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MICROTUBULE ASSEMBLY: Some Possible Regulatory Mechanisms

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Microtubule polymerization in vitro was examined using material purified from porcine brain tissue by a reversible temperature dependent assembly procedure, and was characterized by electron microscopy, viscometry, and sedimentation. The reaction was endothermic, colchicine sensitive, and occurred at neutral pH and moderate ionic strength. Divalent cations (calcium, magnesium) were inhibitory at millimolar concentrations, but stimulated polymerization at the micromolar level. Nucleoside triphosphates were required for assembly of purified subunits. As determined by quantitative sedimentation analyses, the reaction was an equilibrium process. Below a critical concentration of tubulin no assembly occurred. Analytical ultracentrifugation studies indicated that tubulin species with $s^{\circ}_{20,w}$ of 6S and 30S were in equilibrium with each other, and that both were incorporated into microtubules. Electron microscopic analyses suggested that disc (or ring) structures might be intermediates in assembly, and that they were primarily utilized early in the polymerization process. Assembly could be seeded by mixing microtubular fragments from brain or flagella with brain microtubule subunits; depending on conditions of temperature and protein concentration, addition of subunits occurred either with unipolar or biased polar directionality. The possible significance of these properties of the polymerization reaction for control of assembly is discussed.

INTRODUCTION

Microtubules serve essential functions in the maintenance of cell form and in the process of cell division (see 1, 2, 3 for reviews). As originally noted by Porter (4), the appearance of microtubules apparently follows a spatial and temporal program, both in developing cells and throughout the cell cycle. Since the assembly of the mitotic spindle and other microtubule-containing organelles such as cilia and flagella can occur without concomitant protein synthesis, it is evident that the principal control of microtubule formation operates at the level of polymerization from pre-existing subunits. Therefore, explanations are required for the mechanisms that determine the initiation, growth, directionality, and spatial localization of these structures. To experimentally analyze these problems, attempts have been made to establish polymerization systems in vitro in which the molecular components regulating microtubule assembly might be identified. Because of the biochemical nature of this problem, brain tissue, which contains abundant microtubule protein, was chosen as the experimental material. Comparative biochemistry has shown that tubulin from diverse cell types has similar properties (see 3, 5, 6 for reviews): therefore, studies on brain tubulin may well be applicable to cytoplasmic microtubules in general.

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The purpose of this report is to describe further the characteristics of microtubule polymerization in vitro, and in particular, to attempt to identify some of the factors which might be involved in the general regulation of the assembly process. In these studies, the following points have been specifically considered: 1) how do environmental conditions, such as ionic strength and pH, affect polymerization; 2) do divalent cations inhibit or stimulate assembly; 3) what role do nucleotides have in polymerization; 4) is tubule assembly in vitro an equilibrium process; 5) are intermediates involved in the initiation and growth of tubules; and 6) is the growth of tubules directionally determined.

MATERIALS AND METHODS

Preparation of Microtubule Protein

Purified microtubule protein was prepared from fresh porcine brain tissue using minor modifications of the reversible temperature dependent assembly scheme described previously (7). For the preparation of extracts, brain cortex (g wet weight) was homogenized at 0°C with 1.5 volumes (ml) of P or PE buffer* (0.1 M PIPES or 0.1 M PIPES containing 1.0 mM EGTA, pH 6.94 at 23°C) supplemented with 0.1 mM GTP. Following an initial centrifugation to remove large cellular debris (15,000 × g for 15 min), the supernatant was decanted, made to a final GTP concentration of 1.0 mM, and then centrifuged at 4°C at 25,000 × g for 45 min to obtain the supernatant (extract). Addition of GTP after the initial pre-spin to a final concentration of 1.0 mM gave polymerization similar to that obtained in the previous extract preparations made with 2.5 mM GTP (8); however, this modification allowed the use of a lower total amount of nucleotide for the preparative procedure. Similarly, omission of EGTA from the homogenization buffer did not affect the extent of polymerization.

Microtubule protein in the extracts was purified by successive cycles of assembly and disassembly. One cycle consisted of: a) polymerization at 37°C; b) centrifugation at 37°C [to obtain supernatant (HS) and pellet (HP) fractions]; c) resuspension of pelleted microtubules and depolymerization at 0°C; and d) centrifugation at 0°C [to obtain supernatant (CS) and pellet (CP), fractions] (7). In the experiments reported here, the resuspension buffer was generally P or PE solution containing 1.0 mM GTP, and experimental material was obtained by a minimum of two cycles of purification (subscript denotes number of cycles). For storage, pelleted protein (H_2P) was frozen in liquid nitrogen and placed at −80°C; no loss of activity was observed after storage for periods of up to one month. Experimental material was obtained by resuspension of the pellets immediately before use to the desired protein concentration (usually 3 to 10 mg/ml), followed by depolymerization and centrifugation at 0°C (C_2S fraction).

Gel Electrophoretic Analysis

Samples and gels (7.5% acrylamide, 0.6 mm × 8.0 cm) were prepared and run

*Abbreviations used are: PIPES, piperazine-N-N′-bis (2 ethane sulfonic acid); EGTA, ethylene glycol-bis-(β-aminoethylether) tetraacetic acid; GMPPCP, β, γ, methylene guanosine triphosphate; AMPPNP, adenylyl imidodiphosphate; P buffer, 0.1 M PIPES, adjusted to pH 6.94 at 23°C; PE buffer, P buffer containing 1.0 mM EGTA; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl-.

according to the methods of Shapiro et al. (9). Gels were stained for protein with Coomassie brilliant blue, and the areas under the stained peaks quantitated by densitometry and planimetry.

Assay Procedures for Microtubule Polymerization

Polymerization of microtubules was induced by elevating the temperature of extracts or purified material from 0°C to 37°C, and was measured either by viscometry or sedimentation. Viscometric determinations were made as described previously (8) and used to obtain rate and extent data. The extent of polymerization was also assayed by a quantitative sedimentation method (K. Johnson, unpublished results). For this procedure, 0.5 ml samples of purified protein were incubated at 37°C for 30 min. The incubated samples were then centrifuged at 28,000 × g for 20 min at 37°C, and the protein concentration of the resuspended pellet and the supernatant fractions determined by the method of Lowry et al. (10), using bovine serum albumin as a standard.

Analytical Ultracentrifugation

Purified microtubule protein was analyzed by sedimentation velocity using a Spinco Model E analytical ultracentrifuge equipped with Schlieren optics. $s^{\circ}_{20,w}$ values were determined for samples sedimented in 12 or 30 mm double sector cells: $s^{\circ}_{20,w}$ values were obtained by extrapolation of the sedimentation coefficients to infinite dilution. The mass fraction of each sedimenting component was determined by planimetry of tracings derived from photographs taken at 2 or 4 min intervals during the run. For some studies, varying amounts of mineral oil were layered over the samples in the cells so that at a constant speed a range of pressure values was obtained (see 11 for methods and theory).

Electron Microscopy

Samples for electron microscopy were prepared by negative staining. A 5 μl sample was placed on a 100 or 400 mesh carbonized formvar-coated grid for 30 sec and then displaced successively with 3 to 4 drops of each of the following solutions: 1 mg/ml aqueous cytochrome c, water, 1% aqueous uranyl acetate. Excess stain was withdrawn from the side of the grid with filter paper, and the sample air-dried. For quantitative studies, the number of particles per 400 mesh grid square was counted by making uniform scans at a total magnification of 350,000 (instrument magnification of 35,000 viewed through a 10 × binocular). Microtubule length was measured at 35,000 (instrument magnification) relative to a 4 cm diameter circle inscribed on the viewing screen.

DEAE Dextran Decoration of Tubule Fragments

Purified tubulin (C_2S, 4.0 mg/ml) resuspended in 0.05 M PIPES, 2.5 mM GTP, pH 6.94, was incubated at 37°C for 20 min. The polymerized material was then shifted to room temperature and mixed with an equal volume of 0.7 mg/ml diethylaminoethyl (DEAE) dextran (MW 2×10^6; Sigma Chemical Co.) in the same buffer. After 5 min at room temperature, the mixture was centrifuged at 39,000 × g for 30 min at 25°C. The resultant pellet was resuspended with a volume of 0.05 M PIPES, 2.5 mM GTP, pH 6.94,

equal to one-half the volume of the incubated mixture. The resuspension was sheared by forcible passage 10 times through a 4 inch, 22 gauge hypodermic needle to obtain fragments of DEAE decorated tubules less than 2 μ long.

Isolation of *Chlamydomonas* Axonemes

The isolation of *Chlaymdomonas* axonemes and details for the experiments on the polarity of tubule addition are described elsewhere (7, 12; Allen and Borisy, manuscript in press).

