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THE ROLE OF RING AGGREGATES AND OTHER STRUCTURES IN THE ASSEMBLY OF MICROTUBULES

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Beef brain tubulin isolated by cycles of polymerization and depolymerization contains two components, 6S subunit and a 25–35S boundary containing ring-shaped aggregates of tubulin. The rings disappear during microtubule polymerization, and the incorporation of ring tubulin into microtubules has been investigated by studying the changes in the sedimentation of tubulin which occur during polymerization. The "30S" boundary was separated from the 6S boundary by sedimentation at low temperatures. The temperature was then raised by letting a small amount of air into the vacuum chamber and the changes in sedimentation rate and concentration of each component determined as the tubulin polymerized. The 30S material polymerizes preferentially as determined by its decrease in concentration at polymerizing temperatures. Simultaneously with its decrease in concentration the 30S also decreases in sedimentation rate. The decrease in concentration of the 30S correlates well with polymerization while the decrease in sedimentation rate can occur independently of polymerization. The results indicate that the rings are not transformed directly into microtubules, but break down into subunits or small aggregates and these then assemble into microtubules. The rings may serve as a "storage aggregate" of active subunits. The presence of a possible storage aggregate in a dividing cell, the eggs of the surf clam, Spisula solidissima, has been indicated by measurements of particulate tubulin changes during the cell cycle. Microtubule assembly in vitro in homogenates of these eggs indicates that the amount of tubulin which forms microtubules may be controlled by the functioning of the microtubule organizing center.

INTRODUCTION

The pattern of formation and breakdown of microtubules in a living cell can be remarkably elaborate and precise, and is clearly subject to accurate cellular control as to the time and location of microtubule assembly. The ability of isolated tubulin to polymerize in vitro (1) makes a systematic study of the possible control mechanisms experimentally feasible. Such a study requires knowledge of the mechanism of microtubule assembly and the influence of other components (such as calcium or microtubule organizing material) on assembly.

In the work to be discussed here two approaches to the problem of microtubule formation and its control have been taken. In order to study the mechanism of assembly, and in particular how intermediate aggregates may be involved, the changes in the

sedimentation properties of tubulin which occur during the process of polymerization have been examined. This has been done by sedimenting tubulin solutions at low temperatures, under conditions in which tubulin exists in at least two states, monomer and a ring-shaped aggregate, and then raising the temperature of the rotor until polymerization occurs. In the second set of experiments we have attempted to examine the actual changes in tubulin organization in a dividing cell, and using in vitro polymerization experiments which can partially duplicate the behavior of microtubules in vivo to determine how these changes could be controlled.

METHODS

Tubulin was prepared essentially by the method of Shelanski et al. (2) and was stored in 8 M glycerol below 0°C. Glycerol was removed prior to experimentation by passing the solution through G-25 Sephadex medium in 0.1 M MES buffer (3) at pH 6.5.* Polymerization was performed in this buffer with 0.5 mM GTP, 1 mM EGTA, and 0.5 mM $MgCl_2$ added, and at a temperature of 35°C unless otherwise indicated. Temperature shift experiments were performed by the following procedure using a Spinco Model E equipped with electronic speed control, and the UV scanner: a) The rotor and sample were initially at a temperature of 10 to 12°C, and were run at this temperature until the two observable boundaries were clearly separated (about 15 min). All runs were done at 52,000 rpm. b) About 5 min before the temperature jump the diffusion pump and the refrigeration were turned off. c) At the desired time the mechanical pump was unplugged from its outlet on the rear panel. d) The mechanical pump was switched to OFF. e) A *very small* amount of air was admitted into the vacuum chamber. This was done by slowly turning the air valve until the pressure began to rise (but not above about 75 microns) and then rapidly closing the valve again. f) The mechanical pump and the diffusion pump were switched to the ON position. g) The drive was restarted by turning the voltage control to zero and then back to its running position. h) The diffusion pump was turned off again. i) The heater was turned on by adjustment of the temperature control unit. During the run the pressure might have gradually increased and if the temperature began to rise too rapidly the mechanical pump was turned on briefly. The samples were usually scanned at a wavelength of 294 nm.

Experiments using the eggs of the surf clam were performed as described previously (4, 5). In the in vitro polymerization experiments the eggs were washed twice in 1 M glycerol, 1 mM sodium phosphate at pH 8, and homogenized in 1.5 ml (per ml of eggs) of 0.5 M MES buffer usually containing 1 mM EGTA at pH 6.5. In experiments involving stabilization of tubulin aggregates the eggs were homogenized in 10 ml of 1 M hexylene glycol, 0.01 sodium phosphate at pH 6.2 (HGL solution) per ml of eggs (6). For electron microscopy the polymerized homogenates were fixed in 3% glutaraldehyde and post-fixed in osmium tetroxide; sections were stained with uranyl acetate and lead citrate.

RESULTS

Tubulin prepared by cycles of polymerization and depolymerization (2) contain

*Abbreviations used are: MES [morpholino-N-(2 ethane sulfonic acid)] and EGTA [ethylene glycol-bis-(β-aminoethylether) tetraacetic acid].

