Absence of Microtubule Sliding and an Analysis of Spindle Formation and Elongation in Isolated Mitotic Spindles from the Yeast *Saccharomyces cerevisiae*

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ABSTRACT Mitotic spindles were isolated from a cell division cycle mutant of the budding yeast *Saccharomyces cerevisiae* by the lysis of sphaeroplasts on an air:buffer interface and were negatively stained with 1% gold thioglucose. Isolated spindles were incubated under conditions which promoted the sliding disintegration of parallel preparations of *Tetrahymena* axonemes, namely the addition of ATP to 20 μM. In no experiment was a corresponding change in microtubule organization of the spindle observed, even when spindles were first pretreated with either 1–10 μg/ml trypsin or 0.2–2% Triton X-100.

During these experiments a number of spindles were isolated from cells that had passed through the imposed temperature block, and from the images obtained a detailed model of spindle formation and elongation has been constructed. Two sets of microtubules, one from each spindle pole body (SPB), completely interdigitate to form a continuous bundle, and a series of discontinuous microtubules are then nucleated by each SPB. As the spindle elongates, the number of microtubules continuous between the two SPBs decreases until, at a length of 4 μm, only one remains. The spindle, composed of only one microtubule, continues to elongate until it reaches the maximal nuclear dimension of 8 μm. The data obtained from negatively stained preparations have been verified in thin sections of wild-type cells. We suggest that, as in the later stages of mitosis only one microtubule is involved in the separation of the spindle poles, the microtubular spindle in *S. cerevisiae* is not a force-generating system but rather acts as a regulatory mechanism controlling the rate of separation.

The accurate segregation of duplicate sets of chromosomes during anaphase involves two mechanistically distinct events (31). The movement of the chromosomes to the spindle poles, anaphase A, is associated with the shortening of chromosomal fibres (12), while anaphase B, the separation of the spindle poles, is mediated through the elongation of the central spindle. Several of the models put forward to account for the latter envisage a sliding interaction between the microtubules from opposite poles linked by some form of mechanochemical coupling (19, 23, 28). Perhaps the most convincing evidence in favor of such a mechanism comes from electron microscope observations of spindle elongation in diatoms, where the extent of anaphase B separation is directly related to the degree of overlap of the two half-spindles (28). Recently, functional evidence has been obtained from lyzed models of PtK_1 cells that suggest that the motor for this sliding reaction might be the Mg^{2+}-ATPase dynein (5) and that the mechanism of anaphase B is analogous to that responsible for ciliary and flagellar beating (35, 36), despite the fact that the intrinsic polarity of the microtubules in the two systems is different. In a cilium, the outer doublets are polymerised in a unidirectional manner from a single organizing center, and sliding occurs between parallel microtubules (34); while, in the central spindle, microtubules are nucleated from two poles, and, therefore, if sliding occurs it must be between antiparallel microtubules (20).

One way to test the hypothesis that force generation during both anaphase B and ciliary beating are based on similar molecular mechanisms would be to develop an in vitro assay for microtubule sliding in mitotic spindles comparable to that already established for cilia and flagella (37). There are, however, at least two major problems in applying axoneme technology to spindles. First, mitotic spindles are notoriously labile, such that they are difficult to isolate in a normal physiological state (22). Second, they are often extremely complex, being composed of up to several thousand microtubules. Thus, even if controlled changes in microtubule organization were realised, detailed analysis of spindles at the electron microscopic level would present considerable difficulties.

One way of overcoming both obstacles would be to use as an experimental system mitotic spindles that are composed of relatively few microtubules and which, in addition, possess some inherent natural stability. We recently described the isolation of intact spindles with both of these properties from a cell division cycle (*cdc*) mutant of the budding yeast *Saccharomyces cerevisiae* (10, 11, 16).[1] We now report our attempts to induce microtubule sliding in these preparations using conditions which support the sliding disintegration of parallel preparations of *Tetrahymena* axonemes. In the course of these studies we isolated spindles from other stages in mitosis and have used these to construct a detailed model of the sequence of events comprising mitosis in this yeast. Preliminary accounts of this work have been published previously (10, 11).

MATERIALS AND METHODS

Spindles were isolated from the temperature-sensitive cell division cycle (*cdc*) mutant of *S. cerevisiae, ts*327, which is defective in the gene *cdc* 6.1 (7) and derived from the haploid wild type A364A. Cells were arrested in medial nuclear division by incubation at the restrictive temperature of 36.5°C for 5 h. Although in yeast it is difficult to apply conventional terminology, this stage in mitosis is thought to be roughly equivalent to metaphase (27). Cell walls were removed by treatment with 0.1 M dithiothreitol, 0.02 M EDTA, 0.2 M Tris(hydroxymethyl)methyl-ammonium chloride, pH 8.1, followed by incubation in 4% β-glucuronidase (Type H-2; Sigma Chemical Co., St. Louis, MO) for 45 min at 37°C. The resulting sphaeroplasts were lyzed on an air:buffer interface, and the isolated spindles were collected by touching ionized Formvar-coated 200-mesh grids to the buffer surface (8).[1]

Ciliary axonemes were prepared from *Tetrahymena thermophila* BIII by the method of Mitchell and Warner (24). Cells were grown in 2% proteose peptone to a density of 10^5–10^6 cells/ml, harvested by centrifugation, and deciliated by the addition of dibucaine HCl (Sigma Chemical Co.) to a final concentration of 1.3 mM. After centrifugation to remove the cell bodies, cilia were pelleted and then demembranated with 0.2% Triton X-100 in 5 mM $MgSO_4$, 0.5 mM EDTA, 100 mM KCl, 10 mM HEPES, pH 7.4 (HMEK). Axonemes were washed in 5 mM $MgSO_4$, 10 mM HEPES, pH 7.4 (HM) and pelleted at 19,000 *g* for 15 min before resuspension in HM buffer, using gentle homogenisation to disperse the clumps.