RESULTS

Purification of Tubulin

To isolate tubulin for characterization studies, a purification scheme based on the specific association of microtubule subunits was developed (7). As shown in Fig. 1, tubulin was a predominant component in the brain extract, and as determined by quantitative gel analysis, it represented approximately 25% of the total supernatant protein. During fractionation, the tubulin was enriched, and after two cycles of purification, a final preparation (C_2S) was obtained which contained two major components: 79% of this material was tubulin, and 17% was confined to bands of high molecular weight. Further analysis of the purified material on 5% gels indicated that the high molecular weight component could be separated into four bands; two of these had mobilities similar to those of dynein (13), and the other two had mobilities corresponding to molecular weights close to 300,000 (7, and unpublished results).

The procedure described here for tubulin purification differs from that developed by Shelanski et al. (14) in that no glycerol was employed to augment polymerization or to stabilize the tubules. In addition,the tubules remained cold-labile throughout all procedures, and at high protein concentration, greater than 95% of the isolated protein was competent to polymerize (see subsequent results). However, the procedures also differed with respect to the association of high molecular weight material with the purified tubulin. In our preparations, the high molecular weight material was enriched in the initial step, and thereafter the relative proportions of these components to tubulin were not altered through six cycles of polymerization. Using the glycerol isolation, Shelanski et al. (14) reported 95% purity of tubulin and no other discrete components in the remaining 5%. Since both of these preparations are competent to form tubules, one might conclude that the high molecular weight components are not absolutely required for tubule assembly. Although the copurification of these proteins with tubulin suggests that they are specifically associated with microtubules, it is not yet known if these components might have a role in microtubule formation.

Properties of Microtubule Assembly in Extracts and Purified Material.

In order to optimize polymerization in vitro, the conditions required for microtubule assembly were first explored in extracts (8). Similar investigations were also performed on the purified material to determine: 1) whether the purified material retained the properties of colchicine and temperature sensitivity typical of both mitotic spindle forma-

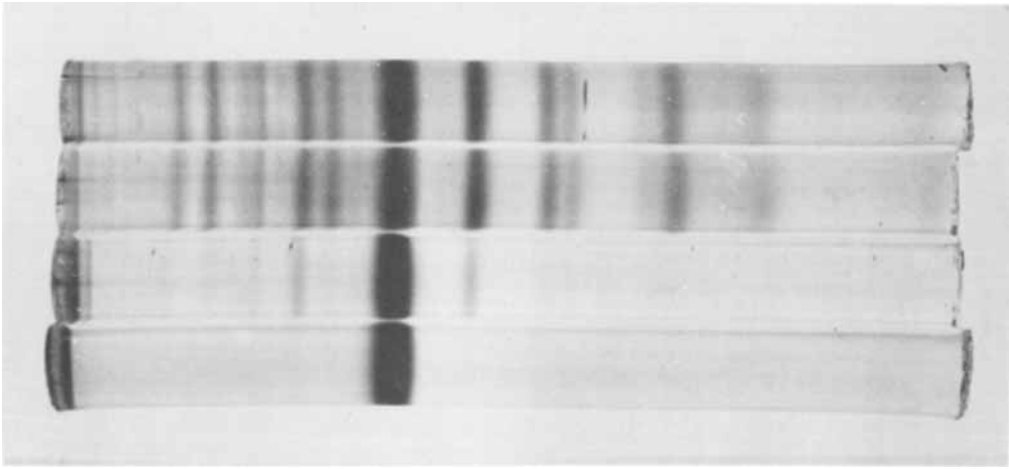

Fig. 1. Acrylamide gels of purification fractions. Extracts were prepared in PE containing 2.5 mM GTP and purification carried out as described in the text. The gels represent the following fractions (top to bottom): extract, H_1 S, H_1 P, C_2 S. The dense band is the microtubule protein.

tion in vivo (15) and microtubule assembly in extracts (8); and 2) if there were changes in the polymerization characteristics of tubulin upon purification which might reflect elimination or alteration of factors important in the cellular regulation of tubule assembly. As summarized in Fig. 2, the properties of microtubule polymerization in these two types of preparations were similar, and in both instances, the reaction occurred at neutral pH, moderate ionic strength, and was both endothermic and colchicine sensitive. However, some differences were observed between microtubule formation in cellular extracts and purified preparations with respect to the nucleotide requirements and protein concentration dependence of the reaction.

Guanosine nucleotide is generally associated with purified tubulin (16–19) and the presence of GTP has been postulated to be important in stabilizing the protein (20–22). Therefore, in characterizing the conditions for assembly in extracts, the role of nucleotides in polymerization was investigated. Although a low level of polymerization occurred in the absence of added nucleotide, GTP substantially augmented the reaction, and to a greater degree than other nucleoside triphosphates or nucleotides (8). However, the absolute requirement of GTP in this reaction could not be determined due to the unknown levels of nucleotides and hydrolase activity in the extracts. With the purified material, no polymerization occurred in the absence of a nucleoside triphosphate (7) and a nonhydrolyzable analog of GTP, β, γ, methylene guanosine triphosphate (GMPPCP), competitively inhibited GTP-dependent polymerization. These data suggested that hydrolysis was associated with the formation of microtubules. However, the extent of polymerization was essentially equivalent in the presence of any nucleoside triphosphate (7), and therefore the greater specificity of tubule assembly for GTP indicated from the experiments on the extracts was not observed in the purified preparations.

It has been suggested from studies on actin that nucleotides may act as kinetic regulators of assembly, since ATP accelerates actin polymerization, and the G to F transformation occurs with the hydrolysis of equimolar concentrations of ATP [see Oosawa and Kasai (23) for review]. However, recent experiments have indicated that this

Properties of Microtubule Assembly

	Extract	Purified
pH	6.9	6.9
GTP	2.5 mM	1.0 mM
Protein concentration	>4.0 mg/ml	>0.2 mg/ml*
Colchicine	inhibits (>1 μM)	inhibits (>1 μM)
Calcium; Magnesium†	inhibits (>1mM);(>10mM)	inhibits (>2mM);(>20mM)
Sodium ; Potassium†	inhibits (>150mM)	inhibits (>150mM)

Fig. 2. Summary table of the properties of microtubule assembly. Data from extracts were derived from experiments described elsewhere (8); similar viscometric assays were used to determine characteristics of polymerization in purified material (C_2S) which had been made in PE containing 1.0 mM GTP. The tubulin content of both types of preparations was 3.0 to 4.0 mg/ml. *Data obtained from sedimentation assay using protein prepared in P buffer containing 0.5 mM GTP and 0.1 mM $MgCl_2$ (see text and Fig. 4). †All ion concentrations represent total concentrations added to the respective types of material; for the experiments on divalent cations, no EGTA was present.

hydrolysis may not be causally related to the rate phenomenon since a nonhydrolyzable analog of ATP, adenylyl imidodiphosphate (AMPPNP), will also promote rapid polymerization of actin (24). Similar studies on the role of nucleotide binding and hydrolysis in the formation of microtubules should indicate whether these processes have a regulatory function in microtubule polymerization.

In both extracts and purified material, a minimum critical concentration of protein was required for assembly. In extracts, there was no viscosity increase at concentrations of protein lower than 4.0 mg/ml (8) and, on the basis of electrophoretic analysis, this corresponded to a tubulin concentration of 1.0 mg/ml. In purified preparations, critical concentrations of 1.0 mg/ml (viscosity assay) and 0.6 mg/ml (sedimentation assay) were obtained; however, under other solution conditions (see subsequent results) critical concentrations as low as 0.2 mg/ml were observed. Therefore, although it was established that there was a critical concentration required for microtubule polymerization in both extracts and purified material, determination of the absolute value for these two types of preparations depended on the assay and solution conditions.

The extent of polymerization was also examined in the two types of preparations and found to differ at a given protein concentration. In extracts prepared in PE solution containing 1 mM GTP, between 20 and 30% of the tubulin polymerized, whereas under the same conditions and at equivalent tubulin concentrations (3.0 mg/ml), 75% of the purified material was assembled. It is possible that this disparity in extent of polymerization might reflect the presence of inactive tubulin in the extract; as this protein was eliminated during the course of purification, a greater amount of polymerization would occur at the same total tubulin concentration.

