

# NUCLEATION OF MICROTUBULES IN VITRO BY ISOLATED SPINDLE POLE BODIES OF THE YEAST *SACCHAROMYCES CEREVISIAE*

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## ABSTRACT

Spindle pole bodies (SPBs) were isolated from the yeast *Saccharomyces cerevisiae* by an adaptation of the Kleinschmidt monolayer technique. Spheroplasts prepared from the cells were lysed on an air-water interface. Spread preparations were picked up on grids, transferred to experimental test solutions, and prepared for whole-mount electron microscopy. Using purified exogenous tubulin from porcine brain tissue, the isolated SPBs were shown to nucleate the assembly of microtubules in vitro. Microtubule growth was directional and primarily onto the intranuclear face of the SPB. Neither the morphology nor the microtubule-initiating capacity of the SPB was affected by treatment with the enzymes DNase, RNase, or phospholipase although both properties were sensitive to trypsin. Analysis of SPBs at various stages of the cell cycle showed that newly replicated SPBs had the capacity to nucleate microtubules. SPBs isolated from exponentially growing cells initiated a subset of the yeast spindle microtubules equivalent to the number of pole-to-pole microtubules seen in vivo. However, SPBs isolated from cells in stationary phase and therefore arrested in G1 nucleated a number of microtubules equal to the total chromosomal and pole-to-pole tubules in the yeast spindle. This may mean that in G1-arrested cells, the SPB is associated with microtubule attachment sites of the yeast chromatin.

**KEY WORDS** yeast · microtubules · spindle pole bodies · nucleation · in vitro · electron microscopy

The essential properties of mitosis in all eukaryotic cells are the development of a bipolar spindle and the subsequent segregation of the chromosomes. Although the details of mitosis may vary in particular organisms, the bipolar mitotic spindle consisting of microtubules is a constant feature (20). Efforts to understand the role of spindle microtubules in chromosome segregation, how-

ever, have been hampered by their complexity and disordered arrangement. One way to circumvent this problem is to concentrate on so-called simpler systems, organisms in which the spindle is composed of relatively few microtubules and which are amenable to combined biochemical, ultrastructural, and genetic analysis. In the yeast, *Saccharomyces cerevisiae*, the organization of the mitotic spindle reveals features common to many other lower eukaryotes (11, 13). The yeast spindle consists of a small number of continuous microtubules extending between two opposed plaquelike

structures, designated spindle pole bodies (SPBs), embedded in the nuclear membrane, and a larger number of so-called discontinuous or chromosomal microtubules which extend between the SPBs and the replicating chromatin (4, 5, 15, 16, 18, 21). The SPBs arise at the commencement of bud emergence by the duplication of the single pole body seen in the interphase nucleus (4, 5, 18). As the bud enlarges, microtubules form at the intranuclear surface of the daughter SPBs which migrate around the nuclear membrane to their polar positions, the microtubules from each pole interdigitating to form the complete spindle.

The number of microtubules that comprise the yeast spindle is a function of the ploidy of the strain examined (18, 20, 29). Serial thick sections through whole or partial spindles, examined in the high voltage electron microscope, indicate a one-to-one correlation between the number of linkage groups per nucleus (17 in the haploid strain; [17]) and the number of discontinuous microtubules (18). The number of continuous or pole-to-pole microtubules is variable but small (usually 5-10) and shows no convincing variation with chromosome number.

One unusual feature of mitosis in *S. cerevisiae* is that the chromosomes do not undergo the characteristic sequence of coiling and condensation and hence are not attached to the spindle by recognizable kinetochores. Despite this, mitosis in yeast is regarded as being essentially orthodox and proceeds by stages recognizable as metaphase and anaphase (18).

With these factors in mind, we have initiated a program designed to analyze spindle formation and mitosis in this organism. *S. cerevisiae* is particularly well suited to such a study, not only because of the wealth of available ultrastructural information mentioned previously, but also because it can be grown in large quantities in a simple, defined medium and because temperature-sensitive mutants which are blocked in various stages of mitosis have been isolated (10). In addition, through the use of exogenous tubulin purified from brain tissue, it has recently become possible to test directly the microtubule nucleation functions of preformed structures. Both the centrosomes and chromosomes of mammalian cells, either *in situ* in lysed cell preparations (14, 23) or isolated onto electron microscope grids (7, 8, 25), have been shown to initiate the assembly of microtubules *in vitro*. We have attempted to apply this *in vitro* assembly assay to the yeast system to investigate spindle

formation in a simple eukaryote cell. In this first report, we describe a procedure for the isolation of the SPBs, an analysis of their function as microtubule organizing centers *in vitro*, and some preliminary chemical characterization. A brief account of some of this work has been published previously (3).

## MATERIALS AND METHODS

### *Growth of Yeast*

The strains of *S. cerevisiae* used in these experiments,  $\alpha$  *his-4* (haploid) and *S-41* (diploid), were kindly provided by Dr. B. Littlewood and Dr. H. O. Halvorson, respectively. Stock cultures were maintained at 4°C on yeast extract peptone medium (6) solidified with 1% agar. Growth was carried out in the same medium lacking agar at 28°C on a rotary shaker and monitored by counting cells in a hemacytometer or by measuring cell density in a spectrophotometer at 650 nm. Cells were harvested at various stages of the growth cycle as specified in the text by centrifugation at 800 g for 5 min.

### *Preparation of Spheroplasts*

Spheroplasts of yeast cells were prepared according to the procedure of Hutchison and Hartwell (12). Cells were washed once with distilled water and resuspended to a density of  $10^8$ - $10^9$  cells/ml in 1 M sorbitol containing 1% glucylase (Endo Laboratories, Inc., Garden City, N.Y.). The suspension of cells in the enzyme-sorbitol mixture was incubated for 90 min at room temperature and then washed free of enzyme by centrifugation and resuspension in 1 M sorbitol. Spheroplasts were stored at room temperature in 1 M sorbitol for up to 4 h before use.

### *Isolation of Spindle Pole Bodies*

SPBs were collected on electron microscope grids by a modification of the Kleinschmidt monolayer technique as described previously (9, 18). Spheroplasts in 1 M sorbitol were harvested by centrifugation and resuspended in distilled water to a density of  $5 \times 10^8$ - $2 \times 10^9$  cells/ml. Under these conditions, the spheroplasts began to swell and would be completely lysed by 4 min after resuspension. Before lysis (2-3 min), aliquots were removed from the suspension of swollen spheroplasts, and small droplets were placed on an air-water interface where lysis and spreading occurred. The spread preparations were picked up on ionized 200-mesh grids coated with 0.25% Formvar and carbon or 0.5% Formvar alone.