In attempts to induce a sliding reaction, spindles and axonemes were incubated in 20 μM ATP either directly or after pretreatment with either 1–10 μg/ml trypsin (Sigma Chemical Co.; 12,000 benzoyl arginine ethyl ester BAEE U/mg protein) or 0.2–2% Triton X-100. In some experiments spindles were isolated directly into a solution containing ATP before collection on grids, and in others spindles and axonemes were mixed on the same grid before the addition of ATP. All solutions were made in HM buffer. Spindle preparations were negatively stained with 1% gold thioglucose, as were the spindle/axoneme mixtures. Axonemes alone were routinely stained with 2% uranyl acetate.

Samples were prepared for thin-section electron microscopy by a modification of the method of Byers and Goetsch (3).[1] Gold or silver sections were cut with a diamond knife on an LKB 2128 UMIV Ultratome ultramicrotome (LKB Instruments, Inc., Rockville, MD) and stained with uranyl acetate and lead citrate (30). All specimens were examined in a Siemens Elmiskop EM102 electron microscope, fitted with a goniometer stage, and calibrated by means of a replica grating.

RESULTS

The structure of mitotic spindles isolated intact from *S. cerevisiae ts*327, after temperature arrest at 36.5°C, has been described in detail elsewhere (10, 11, 16) and only a brief description will be presented here.[1] Spindles were composed of two quadrilaminar spindle pole bodies (SPBs), 160 nm in diameter, separated by a bundle of five to ten continuous microtubules between 1.5 and 2 μm in length. In addition, the intranuclear surface of each SPB was associated with up to 17 discontinuous microtubules while one to three cytoplasmic microtubules emanated from the extranuclear face. These spindles were stable and even survived sphaeroplast lysis in distilled water.

To examine whether an active sliding reaction could be induced in such preparations, spindles were immobilized on Formvar-coated electron microscope grids and incubated in 20 μM ATP in HM buffer, conditions which supported a sliding disintegration of parallel preparations of ciliary axonemes from *T. thermophila* BIII. Sliding in cilia was monitored by change in turbidity of the axoneme suspension (ΔA_{350}, Fig. 1 *a* and *b*) or by negative-stain electron microscopy (Fig. 1 *c*–*e*). Axoneme preparations placed directly into 20 μM ATP showed a decline in absorbance of 23% (Fig. 1 *a*). Those pretreated with 1 μg/ml trypsin for 1 min revealed an initial decrease in absorbance of 20% followed by a further 36% drop upon addition of the nucleotide (Fig. 1 *b*). When we examined them by electron microscopy, we found ~100% of the axonemes treated by both methods showed some stages of dissociation into their component doublet microtubules.

When isolated mitotic spindles (Fig. 2 *a*) were incubated in the presence of 20 μM ATP, no rearrangement of spindle microtubules was observed in the 59 examples examined. As the control cilia had been demembranated with Triton X-100, which may have removed some detergent-soluble component, spindles were also pretreated with 0.2–2% Triton X-100 (35 examples), or 1 μg/ml trypsin (Fig. 2 *b*, 28 examples) before the addition of ATP (Fig. 2 *c*, 13 and 52 examples, respectively). No discernible effect on spindle organization was realized in any preparation after these treatments. Spindles were also incubated in other more complex reactivation media, i.e., that used for *Chlamydomonas* axonemes (2, 9), but again no change in spindle structure was observed.

To demonstrate that the yeast lysate contained no inhibitors of ATP-induced microtubule sliding, mixed preparations of spindles and axonemes were prepared on the same electron microscope grids before treatment as described above. Under these conditions axonemes retained their capacity to undergo a sliding reaction, indicating that no inhibitors of dynein-mediated microtubule-microtubule interaction were present in the isolated spindle preparations.

One possible explanation for the lack of sliding in our experiments is that the spindles used here were not competent to undergo elongation, i.e., some functional component of the sliding machinery is incorporated into the spindle after the point in the mitotic sequence when the *cdc* 6.1 mutation causes the cells to arrest. To eliminate this possibility, sphaeroplasts were returned to the permissive temperature (25°C) for various periods before lysis and spindle isolation as described above. After 2-h, spindles had elongated to a mean length of 4.13 ± 1.5 μm compared to 2.02 ± 0.77 μm for those isolated directly from temperature-arrested cells, thereby ensuring that spindles were in the process of elongation at the point of isolation. The addition of ATP to such preparations with or without treatment with either trypsin or Triton X-100 again produced no detectable change in spindle organization.

Although the *cdc* mutation results in homogeneous populations of spindles, occasionally spindles were isolated from cells that either had not reached or had passed through the imposed temperature block. Images of spindles at all stages of mitosis were thus accumulated (Figs. 3 and 4). Stages in spindle formation are shown in Fig. 3. The spindle initially comprised two sets of microtubules, one from each SPB, that completely

