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Stoichiometry of GTP hydrolysis and tubulin polymerization

(microtubules/brain/GTPase)

RICARDO MACCIONI AND NICHOLAS W. SEEDS

Department of Biophysics and Genetics, University of Colorado Medical Center, Denver, Colo. 80262

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ABSTRACT **Microtubule formation from lamb brain tubulin isolated by affinity chromatography and freed of exchangeable nucleotide requires GTP for maximal rate and extent of polymerization. The nucleotide analogs guanylylmethylenediphosphate and guanylylimidodiphosphate fail to replace GTP; in addition, neither the presence of microtubule associated proteins nor 5 M glycerol relieves the GTP requirement. The relation of GTP concentration and microtubule formation shows an association constant $K = 1 \times 10^4\ M^{-1}$; furthermore, GDP and guanylylimidodiphosphate are competitive inhibitors of GTP for polymerization. Using a rapid filter assay for microtubule formation that allows the quantitative analysis of early polymerization kinetics and correcting for GTP hydrolysis uncoupled from tubulin polymerization, a stoichiometry of two molecules of GTP hydrolyzed per mole of tubulin dimer incorporated into microtubules has been found.**

Microtubules are ubiquitous organelles of virtually all eukaryotic cells and have been implicated in various cell functions, including motility, mitosis, secretion, cell shape, and modulation of surface receptors; therefore, the regulation of their formation has become an area of active interest (1). Tubulin, the subunit protein of microtubules, is a 6S dimer of 110,000 daltons. Both flagellar (2) and cytoplasmic (3) tubulin contain 2 mol of guanine nucleotide per 110,000 g of protein. One mole of bound guanine nucleotide is readily exchangeable with free GTP while the other is not (3), thus establishing two different binding sites, an exchangeable (*E*) site and nonexchangeable (*N*) site. The bound nucleotide also stabilizes tubulin against inactivation (3, 4). Although investigators have been cautious about proposing a role for GTP in polymerization, the first polymerization of tubulin *in vitro* from crude extracts of brain contained GTP (5, 6). Additional studies in several laboratories (7–9) with more homogeneous tubulin preparations demonstrated that GTP was required for polymerization and that GTP analogs, guanylylmethylenediphosphate [GMP-P(CH_2)P] and guanylylimidodiphosphate [GMP-P(NH)P], were inactive. These results suggested that hydrolysis of the γ-phosphate may be necessary for microtubule formation. In addition, when incubated with [γ-^{32}P,^{3}H]GTP, tubulin binds equal molar amounts of ^{32}P and ^{3}H; however, microtubules isolated after polymerization contain only ^{3}H, indicating hydrolysis of the γ-phosphate (10, 11).

Morgan (10) has shown that tubulin purified by affinity chromatography possesses a polymerization dependent GTPase activity that can be distinguished from the uncoupled GTPase activity observed in the presence of colchicine or under other conditions that prevent tubulin polymerization. However, the absence of a quantitative assay for polymerization kinetics prevented determination of the stoichiometry.

We have developed a rapid filter assay for quantifying the moles of tubulin incorporated into microtubules at very early times of incubation*. This assay shows rates and extents of reaction that are identical to those obtained by turbidimetric (7) and sedimentation (12) assays, respectively. Using this kinetic assay it is now possible to determine the stoichiometry of GTP hydrolysis and tubulin polymerization.

MATERIALS AND METHODS

Materials. [γ-^{32}P]GTP (30 Ci/mmol) was purchased from New England Nuclear (Boston). Nonradioactive GTP (Types II and IV), ATP, and cyclic 3′:5′-GMP (cGMP) were purchased from Sigma Chemical (St. Louis); other nucleotides [GMP, GDP, GMP-P(NH)P and GMP-P(CH_2)P] were obtained from P-L Biochemicals (Milwaukee). All other reagents were of the most pure analytical grade available.

