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Kinetic Analysis of Cooperativity in Tubulin Polymerization in the Presence of Guanosine Di- or Triphosphate Nucleotides†

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ABSTRACT: In vitro polymerization of pig brain tubulin, highly purified and deprived of microtubule-associated proteins, was followed by turbidimetry. Treatment of the data yielded the relation existing between the observed turbidity and the amount of polymer formed. This allowed a kinetic analysis, according to Oosawa's theories, of the polymerization process, which consisted of a slow spontaneous nucleation followed by the growth process. The apparent elongation rate constant was closely related to the nucleation process and exhibited a highly cooperative variation with tubulin concentration. The cooperativity was indicative of the size of the nucleus which appears to remain the same whether sheets or microtubules are formed. Magnesium ions appear to play a role in the polymorphism of tubulin polymers, the proportion of microtubules to sheets increasing with magnesium ion concentration. From kinetic experiments evidence was provided for GDP binding in competition with GTP, with a sixfold lower affinity. The tubulin–GDP complex could participate in microtubules elongation, but was not able to form nuclei. The critical concentration of tubulin in the presence of GDP was roughly twice as high as in the presence of GTP.

Highly pure tubulin from mammalian brain is able to polymerize in vitro in the absence of any associated protein, and in a variety of buffers containing magnesium ions, GTP and glycerol (Lee & Timasheff, 1975, 1977). A thorough thermodynamic study of the equilibrium microtubules ⇌ tubulin has been performed under these medium conditions, but until now the kinetics of polymerization has not been used to obtain information about the mechanism and pathways of pure tubulin self-assembly. Turbidimetry measurements of pure tubulin self-assembly have been analyzed in this work with the kinetic theories of protein polymerization developed by Oosawa.

From the data found in the literature, GTP seems to play a puzzling role in tubulin assembly: GTP is necessary for assembly but is hydrolyzed during polymerization and at the ends of microtubules (Maccioni & Seeds, 1977; David-Pfeuty et al., 1977). Isolated microtubules contain only GDP at the exchangeable (E) site (Berry & Shelanski, 1972; Jacobs et al., 1974) and seem to be stable. Two classes of models can account for this result: either GDP is able to promote microtubules assembly, or GTP hydrolysis is necessary for polymerization. However, Weisenberg's experiments (Weisenberg et al., 1976) do not agree with the former hypothesis, while data from other authors indicate that nonhydrolyzable GTP analogues can induce polymerization, which eliminates the latter hypothesis (Kobayashi, 1974; Kobayashi & Simizu, 1976; Arai & Kaziro, 1976). In this paper, attempts have been made to clarify some of these points by a kinetic analysis of the observed effects of GTP and GDP on tubulin polymerization.

Materials and Methods

Reagents. 2-*N*-Morpholinoethanesulfonic acid (Mes)[1] was purchased from Calbiochem, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA) from Sigma, and fibrous cellulose phosphate P 11 from Whatman. Guanosine 5′-triphosphate trilithium salt from Boehringer and guanosine 5′-diphosphate sodium salt I from Sigma were used after checking purity by chromatography on PEI-cellulose Polygram cell 300 (Macherey & Nagel). All other reagents were Merck's analytical grade. Radioactive nucleotides were purchased from Amersham.

Tubulin Purification. Tubulin was purified from fresh pig brain according to Shelanski et al. (1973) by three consecutive cycles of polymerization. Purification was achieved by passage of this tubulin preparation through a phosphocellulose column equilibrated in 0.05 M *N*-morpholinoethanesulfonic acid (Mes) buffer, pH 6.6, containing 0.25 mM $MgCl_2$, 0.5 mM EGTA, and 0.1 mM GTP. Tubulin was thus separated from the microtubule associated proteins (Weingarten et al., 1975). Purity of tubulin was checked by polyacrylamide gradient gel electrophoresis on PAA 4-30 Pharmacia Slabs in $NaDodSO_4$ buffer, pH 8.2. To the eluted tubulin fraction was then added 3.4 M glycerol and this solution was concentrated using an Amicon concentrating cell Model 52 equipped with a PM 30 Diaflo membrane. The resulting tubulin solution (5–10

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[1] Abbreviations used: Mes, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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mg/mL) containing 0.1 mM GTP was then stored at −30 °C and could be used within a week without any appreciable loss in its ability to polymerize.

Sephadex G-25 Gel Filtration. A 1 × 30 cm Sephadex G-25 medium column, equilibrated in 0.05 M Mes buffer (pH 6.6) containing 0.5 mM EGTA, 3.4 M glycerol, and 0.25 mM $MgCl_2$ was used to separate the protein from the major part of the 100 μM GTP present in the stock tubulin solution (1-mL aliquots of tubulin solution were used). Because of the existence of a "non exchangeable" site for GTP and of the high affinity of the "exchangeable site" (Jacobs et al., 1974; Arai & Kaziro, 1976), 2 molecules of GTP remained bound to the tubulin dimer molecule eluted from the column. The exact concentration of nucleotides present in the tubulin fraction was determined in the following way: free and exchangeable GTP present with tubulin was labeled with [^{3}H]GTP before applying to the G-25 column, the radioactivity of the eluted tubulin fractions was obtained, and the protein concentration was determined from the UV spectrum. Total nucleotide concentration (free, exchangeable, and nonexchangeable GTP) was determined before and after the G-25 column by precipitating tubulin with 4% $HClO_4$, and spectrophotometric determination of the concentration nucleotides present in the supernatant using an extinction coefficient at 256 nm of 12.4 mM^{-1} cm^{-1} at pH 1) in this medium.

Protein Concentration Measurements. When nucleotides were present, tubulin concentration was determined by the method of Lowry et al. (1951) using the correction of 11% excess coloration as compared with the bovine serum albumin standard (Pfeuty et al., 1977). In the absence of free nucleotides, tubulin concentration was determined spectrophotometrically using an extinction coefficient of 1.2 mL cm^{-1} mg^{-1} (H. P. Erickson, personal communication). When used simultaneously, these two methods gave the same result.