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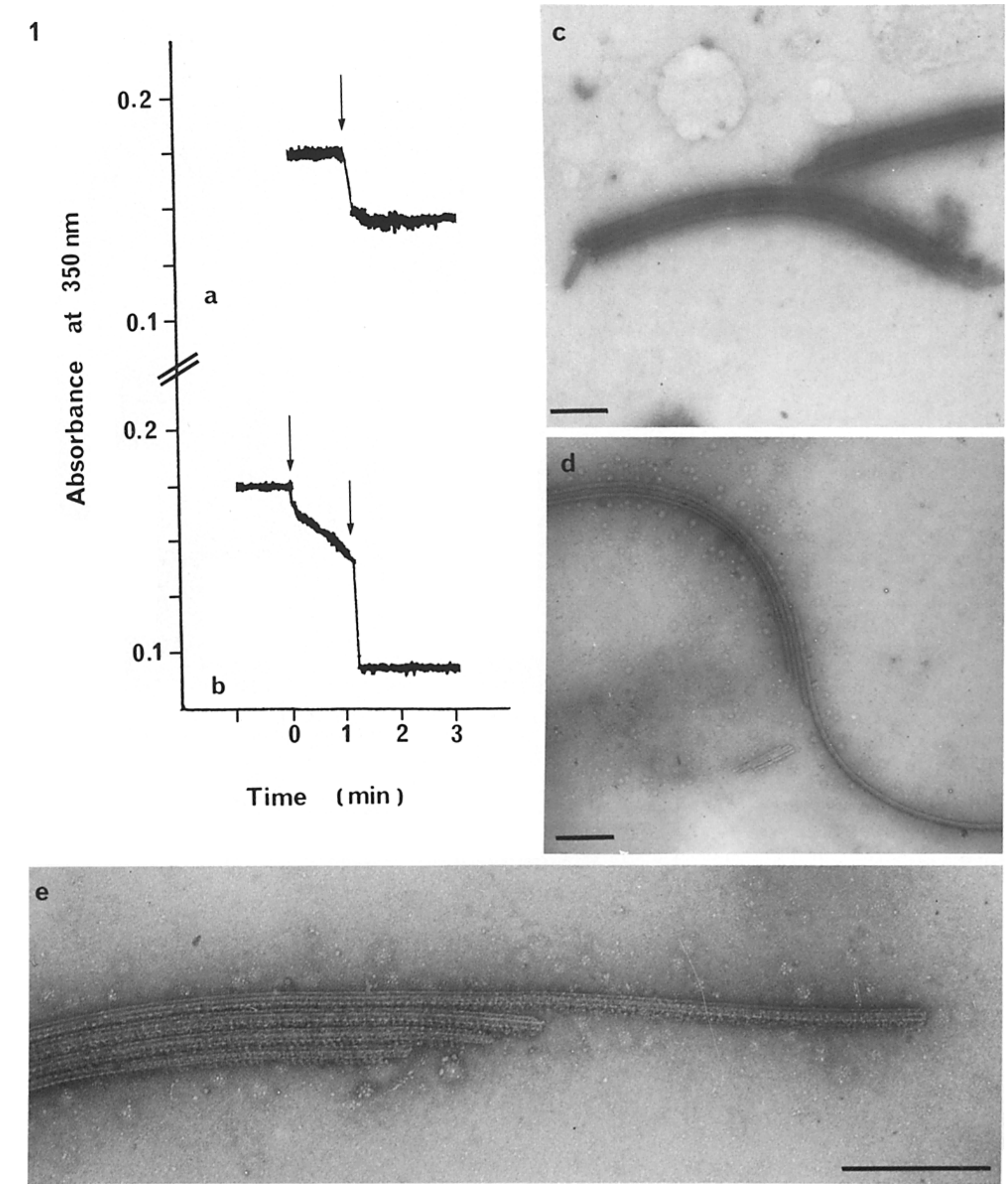


FIGURE 1 The sliding reaction of *Tetrahymena* axonemes: (*a* and *b*) Absorbance trace at 350 nm. (*a*) After 1 min, ATP was added to 20 μM and produced a decline in optical density of 23%. (*b*) Axonemes were pretreated for 1 min with 1 μg/ml trypsin before the addition of ATP, causing a 36% increase in absorbance change. (*c–e*) Electron micrographs showing the ATP-dependent sliding disintegration. (*c*) Control: no ATP treatment; (*d*) treated: 20 μM ATP for 1 min; (*e*) higher magnification of an axoneme after ATP treatment. The dynein arms crossbridge adjacent doublet microtubules. Bars: (*c* and *d*) 1 μm; (*e*) 0.5 μm. (*c* and *d*) × 10,000. (*e*) × 50,000.

interdigitated (Fig. 3*a*) to form a bundle of continuous microtubules (Fig. 3*b*). The discontinuous microtubules were then polymerized (Fig. 3*c*) to a maximal length of 0.76 μm (Fig. 3*d*). The subsequent stages in mitosis involve the shortening of the discontinuous microtubules and the separation of the spindle poles (Fig. 4). The identification of spindles with lengths ranging from 0.8 to 8.6 μm allowed us to accurately reconstruct the entire sequence of elongation. Analysis of these preparations revealed a decline in the number of continuous microtubules with increasing spindle length (Fig. 4*a* and *b*)