Preparation of Tubulin. Microtubules were isolated by two cycles of polymerization from lamb brain homogenates as described (9). The crude microtubule pellets were stored at −75° without resuspension until further use. Tubulin was isolated from the microtubule pellet by deacetylcolchicinic acid (DAC)-affinity chromatography as described (9, 13). The purified tubulin was desalted by Sephadex G-25 column chromatography and concentrated in an Amicon PM-10 ultrafiltration apparatus. The concentrated tubulin was centrifuged at 14,000 × *g* to remove aggregates and used immediately. The protein concentration was determined by the procedure of Lowry *et al.* (14).

Nucleotide Removal. Tubulin preparations were routinely freed of unbound nucleotide or nucleotide bound to the exchangeable site by two successive extractions with 0.5 ml of a 1 mM EDTA, charcoal (prewashed in imidazole-glycerol buffer containing 2% serum albumin, followed by a wash in only imidazole-glycerol buffer) solution per ml of tubulin sample.

The residual guanine nucleotide retained by these charcoal-treated tubulin samples was extracted with 0.5 M $HClO_4$, and separated by PEI-cellulose thin-layer chromatography. Absorbance determinations at 260 and 280 nm were compared to a 1% GTP standard solution subjected to the same experimental conditions.

Preparation of Microtubule Associated Protein Eluted from DEAE-Sephadex. A fraction of microtubule associated proteins (MAPs) that enhance tubulin polymerization was obtained by DEAE-Sephadex chromatography of the protein not retained by the DAC-agarose resin. The DEAE-resin was equilibrated with a buffer containing 50 mM KCl, 50 mM imidazole:Cl (pH 6.8), 0.1 mM GTP, 0.5 mM $MgCl_2$, and 1 M glycerol. A fraction that enhanced tubulin polymerization was eluted with buffer containing 300 mM KCl. This active fraction (DE-MAP) was desalted and concentrated in an Amicon PM-10 ultrafilter.

Polymerization Assay. Microtubule formation was measured

Abbreviations: DAC-, deacetylcolchicinic acid; DE-MAP, microtubule associated proteins eluted from DEAE-Sephadex with 0.3 M KCl; GMP-P(CH_2)P, guanylylmethylenediphosphate; GMP-P(NH)P, guanylylimidodiphosphate; EGTA, ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*′-tetraacetate.

* R. Maccioni and N. W. Seeds, unpublished data.

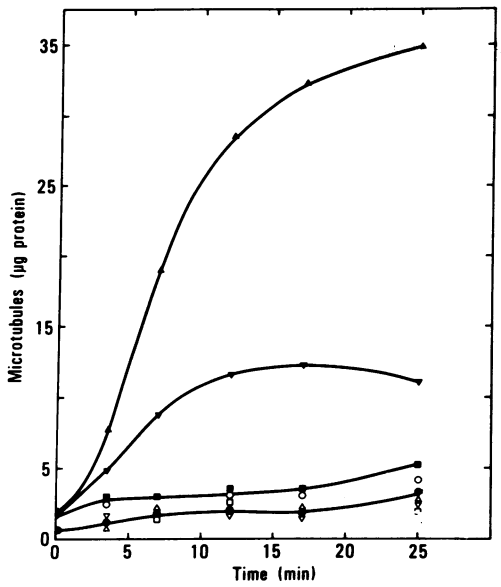


FIG. 1. GTP dependence of tubulin polymerization. Tubulin purified by affinity column and treated with charcoal and DE-MAP prepared as described in *Materials and Methods* were mixed at 4° in a total volume of 0.6 ml of polymerization buffer to give final concentrations of 3.0 and 0.45 mg/ml, respectively. The separate mixtures containing 1 mM of the indicated nucleotides were then incubated at 37°. Aliquots of 60 μl were taken at times indicated and assayed for polymerization (see *Materials and Methods*). Separate control tubes containing 1×10^{-4} M colchicine were treated similarly. The nucleotides used were: GTP (▲), ATP (▼), GMP-P(NH)P (■), GMP-P(CH_2)P (●), GDP (△), GMP (□), cGMP (○), and none (▽).