Effect of Divalent Cations on Microtubule Polymerization

The initial observations on the polymerization of microtubules in vitro suggested that low concentrations (6 μM) of calcium ion inhibited assembly, and that this ion might be important in the regulation of microtubule formation in vivo (25). Therefore, the possible role of divalent cations in the polymerization of purified material was

examined. In our experiments on extracts, added concentrations of calcium greater than 1 mM were required to completely inhibit polymerization (8); at tubulin concentrations equivalent to those in the extract (3.0 mg/ml), similar concentrations (2 mM) were inhibitory in purified preparations (data not shown). If a strong calcium chelator, EGTA, was added over a concentration range of 10^{-7} to 10^{-3} molar, neither the rate nor extent of polymerization of purified material was affected. Therefore, in contrast to the previously reported calculations (25), these results indicated that micromolar concentrations of calcium were probably not involved in the inhibition of tubule assembly. The inhibition at millimolar levels of calcium is of uncertain physiological significance since these concentrations are three orders of magnitude higher than those observed in the regulation of other cellular processes such as muscle contraction (26).

Similar experiments were carried out to determine the effect of magnesium ion on polymerization. Magnesium was totally inhibitory at concentrations greater than 20 mM; however, at lower concentrations (0.1 to 1.0 mM) added magnesium markedly increased the rate of polymerization (data not shown). EDTA, which strongly chelates both Mg^{2+} and Ca^{2+} ions, inhibited polymerization half-maximally at 0.3 mM and totally at concentrations greater than 1.0 mM. Since removal of calcium by EGTA had had no effect on polymerization, it could be inferred from these experiments that magnesium not only increased the rate of polymerization, but was absolutely required for the reaction. However, to test whether the effect of EDTA was due to the chelation of divalent cations rather than to other effects on the protein, the reversibility of inhibition was determined. Protein with an inhibitory concentration of EDTA was incubated with various concentrations of either magnesium or calcium and, as shown in Fig. 3, these experiments demonstrated that the divalent cations could overcome the inhibitory effect of EDTA. Both magnesium and calcium stimulated polymerization at low added concentrations (0.1 mM for Mg^{2+}; 0.8 mM for Ca^{2+}) to an extent equivalent to that of a sample which had been incubated in the absence of EDTA. In addition, at concentrations of added ion in excess of the EDTA, the inhibition profile for each ion was observed. Based on the stability constants of the metal–EDTA complexes [at pH 7.0, $K = 2.52 \times 10^5\ M^{-1}$ for magnesium, $K = 2.0 \times 10^7\ M^{-1}$ for calcium (27)], the calculated free concentrations which would cause maximal stimulation of polymerization were less than one micromolar for either ion.

The marked effect of EDTA but not EGTA in inhibiting polymerization as well as the stimulation of rate on the addition of magnesium suggest that magnesium is required for polymerization, and may be bound to the isolated subunits. In contrast, calcium may be able to substitute for magnesium, but its effect is masked in the presence of the other ion. In the in vitro assembly of actin [see Oosawa and Kasai (23) for review], divalent cations (Mg^{2+}, Ca^{2+}) have been demonstrated to complex with G-actin in molar ratios, and magnesium has been shown to promote assembly to a greater degree than calcium. It is thought that the function of divalent cations in actin polymerization may be involved with the binding and splitting of the adenosine nucleotide. Since it appears that nucleotide hydrolysis may also be associated with tubulin polymerization, magnesium (or calcium) complexed with the protein and/or nucleotide might similarly facilitate this reaction.

Equilibrium Polymerization of Microtubules

The fibers of the mitotic apparatus have been described as existing in a dynamic equilibrium with monomers (15), and by assuming that the birefringence of the spindle was a measure of the extent of polymerization, the thermodynamic parameters for this assembly reaction have been determined (28, 29). Since the majority of the birefringence in the mitotic spindle is thought to be due to the ordered arrangement of microtubules

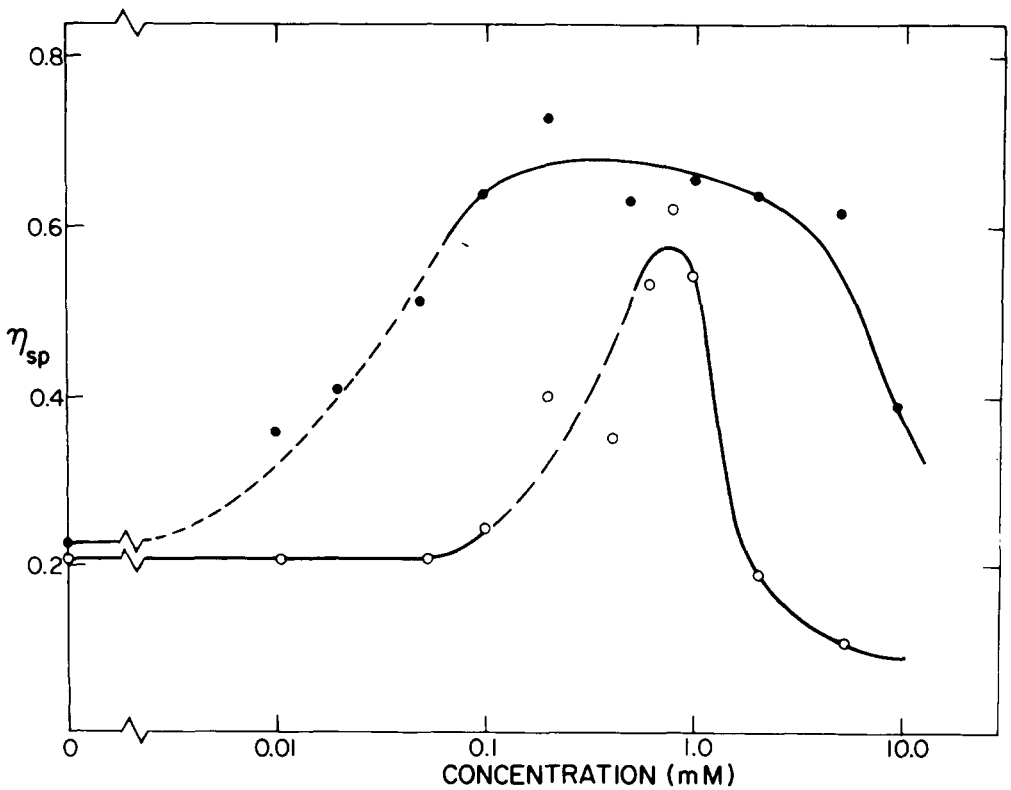

Fig. 3. Viscometric assay of the effect of divalent cations on polymerization in the presence of EDTA. Purified protein (C_2S, 3.2 mg/ml) was prepared in P solution containing 1.0 mM GTP. The samples were then made to 0.9 mM EDTA, and polymerization followed at 37°C in the presence of various added concentrations of $MgCl_2$ (●) or $CaCl_2$ (○) for a total of 40 min. η_{sp} represents the maximum viscosity level reached; points connected by dashed lines indicate reactions in which the maximum rate had been achieved, but in which a plateau was not attained during the course of the experiment.

(15, 30), it was pertinent to examine whether microtubule assembly in vitro was an equilibrium reaction. For these experiments, the extent of polymerization was assayed by centrifugation: tubulin was polymerized, microtubules quantitatively sedimented, and the protein concentration of the pellet (polymer) and supernatant (monomer) fractions analyzed (K. Johnson, unpublished results). Since under some conditions, tubulin is known to aggregate irreversibly into nonmicrotubular structures (31), and since the presence of such aggregates would introduce error into measurements of tubule formation, three criteria were used to evaluate the sedimentation assay: electron microscopy, sensitivity to colchicine, and reversal of polymerization at low temperature. In the presence of inhibitory concentrations of colchicine (100 μM), greater than 95% of the protein incubated was recovered in the supernatant fraction. Similarly, if pelleted material was resuspended and then depolymerized and centrifuged at 0°C, 97% of the protein remained in the cold supernatant.