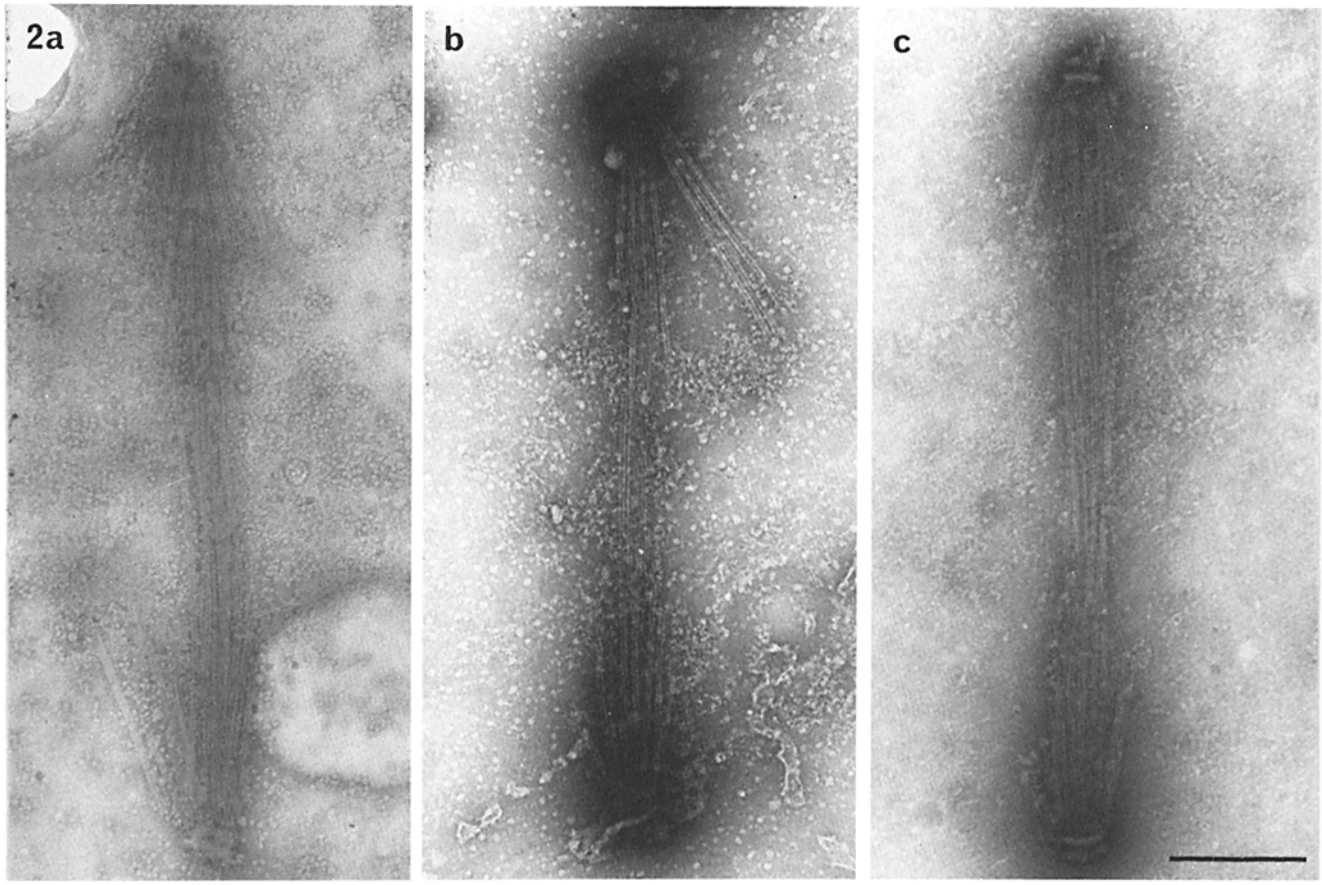


FIGURE 2 Attempts to induce microtubule sliding in isolated mitotic spindles of *S. cerevisiae:* (*a*) control; (*b*) 1 μg/ml trypsin; (*c*) 20 μm ATP for 1 min after pretreatment with trypsin. None of these treatments has any detectable effect on spindle organization. Bar, 0.5 μm. × 35,000.

