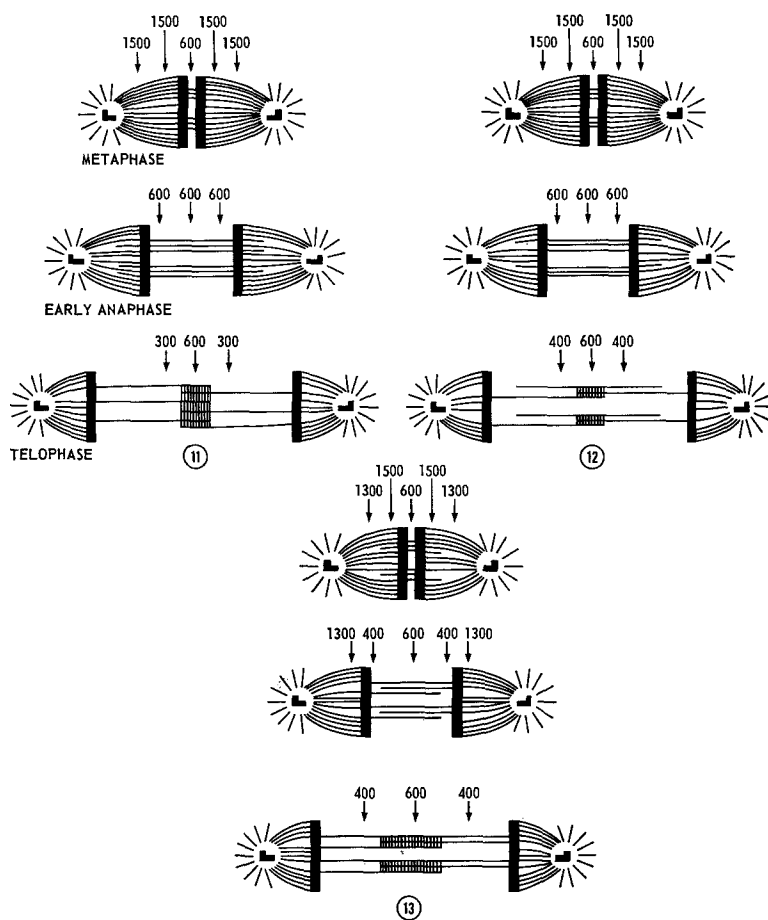


FIGURE 11 Complete sliding-tubule model. If each line represents 100 microtubules, the number of microtubules indicated would represent a fairly close approximation of the spindle of rat kangaroo (PtK<sub>1</sub>) cells. According to this model, the interzone in early anaphase would consist of an equal distribution of 600 microtubules between separating daughter chromosomes. At the end of elongation, a 1:2 ratio would be expected.

FIGURE 12 Partial sliding-tubule model. This model predicts that two-thirds of the microtubules would slide while one-third would remain stable. At the end of elongation, a 3:2 ratio would be obtained. Note, however, that this model also requires an equal distribution of microtubules at all points between separating daughter chromosomes in early anaphase.

FIGURE 13 Nonsliding model for spindle elongation. Interpolar microtubules are overlapped at the cell equator before spindle elongation. As the chromosomes separate at early anaphase, a 3:2 ratio is observed across the interzone. This can be achieved if two-thirds of the interpolar microtubules overlap while one-third of them extend throughout the spindle axis. Elongation, by this model, occurs by the growth of the existing, interpolar microtubules at their polar ends pushing the poles apart. The data presented in Figs. 8 and 9 can best be explained by this model. For simplicity, no effort has been made to illustrate the increase in microtubules in the interzone in late anaphase and telophase.

1955; Mazia, 1961; and Nicklas, 1970). This process is accompanied by a concomitant shortening of chromosomal microtubules as the chromosomes move to the poles. Thus, subunits derived

from the breakdown of chromosomal microtubules may be added to the subunit pool needed for the assembly of the interpolar microtubules during spindle elongation. In this manner, the dynamic

equilibrium of Inoué (1964) between random subunits and polymerized microtubules is maintained in the interpolar microtubules. The formation of a new microtubular system from a pre-existing system in the same cytoplasm has been observed in other organisms. Rosenbaum and Carlson (1969) have shown that in regenerating flagella of *Tetrahymena*, microtubules of the new flagella grow at the expense of polymerized microtubules in the uninjured flagellum. Thus, a direct transfer of microtubule proteins from a polymerized system to the site where newly assembled microtubules are proceeding has been demonstrated by these investigators. It is likely that a similar mechanism accounts for the shortening of chromosomal microtubules and the elongation of interpolar microtubules.

The question of the initial origin of interpolar microtubules at the beginning of mitosis is an important one. It is reported by many investigators that spindle microtubules rarely make contact with the centrioles in animal cells (de Harven, 1968; Robbins et al., 1968). Stubblefield and Brinkley have repeatedly described the direct contact of microtubules with the triplet microtubules of the centriole wall. This is especially evident during the first few moments of recovery after Colcemid inhibition (Brinkley et al., 1967; Stubblefield and Brinkley, 1967; Brinkley and Stubblefield, 1969). It is likely, therefore, that microtubules are generated from both centriole triplets as well as the adjacent pericentriolar matrix (satellites). It would be of considerable interest to determine the position of these two types of microtubules in the interpolar apparatus. Thus, one form may overlap at the equator while the other may extend from pole to pole at metaphase. The apparent increase in interzonal microtubules at anaphase and telophase is also interesting. While this may be due to counting error or intercellular variation in microtubule number, it could also imply that the spindle midzone is a third source of microtubules as in the phragmoplast of plant cells. Additional studies will be necessary in order to elucidate this observation.

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