by a method using glass fiber filters, which is described in depth elsewhere*. Briefly, samples containing tubulin (1.5–7.5 mg/ml) and DE-MAP as indicated were incubated at 37° for the specified times in polymerization buffer of 100 mM imidazole·Cl (pH 6.8), 0.5 mM ethyleneglycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetate (EGTA), 0.5 mM $MgCl_2$, 1 M glycerol with 1 mM GTP, or as indicated, in a final volume of 80 μl. The polymerization reaction was terminated by the rapid addition of 1 ml of buffer [100 mM imidazole·Cl (pH 6.8), 0.5 mM $MgCl_2$, 0.5 mM EGTA, and 5 M glycerol] at 37° and pouring the diluted sample over a Whatman A-glass fiber filter under reduced pressure followed by three 5-ml washes with the same buffer. The entire filtration and washing procedure required <20 sec. The filter was placed in a vial with 0.5 ml of 0.5 M NaOH overnight at room temperature, and a 0.2-ml aliquot (0–35 μg of protein) was assessed for protein content (14). Control determinations containing 10^{-4} M colchicine were performed for each sample, and polymerization is represented by the difference in these protein values. Routinely this colchicine-insensitive background represented 32.3 ± 3.6% of the uninhibited samples. The assay was linear for protein throughout the concentrations used and recovery of protein from the filter was consistently >94%.

Guanosinetriphosphatase Assay. Reaction mixtures were identical to those for polymerization with the addition of [γ-^{32}P]GTP at 0.5–1.0 mM and a final specific activity of 4×10^4 cpm/nmol. Aliquots (50–200 μl) of the polymerization reaction mixture were taken at the indicated times and immediately added to tubes containing 0.5 ml of 0.02 M silicotungstic acid in 0.01 M H_2SO_4 followed by the sequential addition of 0.5 ml of 5% ammonium molybdate·$4H_2O$ in 2 M H_2SO_4 and 1.25 ml of 0.001 M KH_2PO_4 with vigorous mixing. The phosphomolybdate complex was extracted into 2.5 ml of isobutanol:benzene

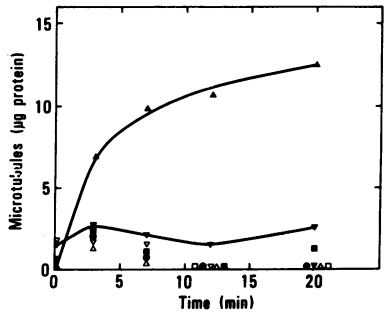


FIG. 2. GTP dependence of tubulin polymerization in 5 M glycerol. Tubulin prepared as in legend of Fig. 1 was incubated at 37° in separate tubes in a final volume of 0.6 ml and with a final concentration of 2.7 mg/ml in the polymerization buffer plus 5 M glycerol and 1 mM of the nucleotides indicated. Aliquots of 100 μl were taken at the times indicated and assayed for polymerization. Separate control tubes containing 1×10^{-4} M colchicine were treated similarly. The nucleotides used were: GTP (▲), ATP (▼), GMP-P(NH)P (■), GMP-P(CH_2)P (●), GDP (△), GMP (□), and none (▽).

(1:1, vol/vol) solution and the organic phase separated by centrifugation at 2000 × *g* for 10 min. The radioactivity of a 2-ml aliquot from the organic phase was determined by liquid scintillation spectrometry after 10 ml of 3a70B (Research Product Internat., Chicago) was added to the vial.

RESULTS

Lamb brain tubulin purified by DAC-affinity chromatography (9, 13) will only polymerize at tubulin concentrations above 4 mg/ml in our standard buffer (9, *); however, an increase in glycerol concentration to 5 M or the addition of DE-MAP permits polymerization at tubulin concentrations from 1.5 to 2 mg/ml*. Thus, it is often advantageous to use DE-MAP, when performing assays with a large number of samples, to conserve tubulin protein; furthermore, this may reflect a more natural mechanism of tubulin polymerization (12).

