Tubulin–Nucleotide Interactions during the Polymerization and Depolymerization of Microtubules†

Richard C. Weisenberg,* William J. Deery, and Peter J. Dickinson

ABSTRACT: The interactions of nucleotides and their role in the polymerization of tubulin have been studied in detail. GTP promotes polymerization by binding to the exchangeable site (E site) of tubulin. The microtubules formed contain only GDP at the E site, indicating that hydrolysis of E site GTP occurs during or shortly after polymerization. Tubulin prepared by several cycles of polymerization and depolymerization will polymerize in the presence of ATP as well as GTP. Polymerization in ATP is preceded by a distinct lag period which is shorter at higher concentrations of ATP. As reported by others, ATP will transphosphorylate bound GDP to GTP. Under polymerizing conditions the maximum level of GTP formation occurs at about the same time as the onset of polymerization, and the lag probably reflects the time necessary to transphosphorylate a critical concentration of tubulin. The transphosphorylated protein can be isolated and will polymerize without further addition of nucleotide. The transphosphorylated GTP is hydrolyzed and the phosphate released during polymerization. About 25% of the phosphate transferred from ATP is noncovalently bound to the subunit as inorganic phosphate and this fraction is also released during polymerization. The nonhydrolyzable analogue of GTP, GMPPNP, will promote microtubule assembly at high concentration. GMPPNP assembled microtubules do not depolymerize in Ca concentrations several fold greater than that which will completely depolymerize GTP assembled tubules; however, addition of Ca prior to inducing polymerization in GMPPNP prevents the formation of microtubules. Thus GTP hydrolysis appears to promote depolymerization rather than polymerization. GDP does not promote microtubule assembly but can inhibit GTP binding and GTP induced polymerization. GDP does *not*, however, induce the depolymerization of formed microtubules. These experiments demonstrate that tubulin polymerization can not be treated as a thermodynamically reversible process, but must involve one or more irreversible steps. Exchange experiments with [^{3}H]GTP indicate that the "E" site on both microtubules and ring aggregates of tubulin is blocked and does not exchange rapidly. However, during polymerization and depolymerization induced by raising or lowering the temperature, respectively, all the E sites become transiently available and will exchange their nucleotide. This observation does not suggest a direct morphological transition between rings and microtubules. The presence of a blocked E site on the rings explains the apparent transphosphorylation and hydrolysis of "N" site nucleotide reported by others.

Isolated brain microtubule subunit protein (tubulin) binds guanine nucleotides with high affinity (Weisenberg et al., 1968; Berry and Shelanski, 1972; Jacobs et al., 1974; Levi et al., 1974; Kobayashi, 1974). There are at least two binding sites per 110 000 molecular weight dimer. At one of these sites (the exchangeable or E[1] site), the nucleotide exchanges rapidly with added nucleotide, while at the other site (the nonexchangeable or N site) the nucleotide exchanges very slowly. These binding sites may contain either GTP or GDP.

In a previous report from this laboratory it was demonstrated that GTP or ATP was required for microtubule assembly (Weisenberg, 1972). The promoting activity of GTP was not unexpected because of the interaction between tubulin and GTP.

A possible explanation for the role of ATP in polymerization came from the observations of Berry and Shelanski (1972) and Jacobs et al. (1974) that ATP could be the substrate for a nucleoside diphosphokinase reaction ("transphosphorylation") which can convert GDP bound to tubulin to GTP. Jacobs et al. have presented data which indicate that transphosphorylation of the bound GDP was sufficient to enable tubulin to polymerize and that this GTP was hydrolyzed during polymerization. Kobayashi (1974), on the other hand, has presented data which indicate that the bound nucleotide on the microtubule is always half GTP and half GDP and he found no evidence for hydrolysis of N site GTP during polymerization. The data of both Jacobs et al. (1974) and the more recent data of Kobayashi (1975) indicate that E site GTP is hydrolyzed during polymerization.

In the experiments reported here we have looked more closely at the interaction of tubulin with GTP and ATP and the kinetics of polymerization in ATP. We have been able to obtain tubulin free of unbound nucleotide which will polymerize and have looked at the bound nucleotide before and after polymerization. We have been able to demonstrate hydrolysis of the E site GTP during polymerization and have also observed apparent changes in the binding of GTP during polymerization and depolymerization. GTP binding and hydrolysis appear to be involved in one or more irreversible steps which occur during polymerization.

Materials and Methods

Tubulin was prepared essentially by the method of Shelanski et al. (1973). All operations were performed at 0–4 °C unless otherwise indicated. All solutions contained 0.1 M Mes (2-(*N*-morpholino)ethanesulfonic acid) at pH 6.6 adjusted with NaOH, 1 mM EGTA, and 0.5 mM $MgCl_2$ unless otherwise indicated. Fresh beef brain was homogenized in an equal volume of Mes buffer containing 25% glycerol and then centri-

† From the Department of Biology, Temple University, Philadelphia, Pennsylvania 19122. *Received August 11, 1975.* Supported by National Science Foundation Grant PCM76-02307 to R.C.W.

[1] Abbreviations used: E site, exchangeable site of tubulin; N site, nonexchangeable site of tubulin; Mes, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; GMPPNP, guanylyl imidodiphosphate.