Polymerization Buffer. Polymerization of purified tubulin was studied in a buffer consisting of 0.05 M Mes (pH 6.6), 0.5 mM EGTA, 3.4 M glycerol and varying GTP and $MgCl_2$ concentrations (Lee & Timasheff, 1975). This buffer was called P buffer.

Turbidity Measurements. Tubulin self-assembly was monitored by turbidimetry. Turbidity of tubulin solutions was measured from the absorbance at 350 nm using a Beckman Acta V recording spectrophotometer, coupled to a (slow) temperature-jump apparatus, build up in the laboratory. Polymerization was started by a jump in temperature (rise time from 4 °C to 37 ± 0.1 °C = 15 s). The thermostated cell contained 100 μL and had a light path of 0.5 cm.

Electron Microscopy Studies. Polymers of tubulin were collected by centrifugation at 37 °C at 30 000g for 20 min in a Beckman Spinco L50 centrifuge. The supernatant was removed and the pellet was processed according to Tilney et al. (1973) with the following modifications: fixation was carried out for 30 min at 37 °C in the polymerization buffer containing 2% glutaraldehyde and 8% tannic acid. Cold buffer without glycerol was used for washing. Postfixation was performed with 1% OsO_4 in Veronal buffer, pH 7.2, for 30 min at 20 °C. The pellets were then dehydrated by acetone and embedded in Araldite. Thin sections were cut with a LKB Ultramicrotome, stained with uranyl acetate and lead citrate, and observed with a Hitachi HU II B electron microscope.

Results

Effect of Tubulin Concentration. Tubulin self-assembly in buffer P containing 5 mM $MgCl_2$ and 0.5 mM GTP was monitored by turbidity (Berne, 1974). The extent of turbidity at different protein concentrations was linearly correlated with the mass of microtubules, as measured by centrifugation, when the tubulin concentration was lower than 1.5 mg/mL, and deviated from linearity at higher concentrations (Figure 1). The "critical concentration" C_C was defined as the extrapolated value of tubulin concentration giving no increase in turbidity. A value 0.3 mg/mL was repeatedly determined for C_C in the presence of 5 mM $MgCl_2$.

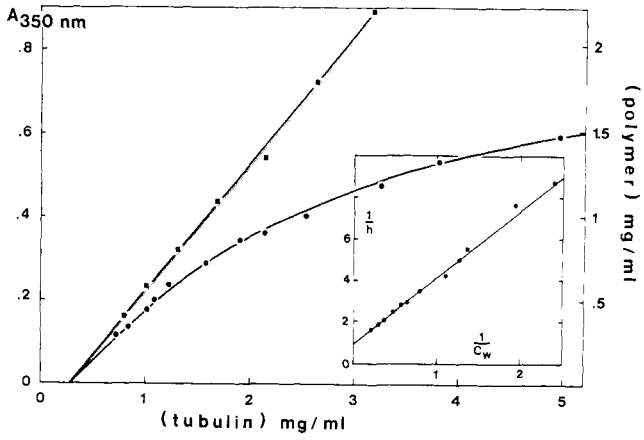


FIGURE 1: Turbidity measurement of tubulin self-assembly. Tubulin was polymerized in buffer P containing 5 mM $MgCl_2$ and 1 mM GTP. The tubulin concentration in the stock solution was determined by the corrected Lowry method. Different dilutions were realized from the stock solution after labelling by trace [γ-^{32}P]GTP and concentration of tubulin in each sample was determined from radioactivity measurement. (●) Turbidity measurement (ΔA_{350nm}). Light path = 0.5 cm. Dotted line = correction of turbidity for concentration effect to eq 1. (■) Concentration of polymerized tubulin in the solution determined by the centrifugation technique. Inset: plots of $1/h$ vs. $1/C_W$, where $C_W = C_{total} - C_C$. The value of C_C was determined graphically from the plots of h vs. C_{total}.

The following empirical relation was established between the observed turbidity h and the weight concentration of microtubules C_W (see Figure 1, insert): α and β are coefficients determined graphically, and $C_W = XC_{total} - C_C$, X being the fraction of polymerizable tubulin ($X \simeq 0.9$). The value of the corrected turbidity $h' = \alpha C_W$ is directly proportional to the amount of polymer and can be calculated from the relationship:

$$h' = \frac{h}{1 - \beta h} \tag{1}$$

The recorded time courses of $h(t)$ were then corrected using eq 1 in order to obtain the true measurement of the evolution of C_W with time.

The data obtained can be described in terms of a lag time τ followed by a pseudo-first-order process of rate constant k. Both $1/\tau$ and k vary in a strongly cooperative manner with tubulin concentration (Figure 2). A plot of log k or log $1/\tau$ vs. log C_{total} has a slope between 5 and 6 (Figure 2, insert).

These results exhibit the distinctive features of a polymerization in which spontaneous nucleation takes place during the lag time and is followed by elongation. During the elongation, no more spontaneous nucleation occurs; therefore the following equation describes the elongation process (Oosawa & Asakura, 1975)

$$dc/dt = (k_+c - k_-)M \tag{2}$$

where c is the concentration of tubulin dimer, M is the concentration of elongating species which here appears to be constant, and k_+ and k_- are the association and dissociation rate constants of one protomer to and from the polymer. The apparent rate constant for the elongation process is $k_{app} = k_+M$. The nucleation and elongation process are related since

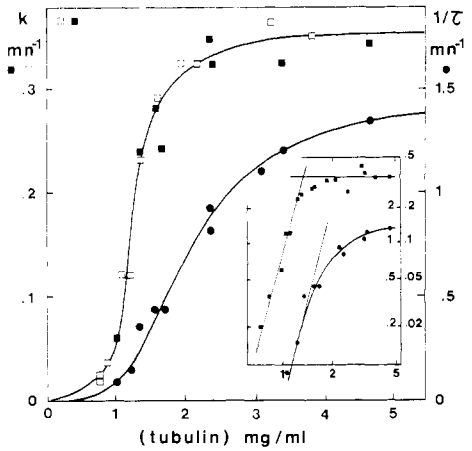


FIGURE 2: Cooperativity involved in the time course of tubulin self-assembly. The experimental conditions were the same as in Figure 1. (□ and ■) Plots of K_{app} vs. C_{total} (two separate experiments). (●) Plots of reciprocal of lag time ($1/\tau$) vs. C_{total}. Inset: replot in logarithmic scales.