### *Preparation of Purified Microtubule Protein*

Microtubule protein was purified from porcine brain tissue using two cycles of a reversible, temperature-dependent assembly scheme (2). For storage, microtu-

bules polymerized in the second cycle of purification were pelleted by centrifugation (39,000 g, 30 min) at 37°C, frozen in liquid nitrogen, and kept at -80°C. Concentrated solutions of tubulin (5–10 mg/ml) were obtained by resuspension of the frozen pellets in polymerization solution (0.1 M piperazine-*N-N'*-bis(2-ethane sulfonic acid) [PIPES], adjusted to pH 6.9 at 23°C with NaOH, containing 0.1 mM MgCl<sub>2</sub> and 1 mM GTP) followed by depolymerization and centrifugation at 0°C. Protein concentrations were determined by the method of Schacterle and Pollack (22) using bovine serum albumin as a standard. Immediately before use, tubulin solutions were diluted with polymerization solution to the final desired concentration which was in the range of 1.0–1.5 mg/ml. In most experiments the tubulin solutions were centrifuged at 230,000 g (50,000 rpm, Spinco type 65 rotor [Beckman Spinco, Palo Alto, Calif.], 2-ml tubes) for 90 min at 0°C to prepare high-speed supernatant solutions which self-initiate assembly only after a long lag (1).

### *Polymerization of Microtubules onto Spindle Pole Bodies*

For testing for microtubule growth onto isolated SPBs, grids of the spread preparations were transferred from the air-water interface to 0.2-ml droplets of the test solutions on blocks of Teflon after first being touched briefly against filter paper to remove excess water. Test solutions were (a) polymerization buffer, (b) tubulin, (c) 100 μM colchicine, and (d) tubulin plus 100 μM colchicine. Solutions b–d were also made up in polymerization buffer. Grids were incubated on the droplets over a range of temperatures (22°–30°C) for various times.

### *Enzymatic Modification of Spindle Pole Bodies*

As a preliminary chemical characterization of the SPBs, grids of spread preparations were exposed to various enzyme treatments before the addition of tubulin. The enzymes used, trypsin, RNase A, DNase 1, and phospholipase A, were obtained from Sigma Chemical Co., St. Louis, Mo., made up as solutions in polymerization buffer lacking GTP, and used within 1 wk of purchase. Trypsin activity was determined by the method of Summers and Gibbons (24) and found to be 700 benzoyl arginine ethyl ester (BAEE) U/mg. Grids prepared as previously described were transferred to 0.2–0.5 ml droplets of enzyme solution on blocks of Teflon. After incubation at 30°C for 15 min, grids were washed twice by floating on 10-ml aliquots of polymerization buffer lacking GTP. Excess buffer was removed by touching the grid briefly against filter paper, after which the grid was transferred to tubulin as described above. After trypsin treatment, grids were floated on 10 ml of an equimolar solution of soybean trypsin in-

hibitor (Sigma Chemical Co.) before washing with buffer and processing as described previously.

### *Electron Microscopy*

Samples on grids taken directly from the air-water interface or after incubation on droplets of experimental solutions were prepared for electron microscopy. The specimens were fixed by flotation or immersion of the grids for 15 s–5 min in 2.5% glutaraldehyde in 0.1 M PIPES, pH 6.9, rinsed with distilled water, stained for 30 min with 1% uranyl acetate, and dried by the critical point method. In many experiments, samples were air-dried directly out of amyl acetate. Specimens were examined in a Philips EM 300 operated at 80 kV. Plate magnifications were calibrated using a replica grating. Lengths of microtubules polymerized onto the SPBs were determined using a map measure on enlargement prints of the electron micrographs.

## RESULTS

### *Identification of the Spindle Pole Bodies*

The identification of SPBs in spread preparations was based largely upon the previous description of Peterson and Ris (18). They described the presumptive SPBs as densely staining bodies from which various numbers of microtubules radiated. These tubules were most likely remnants of the yeast mitotic spindle. The typical trilaminar morphology of the SPB seen in sectioned material (4, 5) was generally not apparent in these preparations although better detail was observed in fixed spreads embedded in a thick layer of negative stain.

In our experiments, grids collected from the water interface after cell lysis revealed the presence of distinct regions of cellular debris and chromatin fibers which had evidently derived from the disruption of single cells. Such regions, which we have called "spreads", were examined for the presence of structures consistent with the description of the SPB noted above. In our initial screening of the spreads, we looked for the presence of native yeast microtubules to facilitate identification of the SPBs. Structures identifiable as partial spindles of *S. cerevisiae* (Fig. 1 *f-h*) were found with low frequency (<1%), but, in the great majority of cases (>95%), spreads failed to reveal any evidence for the presence of native microtubules. The partial spindles consisted of a pair of SPBs connected by a parallel bundle of 5–10 microtubules, 1.0–1.4 μm in length. In favorable instances, the microtubules could be traced from pole to pole and thus presumably represent the

continuous spindle microtubules. In some cases, shorter fragments of radially projecting microtubules, presumably remnants of the discontinuous or chromosomal microtubules, also remained associated with the SPB (Fig. 1 *h*).

The low incidence of native microtubules prompted us to reexamine the spreads for the presence of SPBs not associated with obvious spindle remnants. In 20–40% of spreads prepared from either the haploid or diploid strains, one or two densely staining, discoidal structures, ~170–200 nm in diameter, were observed (Fig. 1 *a*, *b*, and *e*). These putative SPBs were indistinguishable in morphology from the dense bodies obviously serving as foci for microtubules. In most cases, these structures lacked any association with microtubules although short fragments (0.075–0.6  $\mu\text{m}$ ) of one or two microtubules were occasionally observed (Fig. 1 *c* and *d*). We provisionally conclude from these observations that the microtubules of the yeast spindle are labile and depolymerize after the lysis of the cell whereas the SPBs themselves are more stable structures. This conclusion was confirmed by experiments (see next section) in which some putative SPBs served as foci for growth of microtubules.

The morphology of the SPBs varied with their

orientation to the electron microscope grid, but two views appeared to predominate which could be interpreted in terms of the structure as seen in thin sections. In one view, the SPBs appeared as knobby but circular disks corresponding to face-on views (Figs. 1 *a–e* and 3 *b* and *d*) where the observer is sighting along a direction perpendicular to the plane of the disc. In more favorable orientations, the SPBs show an obvious multilayered organization (Fig. 3 *a* and *c*), and these correspond to side-on views obtained by sighting horizontal to the axis of the disk.