Since we have previously demonstrated that polymerization of tubulin purified by DAC-affinity chromatography requires GTP for maximal increases in viscosity (9), the influence of GTP on tubulin polymerization in the presence of DE-MAP or 5 M glycerol was examined with the filter assay for microtubule formation. To remove any free nucleotide or nucleotide bound at the exchangeable site, we treated the purified tubulin preparations with activated charcoal in 1 mM EDTA. Perchloric acid extraction of this charcoal-treated tubulin indicated <1.1 mol of residual guanine nucleotide, 90% as GDP presumably in the nonexchangeable binding site. Although ATP was capable of partial stimulation, tubulin polymerization in the presence of DE-MAP requires GTP for maximal polymerization (Fig. 1), and other guanine nucleotides [GMP-P(NH)P, GMP-P(CH_2)P, GDP, GMP, and cGMP] fail to promote appreciable microtubule formation. These results were confirmed by electron microscopic observation of the samples both before and after filtration.

Tubulin has been reported to polymerize in high concentrations of glycerol without the addition of GTP (15); however, the charcoal-extracted tubulin used in these studies requires GTP for polymerization even in the presence of 5 M glycerol (Fig. 2). Other guanine nucleotides were virtually inactive for tubulin polymerization. However, the curves in Fig. 2 may be somewhat misleading, since they have been normalized by subtracting the protein retained by the filter from samples containing 10^{-4} M colchicine. In contrast to Fig. 1, all the samples in 5 M glycerol displayed an increase in protein trapped by the filter as a function of time at 37°, including the samples

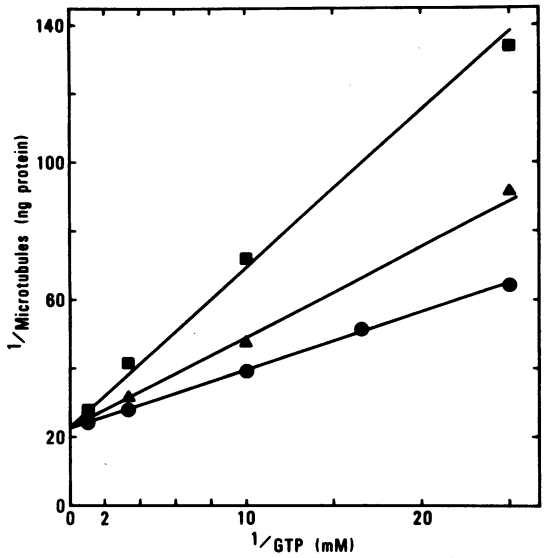


FIG. 3. Double reciprocal plot of tubulin polymerization as a function of GTP concentration and inhibitory effect of GDP and GMP-P(NH)P. Tubulin prepared as in *Materials and Methods* was incubated at 37° for 10 min in separate tubes with 60 μl of polymerization buffer with 3 mM $MgCl_2$ to give a final protein concentration of 5.6 mg/ml. The GTP concentration was increased from 1×10^{-5} to 2×10^{-3} M (●). Control samples containing 1×10^{-4} M colchicine were incubated at the same concentrations indicated. The reaction was terminated and assayed for polymerization as described in *Materials and Methods*. Two other sets of tubes were incubated for 10 min at 37° at the GTP concentrations indicated and with either 1×10^{-3} GDP (■) or 2×10^{-3} GMP-P(NH)P (▲). Control samples containing 1×10^{-4} M colchicine were treated as described above.