TABLE I: Kinetics of Polymerization in ATP and GTP.[a]

Nucleotide Concn (mM)	Lag Time (s)		Initial Rate OD Change per min		Total OD Change	
	ATP	GTP	ATP	GTP	ATP	GTP
1.0	25		0.070		0.09	
0.5	35	15	0.042	0.06	0.08	0.11
0.01	105	20	0.032	0.042	0.08	0.11
0.005	120	20	0.025	0.037	0.08	0.11
0.001		40		0.030		0.08

[a]Tubulin was at a concentration of 1.2 mg/ml and was prepared by three cycles of polymerization and depolymerization.

fuged for 30 min at 13 000 rpm in a Sorval GSA rotor. The supernatant from this centrifugation was further centrifuged for 30 min at 40 000 rpm in a Spinco 50 Ti rotor. To the supernatant from this centrifugation was added 0.5 mM GTP and polymerization induced by incubating at 35 °C for 30 min. Microtubules were collected by centrifugation at 40 000 rpm for 30 min at 25 °C. The microtubule pellet was resuspended in about half the initial volume of solution and depolymerized for 30 min at 0 °C. The protein was stored after two cycles of polymerization and centrifugation in 25% glycerol at −10 °C. Prior to use, a third cycle of polymerization and centrifugation was performed using either GTP or ATP to induce polymerization. After the final clarification the tubulin solution was generally passed through at least 10 volumes of packed Sephadex G-25 medium (Neal and Florini, 1973) to remove remaining traces of glycerol and nucleotides.

Several techniques were used to separate free from bound nucleotide and these are described below.

Gel Filtration Chromatography. Unless otherwise indicated the amount of bound nucleotide was determined by chromatography on 25 × 1 cm columns of Sephadex G-25 medium to which 0.5 ml of tubulin solution was applied. The void volume was obtained within 10 min of starting the column.

Ammonium Sulfate Precipitation. A 0.5-ml solution of tubulin was added to 0.5 ml of saturated ammonium sulfate at 0 °C. The precipitate was collected by centrifugation at 20 000 rpm for 15 min. The precipitate was washed once in 50% saturated ammonium sulfate and the pellet was extracted with 5% perchloric acid to remove bound nucleotide and phosphate.

Ultrafiltration. Conical filters with an exclusion limit of 50 000 daltons (Amicon "Centriflo" membrane cones) were used to obtain a rapid recovery of unbound nucleotide. A 1-ml sample of tubulin was placed in the filter which was then centrifuged at 2000 rpm for 1 min. Approximately 0.1 ml of filtrate was recovered which was then assayed for nucleotide and phosphate.

Centrifugation. Microtubules were collected by centrifugation for 30 min at 40 000 rpm in a Spinco 40 rotor at 25 °C. In quantitative experiments the sample was centrifuged through a 2-ml layer of 10% sucrose to prevent contamination of the pellet by unbound nucleotides.

Rapid Gel Filtration. Approximately 10 ml of packed Sephadex G-25 was prepared in a 50-ml centrifuge tube as described by Neal and Florini (1973). A 1.0-ml sample of tubulin was applied to the packed gel which was then centrifuged for 1 min at 1500 rpm. The voided material was quickly recovered and used immediately for experimentation.

Nucleotides were isolated from perchloric acid extracts of tubulin by adsorption onto activated charcoal, followed by water washes and elution in 50% ethanol and 10% ammonium hydroxide. Inorganic phosphate was determined by the amount of ^{32}P which did not adsorb onto activated charcoal. Thin-layer chromatography of nucleotides was performed on Brinkman PEI plates with fluorescent indicator using the method of Gonzales and Geel (1975). Spots were eluted from the chromatograms in 1 M formic acid.

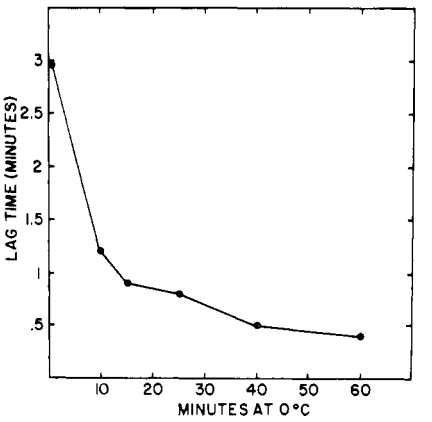

FIGURE 1: Effect of preincubation at 0 °C on the lag in ATP induced polymerization. Tublin (2 mg/ml) was incubated at 0 °C in 0.1 mM ATP, 0.1 M Mes, 0.5 mM $MgCl_2$, 1 mM EGTA, pH 6.6. Polymerization was initiated at the indicated times by transferring the sample to a water-jacketed cuvette at 35 °C.