### Growth of Microtubules onto SPBs In Vitro

The immobilization of both SPBs and chromatin on electron microscope grids provided a simple and direct method of assaying the ability of each to initiate the assembly of microtubules in vitro. Grids were transferred to solutions of porcine brain tubulin under conditions which minimized the spontaneous formation of microtubules but which allowed growth onto preexisting seeds or organizing centers (1, 2). Following incubation, SPBs were consistently seen to be associated with microtubules (Figs. 2 and 3)

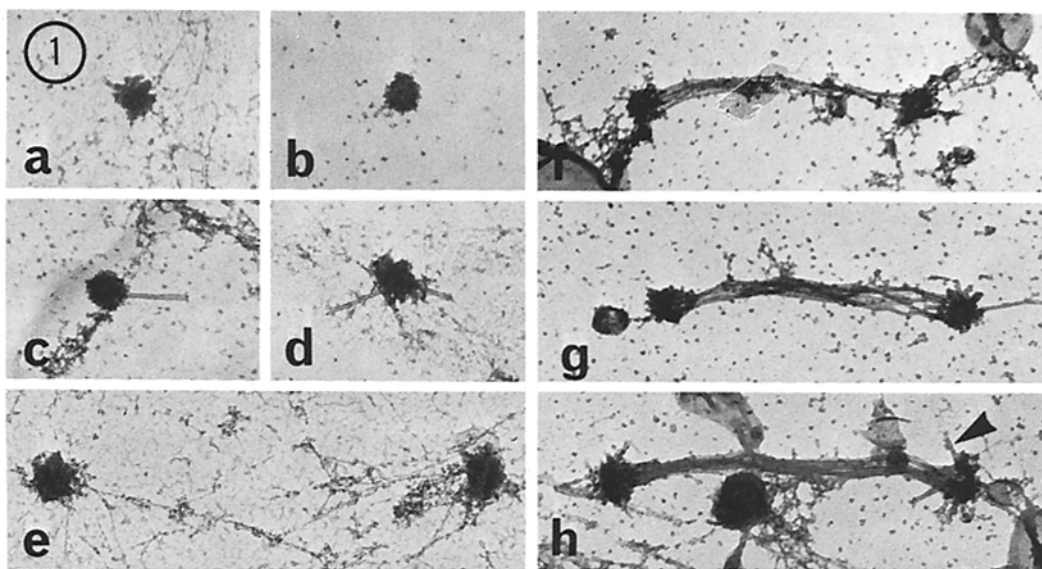


FIGURE 1 Isolated SPBs in spread preparations of diploid yeast cells. Pole bodies appear as densely staining, unstructured bodies (*a* and *b*), occasionally associated with short fragments of one or two native yeast microtubules (*c* and *d*). (*e*) A pair of spindle pole bodies. The fibres in the background are chromatin. (*f–h*) The SPBs remain connected by a parallel bundle of microtubules, presumably the continuous or pole-to-pole microtubules of the yeast spindle. Short fragments of radially projecting discontinuous or chromosomal microtubules are also distinguished (*h*, arrowhead).  $\times 33,000$ .

whereas in no case amongst >200 grids scanned was microtubule growth from a site on the chromatin observed. That the microtubules associated with the SPBs were formed primarily from exogenous brain subunits was suggested by their numbers and their length which varied with the protein concentration and incubation time employed but commonly reached 30  $\mu\text{m}$  (Fig. 2). Since the maximum length obtained by the microtubules of the yeast spindle is  $\sim 8 \mu\text{m}$  (5), *in vitro* microtubule growth had evidently occurred although whether directly onto SPBs or, less interestingly, onto existing fragments of yeast microtubules was not apparent. To distinguish between these possibilities, multiple grids taken from the same spread preparation were subsequently incubated in either polymerization buffer alone, buffer plus colchicine, buffer plus tubulin, or buffer plus colchicine and tubulin. After incubation in each of these solutions, the percentage of spreads containing SPBs was scored and in each case their association with microtubules was recorded. The results of such an experiment are shown in Table I which reveals that SPBs could be detected in spreads with approximately equal efficiency after each of the posttreatments. Only on those grids incubated with buffer plus tubulin was any extensive association of microtubules with SPBs observed. In the absence of added tubulin, or where microtubule assembly was inhibited with colchicine, only 1–2% of the SPBs was associated with microtubules (Table I). Hence we conclude that microtubule assembly in these experiments occurs predominantly by direct addition onto pole bodies, that is, the SPB of *S. cerevisiae* serves as a microtubule-organizing center *in vitro*, although the possibility that short fragments of microtubules, not detected by our method of specimen preparation, remain associated with the SPBs cannot be categorically excluded.

#### *Directionality of Microtubule Growth*

A striking feature of the growth of microtubules onto SPBs *in vitro* was that it was primarily unidirectional, or, in other words, microtubules were seen to emanate from only one face of the SPB (Fig. 4; see also Fig. 3 *b–d*). That this is the intranuclear surface of the SPB is strongly suggested by configurations such as that shown in Fig. 2. Here the orientation of the two SPBs probably reflects their position *in situ*, maintained by persisting native microtubules. Addi-

tional growth from exogenous tubulin has occurred largely onto the surface of each pole body which faces its partner (Fig. 2 *b*). The possibility that this observed directionality is artifactual and imposed experimentally by drying down the grids in a particular way can be discounted on the evidence of Fig. 4 *c* where two parallel groups of microtubules, projecting at right angles to each other, are observed.