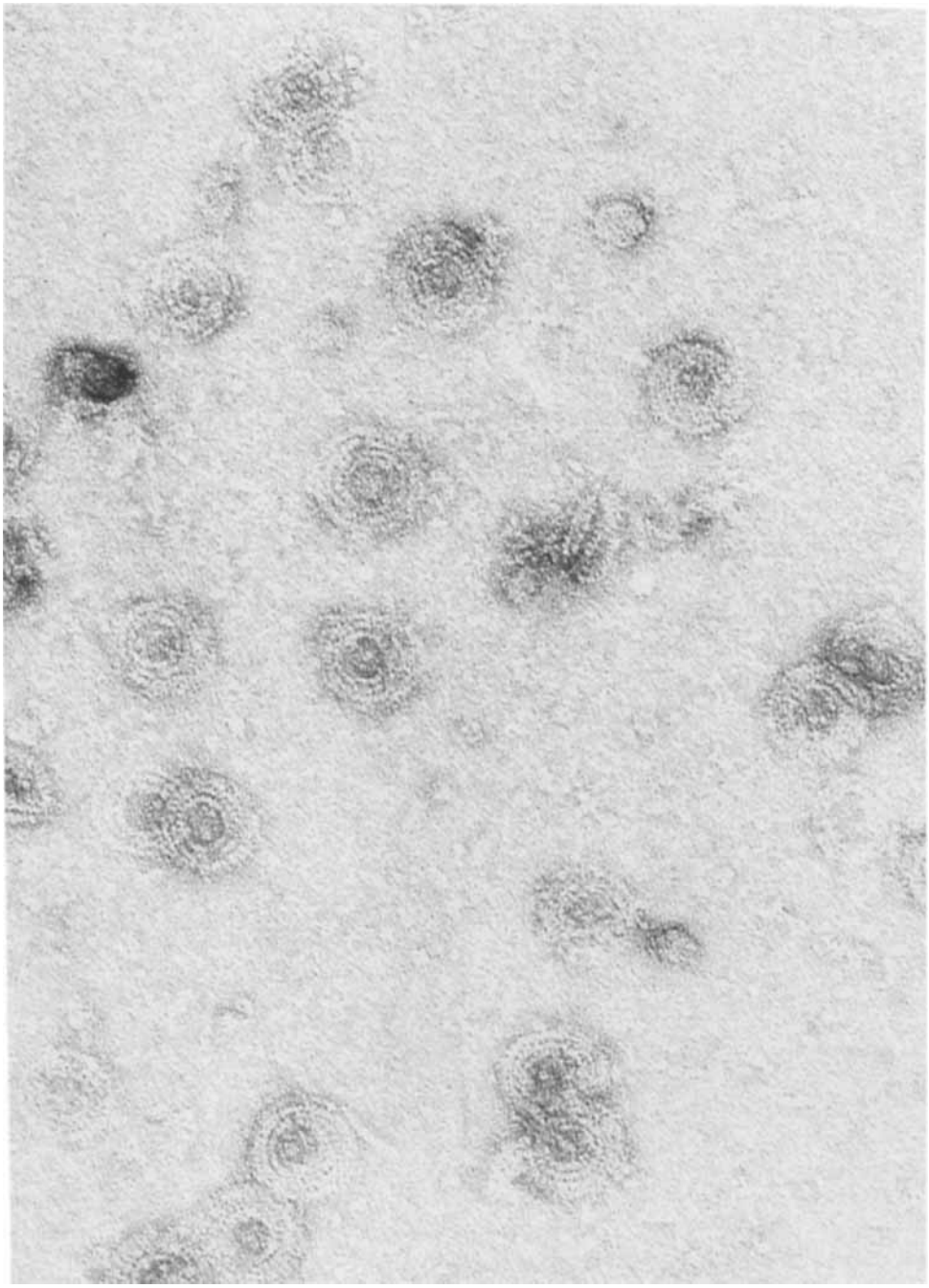


Fig. 1. A field of tubulin rings negatively stained with uranyl acetate. The solution contained 5 mM $CaCl_2$ in addition to the usual components (see Methods). The magnification is about 220,000.

large numbers of ring-shaped aggregates (Fig. 1). The morphology of these structures may vary, but generally consists of two or three concentric layers, although single and four layered rings have also been observed. The inside diameter of the rings is about 23 nm

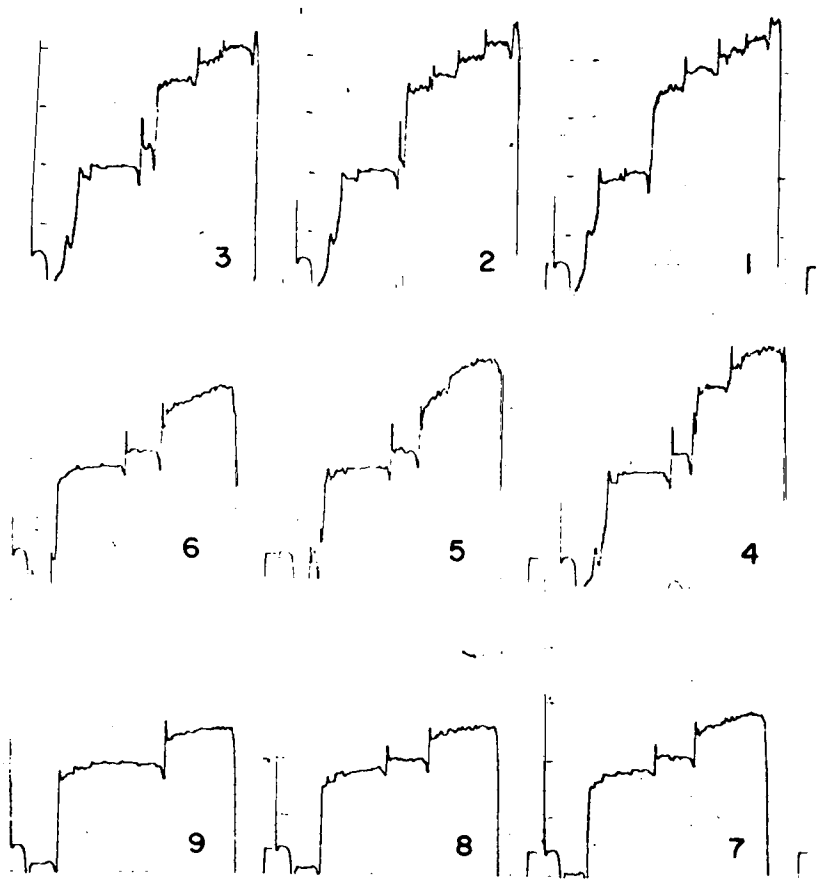


Fig. 2. A selected series of scans taken during a temperature jump experiment in which polymerization of microtubules occurred. The protein was in the standard polymerizing medium and was scanned at 294 nm. In this figure and the ones which follow the top of the solution is to the left and the scans are numbered in the order in which they were performed. These scans were done at the following times (in min) after starting the run: 1, 21; 2, 24; 3, 26.5; 4, 30; 5, 31.5; 6, 33.5; 7, 35; 8, 37; 9, 38.

