Mechanism of Tubulin Assembly: Role of Rings in the Nucleation Process and of Associated Proteins in the Stabilization of Microtubules†

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ABSTRACT: Several types of experiments were designed to elucidate the mechanism of initiation of tubulin assembly in the presence of microtubule-associated proteins (MAPs): (1) The evolution of the proportion of the double ring species with temperature was examined in the ultracentrifuge, under nonpolymerizing conditions (guanosine diphosphate = 0.5 mM). A net dissociation of rings into dimers occurred when temperature increased. The transition took place above 20 °C. (2) The kinetic parameters of the exchange at equilibrium between free tubulin dimers and tubulin in rings were studied at different temperatures, using the technique of isotopic exchange at equilibrium with radioactively labeled tubulin. The values found for the half-time of the exchange reaction varied between ~100 min at 0 °C and 10 min at 22 °C. (3) The relative participation of unlabeled rings and labeled dimers to microtubules in the time course of assembly was studied at 22 °C. The time dependence of microtubule specific radioactivity showed that tubulin incorporated in the initial stages of assembly came predominantly from rings (85–90%). This result indicates that oligomers directly issued from rings are the first intermediates in the assembly process and suggests that at the beginning of polymerization incorporation of isomers or fragments of rings proceeds at a faster rate than their dissociation into dimers. (4) Polymerization experiments in the presence of MAPs with increasing concentrations of tubulin dimers indicated that MAPs are in rapid equilibrium with the microtubules and are distributed along the wall in a more or less loose lattice depending on the relative concentrations of tubulin and MAPs in the solution.

The mechanism of tubulin self-assembly in vitro has been extensively investigated in the past few years [for reviews, see Kirschner (1978), Scheele & Borisy (1979), and Timasheff (1980)]. In the absence of associated proteins, tubulin is able to self-assemble through spontaneous nucleation followed by elongation, and the thermodynamics (Lee & Timasheff, 1977; Erickson & Pantaloni, 1980) as well as the kinetics (Carlier & Pantaloni, 1978) of these processes have been analyzed. One of the simplest ways to account for the experimental results and to understand the assembly reactions within thermodynamics concepts is to consider a series of bimolecular reactions which leads to the formation of a longitudinal protofilament containing N molecules of tubulin, defined as the critial nucleus (Erickson & Pantaloni, 1980) because it is long enough to be able to undergo stable lateral interactions which generate the bidimensional lattice. This nucleation process occurs during the lag time observed in the time course of the polymerization process. The evolution of this lag time with tubulin concentration was very cooperative as a consequence of the N steps needed in building up the nucleus. In the presence of microtubule associated proteins, however, the situation was less clear. A kinetic study of Johnson & Borisy (1977) conclusively demonstrated that the elongation process essentially involved the sequential addition of tubulin dimers only onto microtubule ends and ruled out the possible participation of rings in the elongation process. Yet the intricate mechanism of nucleation of microtubules and the possible relative participation of different tubulin oligomer species in the initiation steps have not been elucidated. A very weak degree of cooperativity (1–2) was exhibited in the variation of the lag time and rate of polymerization with tubulin concentration (Gaskin et al., 1974a; Engelborghs & DeMaeyer, 1977; Johnson & Borisy, 1977) which indicated that nucleation might not be a rate-limiting reaction as in the case of assembly from pure tubulin dimers. Rings have been proposed as preformed first intermediates or nuclei in the assembly process. This model assumed that polymerization was the actual reverse reaction of the depolymerization process, which is now known not to be strictly valid since guanosine triphosphate (GTP)[1] is irreversibly hydrolyzed during assembly.

Electron micrographs of depolymerizing microtubules show that profilaments seem to coil into spirals of the same diameter as the rings observed after complete equilibration of the solution (Kirschner & Williams, 1974; Gaskin et al., 1974b; Erickson, 1974). These data together with the observation of a quantitative correlation between the decrease of rings and increase of microtubules at the beginning of polymerization were suggestive supports of a model in which rings would initially open, uncoil, and form the 13 protofilaments initiating a microtubule wall by lateral interaction (Erickson, 1974; Kirschner et al., 1975; Borisy et al., 1976; Sloboda et al., 1976). The experiments indicated that rings were involved in initiation of assembly, but it was not clear whether their integral structure was needed for nucleation or whether they acted only as bearers of the microtubule-associated proteins (MAPs) which were later shown to be required factors in nucleation (Murphy et al., 1977a,b; Stearns & Brown, 1979). However, at least one of the kinetic steps leading to the nucleation of microtubules is unknown. Obviously the MAPs are involved, but whether the rings directly open into a protofilament or rapidly break down into free tubulin dimers and MAPs before rebuilding another polymeric structure which would be the nucleus remains a matter of discussion (Engelborghs et al., 1980; Weisenberg, 1980). This paper reports an investigation of the equilibrium and of the kinetic parameters of the exchange between free tubulin and tubulin in rings at different

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[1] Abbreviations used: GTP, guanosine triphosphate; MAP, microtubule-associated protein.

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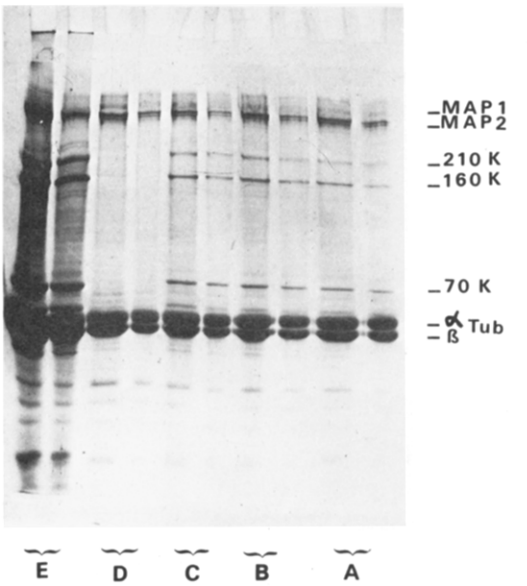

FIGURE 1: Polyacrylamide slab gel electrophoresis in the presence of $NaDodSO_4$ showing the elimination of neurofilament proteins (210K, 160K, 70K) from the prepartion of Shelanski tubulin. Two different amounts of proteins were deposited for each sample. (A, B) Shelanski tubulin (two cycles of polymerization); (C) Shelanski tubulin kept in 1 M NaCl for 1 h; (D) dialyzed supernatant of the centrifugation in 1 M NaCl showing the disappearance of neurofilaments; (E) pellet of the centrifugation in 1 M NaCl.

temperatures and a study of the relative participation of both species in the formation of microtubules.