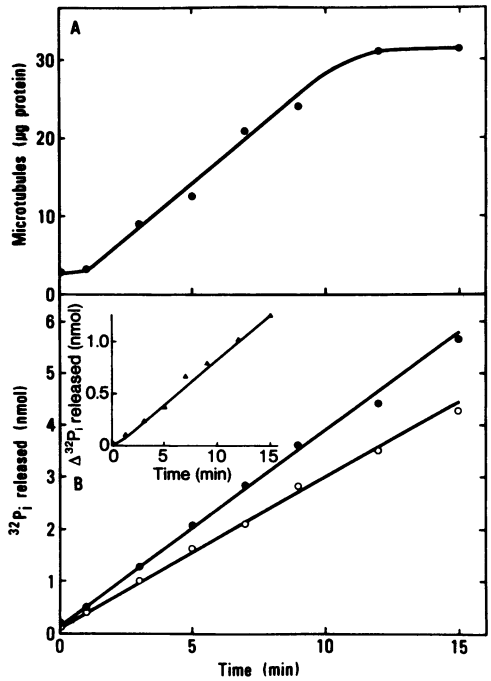


FIG. 4. Kinetics of tubulin polymerization and GTPase activity. Tubulin (7.1 mg/ml) prepared as in legend of Fig. 1 was incubated at 37° in the polymerization buffer with 0.7 mM [γ-^{32}P]GTP (specific activity 42,500 cpm/nmol). Two aliquots of 55 μl each were taken from a single tube at the times indicated and one was assayed for polymerization (A) while the other was assayed for GTPase activity (B). Similarly, control samples containing 1×10^{-4} M colchicine were processed at each time. (A) The values (●) represent the net differences in the amount of protein in the absence and presence of 1×10^{-4} M colchicine. (B) The values for $^{32}P_i$ release were measured in the absence (●) or the presence (○) of 1×10^{-4} M colchicine. The insert represents the differences (▲) in the values for P_i release in the absence and presence of 1×10^{-4} M colchicine.

containing 10^{-4} M colchicine. This increase in retained protein with time was 30% that observed in the presence of GTP without colchicine. Electron microscopic observations showed the material formed in the presence of colchicine to represent amorphous aggregate.

The GTP concentration needed to saturate tubulin for microtubule formation was investigated. Polymerization was measured at GTP concentrations from 10^{-5} to 10^{-3} M after 10 min of incubation, when the rate of microtubule formation was still linear in samples containing 2×10^{-3} M GTP. An association constant† $K = 1.15 \times 10^4$ M^{-1} has been determined (Fig. 3). Identical results were found in more extensive studies at ten different GTP concentrations (not shown). Although this GTP association constant for polymerization is two orders of magnitude lower than those obtained for the direct binding of guanine nucleotide to the *E* site of porcine brain tubulin (16), previous studies in our laboratory using a Millipore filter assay for direct binding of [^{3}H]GTP to lamb brain tubulin have given equilibrium constants of 1.25×10^4 M^{-1}, as analyzed by Klotz plots and Scatchard plots (10).

Since GDP and GMP-P(NH)P did not promote polymerization of tubulin (Fig. 1), but have been shown to bind to the exchangeable site of tubulin (16, 17), it was of interest to examine whether they could compete with GTP during tubulin polymerization (Fig. 3). Both GDP and GMP-P(NH)P demonstrated a competitive inhibition with respect to GTP for tubulin polymerization. Additional studies at several GDP (0.19, 0.49, and 1.0 mM) and GMP-P(NH)P (0.6, 1.0, and 2.0 mM) concentrations permitted the determination of inhibition constants for GDP and GMP-P(NH)P of 6.4×10^{-4} M and 2.2×10^{-3} M, respectively. Since the imidiophosphate analogs bind Mg^{2+} more tightly than their oxyphosphate counterparts (18), a high Mg^{2+} concentration (3 mM) was used in these studies to prevent a nucleotide inhibition of polymerization by a simple sequestering of divalent cation; therefore, at pH 6.8 there should be an excess of free Mg^{2+}.

As previously described (9, 11), purified tubulin preparations possess a small GTPase activity that can be also observed under conditions that do not favor polymerization, i.e., colchicine, low tubulin concentration (10), or Ca^{2+} (11). The relation of GTP concentration to tubulin GTPase activity (coupled and uncoupled) displayed an apparent $K_m = 6 \times 10^{-4}$ M (data not shown), which is the same order of magnitude as the association constant *K* for GTP in microtubule formation.