(± 2, n = 30), while the outside diameter of the first layer is about 35 nm, and that of the second layer is 47 nm. The width of each layer is about 6 nm, which is slightly larger than spacing between protofilaments in the wall of the microtubule, which is about 5 nm. Ultracentrifugation of these preparations of tubulin reveals the presence of two boundaries, the slower one migrating at about 6S, which is the tubulin subunit, and a heterogeneous boundary which can migrate at about 25 to 35S depending upon conditions. The fast boundary, which contains the rings, will be referred to as the "30S" boundary, although its measured sedimentation rate depends upon temperature, the concentration of divalent cations and total tubulin concentration, since it appears to be in reversible equilibrium with subunit.

The rings and the 30S boundary are both absent or greatly reduced in amount after polymerization of microtubules, suggesting that the ring tubulin is directly incorporated into microtubules. However, it is not possible to determine clearly by electron microscopy alone whether the rings associate directly in some fashion to form microtubules, or

whether they break down to subunits or small aggregates, and these subsequently assemble into microtubules. If the rings assemble directly into microtubules their sedimentation rate may be expected to increase, or remain relatively constant as microtubules form. If, on the other hand, they first break down to smaller structures, their sedimentation rate may be expected to decrease prior to microtubule formation. The results of a typical temperature shift experiment, designed to resolve this question, are shown in Fig. 2. As the temperature of the rotor is increased during centrifugation a number of phenomena are observed. The first change which occurs as the temperature begins to increase is a "hypersharpening" of the existing boundaries, and at about the same time a variable number of small, sharp boundaries appear. Both of these phenomena are a result of convective disturbance caused by temperature gradients within the solution and are unrelated to tubulin aggregation. It is always possible to identify the original boundaries, although the convective boundaries may make it necessary to take an average value for the concentration in the "plateau" region.

At a rotor temperature of approximately 18°C a new boundary appears on the trailing edge of the 30S boundary. This new boundary can only be explained by a breakdown or structural change in a fraction of the 30S material. This new boundary migrates at 8.5S if the temperature is maintained at 18 to 20°C, although its sedimentation rate will generally vary during the course of a temperature jump experiment. For convenience this breakdown product of the 30S material will be referred to as the "9S" boundary.

As the temperature of the rotor increases further two phenomena occur nearly simultaneously: the concentration of protein under the 30S boundary decreases, and the sedimentation rate of the 30S boundary drops (Fig. 3). These changes are first detectable at a temperature of about 25°C and are nearly complete at about 35°C. The 30S may decrease in height until it disappears completely, or it may leave a residual boundary which sediments at about 5S. Unfortunately a number of problems make it very difficult to measure sedimentation rates accurately during this part of the experiment. Sedimentation rates were measured by movement of the midpoint of the boundaries, and this point must necessarily change as the concentration decreases. Also, rapid sedimentation of microtubules to the bottom appears to increase convective disturbance in the solution. It is therefore very difficult to determine the correlation between the decrease in concentration and the decrease in sedimentation rate. The loss of 30S material correlates well with microtubule assembly and does not occur if microtubule formation is prevented by the addition of Ca, or the removal of Mg, or the lack of GTP. The presence of microtubules in the pellet in experiments in which the 30S decreased has been confirmed by electron microscopy.

Although the loss of protein from the 30S boundary correlates well with microtubule assembly, the decrease in its sedimentation rate does not, and the decrease in sedimentation rate can occur even if no microtubules form. The extent to which the 30S boundary slows down depends, at least in part, upon the concentration of divalent cations. Higher concentrations of Mg or Ca stabilize the boundary at higher temperatures (Figs. 4 and 5), while the removal of all divalent cations by EDTA results in complete breakdown of the rings by about 30°C (Figs. 6 and 7).

Although the 30S boundary undergoes the most pronounced changes in concentration and sedimentation rate during polymerization, the "9S" boundary usually changes in similar ways. This has been demonstrated most clearly by performing a double temperature jump experiment. The temperature was raised until the 9S boundary appeared, and was then held constant at that temperature until the 30S boundary had migrated to the base

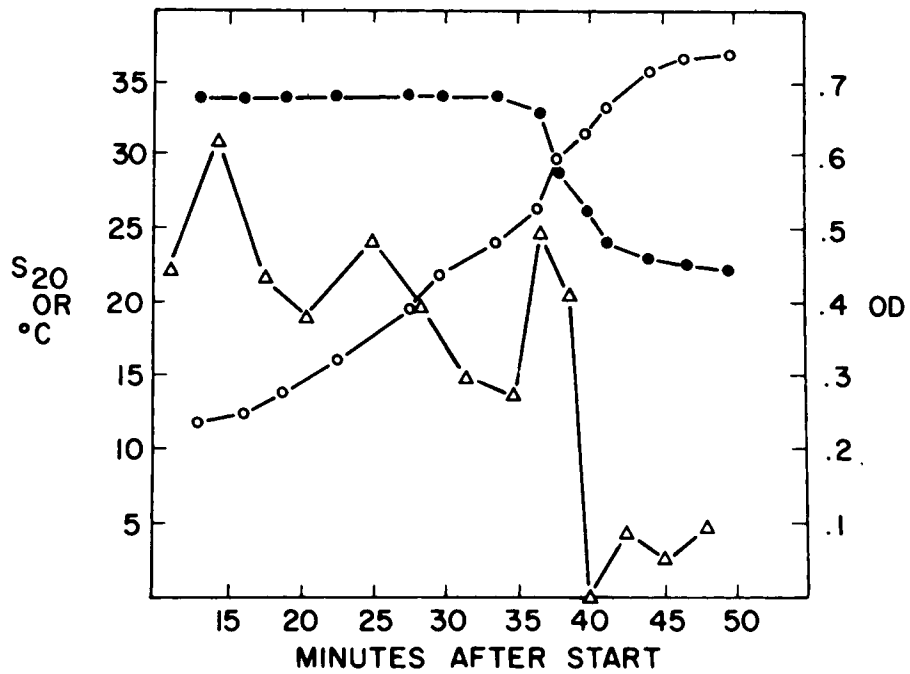


Fig. 3. The changes in sedimentation rate and concentration of the 30S boundary during the experiment shown in Fig. 2. The position of the boundary was measured to the midpoint of the hypersharp region. The concentration was measured 5 mm from the boundary, although greater changes in concentration occur further from the boundary. Filled circles indicate concentration in optical density units at 294 nm. Triangles indicate sedimentation rate corrected to 20°C. Open circles indicate the temperature of the rotor.

of the cell. A second temperature jump was then performed so that the behavior of the 9S material could be followed independently of the 30S. In this experiment the 9S boundary underwent a distinct drop in both concentration and sedimentation rate (Figs. 8 and 9). The 6S boundary occasionally decreases slightly in concentration during a temperature jump, although it has not been possible to correlate this behavior with microtubule formation.