Results

Polymerization and Transphosphorylation in ATP. To study the kinetics of microtubule assembly, solutions of tubulin were incubated for 5 min at 35 °C in a water-jacketed spectrophotometer cell and polymerization was then initiated by addition of GTP, ATP, or EGTA. Polymerization was followed by the increase in turbidity at 340 nm (Gaskin et al., 1974a). After addition of ATP polymerization does not begin until a distinct lag period has passed. The length of the lag is decreased by preincubation with ATP at 0 °C, and the longer the preincubation time at 0 °C the shorter is the observed lag (Figure 1). The brief lag observed after 60-min incubation probably reflects the warming time for the sample. Decreasing the concentration of ATP results in longer lag times and slower rates of polymerization, but little change in the final level of polymerization (Table I). Addition of preformed microtubules with the ATP had no effect on the length of the lag and the lag therefore does not appear to be the result of a slow nucleation step. At all concentrations of GTP little or no lag is observed and the initial rates and final levels of polymerization are slightly greater (Table I) than in ATP.

Further purification of tubulin by additional cycles of polymerization and depolymerization results in a preparation with a greater lag time at the same ATP and protein concen-

TABLE II: Effect of Purification on Lag Time and Transphosphorylation.[a]

No. of Cycles of Polymerization and Centrifugation	Lag Time (min)	Transphosphorylation (mol of ^{32}P Bound per mol of Tubulin)
2	1.4	0.41
3	2.9	0.37
4	5.6	0.28

[a]The tubulin concentration was 1.5 mg/ml and the ATP concentration was 0.07 mM. The amount of transphosphorylation was determined after 15 min at 0 °C by ammonium sulfate precipitation.

trations (Table II), which suggests that the lag is produced by a protein other than tubulin.

The lag in the onset of polymerization is not the result of a slow binding of ATP to tubulin. We have been unable to demonstrate any significant binding of ATP to tubulin using either equilibrium gel filtration with Sephadex which had been preequilibrated with radioactive ATP, or by the use of ultrafiltration to separate free from bound nucleotide; nor is any competition observed between ATP and GTP for binding to tubulin. Instead of competition, a slight increase in GTP binding to the exchangeable site is observed in the presence of even a tenfold excess of ATP. A similar observation has been reported by Jacobs et al. (1974).

The results of Jacobs et al. (1974) indicate that ATP promotes polymerization by the action of a transphosphorylase (nucleoside diphosphokinase) which converts tightly bound GDP to GTP. If this is true the kinetics of transphosphorylation and polymerization should be related. We have found it possible to assay transphosphorylation at short times by the use of ammonium sulfate precipitation. Ammonium sulfate precipitates of tubulin contain both N and E site nucleotide; however, only about 50% of the original bound nucleotide (as compared with gel filtration assay) is recovered in the precipitates. Nevertheless we have found it a useful and reliable tool to determine the relative extent of transphosphorylation and, since ammonium sulfate precipitation stops any further transphosphorylation, it allows the extent of reaction at short times to be determined.

Under polymerizing conditions the rate of transphosphorylation is initially very rapid and the extent of transphosphorylation reaches its maximum level about 2 min after addition of [^{32}P]ATP (Figure 2). After 2 min the amount of bound [^{32}P]GTP drops. Note that the peak of transphosphorylation occurs at about the same time as the onset of polymerization under the same conditions. Under polymerizing conditions the amount of transphosphorylation never exceeded 0.4 mol of labeled GTP per mol of tubulin, and the final level was usually observed to be about 0.2 mol per mol. At 0 °C the initial rate of transphosphorylation is slower than it is at 35 °C, but a final level is achieved which is greater than that at 35 °C (Figure 3). However, we have not been able to obtain more than about 1.0 mol of labeled GTP formed per mol of tubulin at 0 °C. These numbers have been corrected for the apparent 50% loss of bound nucleotide during ammonium sulfate precipitation. The presence of transphosphorylation at 0 °C differs from the observations of Jacobs et al. (1974) who reported no transphosphorylation at 0° C.

The formation of bound [^{32}P]GTP after transphosphorylation has been confirmed by thin-layer chromatography. However, not all of the ^{32}P bound to the ammonium sulfate

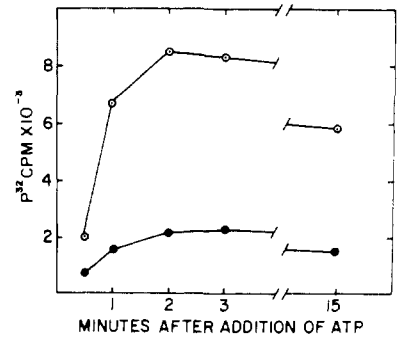

FIGURE 2: Formation of bound [^{32}P]GTP and [^{32}P]PO_4 under polymerizing conditions. Tubulin (1 mg/ml) was incubated in 0.1 mM [γ-^{32}P]ATP (8.5×10^{12} cpm/mol) at 35 °C in the standard polymerization medium, and at the indicated times aliquots were precipitated with ammonium sulfate and total and inorganic phosphate determined after perchloric acid extraction. (○) Total bound ^{32}P; (●) bound inorganic ^{32}P.

precipitated protein is present as GTP. A significant fraction of noncovalently bound inorganic phosphate is also present. This fraction is identified by being solubilized in cold perchloric acid, but is not absorbed onto activated charcoal. This fraction of bound phosphate appears to be present at a nearly constant ratio to bound labeled GTP and represents about a fourth of the total perchloric acid extractable label under polymerizing conditions (Figure 2).