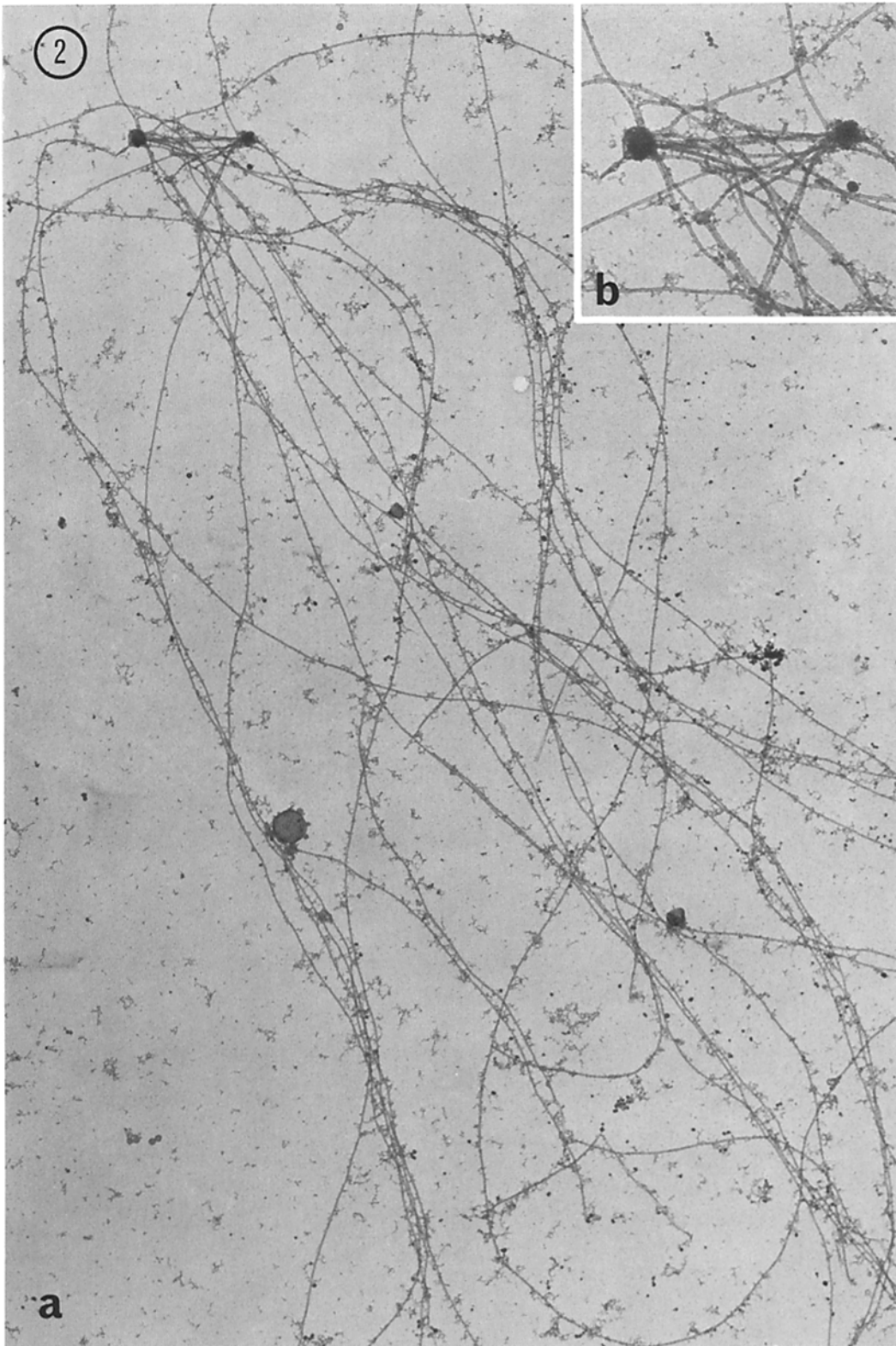
#### *Enzymatic Sensitivity of the SPB*

Another problem that one may attempt to study using the SPB of *S. cerevisiae* as a model is the identity of molecules comprising a microtubule-organizing center. Preparatory to an attempt to isolate the yeast SPB for biochemical analysis, we have approached its macromolecular composition by the less direct method of subjecting the SPBs, immobilized on electron microscope grids, to treatment with various enzymes before their incubation with tubulin. One may ask two questions of the SPBs so treated; does (*a*) the structure of the SPB and (*b*) its ability to nucleate the growth of microtubules *in vitro* exhibit enzymatic sensitivity?

The morphology of the spindle pole body was apparently unaffected by incubation for 15 min at 30°C in 100  $\mu\text{g}/\text{ml}$  of DNase 1, 100  $\mu\text{g}/\text{ml}$  of RNase A (Fig. 3 *c*), and 50  $\mu\text{g}/\text{ml}$  of phospholipase A as shown in Table II. Similarly, the mean number of microtubules initiated per SPB after such treatments was 85–91% of the mean number observed for control grids incubated in buffer alone. However, after treatment with dilute solutions of 1  $\mu\text{g}/\text{ml}$  of trypsin, no SPBs or recognizable fragments of these structures were observed. Associated with this loss of morphological integrity was an absence of any microtubule initiation, suggesting that the whole multilayered SPB including the microtubule initiation sites is sensitive to proteolysis. This was confirmed by experiments in which an equimolar concentration of soybean trypsin inhibitor was added to the trypsin solution. Under these conditions, both the morphology of the SPBs and their ability to support the growth of microtubules *in vitro* were comparable to those of the controls (Table II).

#### *Cell Cycle Dependence of Microtubule Initiation*

The demonstration that the SPB of *S. cerevisiae* serves *in vitro* as a template for the assembly



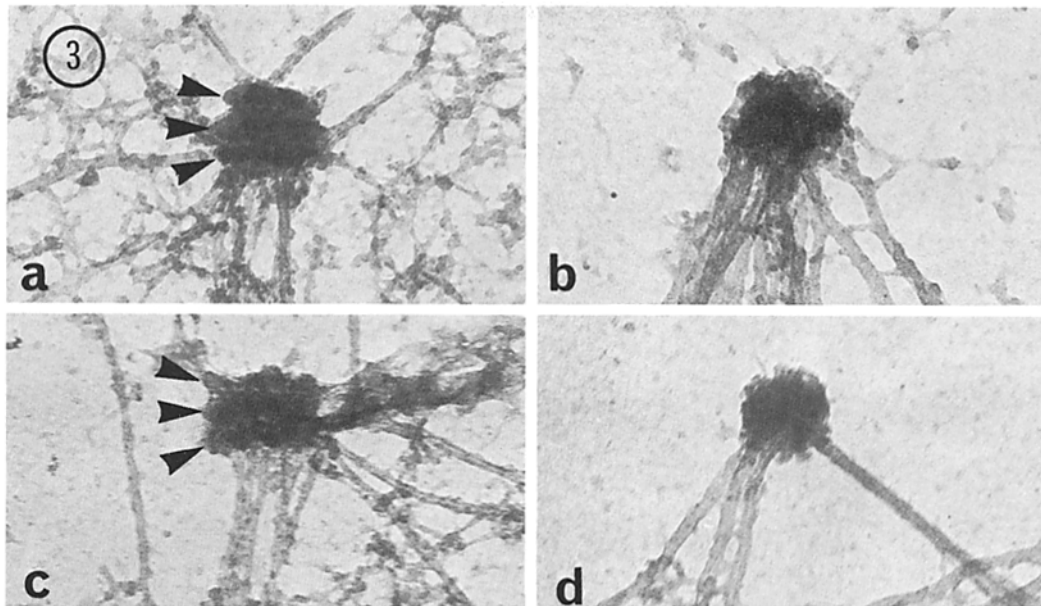


FIGURE 3 Variations in the morphology of the SPB can be interpreted in terms of their orientation to the electron microscope grid. In side-on view, the pole bodies exhibit a rectangular profile composed of three distinct layers as shown by the arrowheads (*a* and *c*). From above, they appear disk-shaped and unstructured (*b* and *d*). The pole body shown in Fig. 3 *c* was treated with RNase before incubation with tubulin and shows particularly clearly that growth of microtubules occurs primarily onto one face of the SPB. All pole bodies are from the diploid strain.  $\times 83,000$ .