Although in vitro studies of polymerization of brain tubulin may reveal important properties of tubulin, which may be related to the functioning of microtubules in the cell, only studies of the behavior of tubulin from a cell undergoing changes in microtubule organization can ultimately reveal the fundamental control mechanisms. For this reason we have been investigating the behavior of tubulin in a dividing cell, the artificially activated eggs of the surf clam, Spisula solidissima (4, 5). The first question which we have attempted to answer is simply what changes actually occur in the organization of tubulin during the formation of the mitotic spindle. These experiments have been performed by homogenizing eggs at various times after activation in a medium which is known to stabilize microtubules, centrifuging the homogenates, and determining the amount of colchicine binding activity which was extractable from the pellets. These results indicated that a significant fraction of the total tubulin (10 to 15% at 23°C) was aggregated into a particulate form prior to activation of the eggs and the initiation of division. The amount of particulate tubulin in unactivated eggs was in fact nearly the

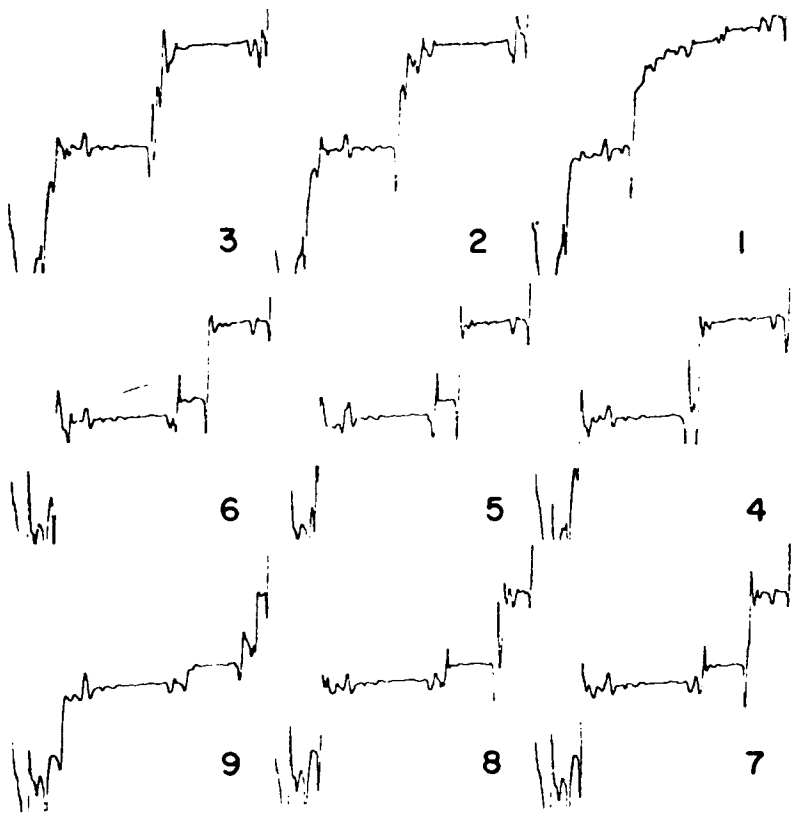


Fig. 4. Scans from a temperature jump experiment in which the solution contained 2 mM $CaCl_2$ to inhibit polymerization. The times of the scans in minutes were: 1, 24; 2, 27.5; 3, 32; 4, 35; 5, 38.5; 6, 40; 7, 43; 8, 45; 9, 46.5.

same as that in the spindle of the metaphase egg. This was a surprising result since the unactivated egg contains no spindle, and few microtubules. Light microscope observations of the unactivated egg homogenates revealed, however, the presence of particles which apparently contain the highly aggregated form of tubulin. These particles generally are composed of a 10–20 micron spherical structure associated with a membranous structure which is probably derived from the egg cortex. Electron microscopy of these particles indicated that they contain relatively few microtubules, particularly when compared to the spindle, but contain large aggregates of approximately 20 nm particles (Fig. 10). Frequently microtubules appear to radiate directly out of these aggregates, suggesting that they may be involved in microtubule assembly.

To study the problem of microtubule and spindle assembly more systematically it is necessary to have an in vitro system which duplicates some of the features of the in vivo process. We have recently been able to obtain such a system using crude homogenates of Spisula eggs in which microtubule assembly results in the formation of structures resembling spindle asters. The extent of polymerization and the organization of the microtubules into asters depends upon the stage of the eggs at the time of homogenization. In homogenates of unactivated eggs few microtubules and no asters are observed after polymerization. However, by 2.5 min after activation distinct aster-like structures are observed.

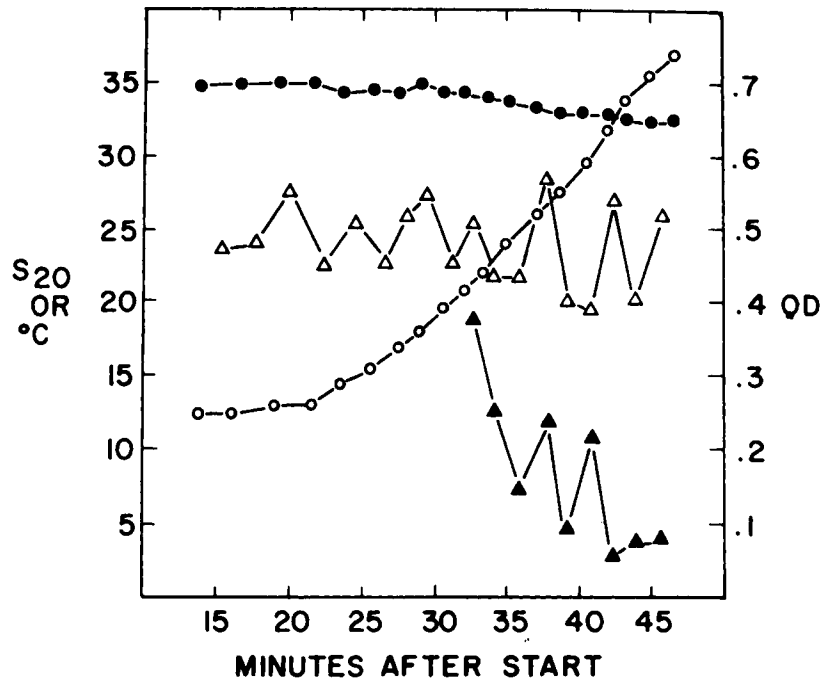


Fig. 5. The results obtained from the experiment of Fig. 4. The filled circles indicate concentration of the 30S boundary. The open triangles indicate sedimentation rate of the 30S. The filled triangles indicate sedimentation rate for the 9S boundary. The open circles indicate temperature.