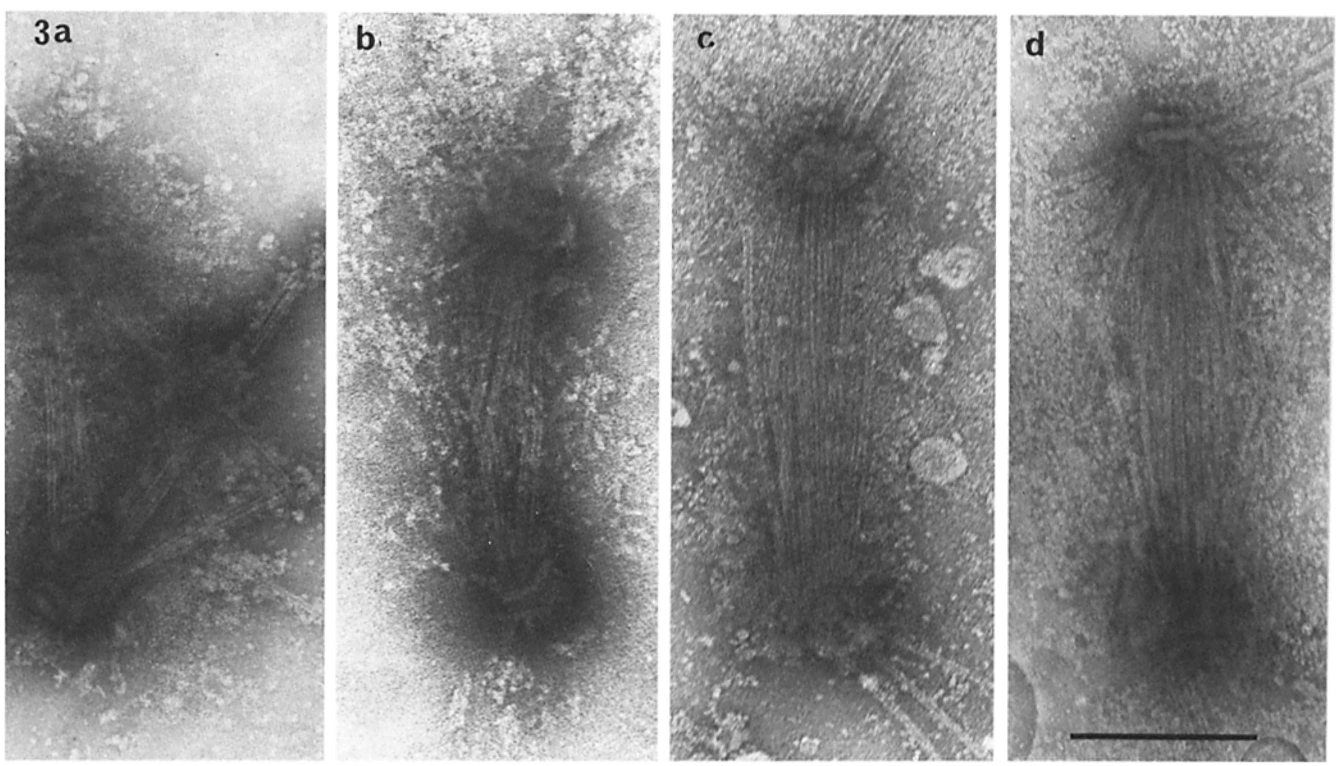
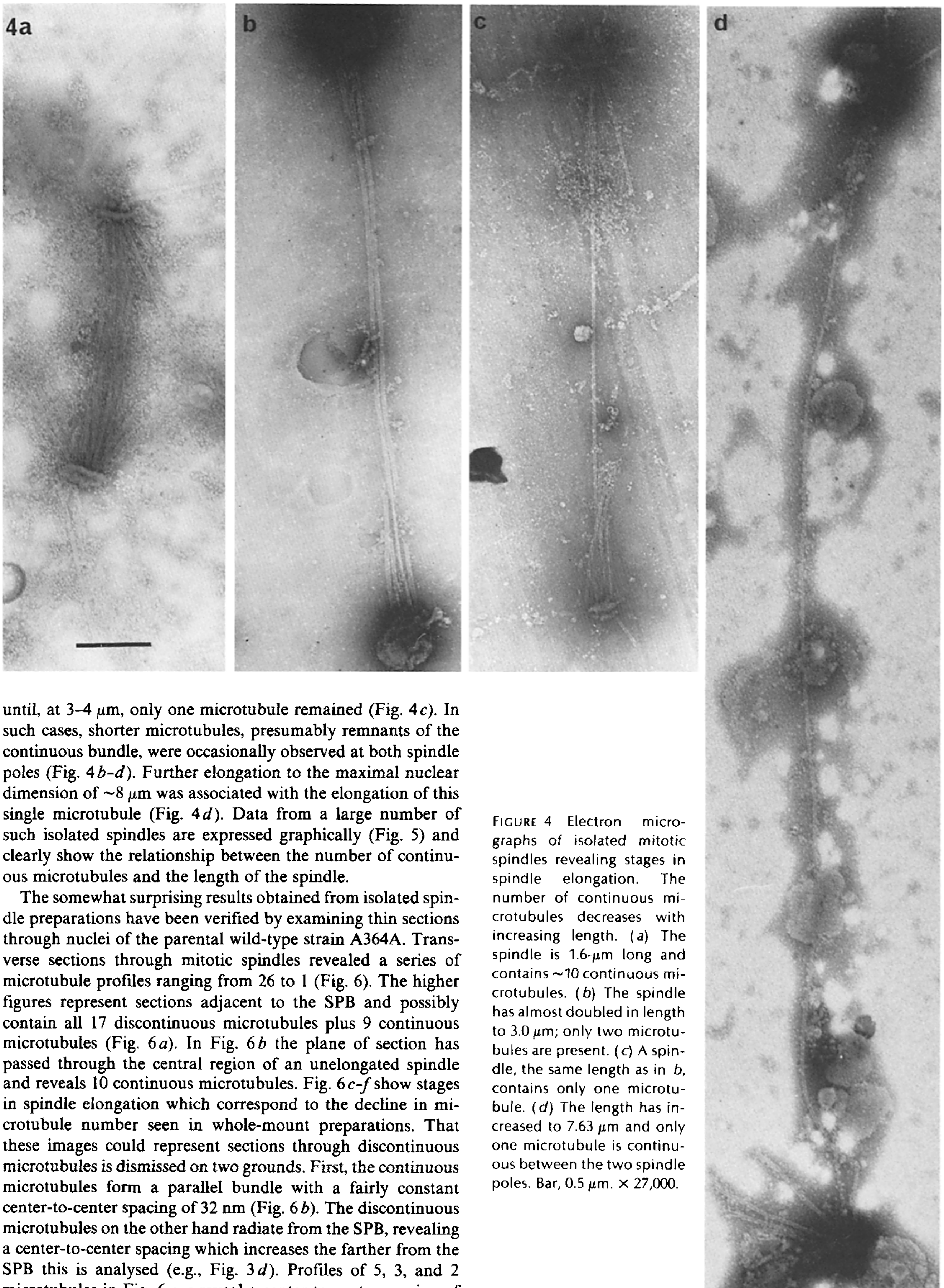


FIGURE 3 Electron micrographs of isolated mitotic spindles showing successive stages in spindle formation. The two sets of microtubules interdigitate (*a*) to form a spindle of continuous microtubules (*b*). Discontinuous microtubules are then nucleated (*c*) to produce a complete spindle (*d*). Bar, 0.5 μm. × 50,000.



until, at 3–4 μm, only one microtubule remained (Fig. 4*c*). In such cases, shorter microtubules, presumably remnants of the continuous bundle, were occasionally observed at both spindle poles (Fig. 4*b–d*). Further elongation to the maximal nuclear dimension of ~8 μm was associated with the elongation of this single microtubule (Fig. 4*d*). Data from a large number of such isolated spindles are expressed graphically (Fig. 5) and clearly show the relationship between the number of continuous microtubules and the length of the spindle.

The somewhat surprising results obtained from isolated spindle preparations have been verified by examining thin sections through nuclei of the parental wild-type strain A364A. Transverse sections through mitotic spindles revealed a series of microtubule profiles ranging from 26 to 1 (Fig. 6). The higher figures represent sections adjacent to the SPB and possibly contain all 17 discontinuous microtubules plus 9 continuous microtubules (Fig. 6*a*). In Fig. 6*b* the plane of section has passed through the central region of an unelongated spindle and reveals 10 continuous microtubules. Fig. 6*c–f* show stages in spindle elongation which correspond to the decline in microtubule number seen in whole-mount preparations. That these images could represent sections through discontinuous microtubules is dismissed on two grounds. First, the continuous microtubules form a parallel bundle with a fairly constant center-to-center spacing of 32 nm (Fig. 6*b*). The discontinuous microtubules on the other hand radiate from the SPB, revealing a center-to-center spacing which increases the farther from the SPB this is analysed (e.g., Fig. 3*d*). Profiles of 5, 3, and 2 microtubules in Fig. 6 *c–e* reveal a center-to-center spacing of

FIGURE 4 Electron micrographs of isolated mitotic spindles revealing stages in spindle elongation. The number of continuous microtubules decreases with increasing length. (*a*) The spindle is 1.6-μm long and contains ~10 continuous microtubules. (*b*) The spindle has almost doubled in length to 3.0 μm; only two microtubules are present. (*c*) A spindle, the same length as in *b*, contains only one microtubule. (*d*) The length has increased to 7.63 μm and only one microtubule is continuous between the two spindle poles. Bar, 0.5 μm. × 27,000.