TABLE I  
*Polymerization of Microtubules onto Spindle Pole Bodies*

Incubation conditions	Percentage of spreads containing spindle pole bodies*	Percentage of spindle pole bodies with microtubules
Buffer	29	2
Colchicine (100 $\mu$ M)	20	1
Tubulin	43	62
Tubulin plus colchicine	30	1

\* Data presented are averages of three experiments, and 50 spreads were examined in each experiment. A spread is defined as an area on an electron microscope grid which can be identified as having been derived from the disruption of a single cell. Cells used in these experiments were of the diploid strain in the exponential phase of the growth cycle.

of microtubules strongly suggests that this is a function for these structures in the organization of spindle microtubules in vivo. It was natural, then, to look for control mechanisms for spindle tubule formation at the level of the structure and activity of the spindle pole body. The ultrastructural work of Byers and Goetsch (5) has delineated the SPB cycle. The SPB is replicated in the early phase of budding which coincides with the period of chromosome replication (S). The parent and daughter SPBs persist for a short time as a double structure and then separate to form the poles of the 1- $\mu$ m spindle at about the completion of S. The morphology of the single SPB remains unchanged through the balance of the cell cycle and into G1 of the next division cycle. In the latter part of G1, the SPB develops a satellite structure before SPB replication and the

FIGURE 2 SPBs from the haploid strain after incubation with tubulin in vitro show extensive addition of microtubules (*a*). In the higher magnification insert (*b*), growth of microtubules can be seen to be primarily onto the intranuclear surface of the SPB. (*a*)  $\times 14,000$ ; (*b*)  $\times 28,000$ .

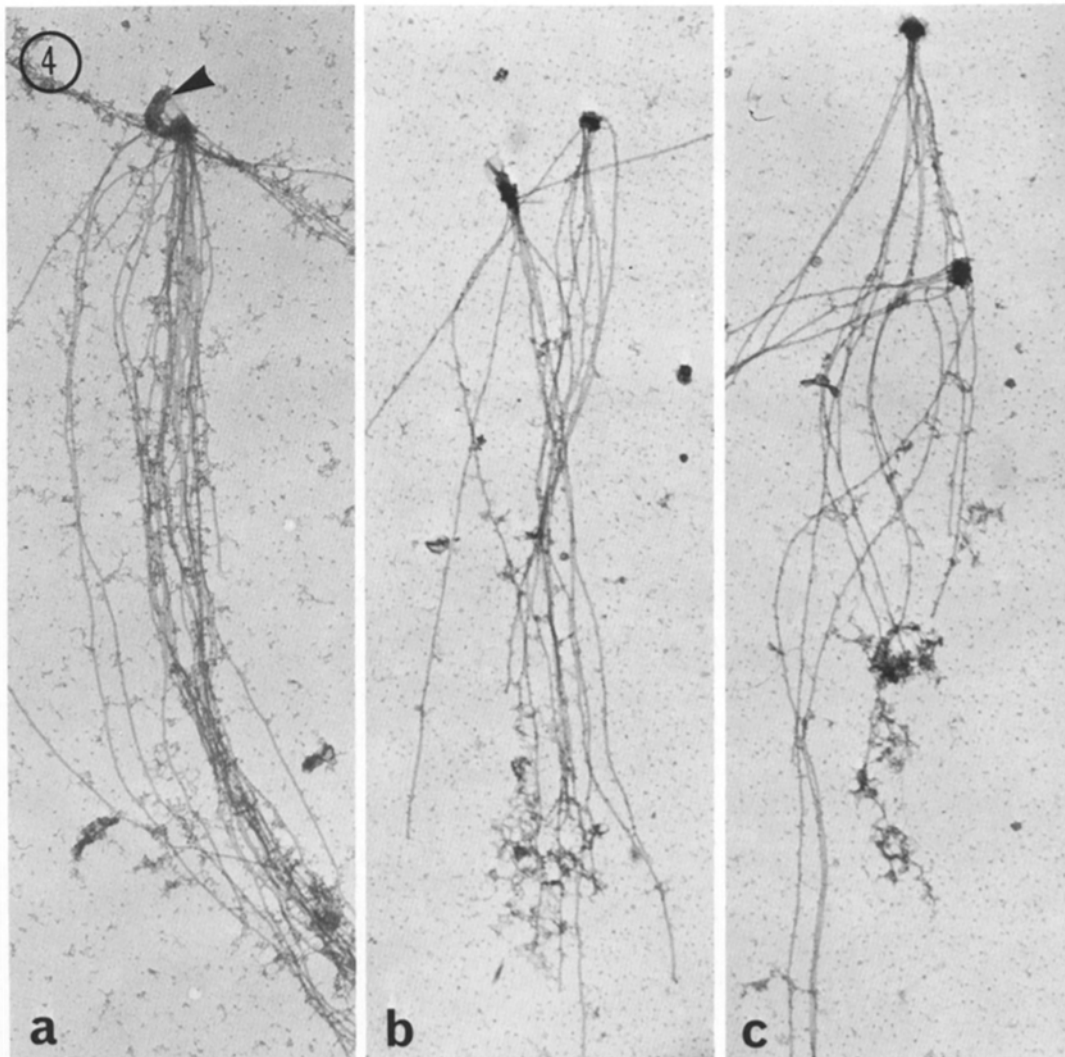


FIGURE 4 Single (a) and pairs (b and c) of SPBs from the diploid strain following incubation with tubulin show the directional growth of microtubules. In Fig. 4 a a fragment of the nuclear membrane (arrowhead) remains attached to the SPB.  $\times 14,000$ .

beginning of a new cycle. Cells arrested in G1 by being grown to stationary phase contain SPBs lacking a satellite and are presumably as yet uncommitted to a new round of replication. Thus, single SPBs in cells arrested in stationary phase may be in a functional state different from that of SPBs in cells undergoing vegetative growth. We therefore assayed the microtubule-initiating activity of newly replicated SPBs and SPBs isolated from exponentially growing and stationary-phase-arrested populations of cells.