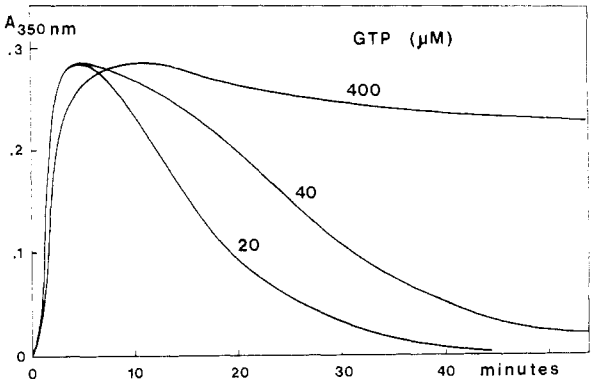


FIGURE 3: Role of GTP and GDP in the stability of microtubules. Phosphocellulose tubulin was freed from nucleotides by passage through a Sephadex G-25 column equilibrated with P buffer (see Materials and Methods). To the eluted tubulin at the concentration of 20.8 μM (2.29 mg/mL) were added 5 mM $MgCl_2$ and GTP at the indicated concentrations: 20, 40, and 400 μM. The time course of the evolution of turbidity at 37 °C was recorded and corrected for the slow formation of some aggregates.

k_{app} is proportional to the concentration of nuclei which are formed during the lag time.

Effect of Magnesium Ion Concentration. The same cooperativity was observed in the kinetics of self-assembly at various magnesium concentrations from 2 to 15 mM. The value of the slopes d log k/d log c_{total} or d log $(1/\tau)$/d log c_{total} was independent of the magnesium ion concentration up to 12 mM. The critical concentration appeared to increase at low Mg^{2+} concentrations, and Mg^{2+} increased the rate of polymerization, as already mentioned by other authors (Lee & Timasheff, 1977; Herzog & Weber, 1977). However, it was observed that, at Mg^{2+} concentrations higher than 5 mM, the formation of amorphous aggregates increased with Mg^{2+} and represented as much as 40–50% of the extent of the turbidity at 15 mM Mg, and 60–70% at 20 mM Mg, as measured from the percentage of reversibility of self-assembly when cooling and from electron microscopy. For that reason, further experiments were performed with 5 mM Mg^{2+}.

Role of Nucleotides in the Self-Assembly of Tubulin. As already seen by several authors, tubulin freed from nucleotides after passage through G-25 column (see Materials and Methods) at a concentration of 2–2.5 mg/mL could not polymerize unless more GTP was added. Figure 3 shows three time courses of polymerization of tubulin (concentration 19 μM) in P buffer containing 5 mM $MgCl_2$ and 20, 40, and 400 μM GTP, respectively. The rapid increase in turbidity was the same in the three experiments, but, after the maximum turbidity was reached, turbidity decreased slowly, showing depolymerization of microtubules, in the cases when GTP concentration was low. The decrease in turbidity was faster and the extent of depolymerization was greater when the concentration of added GTP was lower. Complete depolymerization occurred when polymerization was performed with 20 μM GTP. After this spontaneous depolymerization it was not possible to polymerize again unless more GTP was added. At magnesium concentrations higher than 5 mM, the extent and rate of depolymerization both increase at a given GTP concentration. Depolymerization was also checked by measurement of the protein concentration in the supernatant after centrifugation. Under these glycerol + Mg medium conditions, tubulin exhibits a characteristic GTPase activity which is polymerization dependent (Pfeuty et al., 1977). The depolymerization shown above must then be attributed either to the lack of GTP (this nucleotide would then be necessary to stabilize assembled microtubules), or to the GDP produced which would depolymerize microtubules. In order to answer this question, tubulin was polymerized as above with 20 μM GTP, and various concentrations of GDP were added when the maximum turbidity was reached. The volume of the added GDP solution was small enough to avoid dilution of more than 2% of the tubulin solution. Depolymerization followed the addition of GDP. The extent of depolymerization was a decreasing function of GDP concentration, and reached a stable minimum at high GDP concentration, showing that microtubules could be stabilized by GDP in the absence of GTP. This result can be explained by the following chemical equilibria:

$$\text{microtubules} \overset{K'_p}{\rightleftharpoons} \text{tubulin-GDP}$$

$$\text{tubulin-GDP} \overset{K_{GDP}}{\rightleftharpoons} \text{tubulin} + \text{GDP}$$

and

$$\text{microtubules} \overset{K_p}{\rightleftharpoons} \text{tubulin-GTP}$$

$$\text{tubulin-GTP} \overset{K_{GTP}}{\rightleftharpoons} \text{tubulin} + \text{GTP}$$

The two equilibrium constants K_p and K'_p are the reciprocals of the critical concentrations for assembly measured in the presence of saturating concentrations of GTP and GDP, respectively. The critical concentration $C' = 1/K'_p$ was measured by centrifugation (Figure 4) after polymerization with a minimum amount of GTP and depolymerization by 1 mM GDP. The value of $1/K'_p$ was found to be about twice as large as that of $1/K_p$ determined in the presence of 1 mM GTP. The relative values of K_{GTP} and K_{GDP} can be obtained from competition experiments as described below.