These consist of relatively few microtubules which radiate directly from a dense hollow cylinder which is probably a centriole precursor (Fig. 11). By 4.5 min after activation the asters formed in vitro have a more developed structure. They now contain a distinct centriole, containing triplet tubules, and microtubules radiate out of a more diffuse granular region surrounding the centriole. In homogenates of metaphase eggs the asters formed in vitro look nearly identical to the asters in the fixed cell. The granular material is much more diffuse, to the extent that it appears to have only a tenuous association with the centriole, and few microtubules appear to associate directly with the centriole. (Fig. 12).

The morphology of the in vitro asters suggests that more tubulin polymerizes in homogenates prepared at later times after activation, and this has been confirmed by measurements of polymerized tubulin at various times (Fig. 13). This increase in the amount of tubulin which polymerizes could be a result of changes in the tubulin subunit itself, of changes in some other cytoplasmic component, or of changes in the microtubule organizing center. Although no definite conclusion can be reached yet, evidence has been obtained which indicates that it is the organizing center which is determining the extent of polymerization. The organizing center can be separated from the nonparticulate components of the homogenate, including tubulin, by centrifuging at low temperatures (15,000 rpm for 15 min in a Sorval SS-34 rotor). The pellet obtained from eggs at metaphase will induce aster formation when remixed with its supernatant, or when mixed with a supernatant obtained from unactivated eggs. A pellet obtained from unactivated eggs will not induce aster formation regardless of the source of the supernatant. It is

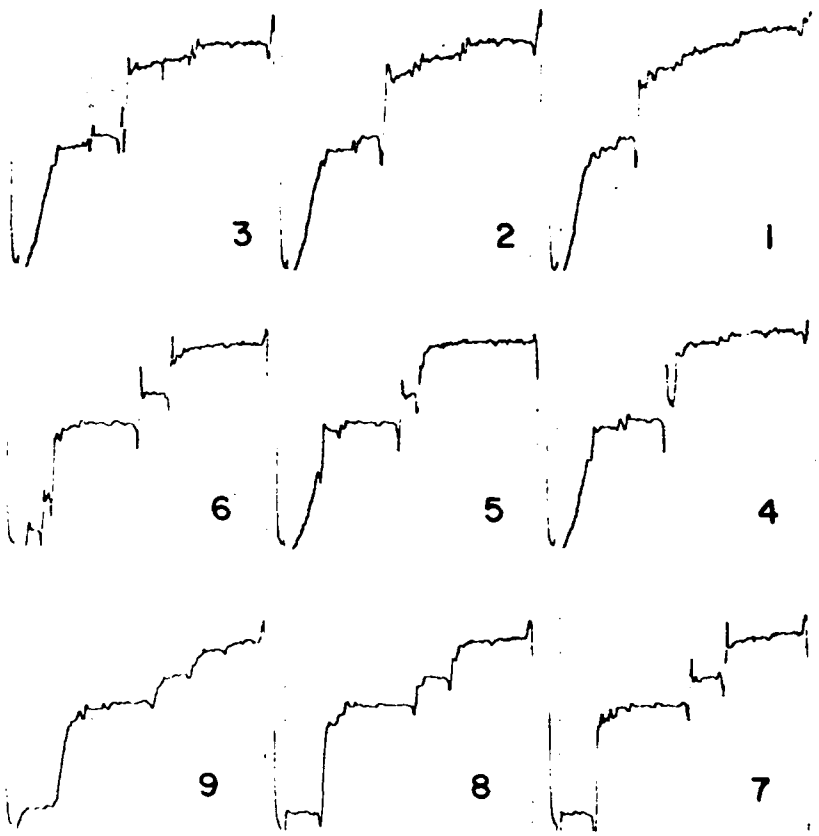


Fig. 6. Scans from a temperature jump experiment in which the solution contained 1 mM EDTA to inhibit polymerization. The times of the scans in minutes were: 1, 21.5; 2, 25; 3, 26.5; 4, 29.5; 5, 33; 6, 36; 7, 41; 8, 44 (the vacuum pump was restarted just prior to this time. Note the loss of the hypersharp spikes on the 9S and 30S boundaries); 9, 46.

clear from this experiment that tubulin from an unactivated egg is capable of polymerizing, and will do so if the organizing center is present.

DISCUSSION

The presence of ring-shaped aggregates of tubulin appears to be functionally related to the ability of tubulin to polymerize. The results which have been obtained are quite complicated, but indicate that microtubules are preferentially formed in the region of the centrifuge cell containing rings. However, the interpretation of this observation is not clear. It is difficult to see how rings could be directly transformed into microtubules by any simple structural modification, nor do the present observations indicate that this is the situation. The other probable explanation is that the rings are composed of tubulin which is in the conformation most able to polymerize into microtubules. Under conditions of polymerization the rings may break down to subunit or small aggregate and these may then form microtubules. The evidence for this model is indirect and is based