#### *Activity of Newly Replicated SPBs*

Spheroplasts were prepared from exponentially growing populations of cells and lysed as described in Materials and Methods. Although exponentially growing cultures are expected to contain cells in all phases of the cell cycle, on the basis of thin-section studies (5), newly replicated SPBs should be recognizable as doubled structures connected by a bridge. Spreads incubated with or without tubulin revealed, as expected,



the presence of pairs of SPBs connected by a ribbon of less structured material (Fig. 5). Thus, the pairs of replicated pole bodies exist for some period as an integral structure before separating and commencing their migration to their positions at the spindle poles. In the spreads incu-

bated with tubulin (Fig. 5), microtubules were observed arising from the newly replicated SPBs. The number and average length of microtubules per pole body in the pair were the same as for single SPBs in the same preparation. Thus, the ability of SPBs to nucleate microtubules seems to appear immediately upon their replication, and a latent period before their maturation does not seem to be evident.

TABLE II  
*Enzymatic Sensitivity of Spindle Pole Bodies*

Treatment	Spindle pole bodies	Microtubule initiation
		%
Buffer	+	100
Trypsin (1 $\mu\text{g/ml}$ )	-	0
Trypsin + soybean trypsin inhibitor (1 $\mu\text{g/ml}$ )	+	84
RNase A (100 $\mu\text{g/ml}$ )	+	91
DNase 1 (100 $\mu\text{g/ml}$ )	+	91
Phospholipase A (50 $\mu\text{g/ml}$ )	+	85

Microtubule initiation is defined as a SPB displaying one or more microtubules. In these experiments (diploid cells, stationary phase of growth), the range of microtubules associated with each SPB was 14-42 (mean  $28 \pm 7$ ). Data are expressed as a percentage of control (untreated) SPBs.

#### *Activity of SPBs in Exponentially Growing and Stationary Phase Cells*

Spreads were prepared from exponentially growing and stationary-phase cultures of a haploid strain of yeast, the SPBs were incubated with tubulin at the same protein concentration and under identical conditions, and the numbers of microtubules observed to initiate from single SPBs were quantitated. The results of such an experiment, using a protein concentration of 1.75 mg/ml, are shown in Fig. 7. Fig. 6 illustrates the growth of microtubules onto haploid and diploid SPBs from stationary-phase cultures which can be compared with their respective exponential phase equivalents in Figs. 2 and 4 *b* and *c*. For the SPBs from exponentially growing

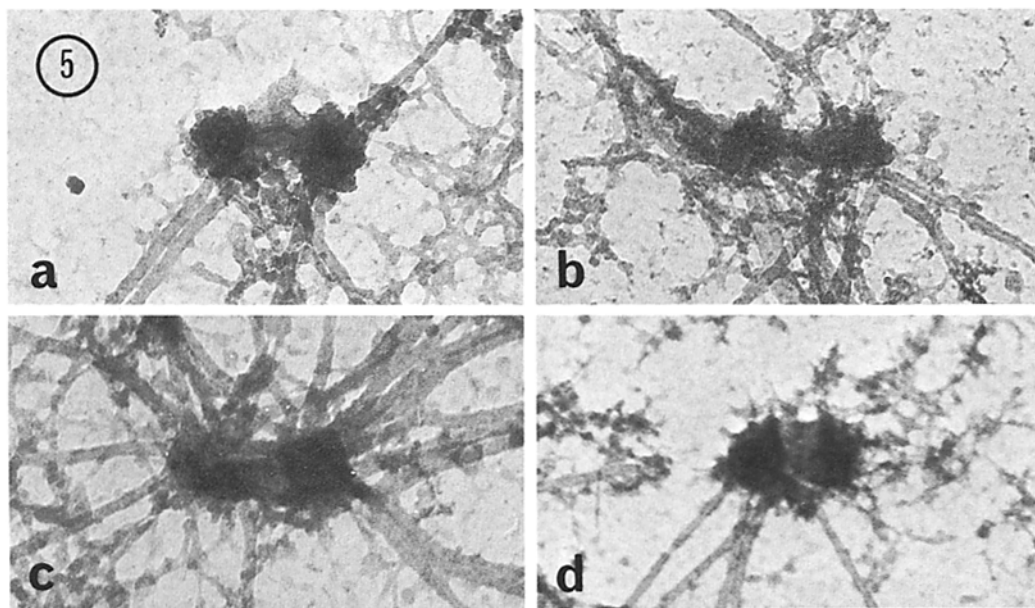
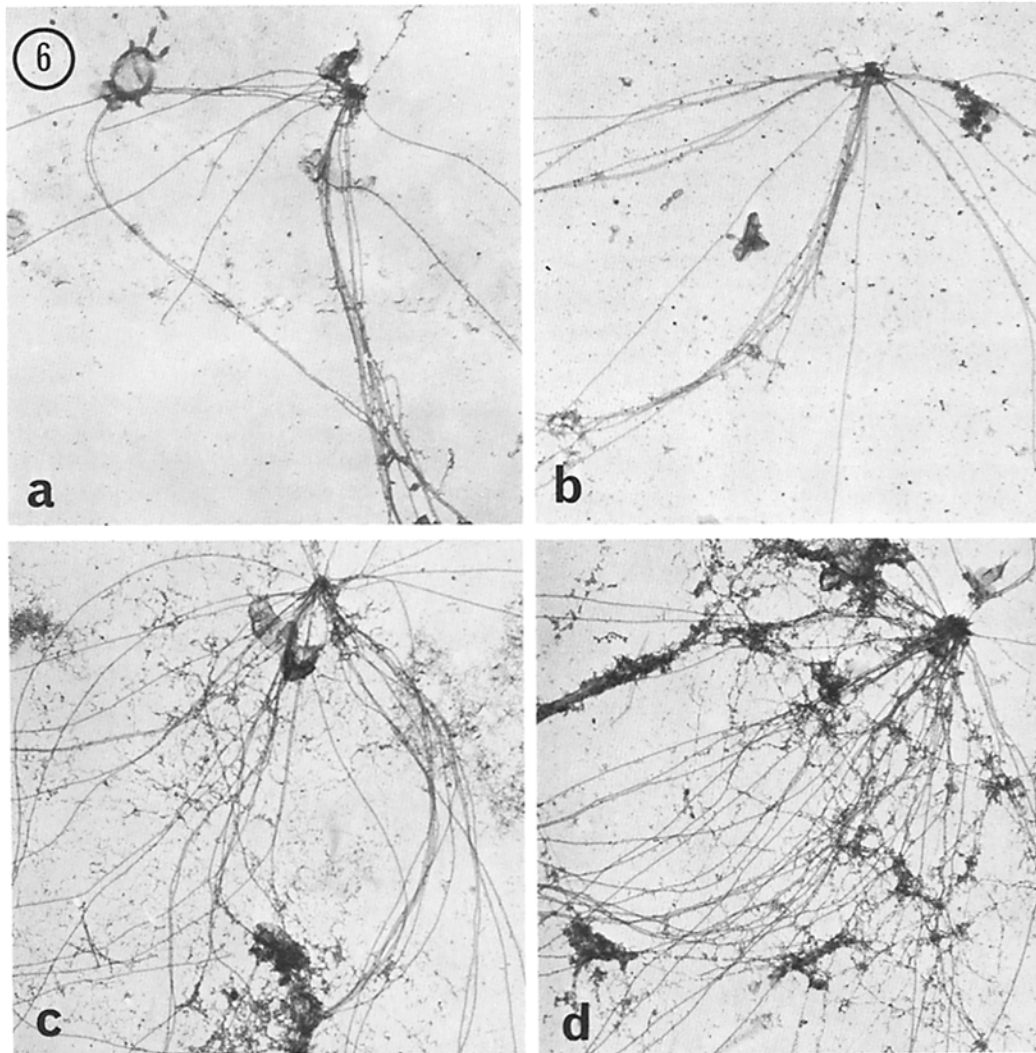