When deprived of GTP after passage through a Sephadex G-25 column, tubulin was found incapable of polymerizing for at least 1 h at 37 °C in polymerization buffer containing 5 mM $MgCl_2$ and 1 mM GDP. However, if microtubules, after hydrolysis of GTP, are stabilized in the presence of 1 mM GDP, it seems thermodynamically necessary that the tubulin-GDP complex should be able to polymerize. In order to solve this paradox, the experiment precedently processed by Weisenberg et al. (1976) was reconducted under our experimental conditions: to the nonpolymerizing solution of tubulin containing 1 mM GDP was added a very small volume (2% v/v) of tubulin

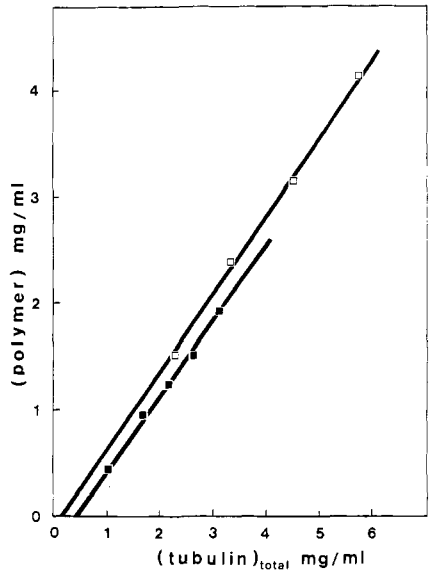


FIGURE 4: Polymerization equilibrium of GDP-tubulin and GTP-tubulin. Several dilutions were prepared from the tubulin fraction eluted from a Sephadex G-25 column. To each sample was added GTP at the same concentration as tubulin. It was checked that the extent of polymerization obtained under such conditions was the same as with a high GTP concentration. When the plateau was reached, 2 mM GDP was added, and incubation at 37 °C was pursued for 45 min, followed by centrifugation at 30 000g for 20 min. The difference in protein concentration of the solution before and after centrifugation gave the weight concentration of the polymer (■); protein concentrations were determined by the method of Lowry). In a control experiment, tubulin at different concentrations was polymerized with 1 mM GTP for the same time and processed in the same way (□).

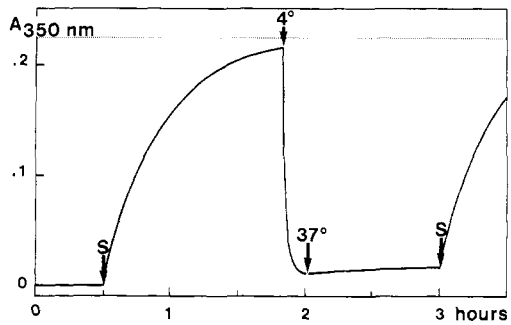
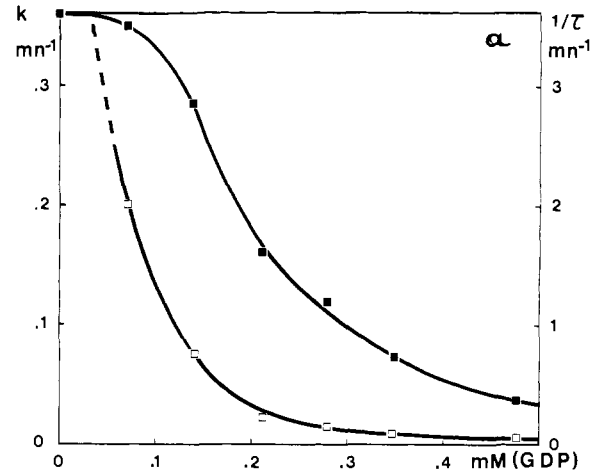


FIGURE 5: Elongation of microtubules promoted by the tubulin-GDP complex. The tubulin solution used was 1.8 mg. After removal of nucleotides and addition of 700 μM GDP, an aliquot of this solution proved unable to polymerize when standing for about 2 hr at 37 °C. At time indicated by the arrow, 5 μL of microtubule seeds (S) was added to another aliquot of this solution at 37 °C. Polymerization was reversible upon cooling to 4 °C. Another polymerization at 37 °C could not be obtained unless some more seeds were added.

at the same concentration and polymerized in the presence of 100 μM GTP was added to the nonpolymerizing solution of tubulin; the seeds thus added immediately promoted the polymerization of tubulin-GDP, without any lag, by a first-order process of rate constant $k = 0.02$ min^{-1} under these conditions (Figure 5). It was verified that the small amount (3 μM) of GTP added with the seeds could not by itself cause polymerization. The pseudo-first-order rate constant was the same when GDP concentration was varied from 0.7 mM to 2 mM and was only dependent upon the concentration of seeds. When the concentration of tubulin was lower than the critical concentration $1/K'_p$, no polymerization occurred after addition of seeds. This may be the reason why Weisenberg et al. (1976)



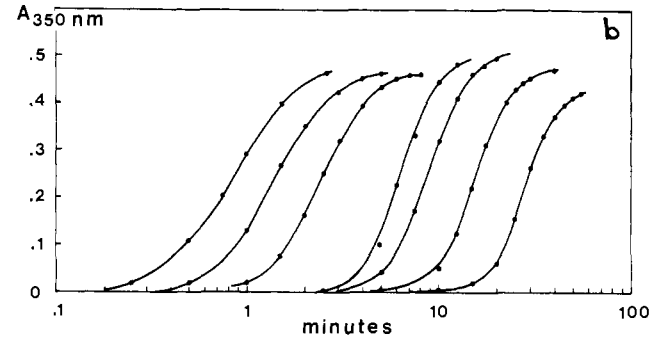


FIGURE 6: Inhibition of the rate of tubulin assembly by GDP. (a) Cooperative decrease with GDP in the apparent propagation constant k (■) and in the reciprocal of lag time $1/\tau$ (□). To a solution of tubulin freed from nucleotides and at a concentration of 17.5 μM (1.92 g/mL) were added 5 mM $MgCl_2$, 50 μM GTP, and variable GDP concentrations. The samples were incubated for at least 30 min at 0 °C before starting polymerization by a temperature jump from 0 to 37 °C. (b) Representation of the time course of self-assembly according to Oosawa (1975), at different GDP concentrations, from left to right: 0, 71, 140, 210, 278, 345, and 475 μM.

did not observe polymerization of the tubulin-GDP complex. No adenylate kinase was present in the purified tubulin preparation: it could catalyze the reaction 2GDP ⇄ GMP + GTP and allow polymerization indirectly.