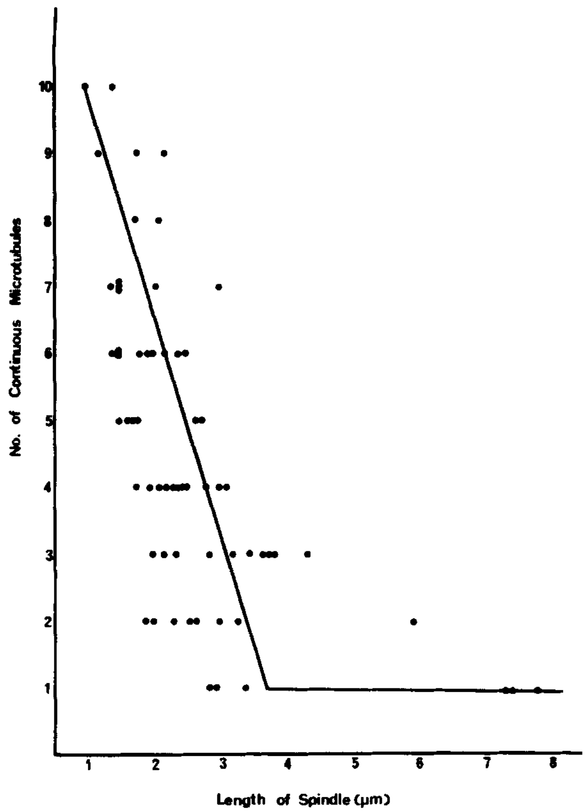


FIGURE 5 A plot of spindle length against the number of continuous microtubules present. As the spindle elongates from 1 to 4 µm the number of microtubules decreases to 1. Subsequent elongation to 8 µm occurs with only one microtubule joining the two SPBs.

32 nm, consistent with their being continuous microtubules. Although such an argument cannot be proposed for Fig. 6*f* which contains only a single microtubule, the geometry of the spindle also precludes that this is a discontinuous microtubule, since any section cutting a discontinuous microtubule perpendicularly must also pass, albeit obliquely, through the continuous microtubules. A lower magnification view of the entire nuclear profile (Fig. 6*g*) shows that this is not the case.

DISCUSSION

The isolation of mitotic spindles from *S. cerevisiae*[1] has, for the first time, allowed spindle chemistry and function to be directly investigated in vitro at the ultrastructural level using negative-stain electron microscopy. We have used this cell-free system to examine the hypothesis that spindle elongation during mitosis is an active process analogous to the dynein-mediated sliding reaction of ciliary axonemes (37). Axonemes from *Tetrahymena* were chosen as controls, for two reasons. First, it has been shown that only low concentrations of ATP (10–30 µM) are required to induce a sliding reaction (37). Although below the physiological levels required for ciliary movement, this concentration might be more consistent with the theoretical calculations of Nicklas (26) for the energy required for chromosome movement at anaphase. Secondly, unlike those obtained from other sources, sliding in *Tetrahymena* axonemes is not dependent upon prior proteolysis, even though such treatment potentiates the reaction (37).

Spindles were incubated under conditions which produced a more or less complete disintegration of parallel preparations of ciliary axonemes, included as an internal control, by the dynein-mediated displacement of adjacent doublet microtubules. No corresponding rearrangement of microtubules was observed in any of the spindles examined, including those pretreated with trypsin or Triton X-100 before exposure to ATP. Spindles were pretreated with Triton X-100 in recognition of the fact that demembranation of cilia with Triton X-100 is required before reactivation and that some detergent-soluble component may uncouple bending from sliding. Our data on the effects of pretreatment on the sliding reaction of *Tetrahymena* axonemes is in good agreement with the results of Warner and Zanetti (37). Although our preparations did not give such large declines in absorbance, when examined by electron microscopy ~100% of the axonemes were observed to have undergone some degree of sliding disintegration. The possibility that spindles isolated from *cdc* 6 were not competent to elongate because cells were arrested before some necessary stage in spindle formation was also examined. Temperature-arrested protoplasts were returned to the permissive temperature for 2 h before lysis to allow the spindles to initiate elongation before incubation in ATP. Such treatment produced no further increase in length over that of control preparations, and from these results we find no evidence to support the hypothesis that spindle elongation in *Saccharomyces* occurs under the conditions required for the reactivation of ciliary axonemes.

Another finding to emerge from this study concerns the sensitivity of the SPB to the protease, trypsin. The microtubule-nucleating capacity of SPBs isolated from stationary-phase cells has been shown to be destroyed by exposure to 1 µg/ml trypsin for 10 min (8). When intact spindles were treated under identical conditions, no change in SPB structure was observed. Such a result suggests that the trypsin-sensitive sites on the SPB are the microtubule nucleating sites which may, to some extent, be protected by the initiation of microtubule assembly.

Whether the data obtained on the lack of sliding in the mitotic spindle of *S. cerevisiae* may be applied to other organisms is, as yet, uncertain. Direct evidence for ATP-dependent movement in mitotic spindles has been obtained in permeabilized models of PtK_1 cells (6) and from the isolated mitotic apparatus of sea urchin eggs (33). Correspondingly, a dynein-like Mg^{2+}-ATPase has been identified in the mitotic spindles of sea urchin embryos (29). Evidence for sliding has also been inferred, indirectly, from the reconstruction of diatom spindles at various stages in mitosis from serial sections (28). Attempts to localize dynein in the spindles of mammalian cells by immunological methods have, however, proved conflicting. Although the mitotic spindles of PtK_1 cells and sea urchin eggs have been reported to react with antisera prepared against a tryptic fragment of dynein 1 (13, 25), other workers using affinity-purified antidynein antisera on PtK_1 cells have reached different conclusions (38).