FIGURE 5 Newly replicated SPBs still connected by a ribbon of unstructured material, after incubation with tubulin. In this doubled configuration, the SPBs tend to lie in face view, and it is not possible to determine whether microtubules arise exclusively from one face of the pole body. Diploid strain.  $\times 83,000$ .



**FIGURE 6** SPBs from stationary-phase cultures of haploid (*a* and *b*) and diploid (*c* and *d*) cells after incubation with tubulin and showing differences in microtubule initiation. In Fig. 6 *a* and *b* the SPBs are associated with 17–20 microtubules whereas in Fig. 6 *c* and *d*, ~40 microtubules have polymerized onto the SPBs. Microtubule growth in Fig. 6 *c* and *d* can be compared with SPBs from exponential phase cultures of the same diploid strain shown in Fig. 4 *b* and *c*.  $\times 9,000$ .

cells, a mean of  $8.5 \pm 2$  and range of 5–12 microtubules were obtained. This result is similar to the average of  $7 \pm 3$  microtubules reported by Peterson and Ris (18) for the pole-to-pole microtubules of the haploid spindle *in vivo*. The *in vitro* distribution was extremely reproducible and was unaffected by raising the protein concentration to 2.6 mg/ml (above which the self-initiation of microtubules became a problem in scoring).

In contrast, a mean of  $15 \pm 4$  and range of 5–

22 microtubules were obtained for the SPBs isolated from stationary cultures of the haploid cells. The mean and, more importantly perhaps, the maximum value are close to the value of 21 for the total number of microtubules in the haploid spindle (18). This coincidence suggests that the SPB in stationary-phase cells has the capacity to nucleate both the chromosomal tubules as well as the pole-to-pole tubules—that is, all the microtubules of the yeast mitotic spindle.

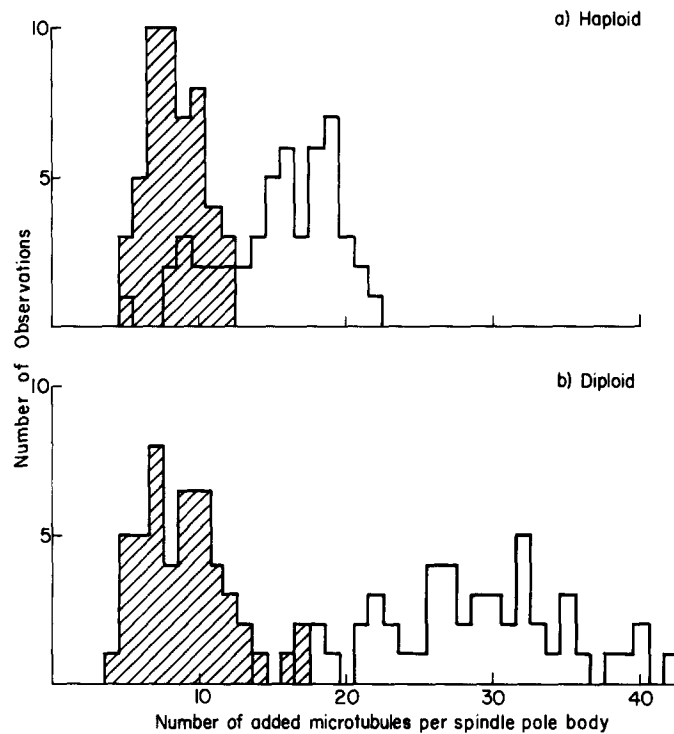


FIGURE 7 Cell cycle dependence of microtubule initiation by spindle pole bodies isolated from haploid (a) and diploid (b) cells. The shaded and open areas of the histogram show the growth microtubules onto SPBs from exponential and stationary-phase cultures, respectively.

To further test this suggestion, the microtubule-nucleating capacity of SPBs from diploid cells was determined and compared with the values for the haploid cells. Peterson and Ris (18) had previously found that the number of pole-to-pole microtubules did not vary significantly with ploidy, reporting averages of  $7 \pm 3$  and  $9 \pm 3$  microtubules for the haploid and diploid, respectively. However, the numbers of discontinuous tubules were found to be proportional to ploidy, with values of 14 reported for the haploid and 29 for the diploid, giving total values of 21 and 38 microtubules for the haploid and diploid, respectively. Since haploid yeast has 17 linkage groups (17), these values compare favorably with the expected values of  $17 + 7 \pm 3 = 24 \pm 3$  microtubules for the haploid half-spindle and  $34 + 9 \pm 3 = 43 \pm 3$  for the diploid. Thus, if the difference in activity of SPBs from exponentially growing and stationary-phase cells indeed represents the sites for initiating the discontinuous microtubules, then the difference in numbers of microtubules initiated by SPBs of diploid cells in the two growth states should

equal approximately the number of yeast chromosomes in the diploid cells, i.e., 34. SPBs were isolated from cultures of a diploid strain in the two growth states as before, incubated with tubulin, and the numbers of microtubules were quantitated. Figs. 4 b and c and 6 c and d illustrate the results, and the quantitative data are shown in Fig. 7 b. A mean of  $9.5 \pm 1.5$  and range of 4-17 microtubules were obtained for the SPBs isolated from exponentially growing cells, whereas a mean of  $28 \pm 7$  and range of 14-42 microtubules were obtained for the stationary-phase cells. Although the range of microtubules initiated by the stationary-phase SPBs was great, it is probably of greater interest to note the maximum number of tubules obtained. As was observed for the haploid SPBs, the number of microtubules initiated by the diploid SPBs isolated from exponentially growing cells corresponded to the number of pole-to-pole tubules, and the number of microtubules initiated by the SPBs from stationary-phase cells corresponded to the total number of microtubules in the yeast half-spindle.