Thus tubulin-GDP, although unable to induce polymerization, can promote the elongation of microtubules when preformed tubules are added as seeds to the solution. In complement to this result, a quantitative analysis of the role of GDP in the tubulin self-assembly was carried out. The rate of polymerization was determined at constant GTP concentration and GDP was varied. For each sample, tubulin was incubated with GTP and GDP at the desired concentrations for at least 30 min at 0 °C to ensure a complete equilibration of the nucleotides with the tubulin. It was observed that when GDP increased, the reciprocal of lag time, $1/\tau$, and the elongation first-order rate constant, k_{app}, both decreased strongly and cooperatively (Figure 6a). The extent of polymerization was, however, independent of GDP concentration. Plots of turbidity vs. log time for each experiment at a given concentration of GTP with varying GDP gave a set of parallel curves (Figure 6b). Thus when GDP is varied, the processes of nucleation and growth remained the same (Oosawa & Asakura, 1975). These results can be interpreted with a model in which GDP binds competitively to the GTP site, and the tubulin-GDP complex can participate in the elongation, but not in the nucleation

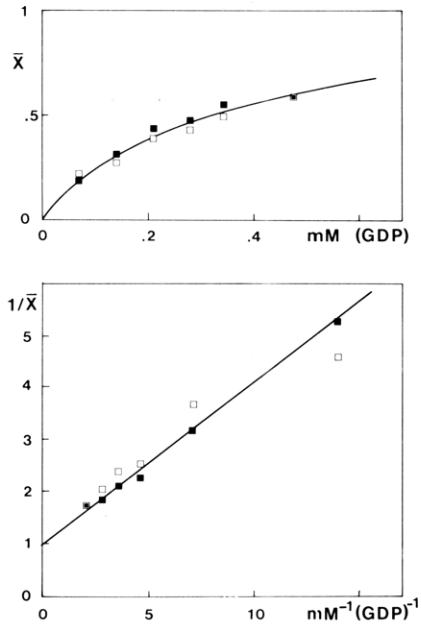


FIGURE 7: Kinetic effect of the competition between GTP and GDP on tubulin. Data from Figure 6 were treated and replotted as follows. The values of k and $1/\tau$ obtained in the presence of GDP at a given tubulin concentration C_0 were compared with the respective calibration curves of k and $1/\tau$ vs. tubulin concentration obtained in the presence of GTP (Figure 2). An "equivalent concentration" C_{eq} of tubulin could thus be attributed to each GDP concentration. The ratio $\bar{X}$ defined as $(C_0 - C_{eq})/C_0$ represented the percentage saturation of tubulin by GDP. Double-reciprocal plots of $1/\bar{X}$ vs. 1/GDP derived from the comparison of either k(GDP) (■) or $1/\tau$ (□) with the corresponding calibration curves gave the same result for $K_{app}(GDP) = 0.3$ mM, in the presence of 50 μM GTP.

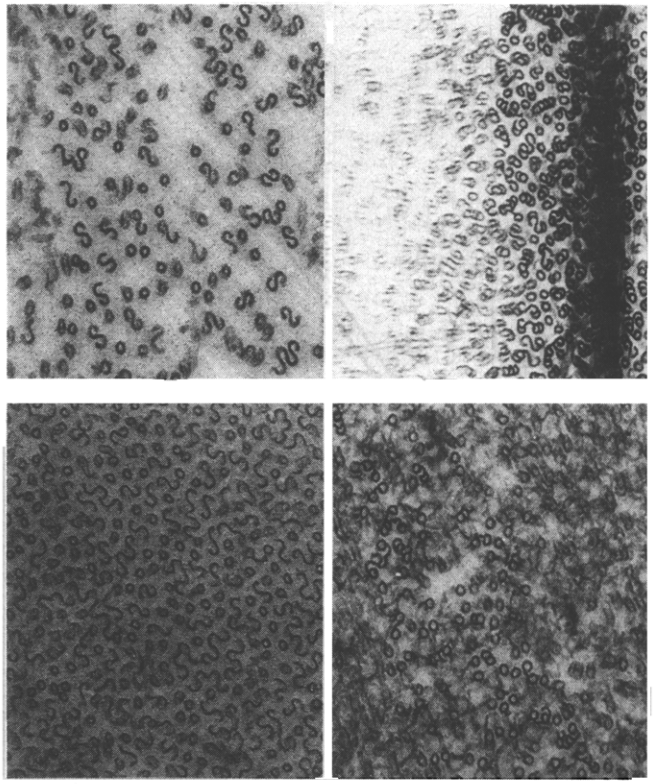


FIGURE 8: Sections of assembled tubulin polymers obtained under different conditions. The experiment was conducted as described in the Materials and Methods section. Magnesium ions and tubulin concentrations were as follows: (top) tubulin concentration in the polymerizing medium = 1 mg/mL; (bottom) tubulin concentration = 8 mg/mL; (left) 2 mM $MgCl_2$; (right) 10 mM $MgCl_2$.

process. Consequently, increasing GDP means decreasing the concentration of tubulin available for nucleation. If tubulin GDP participates in elongation with a rate constant k', the observed elongation process occurs according to the following equation derived from eq 2:

$$-\frac{d(c + c')}{dt} = (kc + k'c')M \qquad (3)$$

where c and c' are the tubulin–GTP and tubulin–GDP concentrations, respectively. Assuming that the exchange of nucleotides on tubulin is fast with regard to the elongation process, c and c' are linked by the relation $c' = \gamma c$, where γ is the ratio of the reduced concentrations of GDP and GTP ($\gamma = (GDP/GTP)(K_{GTP}/K_{GDP})$).