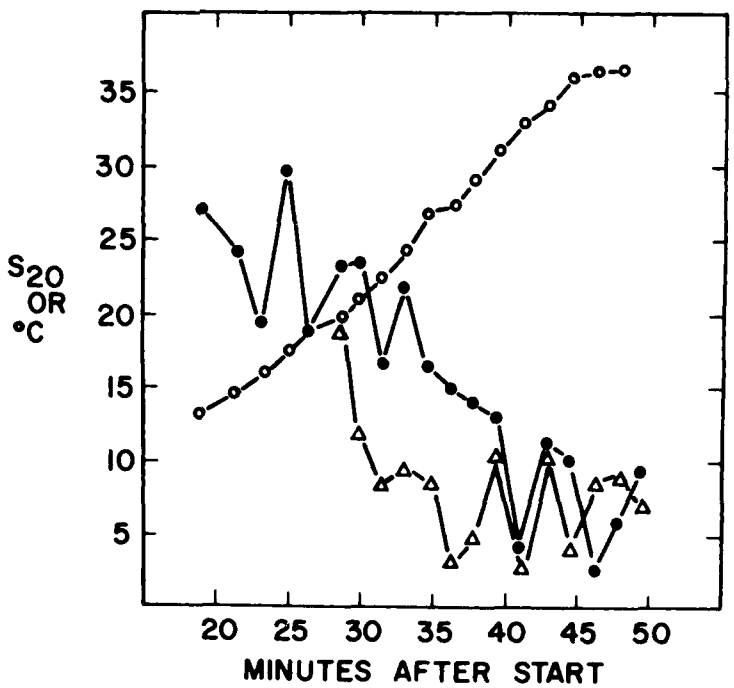


Fig. 7. The results obtained from the experiment of Fig. 6. The filled circles indicate the sedimentation rate of the 30S boundary. The open triangles indicate the sedimentation rate of the 9S boundary. The open circles indicate temperature of the rotor.

upon the behavior of the 30S component during polymerization. In the temperature jump experiment the 30S material decreased in both concentration and sedimentation rate as polymerization occurred. Although some of this decrease in sedimentation rate may be a result of the conditions of centrifugation, it does suggest that rings are not associating directly to form microtubules. Furthermore, it has been demonstrated that the 30S will break down to subunit or small aggregate in the temperature region at which polymerization will occur. It should be pointed out that neither the sedimentation properties of the breakdown products of the 30S nor electron microscopy of solutions of tubulin under similar conditions indicate a simple structural modification of the rings. At polymerizing temperatures, under conditions which prevent microtubule assembly, fewer rings are observed, but we have not been able to identify structures, such as spirals or fibers, which could be interpreted as modifications of the rings. The 9S boundary appears to be formed as a breakdown product of the 30S, and this event occurs at a temperature of about 18°C which is very nearly the temperature at which polymerization can be detected in solution. Although the behavior of the 9S is not as clear as that of the 30S, it also appears to polymerize.

The possible role of high molecular weight aggregates, such as the rings, in the assembly of microtubules, and in particular what function they may have in the living cell is not clear. Nucleation structures are involved in the polymerization of a number of systems, and it is possible that the rings may help to initiate microtubule assembly. However, the number of rings is clearly much greater than the number of microtubules which

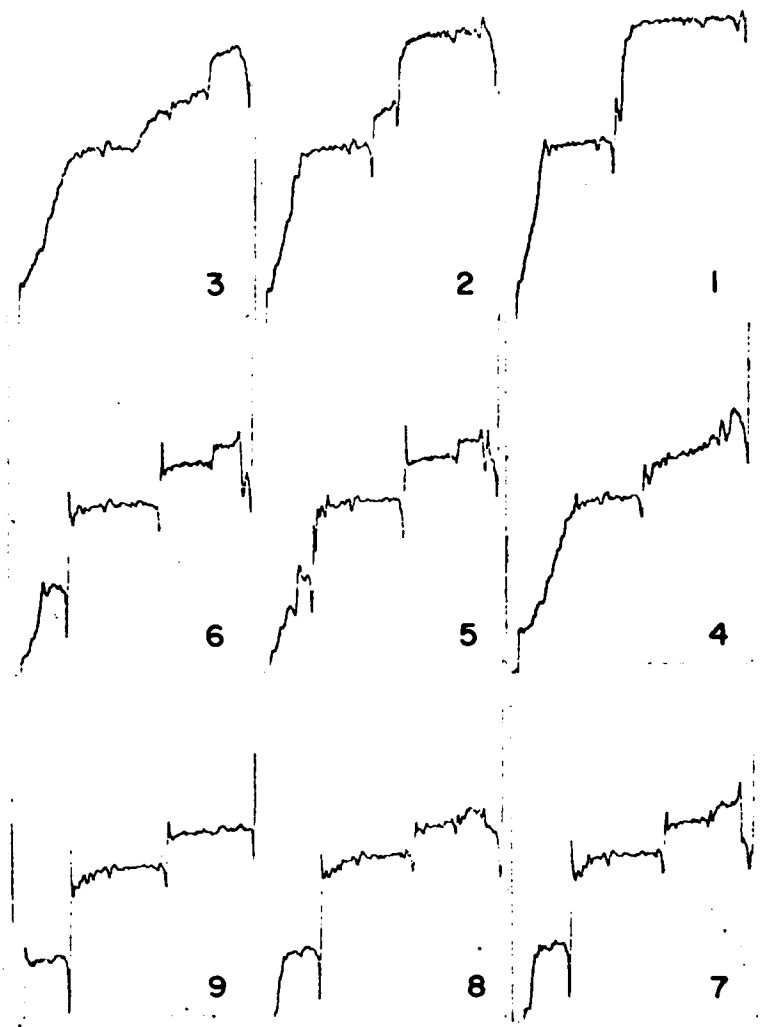


Fig. 8. Scans from a double temperature jump experiment. The temperature of the rotor from scan 1 to scan 4 was maintained at 19°C, and was then raised by letting some air into the vacuum chamber. The times of the scans in minutes were: 1, 29.5; 2, 34; 3, 44; 4, 47.5; 5, 52; 6, 55; 7, 58.5; 8, 60; 9, 65.

form, and it is likely that they have some other role in assembly.