## DISCUSSION

We have demonstrated that the SPB of *S. cerevisiae* can serve to nucleate the assembly of microtubules in vitro, a function which presumably reflects its in vivo role in the formation of the yeast mitotic spindle. This is the first demonstration that the plaque-like structures seen at the spindle poles of many simple eukaryotes are microtubule-organizing centers, although such a role has generally been assumed (19).

The appearance of the isolated SPBs differs slightly from that seen in thin-sectioned material (4, 5) although is more reminiscent of certain images seen in thick sections examined in the high voltage electron microscope (see, for example, Fig. 19 *e* in reference 18). In most of our experiments, the SPBs appeared to lack any defined structure although occasionally they could be seen to be composed of three electron-opaque layers. Thin sections reveal the presence of only two such layers (5) and as yet there is no obvious explanation for the variation in morphology seen with these two different preparative techniques. Two other components associated with the SPB in vivo, namely, the half-bridge and the satellite (5), were not consistent features of isolated pole bodies and, apart from demonstrating that they are not essential for the nucleation of spindle microtubules, these studies provide no additional information as to their function.

Our data also confirm the findings of Peterson and Ris (18) that recognizable spindle fragments can be isolated by the spreading technique. In our hands, isolated spindles appear to be of a fairly uniform size,  $\sim 1 \mu\text{m}$  in length. Although the yeast spindle can extend to  $8 \mu\text{m}$ , Byers and Goetsch (5) have reported that spindles of  $\sim 1 \mu\text{m}$  persist for  $\sim 65\%$  of the budding phase before undergoing a rapid six-eightfold elongation, therefore accounting for our observation that the  $1\text{-}\mu\text{m}$  spindle appears to be the most abundant spindle fragment seen in the lysates. Our findings also suggest that this configuration is quite stable, perhaps because the microtubules span the distance between the poles or perhaps because of as yet undetected cross-bridges between the microtubules in the bundle.

The growth of exogenous porcine brain tubulin in these experiments appears to be predominantly onto the SPBs and not onto persisting fragments of yeast microtubules. It is possible that short

stubs of microtubules ( $0.05 \mu\text{m}$ ) remain associated with the surface of the SPB and are not detected by our method of specimen preparation. However, why the rest of the spindle microtubules should depolymerize and leave a homogeneous population of short pieces is not obvious unless short fragments of microtubules constitute an integral part of the structure of the SPB. Microtubule assembly occurs largely onto only one surface, presumably the intranuclear face of the pole body although, in living cells, cytoplasmic microtubules are also seen to be associated with the extranuclear surface (the "outer plaque" of Byers and Goetsch [5]). Our data do not exclude a small number of microtubules being initiated on the cytoplasmic face; however, it is also possible that the outer plaque is inactivated or becomes detached from the SPB upon cell lysis. Alternatively, the outer plaque may be an attachment site for microtubules extending into the cell bud and not an organizing center. At the moment, it is not possible to distinguish between these explanations.

The biochemical composition of the diverse structures considered under the general heading of microtubule-organizing centers (19) is unknown although they are often assumed to contain a concentration of tubulin subunits. Of the enzymes used in these experiments, the nucleation of microtubule assembly by the isolated spindle pole bodies revealed a sensitivity only to proteolysis. Tubulin in the form of microtubules is quite resistant to digestion with trypsin (26), although it is not known whether this property extends to the subunit form or to other tubulin aggregates. Generally, however, proteolysis by trypsin is a feature more usually associated with elongated proteins (26). Our results might therefore suggest, indirectly, that proteins other than tubulin are involved in the nucleation of microtubule assembly by SPBs in vitro. Alternatively, the apparent trypsin sensitivity of the microtubule initiation sites on the pole body may be a secondary consequence of the destruction of some other portion of the organelle concerned with its other functions such as integration into the nuclear membrane or replication. These latter properties of the SPB might presuppose the involvement of other components such as phospholipids or nucleic acids. Our results suggest that the presence of such macromolecules is unlikely although the possibility that they are present but inaccessible to the enzyme cannot be ruled out. In this regard, Zick-

ler (28) has shown that the pole bodies of another ascomycete, *Ascobolus*, are sensitive to digestion *in situ* with DNase but not RNase.

Previous studies of nucleated microtubule assembly *in vitro* have suggested that the timing of spindle formation in animal cells is regulated by processes involving the centrosome and may involve a maturation event (7, 23, 27). Our results have shown that the ability of the SPBs to nucleate the growth of microtubules also depends on position in the cell cycle, but in a rather different way. The number of microtubules seen to grow from SPBs isolated from exponential phase cultures is small, approximately equivalent to the number of pole-to-pole microtubules in either the haploid or diploid spindle. The same number of microtubules is initiated by newly replicated SPBs or the majority species SPB in exponential cultures. Thus, it seems unlikely that the number of microtubules initiated by the SPBs changes after their initial replication. However, SPBs isolated from cells allowed to accumulate in stationary (G1) phase had the capacity to initiate a larger number of microtubules, sufficient to account for the continuous and discontinuous microtubules in both haploid and diploid strains. From this rather astonishing result, we are obliged to suggest that the spindle pole body in G1-arrested cells contains the microtubule nucleation sites for all the microtubules of the yeast mitotic spindle. It seems clear that the SPBs from the G1-arrested cells are not in the same state as SPBs in actively growing cells. Perhaps the SPBs in the G1-arrested cells are associated with the microtubule attachment sites of the yeast chromatin and are isolated together as a coherent complex. In actively growing cells, the attachment sites might become detached from the SPBs and thus the isolated pole bodies would only display nucleating activity for the continuous microtubules. Although the chromatin in yeast does not condense into chromosomes with morphologically recognizable kinetochores, the postulated attachment sites are functionally equivalent to kinetochores seen in other cells.

We thank Professor Hans Ris for many stimulating discussions during the course of these studies and Dr. Joan Peterson for assistance in the early experiments.

This work was supported by National Institutes of Health grant GM21963 and National Science Foundation grant GM 36454 to Dr. Borisy.

Received for publication 28 December 1977, and in revised form 7 April 1978.

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