The apparent elongation rate constant in the presence of GDP is thus $k'_{app} = (k + \gamma k')M/(1 + \gamma)$. The values of k'_{app} and $1/\tau$ at a given GDP concentration are identical with the couple of values obtained in the presence of GTP at a lower tubulin concentration. Therefore, at each GDP concentration the values of k'_{app} and $1/\tau$ can be placed on the curve given in Figure 2. The tubulin concentration at which the points are placed, at each GDP concentration, is the effective concentration of tubulin–GTP. The amount of tubulin–GDP present is then the total concentration of tubulin minus the equivalent concentration of tubulin calculated in this way. The fraction of tubulin–GDP present is plotted as a function of the GDP concentration in Figure 7 and defines a binding isotherm for GDP binding to tubulin. The apparent dissociation constant for GDP at a given concentration of GTP was obtained from a double-reciprocal plot of the data. Roughly the same isotherm was obtained whether the plots came from the $1/\tau$(GDP) or k'_{app}(GDP) values. This finding indicates that k'_{app}(GDP) was independent of γ, which means $k' \sim k$, and $k'_{app} = kM$. The variation of k'_{app} with GDP was thus only due to the variation of the concentration M of nuclei, which can be formed only from tubulin–GTP. This is a confirmation that the equivalent concentration of tubulin determined by this method for each GDP concentration was indeed the tubulin–GTP concentration present in the solution.

A plot of the apparent dissociation constant of GDP vs. the GTP concentration given was linear but the intercept was too close to zero to permit an accurate determination of the intrinsic dissociation constants K_{GTP} and K_{GDP}. However, from the slope of this plot, the ratio K_{GDP}/K_{GTP} was found to be about 6.

Characterization of the Tubulin Polymers Obtained under Different Conditions by Electron Microscopy. Polymerization of tubulin was performed in buffer P containing 1 mM GTP and various magnesium ion concentrations from 2 to 15 mM. Microtubules were collected by centrifugation, and the pellets were processed for electron microscopy (see Materials and Methods). Figure 8 shows sections of the observed species at two tubulin concentrations and two magnesium concentrations (2 and 10 mM). At 15 mM magnesium, the formation of amorphous aggregates was highly predominant (data not shown). It was observed that several morphologically different species were present among the polymers, essentially microtubules, "S" sheets consisting of about 24–26 protofilaments having the shape of an S and some larger sheets. Such "S" sheets also have been observed with 10 mM phosphate buffer, 12 mM $MgCl_2$, 3.4 M glycerol (H. P. Erickson, unpublished data). The magnesium effect was more pronounced at low tubulin concentration (see diagram, Figure 9).

When tubulin was polymerized with 5 mM magnesium and

the concentration was raised to 10 mM for 1 h after the maximum in microtubule formation was reached, the sections obtained displayed the same repartition of polymer species as when tubulin was polymerized with 10 mM magnesium present at time zero with the tubulin. This result indicates that magnesium can promote the transformation of previously formed sheets into microtubules. This phenomenon occurred without any gross change being observed in the time evolution of the turbidity at the plateau showing that the turbidity of "S" sheets is comparable to that of microtubules. In 10 mM magnesium, doublets and triplets of microtubules quite similar to those that can be observed in the flagella or centrioles are observed. Further investigation on the structure and formation of these doublets is now carried out in this laboratory.

Discussion

The results presented here, concerned with the self-assembly kinetics of purified tubulin, agree with the kinetic theories developed by Oosawa for nucleated helical polymerization of macromolecules. Our data are consistent with a model in which the purified tubulin dimer can form nuclei spontaneously and in which nucleation and elongation are two successive distinct processes.

In the turbidity measurements, we have assumed the hypothesis of Berne (1974) in which turbidity is proportional to the weight concentration of rod-like polymers, independent of their length. This condition appears to be valid in the low concentration range of tubulin ($C < 2$ mg/mL). At higher concentrations, however, some deviation from linearity occurred. This deviation can be explained by a rearrangement of concentrated polymers into a more rigid state, which would result in gel formation. The equation describing the turbidity is empirical; however, it should be pointed out that it is quite similar to a virial expansion of the turbidity:

$$HC_W/h = 1/M_W + 2BC_W + 3CC_W^2 \qquad (4)$$

where h is turbidity, C_W the weight concentration of the solute, M_W its weight average molecular weight, H an optical constant, and B and C virial coefficients. This equation predicts the turbidity should decrease at high concentrations of tubulin (if B and C are positive). Indeed when a solution of microtubules is centrifuged, the pellet obtained, in which the concentration of polymer is as high as 20 mg/mL, is a transparent gel.

The assumption of polymer rigidity in Berne's model is satisfied for all the polymer species observed since the lateral curvature of "S" sheets prescribes the same rigidity for this polymer as for microtubules.

The same apparent cooperativity was derived from the plots of the first-order propagation rate constant or of the reciprocal of lag time vs. the tubulin concentration. The significance of this cooperativity number can be discussed in terms of a simple model in which the nucleation is followed by elongation. The following equation (Oosawa & Asakura, 1975) describes the evolution with time of the concentration of tubulin dimer c_1:

$$\frac{dc_1}{dt} = -c_1\sqrt{k_+k_N}(c_0^n - c_1^n)^{1/2} \qquad (5)$$

where k_+ and k_N are the elongation and nucleation rate constants (reverse reactions being neglected). At the inflexion point of the polymerization time course ($d^2c_1/dt^2 = 0$), the value of dc_1/dt becomes:

$$\left(\frac{dc_1}{dt}\right)_{infl} = -\left(\frac{nk_+k_N}{n+2}\right)^{1/2}\left(\frac{2}{n+2}\right)^{1/n}c_0^{(n/2)+1} \qquad (6)$$

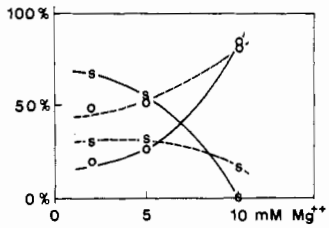


FIGURE 9: Diagram showing the proportion of tubulin incorporated into sheets (S) and microtubules (O) when magnesium concentration was varied. Tubulin concentration: 1 mg/mL (dashed line) and 8 mg/mL (solid line).