A significant fraction of ^{32}P (about 0.2 mol per mol of tubulin) is present which is not extractable by perchloric acid and which is, therefore, assumed to be covalently bound. Phosphorylation of tubulin and associated proteins is being studied by others (Eipper, 1972; Sloboda et al., 1975), and we have not investigated this fraction further.

Further purification of tubulin by additional cycles of polymerization and depolymerization results in a slower rate of transphosphorylation (Table II). This is consistent with the conclusion of Jacobs et al. (1974) that the transphosphorylase is a nontubulin protein.

After incubation with [^{32}P]ATP, about half the bound [^{32}P]GTP does not chase-off with unlabeled GTP. Thus a portion of the labeled GTP would appear to be at the N site. However, as discussed below, analysis of bound nucleotide before and after polymerization indicates that the N site contains unlabeled GTP, and that the tightly bound [^{32}P]GTP is actually on a blocked E site on ring aggregates of tubulin.

Hydrolysis of Bound GTP. Measurements of the release of phosphate during polymerization are ambiguous because of the presence of ATPase activity in these preparations (Burns and Pollard, 1974; Gaskin et al., 1974b). Hydrolysis begins immediately upon addition of ATP or GTP to tubulin at 35 °C under polymerizing conditions. No lag is observed as is observed in polymerization. Nor have we been able to observe any clear correlation between phosphate release and polymerization under conditions where polymerization has been inhibited by Ca or colchicine. However, we have been able to directly demonstrate release of ^{32}P from the transphosphorylated GTP during polymerization. Tubulin was incubated for 1 h at 0 °C in 0.1 mM [^{32}P]ATP to transphosphorylate the bound guanine nucleotide, and free nucleotide was then removed by passing through Sephadex G-25 (Neal and Florini, 1973). Tubulin prepared in this way will polymerize without further addition of nucleotide. Analysis of the bound and free nucleotide and phosphate for one of these experiments is shown in Table III. About 0.5 mol of unbound inorganic phosphate per mol of tubulin is observed after polymerization. The amount of

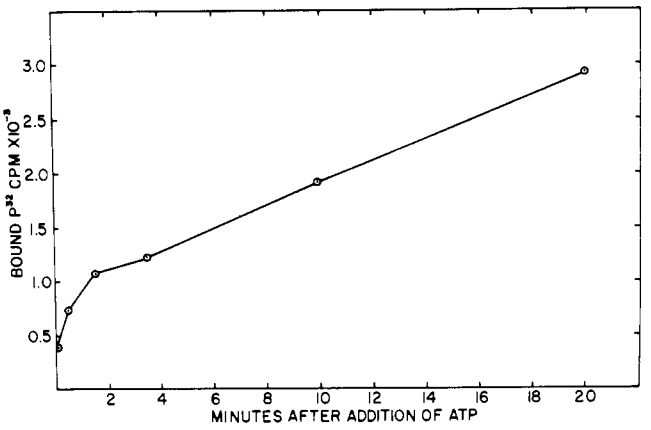

FIGURE 3: Transphosphorylation of tubulin at 0 °C. The tubulin concentration was 2 mg/ml and the ATP concentration was 0.1 mM with a specific activity of 6.0×10^{11} cpm/mol. Other conditions were the same as in Figure 2.

phosphate released is nearly the same as the total moles of noncovalently bound inorganic phosphate and GTP present before polymerization.

Bound Nucleotide of Microtubules. Microtubules polymerized in [^{3}H]GTP contain close to 2 mol of bound nucleotide per mol (110 000 g) of tubulin. In our early experiments, and in results reported by Jacobs et al. (1974) and by Kobayashi (1974), only about 1 mol of nucleotide per mol of polymerized tubulin was observed. We have found, however, that it is difficult to completely extract nucleotide from pelleted microtubules unless they are first dispersed in buffer prior to addition of perchloric acid. Using this procedure we have observed an average of 1.89 ± 0.12 (N = 4) mol of nucleotide bound per mol of polymerized tubulin. Of this, close to half (46 ± 3%, N = 3) of the total bound nucleotide can be labeled with [^{3}H]GTP. This indicates that both the E and N sites are occupied on the microtubule.

Analysis by thin-layer chromatography of nucleotide bound to pelleted microtubules always reveals a mixture of GTP and GDP. The amount of GTP varies from about 25 to 50% of the total bound nucleotide. The GTP bound to the microtubule is not significantly labeled by either [^{32}P]GTP, [^{32}P]ATP, or by [^{3}H]GTP and must be primarily N site nucleotide. The microtubule bound label is 96% in GDP if polymerization is performed in [^{3}H]GTP. Considerable variation is observed in the amount and extent of labeling of bound GDP. However, the amount of bound [^{3}H]GDP was always close to 50% of the total nucleotide. This indicates that all of the E site nucleotide is GDP. The unpolymerized protein incubated with either GTP or ATP at 0 °C contains mostly GTP (a variable amount of GDP is again observed, but generally less than 20% of the total nucleotide). These data indicate hydrolysis of E site GTP during polymerization.