The comparative simplicity of the yeast spindle, relative to those of higher organisms (e.g., 21) has for the first time allowed the entire mitotic sequence to be examined at high resolution in negatively stained, isolated whole-mount preparations. This analysis has revealed several interesting features of mitosis in *S. cerevisiae.* The continuous bundle of microtubules is formed by the complete interdigitation of two distinct sets, so that each microtubule has both ends associated with a SPB. Only when this step has been completed are the discontinuous microtubules then nucleated. This temporal separation of the nucleation of the two families of microtubules has also been inferred from studies which examined the assembly of tubulin from porcine brain onto isolated SPBs in vitro (8).

The most striking feature to emerge from this study, however,

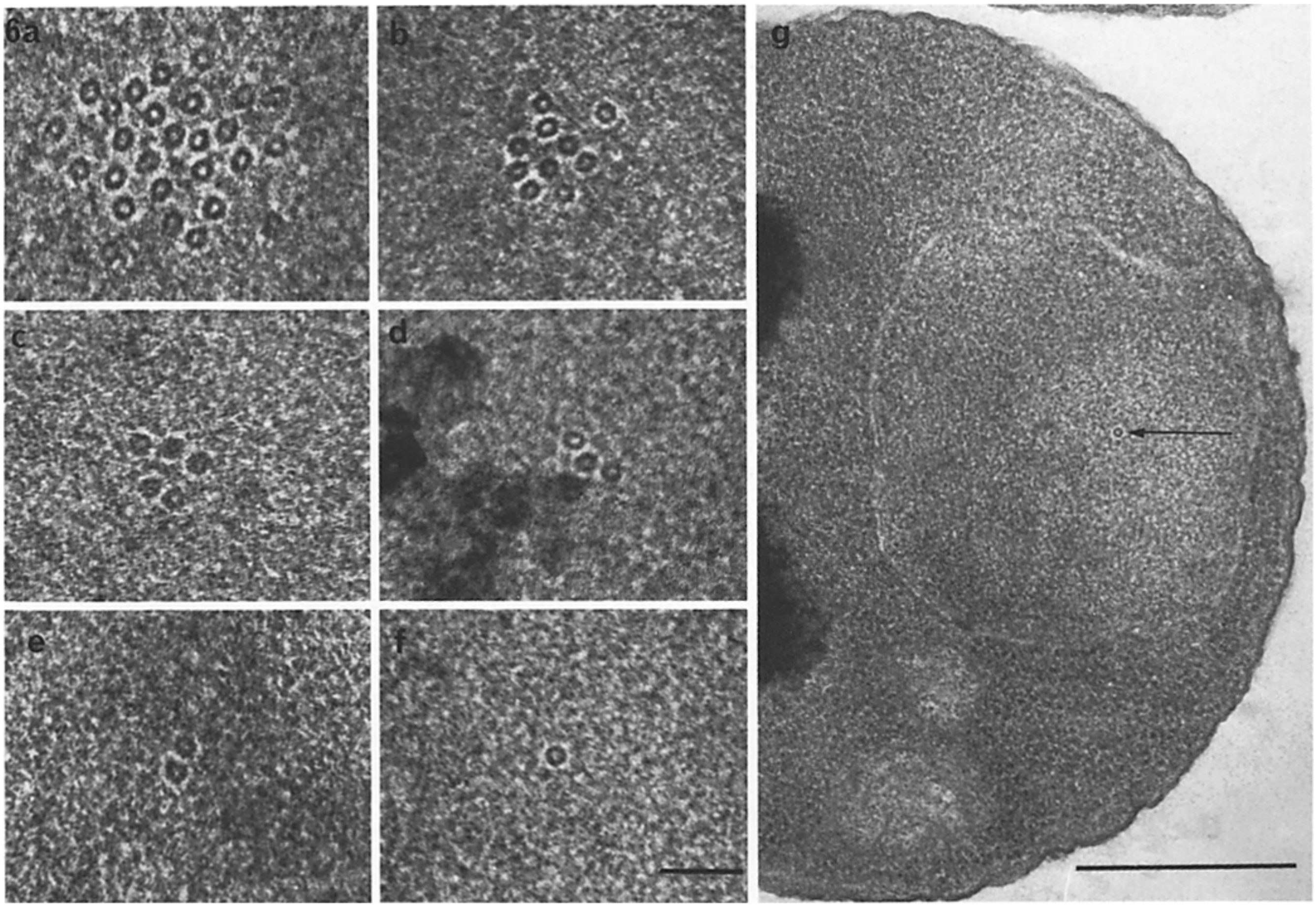


FIGURE 6 Electron micrographs of transverse sections through the mitotic spindle in the parental wild-type strain A364A reveal a variable number of microtubular profiles: (*a*) 26; (*b*) 10; (*c*) 5; (*d*) 3; (*e*) 2; (*f*) 1. *g* shows a section through a nucleus with a single microtubular profile (arrow). Note that no obliquely cut microtubules are present in the section. Bar: (*a-f*) 0.1 μm; and (*g*) 0.5 μm. (*a-f*) × 120,000. (*g*) × 60,000.