The pseudo-first-order rate constant k_{app} measured would thus be proportional to $c_0^{n/2}$, and the cooperativity number found would be equal to $n/2$, n being the size of the nucleus.

The nucleation center is likely to be a protofilament containing about 10 to 12 tubulin molecules. This elementary filament would be the first one which should be able to bind laterally other tubulin filaments, giving rise to the beginning of a sheet (see Appendix). The apparent rate constant k_{app} being related to the size of the nucleus, it is not surprising that both k_{app} and $1/\tau$ vary in a similar manner with tubulin concentration.

Almost the same apparent cooperativity number was derived from experiments performed at different magnesium concentrations, where different species of polymers were observed. This indicates that the nucleus is most presumably the same species whether sheets or microtubules are formed.

At high tubulin concentration, k_{app} reached a maximum value of 0.4 min^{-1}. Under these conditions, it is logical to think that tubulin self-assembly could be kinetically limited either by a conformational change of tubulin before assembly, or by nucleotide exchange on the tubulin which is presumably a slow process (M. Jacobs, personal communication). This hypothesis is supported by the observation that the lag time increased by about 1 min in a second polymerization cycle, independently of tubulin concentration, and under conditions where the GTP concentration was high enough so that the amount of GDP accumulated during the first cycle could not be responsible for this increase in lag time. Indeed a possible explanation is that the GDP produced at the E (exchangeable) site during the first polymerization cycle could not be exchanged in the few minutes of the cooling step, and would be more rapidly replaced by GTP during the first minutes of heating of the second cycle. This first-order process would be kinetically limiting at high tubulin concentration.

Our results show that GTP appears to be the specific ligand for nucleation whereas elongation can occur equally well with GDP as with GTP. This assessment should not be understood in a too restrictive way. Obviously, from a thermodynamic point of view, if GDP can promote elongation, it should be able to promote nucleation. However, in the nuclei formation must be involved the nth power of a rate constant, and if the nucleation rate constant for tubulin–GDP is slightly lower than for tubulin–GTP, the nuclei formation with GDP may not take place in a reasonable interval of time.

The equilibrium dissociation constant for microtubule formation from tubulin–GDP is slightly higher than the dissociation constant for the equilibrium or microtubules with tubulin–GTP. The equilibrium dissociation constant of the tubulin–GDP complex is about sixfold higher than that for tubulin–GTP. Since hydrolysis of GTP occurs during polymerization and at equilibrium in relation with exchange of tubulin at the extremity of microtubules (Pfeuty et al., 1977), depolymerization follows the formation of microtubules when tu-

bulin is polymerized at low GTP concentration, because the concentration of GDP produced by hydrolysis of GTP during assembly is not high enough to maintain the equilibrium microtubules ⇌ tubulin-GDP ⇌ tubulin + GDP shifted to the left. The dissociation constant of the tubulin-GTP complex could not be determined from self-assembly experiments: first, this dissociation constant was most certainly lower than the tubulin concentration range used (10–50 μM); in addition, at GTP concentrations comparable to the tubulin concentration, hydrolysis of the major part of GTP occurred during the polymerization; eventually the variation with GTP of the apparent dissociation constant of tubulin-GDP complex was expected to yield the intrinsic dissociation constants K_{GTP} and K_{GDP}; but these extrapolated values were too low to be accurate enough, and our data only allowed us to assert $K_{GTP} \leq 5$ μM and $K_{GDP} = 6K_{GTP}$. This result obtained under polymerizing condition compares favorably with values published by other authors (Arai & Kaziro, 1976; Jacobs & Kaplow, 1976) under different conditions and using other techniques.

These results suggest that other GTP analogues might promote tubulin self-assembly, at least elongation if not nucleation. Inasmuch as polymerization is highly cooperative, elongation must occur as soon as there is a sufficient concentration of tubulin-GTP analogue complex in solution. Two parameters are involved: the equilibrium dissociation constant for the tubulin-GTP analogue complex; and the dissociation constant for the equilibrium microtubules ⇌ tubulin-GTP analogue. This suggests that discrepancies in the literature about the ability of GTP analogues to promote polymerization may arise from the use of different concentrations of tubulin and nucleotide concentrations.

As has already been observed by other authors (Weisenberg & Timasheff, 1970; Lee & Timasheff, 1975), the essential role of magnesium ions seems to be of an electrostatic nature. Magnesium would reduce the net charge of the protein and, thus, favor tubulin–tubulin interactions. This leads to specific assembly and at high magnesium concentration, to nonspecific amorphous aggregates. In the presence of 3.4 M glycerol, both "S" sheets and microtubules were observed in equilibrium in a proportion varying with magnesium concentration. The curvature of the sections of "S" and microtubules was almost the same, and closed structures having more than 13 protofilaments were not observed. Magnesium ions thus appear to be involved not only in the longitudinal tubulin–tubulin interactions, but also in lateral interactions between protofilaments. A more detailed investigation of these phenomena is underway. Eventually it must be underlined that these kinetic studies are limited to pure tubulin assembly in magnesium and 3.4 M glycerol buffer. Obviously, other buffer conditions, and particularly the presence of variable stoichiometries of microtubule associated proteins which promote assembly, may make a difference.

Acknowledgments

We gratefully acknowledge the excellent technical assistance of Jean Laporte in the electron microscopy experiments. We wish to thank Dr. Yves Engelborghs for helpful and constructive criticism of the manuscript.