Materials and Methods

Reagents. 2-(*N*-Morpholino)ethanesulfonic acid (MES) was purchased from Calbiochem, guanosine 5′-triphosphate trilithium salt was from Boehringer, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) was from Sigma, electrophoresis reagents were from Eastman, phosphocellulose P1 was from Whatman, and Sepharose 6B was from Pharmacia. Bolton and Hunter reagent [3-(*p*-hydroxyphenyl)propionic acid *N*-hydroxysuccinimide ester, mono[^{125}I]iodinated, 2000 Ci/mmol] came from NEN.

Tubulin Purification and Elimination of Neurofilaments. Tubulin was purified from pig brain by two consecutive cycles of polymerization–depolymerization according to Shelanski et al. (1973). Together with tubulin and the high molecular weight microtubule-associated proteins (MAP1 and MAP2), the material obtained also contained contaminating proteins from neurofilaments, in variable amounts from one preparation to the other (15–30% in weight of the total protein). The polydispersity of these neurofilaments has been shown to be the reason for their inefficient removal from cycled tubulin preparations (Berkowitz et al., 1977). These proteins were resolved in polyacrylamide gel electrophoresis as the previously observed triplet 210 000-, 160 000-, 68 000-dalton bands (Runge et al., 1979) which is characteristic of mammalian neurofilaments (Schlaepper & Freeman, 1978; Thorpe et al., 1979). Neurofilaments were eliminated from the preparation by a procedure close to that of Delacourte et al. (1977) as follows: Two times cycled tubulin solution (10 mg/mL) was brought to 1 M NaCl at 0 °C. The total dissociation of rings was checked by electron microscopy. The neurofilaments, which have been shown to be stable in high salt solutions (Keates & Hall, 1975; Delacourte et al., 1977), were spun down by centrifugation at 100000*g* for 3 h at 0 °C. The supernatant was dialyzed overnight against 0.1 M MES buffer, pH 6.6, containing 0.5 mM $MgCl_2$, 1 mM EGTA, and 4 M glycerol. Figure 1 shows that the material obtained was freed of the neurofilament proteins. The pellet of neurofilaments contained also a small amount of high molecular weight MAPs and tubulin. Whether the MAPs may be associated to neurofilaments is a matter of discussion (Komiya & Kurokawa, 1980; Delacourte et al., 1980). After dialysis, the reappearance of rings was checked by electron microscopy; 98% of the initial ability of the solution to polymerize was recovered. The yield obtained by this procedure was higher than by the Bio-Gel A150m chromatography used by Berkowitz et al. (1977).

Labeling of Tubulin with Bolton Hunter Reagent. Radioiodinated (^{125}I)tubulin able to copolymerize with native tubulin with the same critical concentration was obtained as previously described (Carlier et al., 1980). The method can be briefly summarized as follows: Pure DEAE-Sephadex chromatographed tubulin (Murphy et al., 1977a) was labeled, tubulin able to polymerize was selected by one cycle of copolymerization with unlabeled Shelanski tubulin, and microtubules were stored at −30 °C in reassembly buffer (0.1 M MES, pH 6.6, 0.5 mM $MgCl_2$, 1 mM EGTA, and 0.5 mM GTP) containing 8 M glycerol. Before use, tracer-labeled tubulin dimer was prepared by phosphocellulose chromatography of an aliquot of this solution.

Ultracentrifugation Studies. Solutions of tubulin and rings were analyzed in a Beckman Model E analytical ultracentrifuge equipped with an electronic speed regulation unit and a RTIC temperature control. All centrifugations were performed at 60 000 rpm in a standard cell of 12 mm, 2° angle, mounted in a type AV.D. rotor. Kodak metallographic plates were used for the photographs. Distances on the film were measured with a Nikon 6C microcomparator. Sedimentation coefficients were corrected to 20 °C. The mass fraction of each sedimenting component was determined from planimetry of the "Schlieren" patterns with an OTT type II planimeter as the ratio of the area under a particular peak to the total area of the profile. Correction was made for radial dilution.

Separation of Tubulin Dimers and Rings. The separation of tubulin dimers (6 S) and rings (30 S) was achieved by gel filtration at 4 °C on a 0.9 × 45 cm Sepharose 6B column according to Erickson (1974). The same technique has previously been used in our laboratory to study the exchange of nucleotide on rings and dimers (Jacobs & Huitorel, 1979). Rings were eluted in the void volume within 10 mn after deposition of 0.85 mL of a 3–4 mg/mL tubulin + rings solution. Since the half-time for the exchange of tubulin between rings and dimers was around 100 min at 4 °C, the temperature at which the chromatographic separation was performed, a posteriori it was determined that during this 10-min delay only 5% supplementary exchange would take place. Consequently this technique did not introduce a large quantitative perturbation in the measurements. Fractions were collected in a Isco collector equipped with a Isco Model UA 5 absorbance monitor. Protein concentration was determined by the method of Lowry et al. (1951) with the correction previously determined for tubulin (David-Pfeuty et al., 1977). Specific radioactivities of the fractions were derived from the countings performed in a Packard Tricarb Model 3310 liquid scintillation spectrometer.