Changes in Nucleotide Binding during Polymerization and Depolymerization. We have observed changes in the availability of nucleotide binding sites during both polymerization and depolymerization. As noted above microtubules polymerized in [^{3}H]GTP contain bound [^{3}H]GDP and about an equal amount of unlabeled GTP. If microtubules which have been polymerized in 0.1 mM [^{3}H]GTP are chased with a tenfold excess of unlabeled GTP (for 15 min at 35 °C), no significant loss of the bound label is observed. Thus the E site which bound labeled nucleotide during polymerization becomes nonexchangeable on the assembled microtubules.

A more complicated change in binding occurs during depolymerization at 0 °C. Microtubules were collected by centrifugation and then depolymerized at 0 °C in 0.1 mM [^{3}H]GTP. This was followed 15 or 30 min later by a chase with 1.0 mM GTP and after an additional incubation period the amount of bound label was determined by gel filtration. We observed that only about 50% of the bound label could be subsequently chased off if the label was present during depolymerization as compared with an 80–90% chase if the label was added to unpolymerized tubulin. In another series of experiments the order of addition of the labeled and unlabeled GTP was varied. If the label was added during depolymerization and followed by a chase 0.64 ± 0.15 mol (N = 4) of label was bound while, if the unlabeled GTP was added prior to the label, only 0.22 ± 0.1 mol of label bound (incubation time in all cases was 15 min at 0 °C). In an additional experiment tubulin was depolymerized in 0.1 mM [^{3}H]GTP for 15 min followed by a tenfold chase. One half of the sample was then carried through an additional cycle of polymerization and depolymerization while the other half remained at 0 °C. The sample which remained at 0 °C had 6.4 times as much bound label as the sample which went through the additional cycle of polymerization and depolymerization. Since both samples were at the same final conditions (temperature, protein concentration, nucleotide concentration, and specific activity were all identical), the difference in binding can only reflect their different histories.

TABLE III: Hydrolysis of Transphosphorylated GTP during Polymerization.

Fraction	mol of (^{32}P)PO_4 per mol of Tubulin Polymerized	mol of [^{32}P]GTP per mol of Tubulin Polymerized
Unpolymerized[a]	0.095	0.34
Microtubule[b]	0.001	0.003
Unbound[c]	0.48	Negligible

[a]Determined by ammonium sulfate precipitation prior to inducing polymerization. [b]Determined by centrifugation of microtubules at 40 000 rpm at 25 °C. [c]Determined by ultrafiltration of microtubule supernatant.

Fractionation of tubulin by gel filtration on Sepharose 6B yields two major components, the subunit fraction and a fraction which is voided from the column and consists primarily of ring aggregates (Kirschner et al., 1974). If microtubules are depolymerized at 0 °C in [^{3}H]GTP and then chased to remove exchangeable label, nearly all (88 and 86% in two experiments) of the bound label is observed in the ring fraction. By comparison if [^{3}H]GTP is added to unpolymerized tubulin at 0 °C and chromatographed, nearly all (79 and 87% in two experiments) of the bound label is in the subunit fraction. This observation suggests that the ring E site is blocked and does not bind labeled GTP, although it can be labeled during depolymerization of microtubules. Ring aggregates make up about 50% of the total tubulin in these preparations as determined by analytical centrifugation (Weisenberg 1974). Thus only about 50% of the E sites or 25% of the total bound nucleotide should be exchangeable. The maximum fraction of bound [^{3}H]GTP which we have obtained in these preparations is in fact about 25%. This should be compared with 50% exchange obtained in earlier preparations (Weisenberg et al., 1968); however, those preparations did not contain ring aggregates.

The observation that the E site on ring tubulin is not rapidly

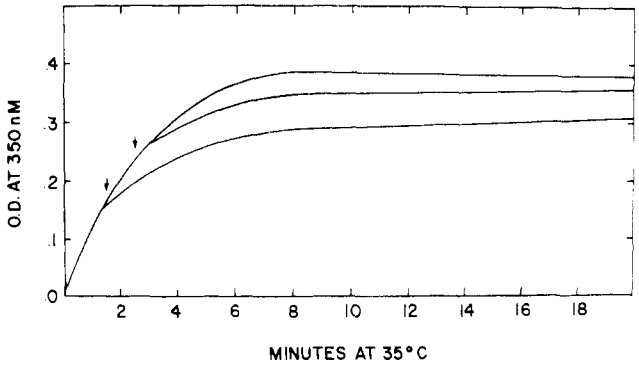

FIGURE 4: Inhibition of microtubule elongation by GDP. Polymerization was initiated in 0.1 mM GTP under standard conditions and microtubule formation followed by the increase in turbidity at 350 nM. At the indicated times (arrows), 1.0 mM GDP was added (by dilution of a 0.1 M stock solution). The slight increase in turbidity at later times seen in this experiment was not generally observed after addition of GDP.