Appendix

Elementary thermodynamic concepts can account for tubulin polymorphism and show that sheet and microtubule formation both must arise from the same nucleation mechanism. Indeed the sheet-shaped wall of microtubules can be considered as a bidimensional polymer having the same helicoidal surface lattice as the closed microtubule. The following equations give the concentrations of different tubulin polymers (i) in solution either filaments, sheets, or microtubules:

$$F_i = b^{-1}(bC_1)^i = b^{-\sqrt{(i+r)r}}(KC_1)^i \quad \text{(A1)}$$

$$S_{im} = a^{-n}b^{-m}(KC_1)^i = b^{-2\sqrt{i/r}}(KC_1)^i \quad \text{(A2)}$$

$$MT_i = b^{-13}(KC_1)^i \quad \text{(A3)}$$

In these equations, C_1 is the free tubulin concentration, K is the equilibrium polymerization constant, i is the degree of polymerization, and r is the ratio of longitudinal (e_b) and lateral (e_a) bond energies, i.e.

$$r = \frac{e_b}{e_a} = \frac{\ln b}{\ln a}, \quad a = \exp\frac{e_a}{RT}, \quad b = \exp\frac{e_b}{RT} \quad \text{(A4)}$$

Demonstration and discussion of these equations will be presented in a following publication.

The shape of the sheet is determined by the ratio of these bond energies: $r = n/m$, where n is the number of tubulin molecules present in a protofilament, and m the number of protofilaments in a sheet. These equations predict that, when the degree of polymerization i is high, microtubules must be the most frequent polymers in solution, i.e., the most stable ones. Sheets appear as the most stable structures when the i value is such that $2\sqrt{i/r} < 13$. When $i/r < 1$, the predominant polymers are protofilaments containing less than r tubulin molecules. Lateral bonding between the tubulin molecules of two adjacent protofilaments occurs with a loose interaction constant a, and the resulting binding constant between two protofilaments interacting through n bonds is a^n.

The physical significance of the above relation, which can be written $a^r = b$, is that as soon as two protofilaments contain r tubulin molecules, they can be bound to each other with r lateral bounds giving the same stability as a longitudinal interaction.

Consequently the nucleation center for the formation of either sheets or microtubules must be the shortest protofilament which would be able to undergo stable lateral interaction. Statistical studies of the length of "S" sheets obtained at 37 °C in buffer P, in the presence of 5 mM Mg, give an average length of 2.8 μm which corresponds to r values of 12 to 14, taking into account the known orientation and dimension of tubulin (Erickson & Voter, 1976). This preliminary number obtained from equilibrium experiments is in good agreement with the size of the nucleation center obtained from the kinetic experiments presented in this paper. Within this model, formation of microtubules would occur from sheets containing 13 protofilaments which allows the accurate binding between the 1st and 13th protofilaments (Erickson & Voter, 1976). According to eq A2 these sheets should contain $i = 13^2r$ tubulin molecules. Microtubules formed from these sheets would be more stable than the sheets having the same degree of polymerization.

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The Blue Membrane: The 3-Dehydroretinal-Based Artificial Pigment of the Purple Membrane†

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ABSTRACT: A blue-colored artificial purple membrane pigment is formed from the recombination of retinal$_2$ with bleached apopigment. The dark- and light-adapted forms of this "blue membrane" have their λ_{max}'s at 593 and 603 nm, respectively, and their extinction coefficients relative to that of light-adapted purple membrane were 0.79 and 0.87, respectively. The circular dichroism spectrum of the blue membrane shows an exciton interaction between the chromophores, similar to that seen in the purple membrane. The ratios of maximum to minimum in the biphasic CD spectrum are larger than those of the purple membrane, the differences probably being due to a larger intrinsic CD band of the blue-membrane pigment. The rate of dark adaptation of blue membrane is faster than that of purple membrane, and the activation energy of dark adaptation of blue membrane is 19 kcal/mol, while that of purple membrane is 24 kcal/mol. The blue membrane's absorption spectrum is more readily affected by changes in pH than the purple membrane. In the purple membrane, irradiation leads to a ~410-nm intermediate designated M. An intermediate similar to this, M^B, is formed by irradiating the blue membrane at −60 °C in 1 M NaCl and at a slightly alkaline pH. The primary photoproduct was observed at liquid nitrogen temperatures, but the quantum efficiency seems to be temperature dependent. Many of the differences between the blue and the purple membrane are due to the differences in the length of π-electron conjugation of the two chromophores. However, other differences such as temperature-dependent photochemistry, different pH stability, and different rates of dark adaptation would suggest a different chromophore–protein relationship in the blue and purple membranes.

The purple membrane of *Halobacterium halobium* absorbs photons and converts the light energy to chemical free energy by the formation of a proton gradient across the membrane (Oesterhelt and Stoeckenius, 1973; Rosenfeld et al., 1977). The chromophore of purple membrane, like visual pigments such as rhodopsin and iodopsin, is retinal$_1$. Upon prolonged incubation in the dark, the purple membrane has its absorption λ_{max} at 558 nm (dark-adapted form). After illumination, the λ_{max} shifts to 568 nm; this species, the light-adapted form, can slowly revert back to the dark-adapted form (Oesterhelt and Stoeckenius, 1971). In the light-adapted form, the retinal$_1$ is in the all-trans conformation, while the dark-adapted form contains both the 13-cis and all-trans conformations (Oesterhelt et al., 1973; Pettei et al., 1977; Maeda et al., 1977). The individual purple-membrane protein molecules are arranged in a rigid, highly ordered, two-dimensional hexagonal lattice with groups of three molecules clustered about a symmetry axis (Henderson and Unwin, 1975). The chromophores of the pigment molecules interact with each other and show a typical biphasic CD spectrum due to exciton interaction (Heyn et al., 1975; Becher and Ebrey, 1976; Ebrey et al., 1977).

In nature, there is another type of retinal, retinal$_2$ (3-dehydroretinal), found in some visual pigments such as porphyropsins and cyanopsins (Wald, 1960); retinal$_2$ has one more double bond in the β-ionone ring than retinal$_1$. Because of this extra conjugation, retinal$_2$-based pigments have a number of altered spectroscopic properties, such as having their λ_{max} at

† From the Department of Physiology and Biophysics, University of Illinois, Urbana, Illinois 61801. *Received November 9, 1977.* Supported in part by United States Public Health Service Grants EYO-1323 and RCDA EYO-0025 and National Science Foundation Grant PCM76-82764.

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0006-2960/78/0417-1915$01.00/0