Polymerization Studies. Microtubules were polymerized at the desired temperature in reassembly buffer and were rapidly separated from the tubulin dimer solution (in less than 40 s) by centrifugation at 160000*g* for 2 min in a Beckman Airfuge. This technique permitted the measurement of the weight of microtubules at short time intervals during the course of polymerization.

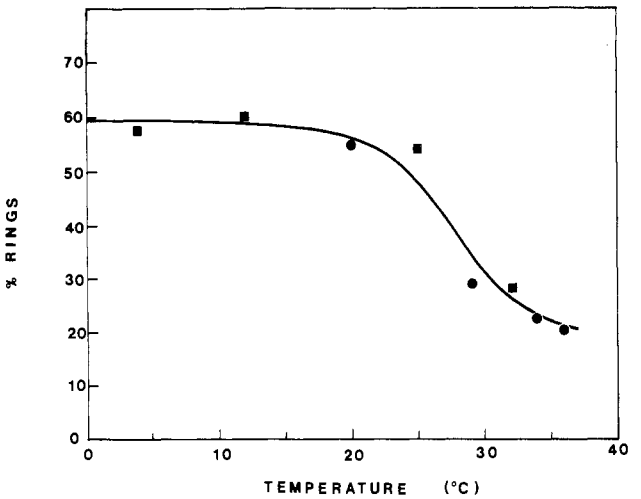

FIGURE 2: Evolution with temperature of the proportion of rings in a solution of tubulin and MAPs. The percentage of ring material is derived from the measurement of the areas under the Schlieren peaks in the analytical ultracentrifuge, as described under Methods. The experiment was performed in reassembly buffer (see Methods) at two different concentrations of tubulin: (■) 2.4 mg/mL; (●) 1.6 mg/mL.

In the determination of microtubule weight, a correction was brought for the occasional formation of aggregates by performing the following control: parallel samples were polymerized during the same time as the assays, cooled, and centrifuged at 20000*g* at 0 °C for 15 min, which ensured the complete depolymerization of microtubules while irreversible amorphous aggregates were spun down as judged by electron microscopy. The amount of microtubules was calculated from the difference between the concentrations of the supernatants of the cold and warm centrifugation. This correction for aggregate formation was always weak.

Results

Ultracentrifugation Studies. The equilibrium between rings and dimers species has been studied at different temperatures in the 4–37 °C range. In order to avoid polymerization of tubulin at temperatures higher than 20 °C, absence of GTP was ensured by processing the tubulin through assembly–disassembly cycles in order to hydrolyze all the GTP present, and 0.5 mM more GDP was added.

It was checked that at 4 °C the equilibrium between the rings and dimers was roughly the same whether GTP or GDP was present (data not shown). Figure 2 shows that the equilibrium between rings and tubulin was shifted toward the dimer species when temperature increased. The transition took place above 20 °C. No intermediary species was observed. Total dissociation of rings into dimers occurred at 37 °C when glycerol was present at 1–2 M in the buffer, which is in agreement with the previous finding that glycerol weakens the interaction between MAPs and tubulin (Olmsted et al., 1974). These data confirm the preliminary observations of Weisenberg (1974) in the ultracentrifuge.

Exchange between Tubulin Dimers and Tubulin in the Rings. The rate of exchange of tubulin between the ring and dimer species was studied by the isotopic exchange at equilibrium technique. ^{125}I-Labeled tubulin was used as a tracer of the 6S species. A very small amount of the tracer was added to the solution of rings and dimers at zero time in order not to perturb the equilibrium. At different time intervals, aliquots were chromatographied on Sepharose 6B, and the evolution with time of the specific radioactivity of rings and dimers was followed until equilibration. Figure 3 shows the evolution of

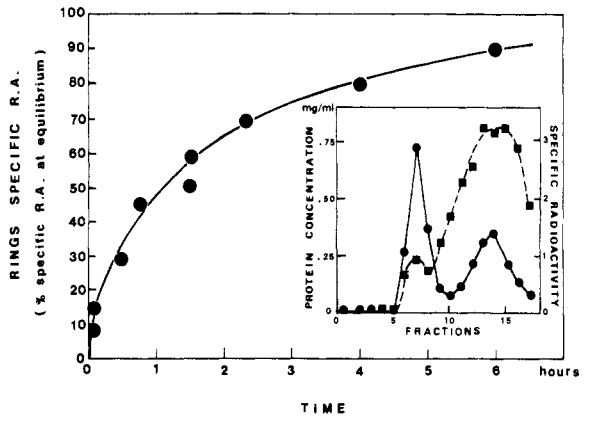

FIGURE 3: Time course of the exchange between labeled tubulin dimers and unlabeled rings at equilibrium. At the indicated times of the isotopic exchange at equilibrium reaction, aliquots of the solution of rings and dimers were chromatographed over Sepharose 6B to separate rings from dimers, and the evolution of specific radioactivity of the rings (●) was followed. The specific radioactivity of the rings reached at equilibrium was 80% of the average specific radioactivity of the bulk tubulin solution due to the binding of unlabeled MAPs to the ring fraction. A typical Sepharose 6B chromatography pattern is shown in the inset. (■) Specific radioactivity of each fraction, in arbitrary units; (●) absorbance at 280 nm.

ring specific radioactivity in a typical experiment performed at 0 °C. According to McKay (1938), the rate of appearance of the label in the rings is described by

$$\ln (1 - f) = V_e \frac{a + b}{ab} t$$

where V_e is the equilibrium velocity or flow, a the concentration of initially labeled tubulin, b the concentration of rings which receive the label, and f the extent of equilibrium, defined by $f = (a_0^* - a^*)/(a_0^* - a_\infty^*) = b^*/g_\infty^*$ where a_0^*, a^*, and a_∞^* are the specific radioactivities of the tubulin dimers at times 0, t, and infinity, respectively, and b^* and b_∞^* are the specific radioactivities of the rings at times t and t_∞ ($b_0^* = 0$ in the experiment).