The protein concentration dependence of polymerization was investigated using the sedimentation assay, and results from one of these experiments are shown in Fig. 4. At total protein concentrations less than 0.2 mg/ml, there was no polymerization,

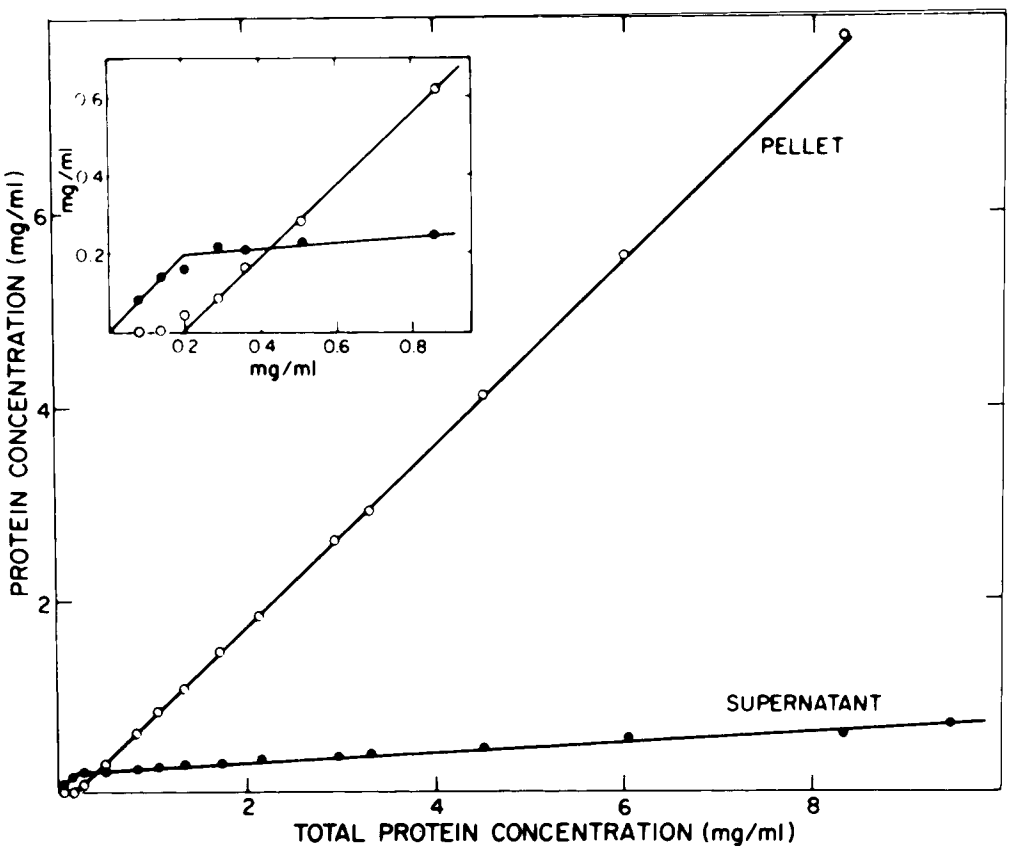

Fig. 4. Sedimentation analysis of the dependence of polymerization on protein concentration. Purified microtubule protein (C_3 S) was prepared in P solution containing 0.5 mM GTP, 0.1 mM $MgCl_2$; immediately before incubation, aliquots were diluted to obtain a range of final protein concentrations. The samples were incubated, centrifuged, and assayed for protein as described in Materials and Methods (sedimentation assay). Ordinate: protein concentration in pellet (○) and supernatant (●). Abscissa: total protein concentration of the incubated aliquot. The insert is an expanded version of the same data at the lower protein concentrations.

whereas above this value, the extent of polymerization (pellet concentration) was proportional to total protein concentration; at 1.0 mg/ml, 75% of the total protein was pelleted, and at 9.0 mg/ml, 94% was polymer. Conversely, the supernatant protein concentration was equal to the total concentration at values less than 0.2 mg/ml; however, at this or higher total concentrations of protein, the supernatant remained nearly constant (4.5% linear increase) at approximately 0.2 mg/ml. This distribution of monomer and polymer was identical if the tubulin was first polymerized and then diluted to various protein concentrations. Since the amount of monomer and polymer obtained at each protein concentration was the same regardless of whether the final state was approached through the polymerization or depolymerization of the tubules, it was concluded that microtubule assembly in vitro was a true equilibrium reaction.

The results obtained on the concentration dependence of tubule assembly are comparable to those derived from studies on the in vitro formation of actin (see 23 for dis-

cussion). For G to F actin transformation, there is an abrupt transition at a critical total protein concentration; below this value, no assembly occurs, whereas at higher concentrations, the amount of monomer remains constant at the critical concentration as polymer increases with the total concentration. This phenomenon has been interpreted as analogous to a phase transition in which all monomer in excess of the critical concentration is incorporated into polymer. In addition, it has been postulated that the critical concentration arises from the cooperative association of 3 to 4 actin subunits, and that this interaction is required for the initiation of assembly. Investigations of the solution conditions under which critical concentrations for tubule formation are shifted may be pertinent in determining factors important in the regulation of microtubule assembly. The following section describes evidence for the presence of multiple oligomeric species of tubulin, and it is possible that the critical concentration required for polymerization is related to the formation of one of these components.

Components in Tubulin Preparations

Purified microtubule protein preparations were analyzed by analytical ultracentrifugation to determine whether there might be intermediates involved in the assembly process. As shown in Fig. 5, samples of purified material were composed of two major sedimenting species; one had an $s^{\circ}_{20,w}$ of 6S and corresponded to the 6S colchicine-binding moiety previously defined as the dimeric subunit of microtubules (20), whereas the other had an $s^{\circ}_{20,w}$ of 30S. Above pH 6.9, an additional peak with $s^{0}_{20,w}$ of 20S was also observed. Using samples at the same protein concentration, the relative proportions of the 6S and 30S species were investigated under conditions where polymerization had been prevented by various treatments (Fig. 5). When assembly was inhibited either by keeping the protein at 0°C or by substituting GMPPCP for GTP, the relative percentage of the two species remained the same. Therefore, although the presence of a hydrolyzable nucleotide was required for polymerization, it was not necessary for the existence of the 30S component. In the presence of 2 mM $CaCl_2$, similar results were obtained except that the S value of the faster sedimenting component increased slightly. In contrast, incubation at elevated temperatures in the presence of inhibitory concentrations of colchicine (100 μM) resulted in the disappearance of the 30S component and a corresponding increase in the 6S fraction. Therefore, colchicine not only prevented polymerization but also caused the disruption of the 30S structure.

Analysis of polymerized material indicated that both 6S and 30S species were depleted after the formation of tubules, and upon depolymerization at 0°C, both components reappeared at the initial levels. Since these data and the fact that greater than 95% of the tubulin could polymerize suggested that both species were being incorporated into tubules, the relationship between the 6S and 30S components was investigated further. As shown in Fig. 6, the amount of 30S component increased with increasing protein concentration and there was a concomitant decrease in the 6S peak; at 1.0 mg/ml the 30S peak represented 9% of the total protein, while at 9.0 mg/ml greater than 57% was in this fraction. The protein concentration dependence of these components suggested that they were in equilibrium, and additional evidence for this equilibrium was derived from experiments on pressure dependence (J. M. Marcum, unpublished results). At a given protein concentration, increasing pressure over the range of 50 to 200 atmospheres resulted in a shift in the mass fraction of material from the 30S into the 6S component. From these results, it can be inferred that the association of 6S to 30S involved a positive

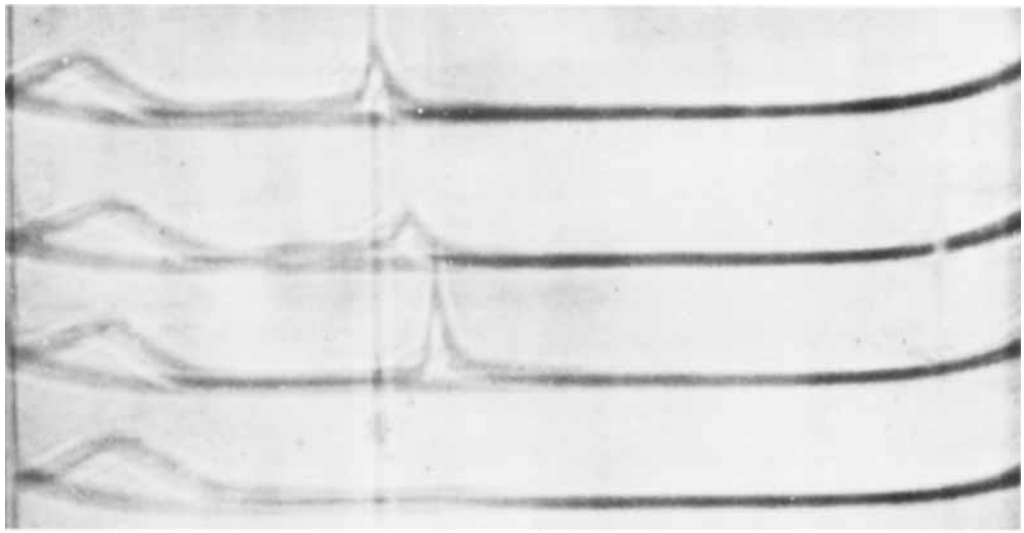

Fig. 5. Sedimentation patterns of purified tubulin preparations. Purified microtubule protein (C_3S, 3.5 mg/ml) was prepared in P solution containing 0.2 mM $MgCl_2$. Part of the preparation was made to 1.0 mM GTP, and an aliquot was held at 0°C. The remainder was incubated at 23°C for 30 min in the presence of either 2.0 mM $CaCl_2$ or 100 μM colchicine, and then returned to 0° for 30 min prior to sedimentation. Another aliquot of the preparation was made to 1.0 mM with GMPPCP instead of GTP, and a parallel incubation carried out. Samples were sedimented at 5°C at 50,480 rpm in 12 mm double sector cells, and photographs were taken 18 min after reaching maximal speed. The patterns correspond to the following preparations (top to bottom): 0°C; 1.0 mM GMPPCP; 2 mM $CaCl_2$; 100 μM colchicine.

volume change as is characteristic of protein association reactions that are entropically driven by the release of water upon polymerization (32).