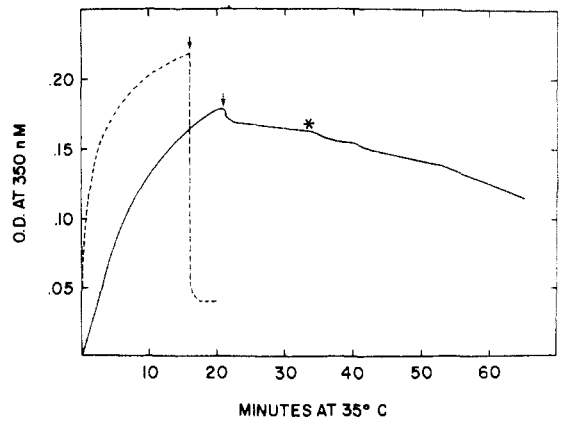

FIGURE 5: Polymerization and Ca stability of microtubules in GMPPNP. Tubulin (2.5 mg/ml) was polymerized under standard conditions in either 0.1 mM GTP (dotted line) or in 2 mM GMPPNP (solid line). At the time indicated by the arrows, 4 mM $CaCl_2$ was added to induce depolymerization. At the time indicated by the asterisk (*), 1 mM GTP was added.

exchangeable gives an explanation of the transphosphorylation data. The fraction of transphosphorylated GDP which is not chaseable is probably bound to the ring E site. This has been confirmed directly by Sepharose chromatography of transphosphorylated tubulin which has been chased with cold GTP This explains the discrepancy between Jacobs et al. (1974) and Kobayoshi (1975) concerning the hydrolysis of the N site. Only E site is in fact hydrolyzed, but simple chase or double-label experiments may suggest that N site is also hydrolyzed.

Although ring E site is not exchangeable, microtubules assembled in [^{3}H]GTP have the E site completely labeled. Since the E site is nonexchangeable on both the ring and the microtubule, this suggests that the ring E site must become transiently available during polymerization. This possibility was tested by the following experiment. The ring E site was labeled with [^{3}H]GTP during depolymerization at 0 °C followed by a tenfold chase with cold GDP. Half the protein was then incubated at 35 °C while the other half remained at 0 °C. The presence of excess GDP prevented any detectable microtubule assembly. Nearly all (82%) of the label which remained bound at 0 °C was chased off after incubation at 35 °C. Thus the transient availability of the ring E site does not appear to require microtubule assembly, but is probably a result of structural or conformational changes in the rings at 35 °C.

Effect of GDP on Polymerization and Depolymerization. Our results, as well as reports from other laboratories (Jacobs et al., 1974; Kobayashi 1975), indicate that assembled microtubules contain only GDP at the E site. From this observation it might be predicted that GDP-tubulin should be capable of polymerization, although perhaps at a slower rate than GTP-tubulin (similar to the differences between ADP-actin and ATP-actin). However, we have been unable to obtain polymerization in GDP at concentrations up to 2 mM. Furthermore GDP inhibits polymerization by GTP, presumably by competitive binding since GDP binds to the E site with only slightly lower affinity than GTP (Arai et al., 1975). GDP (at 1 mM) also inhibits polymerization of tubulin preincubated for 1 h in 0.1 mM ATP.

The inhibitory activity of GDP cannot be explained by a requirement for GTP for the initiation or nucleation of polymerization. The addition of a small volume of GTP assembled tubules does not overcome the inhibition by GDP. Furthermore, if GDP (1 mM) is added after initiation of polymerization in 0.1 mM GTP, the rate of assembly rapidly decreases and a final plateau level of polymerization is reached which is significantly less than that achieved in GTP alone (Figure 4).

The observation that microtubule assembly achieves a plateau level even in the presence of inhibitory concentrations of GDP suggests that GDP does not depolymerize existing microtubules. We have observed in fact that microtubules are completely stable for at least 4 h in concentrations of GDP several times greater than that needed to completely inhibit polymerization (the exact ratio of GDP to GTP is not known since much of the GTP will be hydrolyzed during the experiment). Since concentrations of GDP which will completely inhibit polymerization do not depolymerize preexisting microtubules, then we can only conclude that microtubule assembly is not a reversible reaction with respect to the nucleotide interaction (at least over times on the order of an hour). It should be noted that these preparations rapidly depolymerize upon addition of Ca or upon lowering the temperature, so they do not have unusual stability characteristics.

Polymerization and Depolymerization in GMPPNP. As originally observed by Kirschner and co-workers (Lockwood et al., 1975), the nonhydrolyzable analogue of GTP, guanylyl imidodiphosphate (GMPPNP), will promote the formation of microtubules. Because of the relatively low binding affinity of the analogue (Arai et al., 1975), it is necessary that nearly all competing GDP be removed. The rate and final level of polymerization appear to be somewhat less in GMPPNP than in GTP, but a careful comparison has not yet been performed. The most striking and significant difference between analogue microtubules and GTP microtubules is in their sensitivity to Ca-induced depolymerization (Figure 5). Concentrations of Ca several times greater than that necessary to completely or partially depolymerize GTP microtubules have little or no effect on GMPPNP microtubules. The failure of Ca to depolymerize analogue microtubules has been directly conformed by electron microscopy.

Microtubules formed in GMPPNP contain approximately 1 mol of GMPPNP and 1 mol of GTP (as determined by centrifugation of microtubules through a sucrose cushion followed by extraction and thin-layer chromatography of the nucleotide). If the presence of analogue bound to the E site makes the tubules insensitive to Ca, it might be possible to restore Ca sensitivity by the addition of GTP. Addition of GTP to GMPPNP microtubules which have been previously treated

with Ca does result in a slow but significant rate of depolymerization (Figure 5).