At the concentration of 2.8 mg/mL used in this experiment the rings represented 40% of the protein content. It is known that the structure of the double ring consists of 28 tubulin molecules, 12 dimers in the inner ring and 16 dimers in the outer ring, as is shown in Figure 4 (D. Pantaloni and S. Edelstein, unpublished results; Erickson, 1978). Therefore the concentrations of dimers and rings were $a = 15$ μM and $b = 0.36$ μM, respectively.

The measured apparent rate constant for the radioactivity flow toward the rings at 0 °C was 0.35 ± 0.05 h^{-1} ($t_{1/2}$ = 92–120 min). The technique of isotopic exchange at equilibrium does not, however, permit us to propose any molecular scheme for the exchange of tubulin between the ring and the dimer species since the same logarithmic law for the flux of radioactivity is expected in all cases (McKay, 1938). However, the following scheme for the exchange of the tubulin molecules in the ring is assumed:

$$T_n \underset{k_+}{\overset{k_-}{\rightleftharpoons}} T_{n-1} + T$$

with $[T_{n-1}] \ll [T_n]$ at equilibrium and $n = 28$. Assuming that the 28 molecules of tubulin in the ring are virtually identical, they can exchange with free labeled dimers independently of each other, and the resulting flow of label can be expressed as

$$V_e = C_{28}^1 k_- [T_{28}] = C_{28}^e k_+ [T_{27}][T]$$

where k_- and k_+ are the intrinsic dissociation and association rate constants in the equilibrium between tubulin in the ring

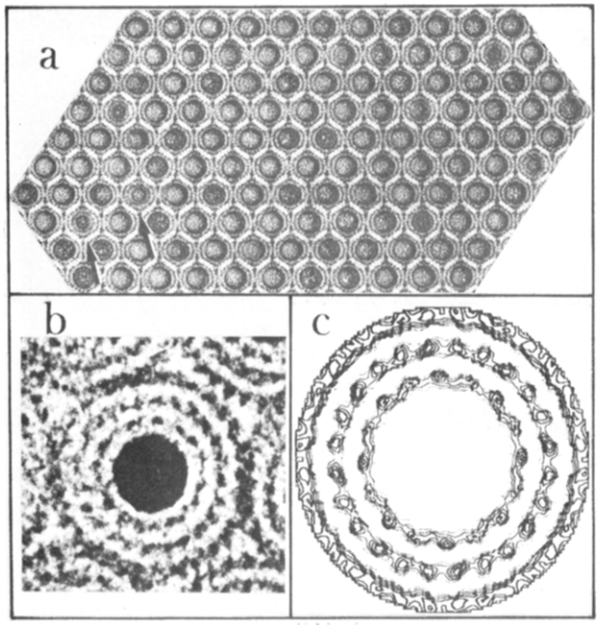

FIGURE 4: (a) Electron micrograph of a crystal of tubulin showing the stacking of coplanar concentric double rings. Arrows indicate some concentric triple rings (×108000). (b) High magnification of a triple ring (×540000). (c) Image reconstruction using selected rotational harmonics to calculate a rotationally filtered image (× 855000) showing a concentric triple ring of 8, 12, and 16 α, β dimers of tubulin (in collaboration with Edelstein et al.).

structures and free tubulin. The apparent microscopic corresponding rate constants are $C_{28}^{1}k_{-} = 28k_{-}$ and $C_{28}^{\circ}k_{+} = k_{+}$. The statistical binomial coefficients C_{28}^{1} and C_{28}° are here to account for the equal probability of exchange of the 28 tubulin molecules in the ring. This situation is an illustration of the theoretical case of isotopic exchange through n parallel reactions studied by Yagil & Hoberman (1969), who showed that the exchange rate is the sum of the n individual rates. The value of k_{-} could be derived from the measurement of the exchange rate constant $k_{app} = V_e(a + b)/ab$, which gave

$$k_{-} = k_{app}\frac{ab}{a+b}\frac{1}{28b} = \frac{1}{28}k_{app}\frac{a}{a+b}$$

At 0 °C k_{-} took the value of 0.012 h^{-1}.

The temperature dependence of the exchange rate was studied by the same technique. Each sample was incubated at the desired temperature for 30 min, after which it was chromatographied on Sepharose 6B at 4 °C. The apparent rate of exchange, k_{app}, was derived from the measurement of the extent of exchange during 30-min incubation and was plotted vs. temperature between 0 and 20 °C. The Arrhenius plot of log $k_{app}(T)$ vs. $1/T$ (Figure 5) yielded an activation energy of 12 kcal/mol. From this plot the $t_{1/2}$ value was 10 min at 22 °C. This plot could not be linearly extrapolated to higher temperatures since an appreciable dissociation of rings into dimers then took place, and the variation of the rate constant, $k_{app} = V_e(a + b)/ab = 28k_{-}(a + b)/a$, is affected by the variation in the ratio $(a + b)/a$. However if the rings represent 40% of the tubulin material at 0 °C and 10% at 37 °C, the value of $(a + b)/a$ equals 15.36 = 1.02 at 0 °C and 23.0/22.9 = 1.00, which gives a 2% variation only. We can thus conclude that the variation in the dissociation state of the rings interferes only slightly with the exchange rate and induce that the half-time for the exchange reaction is fast, i.e., in the minute range at 37 °C.