On the basis of several criteria, the ultracentrifugation studies have indicated that the 30S component is a specific aggregate of tubulin. Both the concentration and pressure dependence studies demonstrated the existence of an equilibrium between the 30S component and the 6S tubulin subunits. In addition, at high protein concentrations, the 30S component represented greater than 50% of the material in purified preparations. Since gel electrophoretic analyses have shown that these preparations are 79% tubulin, it is apparent that the 30S fraction is an oligomer of tubulin. The disappearance of the 30S fraction upon incubation with colchicine and the concomitant increase in the 6S peak further demonstrates the direct association between this species and the tubulin subunits. The sedimentation coefficients of the high molecular weight components are not known and the position of this material in the analytical centrifugation patterns has therefore not yet been established.

Electron Microscopic Observations on Polymerization

The preceding data on sedimentation suggested that intermediates other than subunits might be involved in tubule polymerization. Therefore, electron microscopic observations on tubule assembly were made to determine if morphological structures important in the initiation and growth of tubules could be detected.

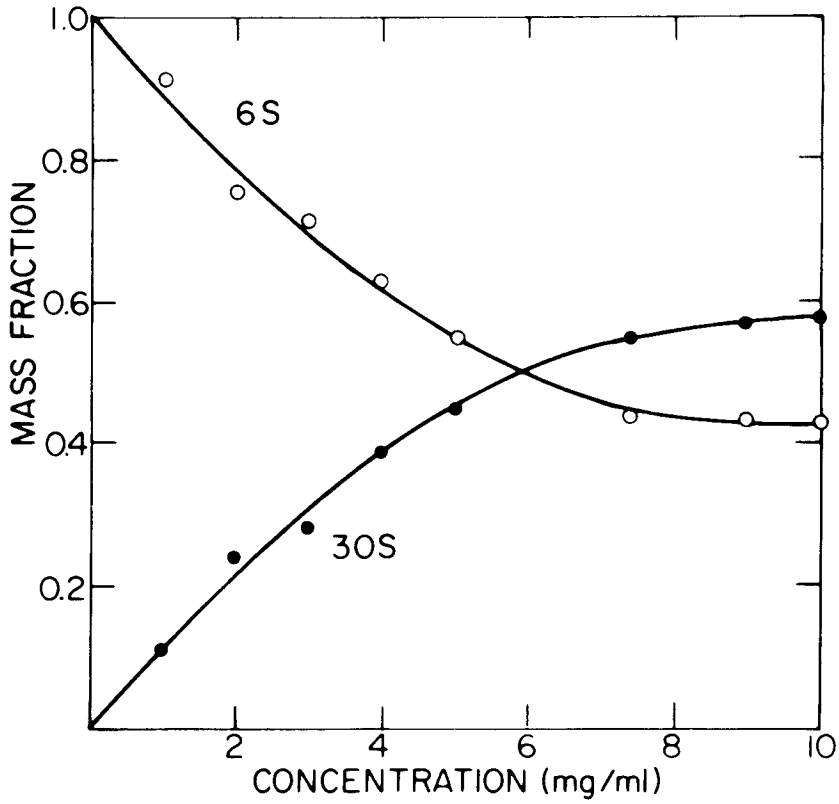

Fig. 6. Analytical ultracentrifugation analysis of the dependence of microtubule protein species on protein concentration. Purified microtubule protein (C_3 S) was prepared in P solution containing 1.0 mM GTP, 0.2 mM $MgCl_2$. Aliquots were diluted immediately before analysis and were centrifuged at 5°C at 50,480 rpm in double sector cells. The amount of material in each sedimenting fraction was determined by planimetry of Schlieren photographs.

The existence of specific particulate fractions required for polymerization had been suggested from experiments in which extracts prepared by centrifugation at low speed polymerized, whereas those obtained at higher speeds did not; the lack of polymerization of the high-speed supernatants was correlated with the absence of a disc structure present in the low-speed extracts (33). In purified preparations, discs (340 Å O.D., 235 Å I.D.) similar to those seen in the extracts were the predominant structure observed in unpolymerized samples (Fig. 7), and their persistence through the purification procedure suggested that they were both incorporated into and derived from microtubules. It is suggested that these discs may correspond to the 30S component observed in sedimentation studies since under conditions where discs existed, the 30S species was also present, and when the 30S component was eliminated by incubation with colchicine, the discs were no longer observed. In addition, calculations indicated that a sedimentation coefficient of approximately 30S was consistent with a structure with the dimensions of the disc.

In addition to individual discs, structures resembling stacks of discs were observed in unpolymerized samples of purified tubulin (Fig. 7). These stacks were composed of

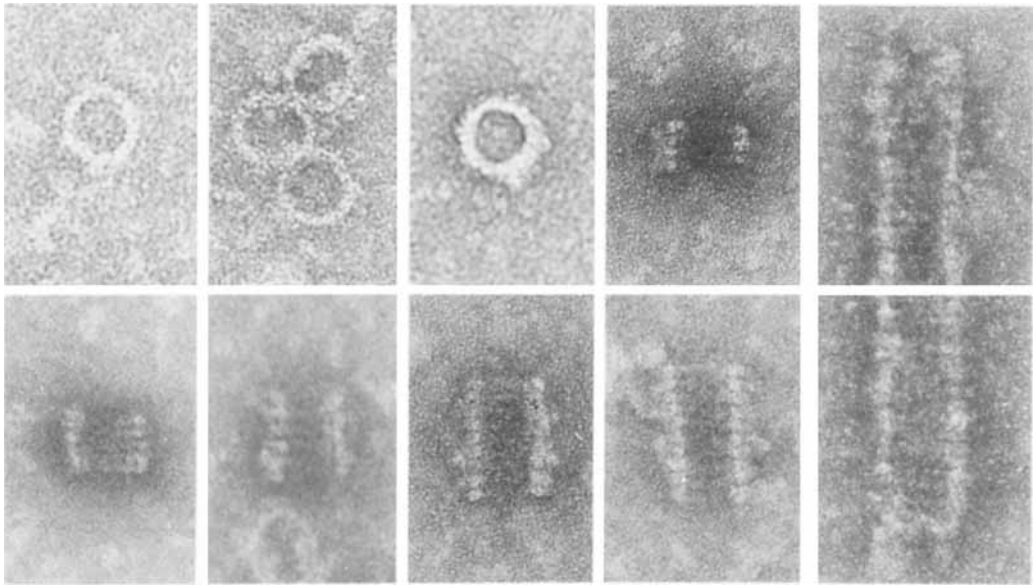

Fig. 7. Electron micrograph of discs and stacks. Negatively stained samples were made of purified tubulin prepared in P solution containing 1.0 mM GTP, 0.2 mM $MgCl_2$ at 0°C. The two upper left-hand panels are individual discs; the other panels are stacks of varying length. The center panel in the upper row is interpreted as a stack in end-on view. Magnification: 294,000 X.

numbers of regular units which gave a cross-striated appearance; although the length varied, structures with numbers of striations between four and eight were most frequently observed. The outer diameter of the stacks was similar to that of the individual discs (350 Å), as was the apparent wall thickness of 50 to 60 Å. Assuming that each striation represented a disc equivalent to those shown in Fig. 7, it was determined that approximately 5 to 10% of the total discs observed were incorporated into these structures.

To determine whether discs, stacks, or other intermediates were directly involved in the processes of tubule initiation and growth, time course observations on polymerization were made. The structures observed earliest in polymerization were a few, very short (less than 0.1 to 0.2 μ) tubules, and within a few minutes of incubation at 37°C, the number and length of these had increased (Fig. 8A); after 15 min of incubation, large numbers of tubules in excess of 20 to 30 μ were observed (Fig. 8B). Throughout the course of polymerization, greater than 80% of the ends of the tubules appeared even, and only very rarely were individual protofilaments or sheets of protofilaments which might be intermediate structures in assembly observed.