Although Ca does not depolymerize microtubules formed in GMPPNP, the addition of Ca prior to inducing polymerization by raising the temperature prevents polymerization from occurring.

Discussion

Before discussing the results of these experiments we want to emphasize the complexity of tubulin and microtubule assembly. Tubulin has at least two nonidentical binding sites for guanine nucleotide (usually distinguished by their exchangeability, but as demonstrated in these experiments exchangeability alone is not always adequate to distinguish these sites). The bound nucleotide may be either GDP or GTP. Tubulin may exist as dimers, as ring aggregates, and as microtubules (and presumably as intermediates between these forms). Microtubules appear to contain proteins other than tubulin (Sandoval and Cuatrecasas, 1976) and a nontubulin protein may be required for ring and microtubule formation (Weingarten et al., 1975). Tubulin may be covalently modified by phosphorylation (Eipper, 1972) and by tyrosine addition at the carboxyl terminus (Arce et al., 1975). Thus tubulin solutions probably consist of a complex mixture of tubulin and associated proteins and of various chemical and aggregated states of tubulin.

In spite of the considerable complexity of tubulin chemistry, we have made a number of observations which allow some important conclusions to be reached. One result which is clear from our data, and which is consistent with the data of Jacobs et al. (1974) and Kobayashi (1975), is that E site GTP is hydrolyzed during polymerization. This is demonstrated most clearly in our experiments by analysis of bound nucleotide before and after polymerization. We have demonstrated hydrolysis directly by analysis of the microtubule-bound nucleotide after polymerization in [^{32}P, ^{3}H]GTP. Essentially all the bound tritium is GDP and little or no [^{32}P]GTP is present. The amount of bound [^{3}H]GDP is close to a mole per mole and is half of the total bound nucleotide. The remainder is presumably bound unlabeled GDP and GTP at the N site. We have also demonstrated hydrolysis by ultrafiltration of tubulin which had been transphosphorylated with [^{32}P]ATP and unbound nucleotide removed. After polymerization nearly all the ^{32}P is present as unbound inorganic phosphate.

The data presented here and the results of Jacobs et al. (1974) both indicate that ATP promotes polymerization by transphosphorylation of a tightly bound GDP which is subsequently hydrolyzed during polymerization. The transphosphorylation of the bound GDP appears to require a nontubulin protein. Our experiments demonstrate the involvement of transphosphorylation by a correlation between the rate of transphosphorylation and the length of the lag in the onset of polymerization. Conditions which decrease the rate of transphosphorylation result in an increased lag, and the peak of transphosphorylation occurs at about the same time as the onset of polymerization. The decrease in the amount of transphosphorylation which occurs after the start of polymerization can be explained by hydrolysis of the bound nucleotide.

During transphosphorylation at 0 or 35 °C under polymerizing conditions, a fraction of the phosphate transferred from ATP is present in noncovalently bound inorganic phosphate. This noncovalently bound phosphate may be released from tubulin at a slow rate and this could explain the low level of ATPase activity observed in these preparations. Polymerization may stimulate the formation or release of this phosphate and this could explain the hydrolysis of GTP during polymerization.

Previous reports have indicated that N site GTP is not hydrolyzed during polymerization (Kobayashi, 1975) and also that it is hydrolyzed (Jacobs et al., 1974). Our results indicate that the N site nucleotide is primarily GTP on the microtubule and is therefore not hydrolyzed. Apparent hydrolysis of the N site nucleotide may be observed because of the nonexchangeability of the E site on tubulin rings.

The variation in the exchangeability of E site nucleotide suggests structural changes in tubulin during polymerization and depolymerization. Microtubules contain a tightly bound E site GDP which does not exchange. (Note: actual exchange rates have not been determined. Fast exchange means that complete turnover of bound nucleotide occurs in a few minutes, the time required for polymerization or depolymerization. Slow exchange or no exchange means no detectable turnover during times on the order of 30 min.) This result suggests that the E site is "blocked" by intertubulin bonds in the microtubule. Exchange of nucleotide may occur at the ends of the microtubule, but this would not be detectable in these experiments. In a similar manner the E site on the rings appears to be blocked. However, during both polymerization and depolymerization, induced by raising or lowering the temperature, respectively, all the E sites are rapidly exchangeable. The simplest explanation for this observation is that transitions between microtubules and rings involve the formation of subunit or a more open aggregate in which E sites are exposed. A direct structural transition between rings and microtubules is not suggested by this observation.

The behavior of tubulin and microtubules in GDP has important consequences. Our data demonstrate that GDP will not promote polymerization by itself and inhibits polymerization by GTP. The failure of GDP to promote polymerization does not reflect a requirement for GTP for nucleation since the addition of excess GDP to growing microtubules results in a rapid inhibition of further assembly. Although GDP inhibits polymerization, it does not depolymerize assembled microtubules. There are two simple explanations for the failure of GDP to act reversibly on tubulin polymerization. One possibility is that assembled microtubules are not in equilibrium with subunit, and thus once formed would be stable even though they contain bound GDP. This idea is not consistent with current theory or experiment, all of which indicate reversible equilibrium.