Relative Participation of Rings and Dimers in the Polymerization of Tubulin in Microtubules. The experiment related above shows that it is technically possible to study the in-

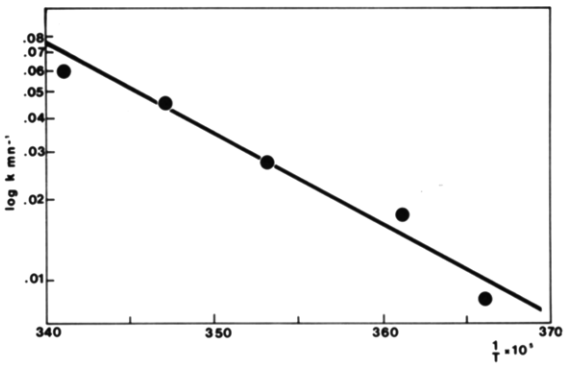

FIGURE 5: Arrhenius plot (log k vs. $1/T$) of the variation of the rate of exchange between tubulin and rings with temperature. From the slope of the plot, a value of 12 ± 2 kcal/mol was determined for the activation energy of the reaction.

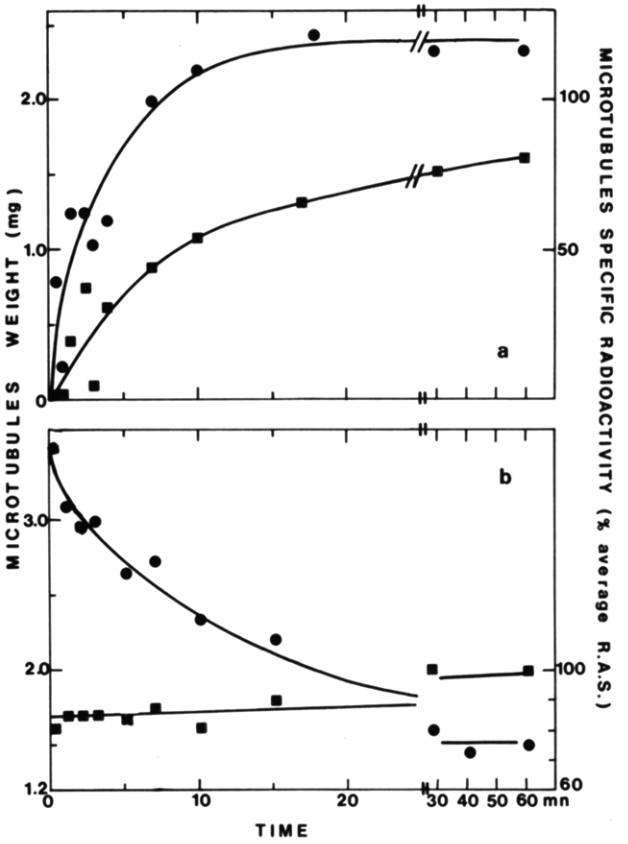

FIGURE 6: Evolution with time of the weight (●) and specific radioactivity (■) of microtubules during polymerization at 22 °C (a) and depolymerization at 13 °C (b). At zero time, a pulse of ^{125}I-labeled pure tubulin was added at 0 °C to the tubulin solution and polymerization was started. At short time intervals, aliquots were centrifuged for determination of the weight and specific radioactivity of microtubules. A separate sample was polymerized at 22 °C and the same parameters were followed during depolymerization.

corporation of rings and dimers in microtubules at 22 °C, a temperature at which the exchange between tubulin and rings is not too fast. The experiment was conducted at follows: Trace amounts of ^{125}I-labeled tubulin were added at zero time to a solution of tubulin and rings at 0 °C at 4 mg/mL in order to label the dimer molecules. The solution was allowed to polymerize at 22 °C, and aliquots were rapidly spun down at short intervals. The amount and specific radioactivity of microtubules were measured at each time (Figure 6a). The data showed a preferential incorporation of unlabeled material, i.e., coming from rings, in the early times of polymerization; then the specific radioactivity of microtubules increased with

time during the assembly process and reached, at equilibrium, about 80% of the average specific radioactivity of the tubulin solution. At time t = 2 min, when the exchange between rings and labeled dimers can be neglected, 1.2 mg/mL tubulin had polymerized and the measured microtubule specific radioactivity was 20–25% of the average tubulin specific radioactivity. At the tubulin concentration of 4 mg/mL used in the experiment, 60% of the material consisted of rings; therefore the specific radioactivity of the labeled dimer fraction (40% of total tubulin) at time zero was 2.5-fold the measured average specific radioactivity of the total tubulin. The amount of labeled dimers participating in the first microtubules present at time 2 min could then be calculated as $x = 1.2 \times (0.20–0.25) \times 1/2.5 = 0.1–0.12$ mg/mL. This means that only 8–10% of the tubulin incorporated in the microtubules formed during the first 2 min of the assembly process came from dimers, while the remaining 90% came directly from rings. This represented as much as 1 mg/mL tubulin from rings incorporated initially in the polymer, i.e., 40–45% of the ring material initially present in the solution at 0 °C. This unlabeled tubulin remains sequestered in the microtubule, which explains why the microtubule specific radioactivity observed when the turbidity plateau has been reached cannot but be smaller than the average specific radioactivity of the solution. However this first phase is followed by a slow increase in the microtubule specific radioactivity which is most likely due to the unidirectional subunit flow through the microtubules at steady state previously demonstrated by Margolis & Wilson (1978). This process is expected to lead to a homogeneous microtubule labeling after a long time.

In order to check that the initial evolution of microtubule specific radioactivity was not an artifact due to a slower polymerization rate of labeled tubulin than the polymerization rate of native unlabeled tubulin, a control experiment was run in parallel in which the labeled dimers had been allowed to completely equilibrate with rings before polymerization was started. In this case, the microtubule specific radioactivity was constant all along the polymerization curve, which confirmed that the labeled tubulin had the same kinetic properties as the unlabeled tubulin in the assembly process.