The possible involvement of discs in the assembly process was determined by quantitative analysis of these structures during tubule formation. In order to obtain numerous data points, observations were made on samples which had been incubated at 24°C to reduce the rate of the reaction. Figure 9 compares the viscosity profile at 24°C with the total polymer (microtubule) length and number of discs during polymerization. After 3 min of incubation, the total extent of polymer could not be determined because a sizeable population of the tubules was not wholly contained within the grid squares; at this time, polymerization corresponded to approximately one-tenth of the total reaction as measured by change in viscosity (lower panel, Fig. 9). As can be seen in the upper panel

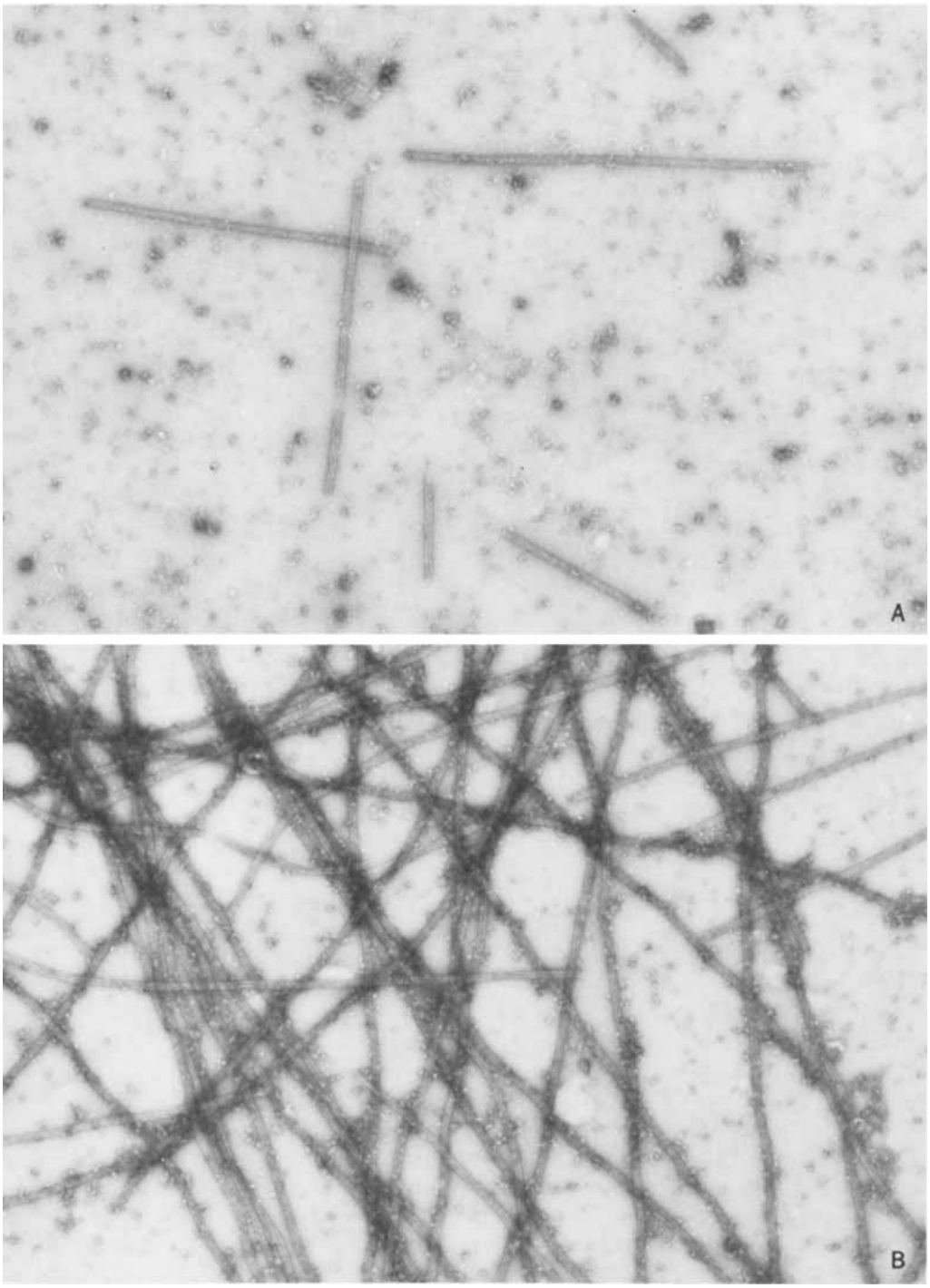

Fig. 8. Electron micrograph of polymerized tubules. Purified tubulin was prepared in PE solution containing 1.0 mM GTP and samples were made after A) 3 min or B) 15 min of incubation at 37°C. Magnification: 28,000 X.

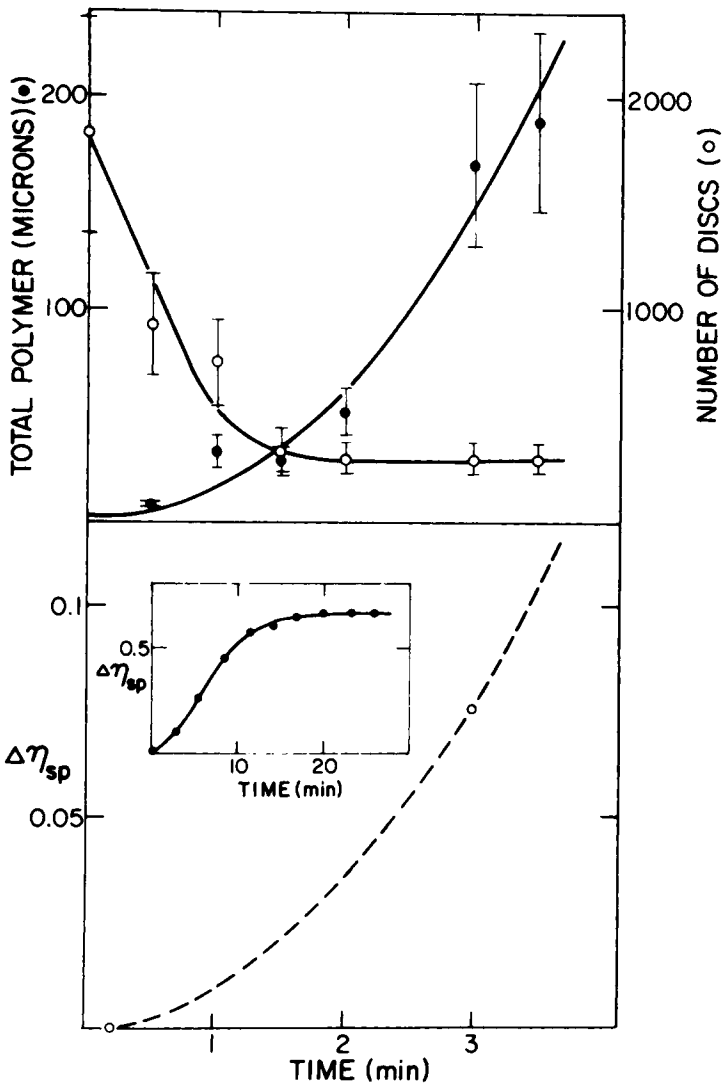

Fig. 9. Distribution of total polymer and discs during polymerization. Purified microtubule protein (C_2S, 3.6 mg/ml) was prepared in P solution containing 1.0 mM GTP, 0.2 mM $MgCl_2$, and incubated at 24°C. Viscosity development was followed, and samples for electron microscopy made every 30 sec during the initial part of the reaction. Bottom panel: change in specific viscosity (η_{sp}) with time of incubation at 24°C; insert shows viscosity development over the total time course of the reaction. Upper panel: (○) number of discs/400 mesh grid square; (●) total length (μ) of microtubule polymer/400 mesh grid square. Data points represent determinations on 2 or 3 grid squares for each point; bars indicate percentage error.

of Fig. 9, the total number of discs (and stacks) decreased early in polymerization, and had fallen to approximately 15% of the original value within 2 min; simultaneously, total polymer length increased rapidly. The decline in disc number was not due to the temperature lability of these structures since under conditions where polymerization was prevented by the omission of a hydrolyzable nucleotide, the disc number remained constant at the initial level. Similarly, if polymerization were slowed by decreasing the magnesium concentration, the more gradual increase in viscosity and total polymer length was paralleled by a more gradual decline in disc number. The majority of discs therefore were utilized early in the polymerization process. It is not yet known to what extent the discs were directly incorporated into polymer or broken down into subunits; however, these data suggest that a certain fraction of the discs or stacks may be primarily involved in the initiation of tubule assembly. These observations do not exclude the possibility that subsequent growth might also occur by the incorporation of discs. However, as described in the next section, microtubule growth can occur solely by the addition of subunits to formed microtubules.