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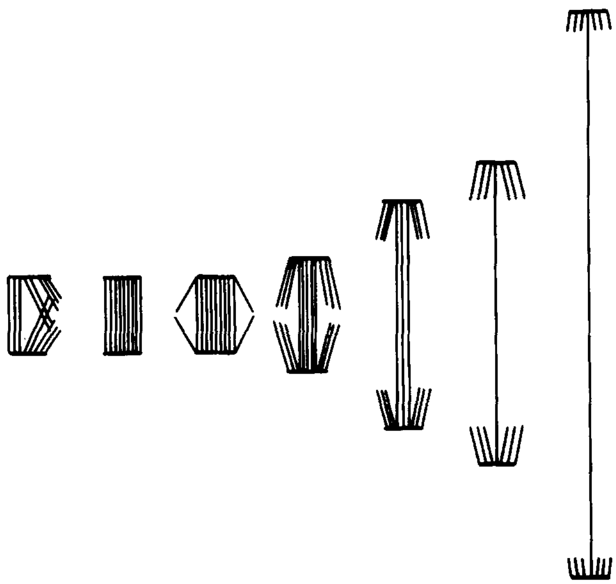

FIGURE 7 The disappearing microtubule model for spindle formation and elongation in *S. cerevisiae*. The spindle is formed by the interdigitation of two sets of microtubules, one from each SPB (thick lines), to form a continuous bundle. Discontinuous microtubules are then nucleated and the complete spindle produced. As spindle elongation occurs the discontinuous microtubules shorten and the number of continuous microtubules decreases to unity.

was the decline during spindle elongation in the number of continuous microtubules from ~10 to 1. Single microtubule spindles continued to elongate, reaching a maximal span of ~8 μm. This sequence of events is depicted diagrammatically in Fig. 7. The most probable explanation to account for this decrease is that the microtubules depolymerize from their distal ends. This is consistent with the observation that short fragments of microtubules are associated with the SPBs during anaphase B and, also, with the finding that the proximal ends of yeast spindle microtubules are "closed" while the distal ends are "open" (4).[1] It is, at the moment, unclear why one microtubule, attached to the central region of both SPBs, is favored to survive, but it is interesting to note that during anaphase A in *Haemanthus katherinae* microtubules disappear from the periphery of the kinetochore (14).

Although this report is the first describing a mitotic apparatus composed of a single continuous microtubule, it is not unique in reporting that the number of spindle microtubules decreases during anaphase and telophase (e.g., 18). The data presented here on the decrease in microtubule number are consistent with the cytological observations of Robinow and Marak (32), who showed that the affinity of the "intranuclear fiber" for acid fuchsin was much greater when the fiber was short and compact than when it was expanded. Our model for mitosis in yeast differs from that of Byers and Goetsch (3) and Peterson and Ris (27) in two respects. First, the interdigitation of two sets of microtubules gives rise to a continuous bundle, the discontinuous microtubules being nucleated after this event; and secondly the number of continuous microtubules decreases to unity during spindle elongation. Spindles composed of a single microtubule have not been observed before in *S. cerevisiae* for two reasons. Their appearance during the cell cycle is transitory: anaphase B occurs within a small fraction of the cycle, while, for instance, the 1-μm spindle of medial nuclear division may remain for upwards of 60% of the cycle time. It also testifies to the difficulty of obtaining complete longitudinal sections through a structure 8-μm long but only 25 nm in width. We observed single microtubule spindles in transverse thin sections only after an exhaustive search through a large number of nuclear profiles. In many instances the microtubules had been cut at an oblique angle and it was necessary to use a double tilt goniometer stage to orient the microtubular profiles.

We have presented two lines of evidence that argue against mitosis in *S. cerevisiae* being based on a sliding microtubule mechanism involving a dyneinlike Mg^{2+}-ATPase. First, functional studies in vitro have shown that isolated spindles do not undergo a sliding reaction analogous to that found in ciliary axonemes. This result is consistent with our failure to detect crossbridges between the continuous microtubules.[1] Secondly, the finding that a single microtubule spindle may undergo a two- to threefold increase in length suggests that microtubule polymerization is of paramount importance during anaphase B in *S. cerevisiae.* Our results suggest a more passive role for the mitotic spindle and are in agreement with information on the position of the spindle in the dividing nucleus (27, 32). After formation the 1-μm spindle of medial nuclear division is positioned oblique to the longitudinal axis of the dividing cell. The nucleus then extends a process that passes through the neck of the bud into the daughter cell. It is not until this stage that the spindle starts to elongate and the spindle poles migrate around the nucleus. Thus, the spindle does not span the long axis of the nucleus until late in anaphase.

It is suggested that, rather than being the force generator of chromosome movement, the spindle of *S. cerevisiae* acts as a governor, limiting the rate of SPB separation. Such a mechanism might be achieved by limiting the rate of polymerization and disassembly of the continuous microtubules. Regulation of this type, limiting the rate of anaphase B to a velocity less than that of anaphase A, would ensure that both daughter nuclei receive the correct chromosome number. This is in accord with studies on spindle elongation in *Fusarium solani* (1) where a laser beam of 0.25-μm diameter was used to destroy selected portions of the central spindle. Disruption of these microtubules caused a threefold increase in the rate of spindle pole separation, while irradiation of certain portions of the cytoplasm close to the SPBs caused this rate to slow. Similar results have been obtained in mammalian cells after the mechanical disruption of PtK_1 spindles using a microneedle to push the dorsal and ventral membranes of the cell together. This generated a hole through the interzonal region of the spindle and increased the rate of spindle pole separation from 1.0 to 1.8 μm/min (17).

In conclusion, the mitotic spindle of *S. cerevisiae* is potentially an exceedingly useful system for studying the movements associated with both anaphase A and B. Isolated spindles are stable and may be examined at high resolution in negatively stained preparations. Also, anaphase B movements in this yeast are highly exaggerated. SPBs may be isolated and used for in vitro studies on microtubule polymerization using either brain tubulin or the recently purified yeast tubulin (15). In addition to the above, there are also the concomitant advantages of using, as an experimental system, an organism for which there is a wealth of genetic and biochemical information and where there also exists a large collection of *cdc* and other mutants.

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