The evolution of the specific radioactivity of microtubules formed at 22 °C from unlabeled rings and labeled dimers was then followed during their depolymerization at 13 °C in the same way as above. If we assume that microtubules depolymerize essentially by one end, an initial increase or decrease in their specific radioactivity can be expected, depending on the unlabeled end (rings initially incorporated) or the "assembly end" (more labeled) being the fastest depolymerizing end. Surprisingly, the specific radioactivity of microtubules remained constant during depolymerization (Figure 6b). A possible explanation again may come from the treadmilling phenomenon which takes place at steady state and results in a displacement of the first incorporated unlabeled material either toward the bulk of free tubulin dimers or toward the body of the microtubule depending upon whether the net assembling end at steady state is or is not, respectively, the same as the fastest elongating end (defined as + end) in the pre-steady-state conditions (Zeeberg et al., 1980). Due to the statistics involved in the treadmilling process and the fluctuations in the microtubule length showing a Poisson distribution, both behaviors might actually result in a quasi-homogeneous distribution of radioactivity in the polymer and dimer phases.

When the same experiment as described above at 22 °C was done at 37 °C, no variation in the microtubule specific radioactivity was observed during polymerization. The equilibrium average value of tubulin specific radioactivity was observed even in the early stages of the polymerization. This result is in agreement with the findings of Terry & Purich (1979). The technique used here does not, however, allow any conclusion to be derived from this result since a rapid equilibration between dimers and rings must take place at this temperature, as seen above.

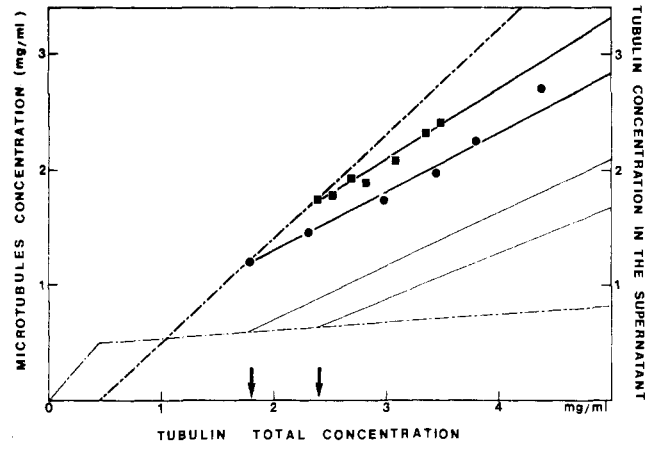

FIGURE 7: Increase in the amount of microtubules formed when various amounts of pure phosphocellulose tubulin was added to Shelanski tubulin at a constant concentration C_s (indicated by the arrow). (●) C_{s_1} = 1.8 mg/mL; (■) C_{s_2} = 2.4 mg/mL. Thick lines represent the evolution of microtubule weight and thin lines the corresponding evolution of free tubulin when the total tubulin concentration is varied; dashed lines are the typical lines which are obtained when Shelanski tubulin is varied in abscissa and solid lines the experimental lines obtained when pure phosphocellulose tubulin was varied and added to a constant amount, C_s, of Shelanski tubulin.

Role of MAPs in Microtubule Assembly and Stability. When pure 6S tubulin dimers were added to a solution of Shelanski tubulin at 3.9 mg/mL containing 60% rings, no increase in the absolute concentration of rings was observed, as judged from measurements of the areas under the "Schlieren" peaks in the ultracentrifuge. This was in agreement with the fact that the equilibrium between rings and dimers is limited by the amount of MAPs (Vallee & Borisy, 1978); therefore increasing the dimer species cannot significantly increase the amount of rings in this medium. It was interesting to study the polymerization of tubulin working with a constant amount of Shelanski tubulin, i.e., a constant concentration of MAPs, and increasing amounts of tubulin dimers. Figure 7 shows that the amount of microtubules polymerized at 37 °C under these conditions increased linearly with the concentration of added pure tubulin dimers. The concentration of free tubulin in the supernatant increased as well, indicating that the stability of formed microtubules decreased. Since the MAPs are distributed along the microtubules, these results show that when pure tubulin dimers are added to a cycled tubulin preparation, the microtubules obtained contain a lower amount of MAPs per tubulin polymerized than in the cycled preparations studied by Amos (1977) and are thus less and less stable with increasing ratio of [tubulin]/[MAPs]. When the experiment was done with labeled tubulin dimers added to Shelanski tubulin, the same specific radioactivity was found in the microtubules and the supernatant, whatever amount of tubulin dimers had been added to Shelanski tubulin. This indicated that unlabeled and labeled added tubulin dimers behaved in an identical manner in the polymerization process, and the microtubule preparation obtained is homogeneous.

When phosphocellulose tubulin was added at 37 °C to already formed Shelanski microtubules at steady state, the amount of microtubules increased up to the same value as

when the same preformed mixture of Shelanski and phosphocellulose treated tubulins were allowed to coassemble by a temperature jump from 0 to 37 °C. This result indicates that the stability of the microtubules is the same in the two experiments; in other words, the distribution of the MAPs on the microtubules is the same whatever pathway has been followed to obtain the polymer.