Polarity of Tubule Assembly

The ordered assembly of microtubules appears to be important in the development and organization of specific cellular structures, e.g., in the outgrowth of neurites, cilia, and flagella, and the arrangement of the mitotic spindle. Therefore, in addition to control by specific nucleation centers, cells require some mechanism to specify the directionality of tubule growth. In studies on flagellar regeneration, it was observed that new material was added only at the distal tip of the flagellum (34). In addition, both in vivo (35) and in vitro (36) studies on the formation of bacterial flagella have demonstrated that subunits add to basal structures (or seeds) unidirectionally. To determine whether microtubule assembly was similarly regulated with respect to the directionality of polymerization, experiments were carried out on the in vitro addition of microtubule subunits to seeds.

The initial experiments were made in a heterologous system in which the microtubule subunits present in high-speed supernatants of brain extracts were mixed with intact axonemal microtubules from *Chlamydomonas* flagella (7, 12; Allen and Borisy, manuscript in press). The outer doublets and central pair microtubules of theflagella were morphologically distinguishable from added neurotubules; in addition, the directionality of associated side-arms on the axonemes permitted the determination of the specific directionality of assembly onto the flagellar seeds relative to the original orientation of the flagellum on the cell. In these experiments it was observed that the addition of brain microtubule subunits to the flagellar seeds was polar and occurred at the distal end of the flagellar fragments. Identical experiments have been carried out using purified microtubule subunits prepared by high-speed centrifugation (230,000 × g for 90 min), and addition was found to be dependent on protein concentration and temperature (Allen and Borisy, manuscript in press). Figure 10 represents the results from such an experiment in which intact axonemes were mixed with varying concentrations of purified subunits, and the number of added microtubules per flagellum was scored as a function of distal and proximal addition to the axonemes. At low concentrations of added brain subunits (0.35 mg/ml) all addition was at the distal end of the flagellum. However, as protein concentration was increased, some tubules were found at the proximal end

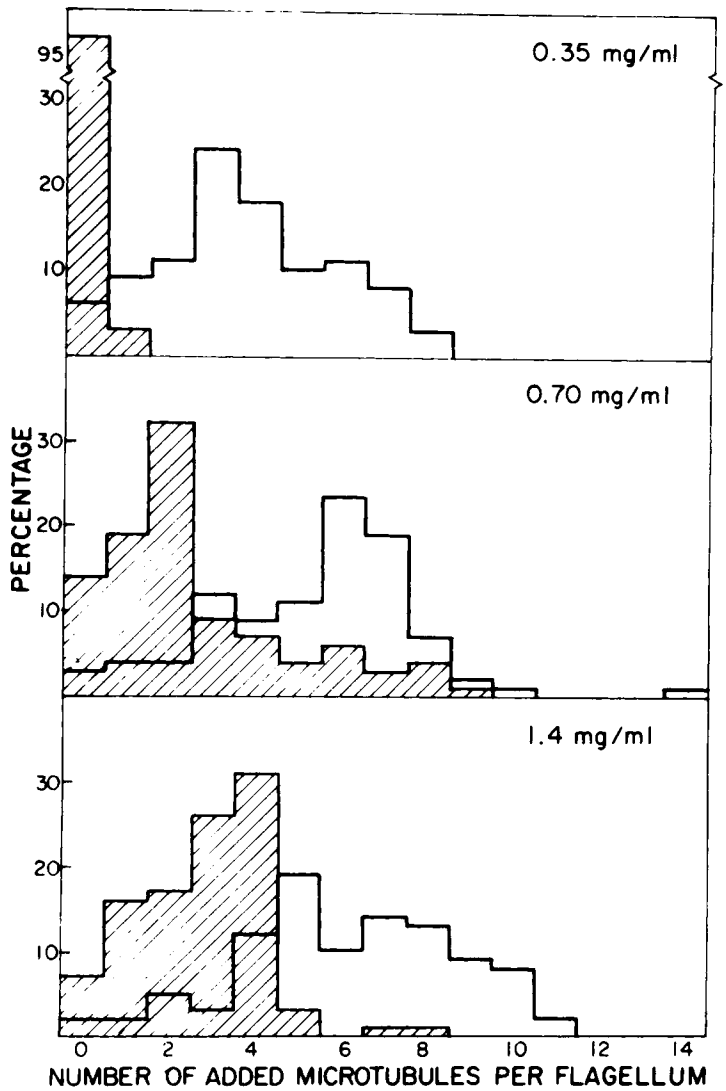

Fig. 10. Distribution of added porcine brain microtubules to isolated *Chlamydomonas* axonemes. Purified microtubule protein (C_2S) was prepared in PE solution containing 2.5 mM GTP, centrifuged at 230,000 X g for 90 min, and then mixed with an equal volume of *Chlamydomonas* axonemes (see Ref. 7). The data represent tubules present after incubation of the mixture for 10 min at 37°C. Hatched area: proximal addition; open area: distal addition. Ordinate: percentage of tubules in each number class.

of the axoneme, although the total number (and length) was significantly lower than those added at the distal end. Similarly, low temperature (25°C) favored unidirectional growth, whereas higher temperature (30°C, 37°C) favored some bidirectionality. Therefore, these data indicated that microtubule assembly in this heterologous system occurred in a "biased" polar manner, since although subunits could add to either end of the pre-existing tubule, addition occurred preferentially at only one end. Since the brain microtubule subunit preparations alone did not polymerize, these experiments further indicated that the addition of subunits to pre-existing tubule fragments would account for the growth of microtubules.

The question of polarity of tubule growth was also examined using a homologous system in which fragments of polymerized tubules from brain were specifically labeled to use as seeds. As described in Materials and Methods, polymerized microtubules were decorated with the polyanion, DEAE dextran, and a population of decorated microtubule fragments less than 2 μ long was obtained (Fig. 11A, B). Upon incubation with high-speed supernatants (230,000 X g for 90 min) of purified microtubule protein, the addition of new polymer was easily distinguished, and occurred in both a polar (Fig. 11C) and bipolar manner (Fig. 11D). Preliminary quantitation indicated that conditions of temperature and protein concentration similar to those investigated in the heterologous assembly system yielded similar results with respect to the biased directionality of tubule assembly. Experiments on the polymerization of chick brain subunits with isotopically labeled seeds from the same source have also indicated that tubule growth is directional (37).

Copolymerization of Tubulins

Studies on the characteristics of microtubules from cilia, flagella, and the cytoplasm have indicated that although the structures differ in stability, tubulin subunits from all these sources are very similar (see 3, 5, 6 for reviews). In addition, recent analyses of tubulin derived from chick brain and sea urchin sperm flagella demonstrated that the amino acid sequences differed at only one position in the first 25 residues from the N-terminus (38). It was therefore postulated that the tubulin molecule was highly conserved during evolution. Functional evidence for this conservatism has been obtained from the recent copolymerization of different types of tubulin. As described in the previous section, subunits derived from mammalian neuronal tissue polymerized in vitro with the tubules from *Chlamydomonas* flagella; similar results were also obtained using chick or rat brain tubulin and *Chlamydomonas* flagella or sea urchin sperm tails (39, 40). Brain tubulin has also been demonstrated to augment the birefringence of the mitotic spindles of *Chaetopterus* oocytes (41), *Spisula* oocytes (42), and mammalian tissue culture cells (43), and it has been inferred from these observations that the exogenous tubulin can be incorporated into the spindles of heterologous cell types. Since these studies all suggested that tubulin from diverse sources could copolymerize, it is probable that some of the characteristics of assembly which have been defined for tubulin from neuronal tissue will be applicable to microtubule formation in other cellular types.