Another possible explanation is that microtubules dissociate (presumably at their ends) into subunits which are able to rebond to the microtubule even though they contain GDP rather than GTP. Some of the energy of hydrolysis and release of phosphate would be used in this case to induce a long-lived conformational change in tubulin. This possibility is consistent with the observation that GMPPNP microtubules differ from GTP microtubules in their sensitivity to Ca. It should be emphasized that these microtubules do not have unusual stability characteristics and are depolymerized within a few minutes by treatments with cold temperatures or Ca (preliminary data also indicate that they depolymerize very rapidly upon dilution). This should be compared with no depolymerization in GDP after an hour.

We have confirmed the observations of Kirschner and coworkers (Lockwood et al., 1975) that tubulin polymerization can be induced by nonhydrolyzable analogues of GTP. Thus hydrolysis of bound GTP does not appear to be required for polymerization. Nevertheless GTP hydrolysis clearly occurs

during the normal polymerization of tubulin, and the reasonable assumption is that hydrolysis has some effect on the properties of microtubules. We have observed one clear difference between GTP and GMPPNP microtubules. GMPPNP microtubules are not depolymerized by concentrations of Ca several times that necessary to completely depolymerize GTP microtubules.

The insensitivity of GMPPNP microtubules to Ca suggests a possible biological function for nucleotide hydrolysis during polymerization. A living cell may need to be able to both assemble and to disassemble microtubules rapidly. Rapid assembly is achieved by using the binding energy of GTP to obtain the desired rapidly polymerizing conformation. Hydrolysis of the GTP then alters the conformation to allow rapid depolymerization by Ca ions. The idea that hydrolysis during polymerization produces a conformationally altered state is supported by the irreversible behavior of microtubules in GDP.

From these data the following picture can be drawn of the interaction of nucleotides with tubulin during polymerization. At 0 °C no polymerization is occurring and only the E site on the free subunit is exchanging its GTP with medium GTP. The ring protein contains either GTP or GDP, but it is not exchanging rapidly with medium nucleotide. The bound GDP may be converted to GTP, however, by transphosphorylation by ATP. Upon raising the temperature, the E site on the ring protein becomes available to medium GTP. The GTP-tubulin polymerizes and forms a stable, Ca-insensitive microtubule. Hydrolysis of the GTP occurs, and the phosphate is released from the protein. The resulting GDP at the E site is unavailable for exchange (except possibly at the ends of the microtubule). The microtubule is now in a state which is Ca sensitive and is in rapid equilibrium with subunit or rings or both. The equilibrium appears insensitive to GDP, suggesting that the protein is in a conformation which is polymerizable even though it contains GDP rather than GTP. Upon depolymerization (at 0 °C) the protein is returned to a GTP-requiring conformation and the E site nucleotide becomes completely available for exchange. Rings then form (or undergo a conformational change) and the ring E site becomes nonexchangeable.

References

Arai, T., Ihara, Y., Arai, K., and Kaziro, Y. (1975), *J. Biochem. (Tokyo) 77,* 647–658.

Arce, C. A., Rodriguez, J. A., Barra, S. H., and Caputto, R. (1975), *Eur. J. Biochem. 59,* 145–149.

Berry, R. W., and Shelanski, M. L. (1972), *J. Mol. Biol. 71,* 71–80.

Burns, R. G., and Pollard, T. D. (1974), *FEBS Lett. 40,* 274–280.

Eipper, B. A. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 2283–2287.

Gaskin, F., Cantor, C. R., and Shelanski, M. L. (1974a), *J. Mol. Biol. 89,* 737–738.

Gaskin, F., Kramer, S. B., Cantor, C. R., Adelstein, R., and Shelanski, M. L. (1974b), *FEBS Lett. 40,* 281–286.

Gonzales, L. W., and Geel, S. E. (1975), *Anal. Biochem. 63,* 400–413.

Jacobs, M., Smith, H., and Taylor, E. W. (1974), *J. Mol. Biol. 89,* 455–468.

Kirschner, M. W., Williams, R. C., Weingarten, M., and Gerhart, J. C. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 1159–1163.

Kobayashi, T. (1974), *J. Biochem. (Tokyo) 76,* 201–204.

Kobayashi, T. (1975), *J. Biochem. (Tokyo) 77,* 1193–1197.

Levi, A., Cimino, M., Mercanti, D., and Calissano, P. (1974), *Biochim. Biophys. Acta 365,* 450–453.

Lockwood, A. H., Penningroth, S. M., and Kirschner, M. W. (1975), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34,* 540.

Neal, M. W., and Florini, J. R. (1973), *Anal. Biochem. 55,* 328–330.

Sandoval, I. V., and Cuatrecasas, P. (1976), *Biochem. Biophys. Res. Commun. 68,* 169–177.

Shelanski, M. L., Gaskin, F., and Cantor, C. R. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 765–768.

Sloboda, R. D., Rudolph, S. A., Rosenbaum, J. L., and Greengard, P. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 177–181.

Weingarten, M. D., Lockwood, A. H., Hwo, S., and Kirschner, M. W. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 1858–1862.

Weisenberg, R. C. (1972), *Science 177,* 1104–1105.

Weisenberg, R. C. (1974), *J. Supramol. Struct. 2,* 451–465.

Weisenberg, R. C., Borisy, G. G., and Taylor, E. W. (1968), *Biochemistry 7,* 4466–4478.