Discussion

The presented results try to get some insight in the mechanism of microtubule initiation in the presence of MAPs. Although no detailed kinetic mechanism can be presented, a few clear features seem to emerge and help to propose a possible pathway for assembly. The stability of rings is weakened when temperature is increased. It is likely that the open ring is very unstable and would dissociate into dimers rapidly, as could be seen in the ultracentrifuge. However transient fragments of rings may exist during the dissociation process; these may act as preformed intermediates in the initiation of microtubules. Initiation of microtubules could thus be understood as the result of a competition between two kinetic events: sequential dissociation of rings into dimers, via transient "fragments", on the one hand and lateral association of these fragments to form the first microtubule wall on the other hand. Later in the polymerization course, dimers and occasionally very short linear oligomers in equilibrium with dimers would be present and participate in the elongation of microtubules. At 22 °C the exchange between tubulin and rings as well as the breakdown of rings were slow enough to allow the measurement of the relative participation of both species, rings, and labelled dimers, in the assembly process, and in this case it is clear that before any total dissociation of rings into dimers can occur, fragments of rings are directly incorporated as first intermediates in the microtubules, and in the initial stages of assembly, at least 85% of the microtubule is made from fragments of rings. Such a mechanism explains that the apparent rate constant for the assembly process under these conditions does not depend on the $(n/2)$th power of the tubulin concentration, as in the case of pure tubulin dimer self-assembly, where n consecutive bimolecular steps are necessary to build up the nucleus. When temperature increases, the extent of dissociation of rings is larger and the rate of exchange between tubulin and rings is higher. Presumably at 37 °C a lesser amount of rings participates in the initiation of microtubules, although we may expect the sequence of events to be qualitatively the same as at 22 °C since the tubulin concentration dependence of the apparent elongation rate constant is the same at both temperatures (Gaskin et al., 1973). In this respect our results, obtained at 22 °C, are in good correlation with the recent findings obtained at 37 °C by Mandelkow et al. (1980) who used the X-ray diffraction technique to follow the early stages of polymerization. Actually these authors also observed the dissociation of rings at 37 °C under polymerizing conditions and point to the transient existence of short fragments of rings which may be involved in the nucleation of assembly. Karr & Purich (1980) and Engelborghs et al. (1980) also reported the dissociation of rings upon warming, but their data do not provide evidence for any nucleation center coming from fragments of rings. Our results also agree with the data of Stearns & Brown (1979) who showed evidence for a preferential incorporation of MAP2 in microtubule initiation. MAP2 is known to be associated with the rings, and if we assume, in the experiment illustrated by Figure 6a, that MAP2 remains associated to the ring fragments in microtubule assembly, at time 2 min about 30% of the total microtubule protein would consist of MAP2. This calculated value is close to the value measured by Stearns and Brown. Finally our data provide some experimental evidence to support one of the possible pathways for assembly proposed by Weisenberg (1980) in a theoretical model.

It should be noted that these experiments have been performed with a material containing only tubulin and MAPs. In particular, the neurofilament proteins have been carefully eliminated. This precaution is necessary in order to avoid a heavy contamination of the ring fraction in Sepharose 6B chromatography and consequently wrong interpretations of the specific radioactivities data. Such a contamination may have been the explanation for the puzzling findings of Weingarten et al. (1975), who earlier raised the problem of the fate of rings in the assembly and disassembly of microtubules and concluded that rings were conserved in these processes. This statement means that MAPs would be irreversibly bound to determined fragments of tubulin protofilaments. Our results rather show that tubulin can go on and off rings stabilized by the MAPs and that MAPs are in rapid equilibrium with the microtubules, since the stability of microtubules formed upon addition of phosphocellulose tubulin to Shelanski tubulin is the same whether pure tubulin is added before or after microtubule assembly. The data therefore indicate that MAPs are not distributed in a definite rigid lattice along the microtubule. When extra tubulin is added, less stable microtubules having a higher critical concentration and along which the MAPs are distributed evenly in a looser lattice can be formed. The distribution of MAPs on the lattice giving one MAP molecule every 12 tubulin dimers (Amos, 1977) in typical cycled microtubule preparations only corresponds to the usually found packing of MAPs on the microtubule wall when tubulin is isolated by a polymerization cycling procedure. In our experiments we could decrease the content of microtubules in MAPs from 1 MAP per 11–12 tubulin dimers down to 1 MAP per 20–25 dimers. This is not a limit since at very high tubulin concentrations microtubules could be obtained without any MAPs bound.

It seems reasonable to think that the energy variation ΔG_{ij} which is associated to the formation of a microtubule consisting of i molecules of tubulin and j molecules of MAPs is equal to the sum of the terms ΔG_1, corresponding to the tubulin–tubulin interactions in the microtubule wall, and ΔG_2, corresponding to the stabilization of the microtubules by MAPs:

$$\Delta G_{ij} = i\Delta G_1 + j\Delta G_2 = i[\Delta G_1 + (j/i)\Delta G_2]$$

Consequently the apparent equilibrium dissociation constant of these microtubules is $K'_c = \exp[-[\Delta G_1 + (j/i)\Delta G_2]/RT] = K_{c_0}\gamma^{j/i}$, where K_{c_0} is the elongation equilibrium constant in the absence of MAPs and γ is the stabilization constant of one molecule of tubulin in the microtubule by one molecule of MAP. Figure 8 shows a linear representation, $\log K'_c = \log K_c + (j/i) \log \gamma$, of the data shown in Figure 7, from which the values $\gamma = 10^6$ and $K_{c_0} = 2.5 \times 10^4$ M^{-1} can be derived. This last number corresponds to a critical concentration $C_{c_0} = 4.4$ mg/mL for tubulin in the absence of MAPs in these medium conditions. This value is in good agreement with the data obtained by Herzog & Weber (1977) in a similar medium, in the absence of glycerol. Our data agree with the conclusions reached by Murphy et al. (1977b) showing that an increase in the high molecular weight MAPs would decrease the depolymerization rate constant without affecting the polymerization rate constant. The results obtained by Stearns and Brown, Murphy et al., and us allow us to propose a simple model for assembly in the presence of MAPs in which protofilaments rich in MAPs would incorporate first, tubulin dimers would then add to the growing microtubule, and the