DISCUSSION

This report constitutes a partial summary of the data which have been obtained in characterizing the process of microtubule polymerization in vitro, and a generalized scheme

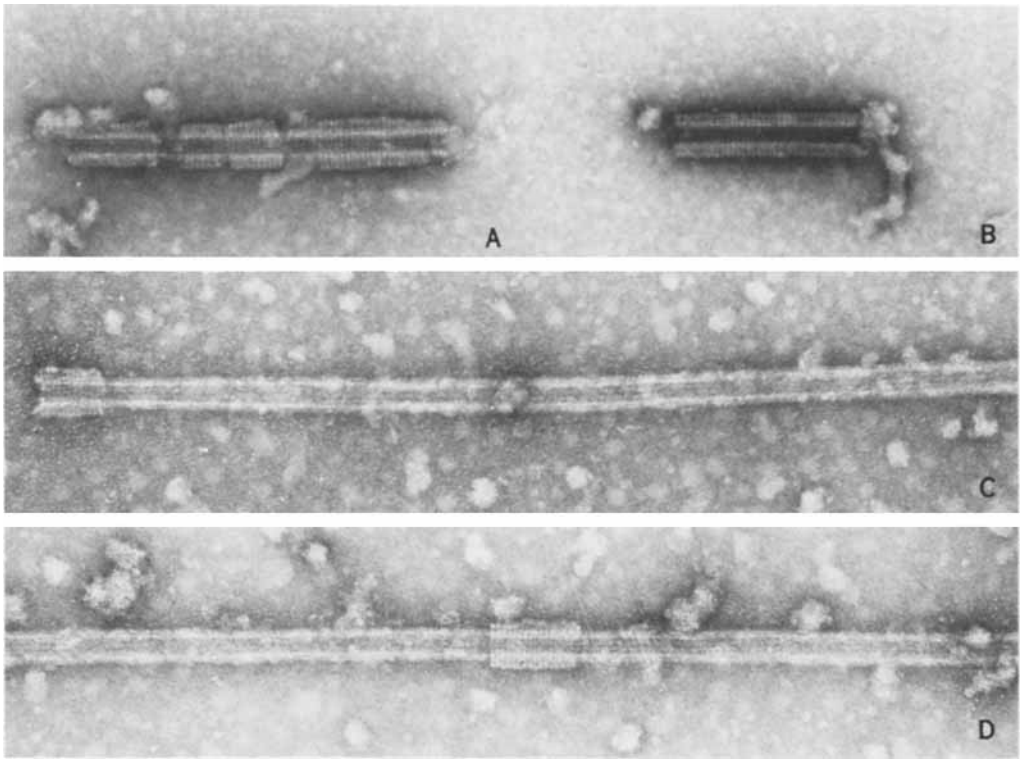

Fig. 11. Addition of microtubule subunits to polymerized microtubule fragments decorated with DEAE dextran. Purified microtubule protein (C_2S, 4.0 mg/ml) was prepared in 0.05 M PIPES, 2.5 mM GTP and centrifuged at 230,000 X g for 90 min. The supernatant was mixed with decorated microtubule fragments, 9:1 (v/v) (see Materials and Methods), and then incubated at 25 or 37°C for 10 min. A, B, DEAE decorated microtubule seeds; C, unidirectional addition of microtubule subunits to decorated seeds at 25°C; D, bidirectional addition of microtubule subunits to decorated seeds at 37°C. Magnification: 168,000 X.

incorporating this information is shown in Fig. 12. The conditions for this reaction were close to physiological in that neither extreme temperature, pH, nor ionic strength was required for polymerization. In addition, as in the in vivo assembly of the mitotic spindle (15), colchicine prevented microtubule formation. Analytical ultracentrifugation and electron microscopic analyses suggested that colchicine inhibition occurred not only by complexing with subunits and directly preventing the formation of polymer, but also by causing the dissolution of an intermediate structure (disc, 30S) involved in the assembly pathway.

The presence of micromolar levels of divalent cations, particularly magnesium, has been demonstrated to be essential for microtubule formation. As discussed previously, the role of these ions may be related to the binding and hydrolysis of the nucleoside triphosphates required for polymerization. The persistence of the 30S component in the presence of inhibitory (millimolar) levels of calcium suggests that at high concentration, calcium complexes with the discs and prevents further assembly.

Microtubule assembly in vitro has been demonstrated to be a reaction in which both tubule formation and dissolution are defined as an equilibrium process, and a critical concentration of monomer for this reaction has been determined. An equilibrium also

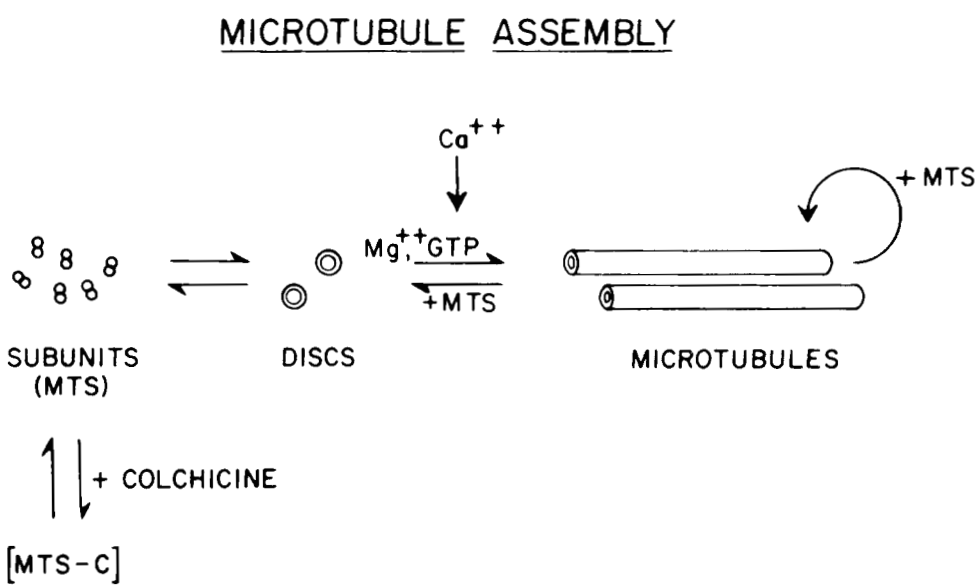

Fig. 12. Summary scheme of microtubule assembly.

existed between the 6S subunits and 30S (disc) components. As outlined in the summary scheme, the discs (30S) are probable intermediates in the assembly process. Several investigators have proposed mechanisms by which the discs or similar ring structures and microtubule subunits are involved in tubule formation.

Kirschner et al. (44, 45) used tubulin preparations isolated in the presence of glycerol, and in ultracentrifugation analyses, observed the existence of 6S and 36S components. Using column chromatography, these preparations were further separated into two fractions. The leading fraction was comprised primarily of the 36S species, and rings consisting of two concentric layers were observed in this material; the inner ring had a diameter (360 Å) similar to that of the discs in our preparations (340 Å), whereas the outer layer was 430 Å in diameter. The trailing fraction was comprised solely of 6S material. From observations that the leading fraction was competent to polymerize, whereas the 6S fraction could only be incorporated into forming tubules, Kirschner et al. (44, 45) proposed a model for tubule assembly based on the existence of tubulin in two states. The leading fraction was composed of Y tubulin which was primarily in an oligomeric form (36S/rings); polymerization of this fraction was postulated to occur by the uncoiling of the rings and the lateral association of the resultant protofilaments to form tubule walls. The trailing fraction was composed principally of X tubulin (6S) which could not initiate polymerization; however, these subunits could be intercalated into the ordered arrays of protofilaments formed from the Y tubulin fraction.

Observations by Erickson (46) on the formation of tubules were similar to those of Kirschner et al. (44, 45). In tubulin preparations separated by column chromatography, the leading fraction was composed of rings and was competent to polymerize, but the trailing fraction (subunits) was unable to polymerize. Electron microscopic observations were interpreted as indicating that assembly proceeded by the lateral association of protofilaments into sheets; these subsequently folded to form microtubules (47).

Analytical ultracentrifugation studies by Weisenberg (48) also demonstrated the

presence of an oligomeric component (approximately 30S) which was diminished upon polymerization.

Our data indicated that diminution of both the 6S and 30S occurred upon polymerization. Since at high concentration greater than 95% of the total tubulin polymerized, this demonstrated that the tubulin molecules in both sedimenting components were incorporated into polymer. However, several observations suggested that the discs (30S) might be primarily utilized in the initial stages of polymerization, and that they were not necessarily involved throughout tubule elongation. The sedimentation experiments on extracts had suggested that discs might be required for tubule formation (33), and quantitative electron microscopic studies demonstrated that disc and stack numbers decreased rapidly early in polymerization. The rare occurrence in our preparations of laterally associated sheets of protofilaments or discs in proximity to the protofilaments in tubule walls suggested that the discs were not necessarily continually integrated into forming and elongating tubules. In addition, the polymerization of subunits on either flagellar or neurotubule seeds demonstrated that tubule growth could occur by the incorporation of monomer alone. Therefore, an alternative model to those which have been proposed is one in which some fraction of the discs or stacks serves as initiation structures, and continued growth occurs primarily by the addition of subunits to preformed tubule fragments. Under conditions which favor polymerization, the rearrangement of subunits in the 340 Å discs (or stacks) could result in the generation of a 250 Å diameter tubule segment. Precedent for an extensive change in diameter of a macromolecular structure has been established by studies on the rearrangement of sheath subunits during contraction of the tail of bacteriophage T4; the diameter of the extended sheath (116 Å) increased to 152 Å upon contraction (49). It has also been suggested that macrotubules (340 Å in diameter) observed in various cell types may be derived from microtubules by a rearrangement of the tubule surface lattice (50). However, we wish to emphasize that the exact mechanism by which discs (or rings) may be involved in tubule formation has not been determined, nor has the existence or nature of an initiation complex been unequivocally established.

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