In many cellular systems microtubules form in specific regions of the cell and the microtubules may have closely defined lengths and distributions. Some of this behavior can no doubt be explained by the functioning of microtubule organizing or nucleating centers but some of it may require further explanation. Such a conclusion was reached by Tucker (6) from studies of microtubule organelles in the ciliate Nassula. Tucker concluded that the morphological events which occur in Nassula "cannot be entirely accounted for by the interaction of randomly diffusing molecules with a set of nucleating sites and mature and growing microtubular organelles." A possible solution to some of the problems

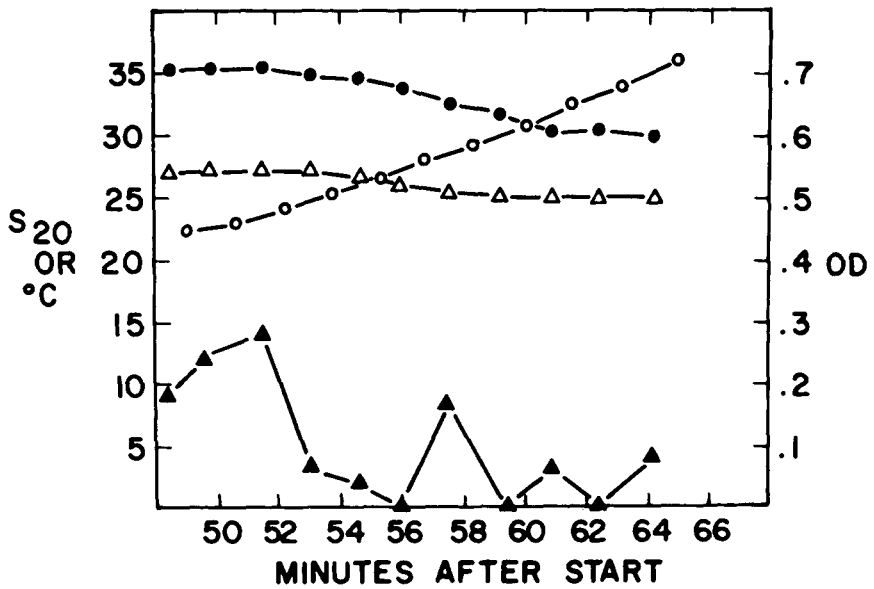


Fig. 9. The results obtained from the experiment of Fig. 8. The filled circles indicate the optical density of the 9S boundary. The open triangles indicate the optical density of the 6S boundary. The filled triangles indicate the sedimentation rate of the 9S boundary. The open circles indicate temperature.

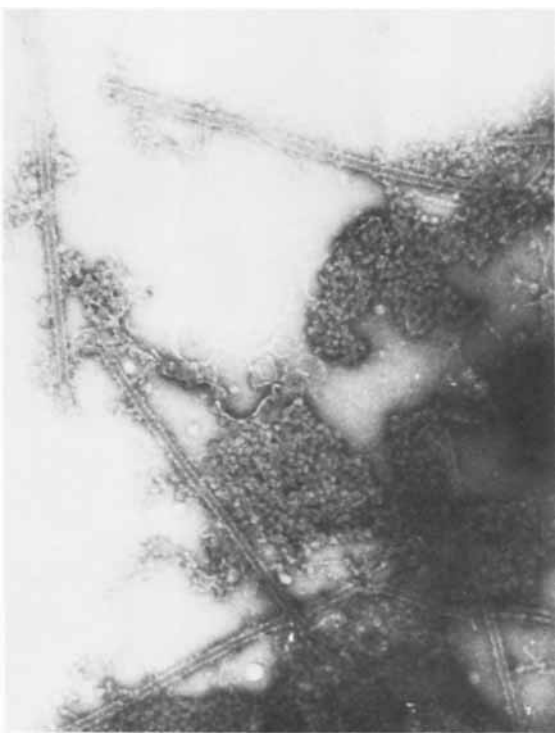


Fig. 10. Electron micrograph of a region of a tubulin containing structure obtained from unactivated Spisula eggs. Negatively stained with uranyl acetate. Magnification about 100,000.

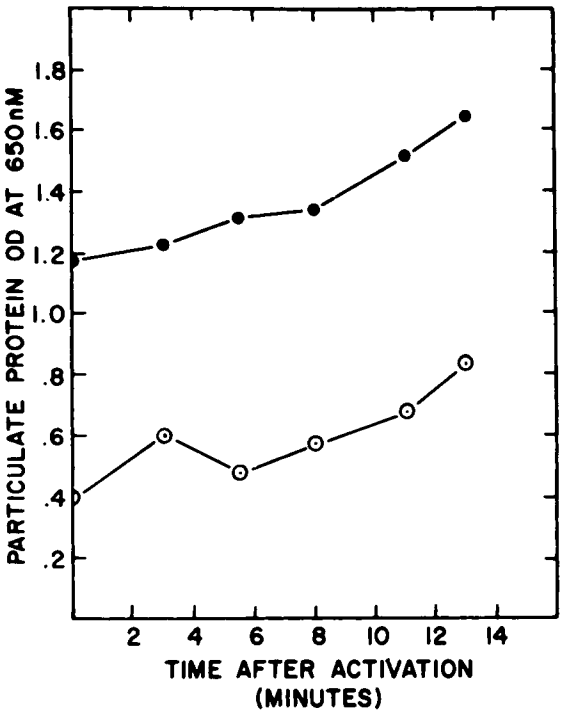


Fig. 13. Changes in the amount of protein which polymerizes in vitro in homogenates prepared at various times after activation. The homogenates were polymerized 15 min at 28°C and were then diluted in 10 volumes of HGL solution to stabilize the microtubules. They were then centrifuged at 2,500 rpm in a Sorval GLC-1 for 15 min (open circles) or at 20,000 rpm in a Sorval RC2-B for 15 min (filled circles). The pellets were extracted with 0.01 sodium phosphate at pH 7 and after re-centrifuging the amount of extracted protein was determined by the Lowry procedure.

of microtubule assembly could be obtained by the existence of a localized aggregate of tubulin which could serve as a nondiffusing pool of active tubulin subunits. In a dividing cell, for example, such an aggregate could provide a locally high concentration of tubulin in the region of the centriole, which could enhance the formation of spindle microtubules as opposed to cytoplasmic nonspindle microtubules.