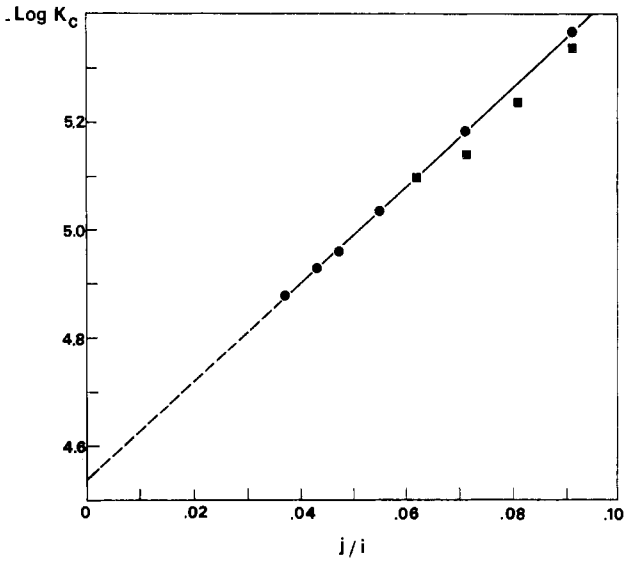

FIGURE 8: Stabilization of microtubules by MAPs. The critical concentration of microtubules obtained when various amounts of phosphocellulose pure tubulin are copolymerized with a constant amount of Shelanski tubulin (data from Figure 6) is plotted vs. the ratio j/i of the number of MAP2 molecules per tubulin dimer in the microtubule. Assuming 15% in weight of MAP2 to be present in the cycled tubulin preparation, a number of 1 MAP2 molecule (M_r 280000) per 11 ± 1 molecules of polymerized tubulin was calculated when Shelanski tubulin alone was polymerized. When pure tubulin was copolymerized with Shelanski tubulin, the evolution of the ratio j/i could be calculated from the increase in microtubule formation.

MAPs would redistribute evenly on the surface of the wall in order to stabilize the polymer. This stabilization energy is proportional to the average amount of MAPs bound to a given amount of polymerized tubulin.

Acknowledgments

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Chemical Differences Distinguish Ciliary Membrane and Axonemal Tubulins†

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ABSTRACT: Tubulin was prepared by exhaustive dialysis solubilization from axonemal A and B subfibers and by detergent solubilization of the membrane of cilia from the scallop *Aequipecten irradians*. The respective α and β chains were isolated by preparative sodium dodecyl sulfate ($NaDodSO_4$)-polyacrylamide gel electrophoresis and then compared by amino acid analysis and high-resolution two-dimensional tryptic peptide mapping. Minor amino acid and peptide differences distinguished A- and B-subfiber-derived tubulin subunits from each other, but far more significant amino acid differences distinguished the membrane-derived subunits from those of the axoneme. Peptide mapping revealed that each membrane tubulin subunit contained two major peptides not coincident with those of the axoneme and vice versa, but many corresponding peptides differed markedly in relative intensity. The α and β subunits from these sources showed virtually identical isoelectric points. Certain $NaDodSO_4$-polyacrylamide gel systems, sensitive to differential detergent binding, allowed the membrane-derived subunits to be distinguished from those of the axoneme. Under nondenaturing conditions, the membrane-derived tubulin bound both anionic and cationic detergents more strongly than axonemal tubulin. These data indicate that ciliary membrane tubulin is a distinct molecule whose subunits have the same molecular weight and isoelectric point as those of axonemal tubulin but differs chiefly in terms of nonpolar, conservative substitutions. These chemical differences argue against the artifactual origin of the protein from breakdown of the axoneme.

In a comparison of membranes from molluscan gill cilia with those from sperm flagella, it was found that the latter were composed chiefly of a 250 000-dalton glycoprotein while the former contained a like amount of 55 000-dalton, mildly PAS-positive (periodic acid–Schiff base-positive)[1] protein. The 55 000-dalton ciliary membrane protein was identified as a tubulin on the basis of electrophoretic separation into two equimolar chains, size distribution of CNBr fragments, tryptic peptide mapping, and colchicine binding (Stephens, 1977a). In a study utilizing a variety of membrane preparation methods, Dentler (1980) showed that, regardless of the method, the ciliary membrane of *Tetrahymena* was also composed chiefly of a 55 000-dalton, PAS-positive protein whose combined equimolar subunits yielded tryptic peptides characteristic of a tubulin dimer. A preliminary vectorial labeling study indicated that molluscan ciliary membrane tubulin was an integral membrane protein (Stephens, 1977b) while a later study demonstrated cross-linkage of both the molluscan and the ciliate 55 000-dalton protein within the membrane bilayer by a lipophilic, bifunctional reagent (Dentler et al., 1980).

By no means is the claim for a 55 000-dalton, tubulin-like major ciliary membrane protein a universal one. Two sets of workers (Adoutte et al., 1980; Brugerolle et al., 1980) find only small amounts of such a protein in membrane vesicles prepared from *Paramecium* cilia. Flagellar membranes typically contain none (Witman et al., 1972; Stephens, 1977a), although there are several reports to the contrary (Otter, 1978; Adair & Goodenough, 1978). The novelty of tubulin—a classic cytoplasmic protein—in a ciliary membrane, the dominance of tubulin as a structural component of the 9 + 2 axoneme, and the near absence of tubulin in membranes of related organelles or organisms raise the obvious question of artifact.

Based upon simple quantitation, it was argued previously that breakdown of the axoneme during isolation was a very unlikely source for the tubulin found in detergent-solubulized membrane fractions (Stephens, 1977a; Dentler, 1980). Even if both labile central pair members dissolved totally, they could account for only one-third of the tubulin found in the membrane fraction. However, the amorphous ciliary matrix might contribute unpolymerized axonemal tubulin awaiting assembly. This protein would be subject to solubilization by detergent or to entrapment during vesicle preparation. Such a protein might be identifiable as arising from 9 + 2 microtubules through some specific chemical property.

This report presents further biochemical characterization of the molluscan 55 000-dalton ciliary membrane protein, concluding that it is a tubulin isotype differing significantly in its chemical properties from the tubulin of the axoneme. A preliminary account of this work was presented at the 24th Annual Meeting of the Biophysical Society, New Orleans, LA, June 1-5, 1980 (Stephens, 1980).

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[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; PAS, periodic acid–Schiff base; DCC, dicyclohexylcarbodiimide; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone.

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