The existence of such an aggregate is suggested by the experiments performed on dividing surf clam eggs. In these cells there does indeed appear to be a nonmicrotubular aggregate of tubulin, and upon fertilization, or parthenogenic activation, of the eggs this aggregate appears to break down and may be incorporated into spindle microtubules. Breakdown of this structure occurs just prior to, or possibly simultaneously with meiotic spindle formation, and the amount of tubulin in this aggregate appears to be nearly the same as the amount of tubulin which eventually appears in the spindle. Experiments by Stephens (7) on sea urchin eggs have demonstrated a phenomenon which may be related to the above. He has found that the apparent "pool size" of tubulin subunits which will form microtubules is determined by the temperature of the eggs at early prophase, prior to spindle formation, and is independent of protein synthesis. The pool size could represent the amount of tubulin in a storage aggregate which eventually provides the tubulin which incorporates into the spindle. Another possibility is that the

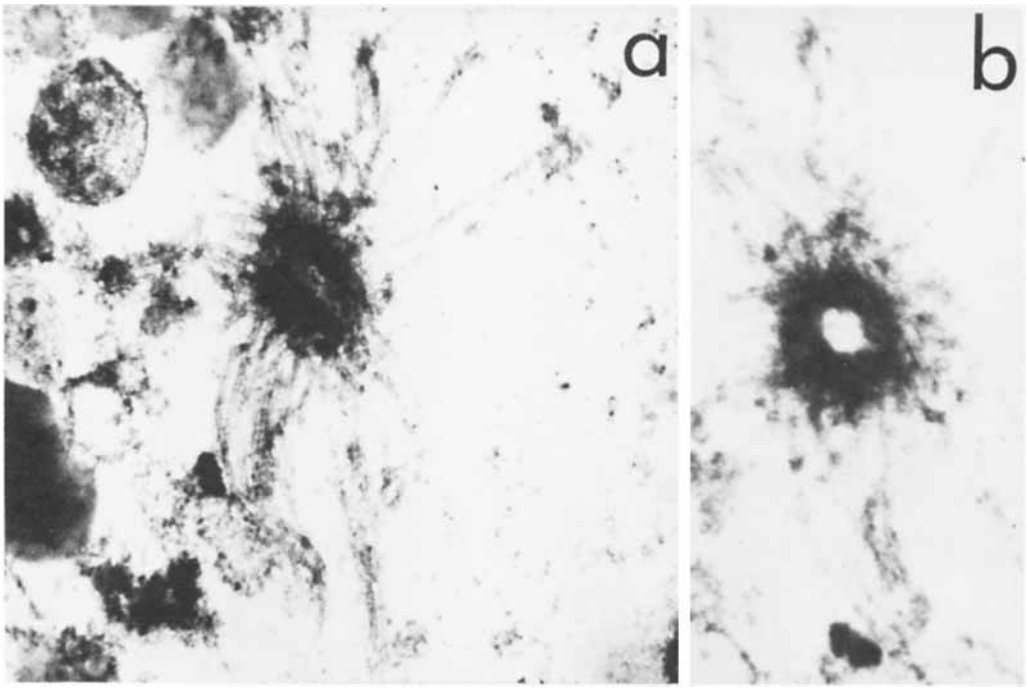
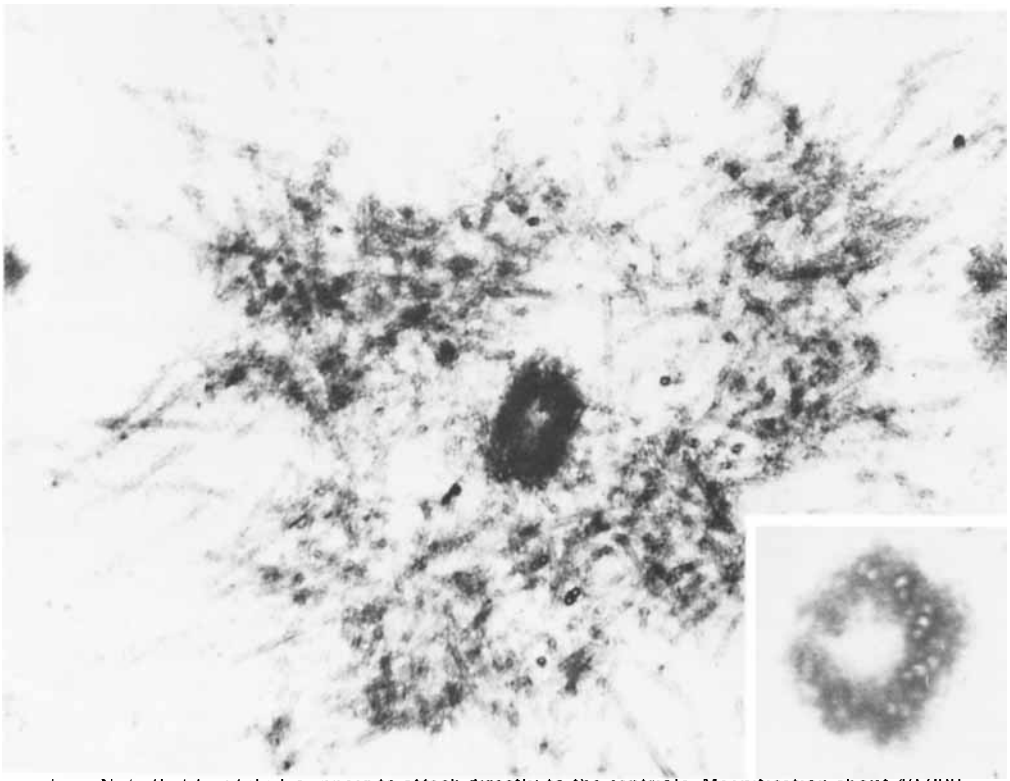


Fig. 11. Electron micrograph of an aster formed in vitro from eggs homogenized 2.5 min after activation. (A) A number of tubules radiating out of a dark central structure. (B) Same as (A) but the section was tilted to show the structure of the center more clearly. Magnification of (A) is about 40,000, and (B) is about 55,000.



tion. Note that few tubules appear to attach directly to the centriole. Magnification about 40,000. Insert: the appearance of the centriole after tilting the section so that it is observed end on. Magnification about 85,000.

apparent pool size is determined by the functioning of the organizing center rather than by the activity of the tubulin. This possibility is raised by the observations on in vitro aster formation in Spisula and the effect of the organizing center on the ability of tubulin to polymerize. It is also difficult to see at this time how the ring aggregates formed by purified brain tubulin relate to any of these problems, but they do suggest a number of questions which only future research can answer.

ACKNOWLEDGMENTS

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