Tubulin preparations also display a polymerization-dependent GTPase activity that is distinguished from the uncoupled GTPase activity by the addition of colchicine to inhibit polymerization (Fig. 4B). If tubulin polymerization is prevented by using a low tubulin concentration (i.e., <3 mg/ml) or by the addition of Ca^{2+}, only a single GTPase activity, identical to that found with colchicine, is observed. Using the filter assay for microtubule formation, which uses a colchicine control to correct for tubulin aggregates, we made simultaneous determinations of microtubule formation and GTPase activity on

† Ligand-induced chain polymerization reactions admit the kinetic relationship $(1/V) = [(1/kc^\circ) + (1/kc^\circ K)\cdot(1/C)]$ where *V* is the initial rate of polymerization, *k* is the specific rate constant for sequential addition of monomer units to the growing polymer chain, *K* is the association constant for binding of the ligand to the monomer protein, c° is the initial concentration of protein; and *C* is the equilibrium concentration of ligand (personal communication, Dr. J. R. Cann).

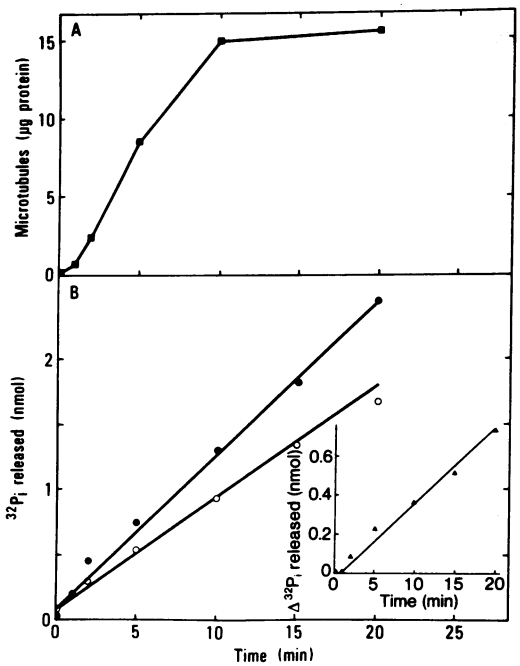


FIG. 5. Kinetics of tubulin plus DE-MAP polymerization and GTPase activity. Tubulin and DE-MAP prepared as in legend of Fig. 1 were mixed at 4° in a total volume of 0.7 ml to give final concentrations of 2.6 and 0.32 mg/ml, respectively. The mixture was incubated at 37° in the polymerization buffer with 0.5 mM [γ-^{32}P]GTP (35,000 cpm/nmol). Two aliquots of 55 μl each were taken at times indicated, and one was assayed for polymerization (A) while the other was assayed for GTPase activity (B). Control samples in the presence of 1×10^{-4} M colchicine were taken at each time. (A) The values (■) represent the differences in the amount of protein in the absence and presence of 1×10^{-4} M colchicine. (B) The values for $^{32}P_i$ release were measured in the absence (●) or the presence (○) of 1×10^{-4} M colchicine. The insert represents the differences (▲) in the $^{32}P_i$ values in the absence and presence of 1×10^{-4} M colchicine.

aliquots of a sample containing 7 mg/ml of tubulin purified by DAC-affinity chromatography in polymerization buffer with saturating [γ-^{32}P]GTP (0.5–1.0 mM) (Fig. 4A and B). After an initial lag the formation of microtubules increases linearly for 8–10 min. The polymerization-dependent GTPase also shows a brief lag, then a linear increase (Fig. 4, insert). The difference in the two rates (slopes) of GTPase activity (Fig. 4B) divided by the rate (slope) of microtubule formation (Fig. 4A) gives a stoichiometry of 2.2 mol of GTP hydrolyzed per mol of tubulin dimer (110,000 g of protein) in microtubules. Additional studies ($n = 6$) with tubulin purified by DAC affinity chromatography have shown an average value of 2.12 ± 0.04 mol of GTP hydrolyzed per mol of tubulin dimer polymerized.

The stoichiometry of GTP hydrolysis during DE-MAP-enhanced polymerization of tubulin was also examined. Samples containing 2 mg/ml of tubulin and saturating DE-MAP (0.25 mg/ml) were monitored for polymerization and GTPase activity (Fig. 5A and B). The kinetics of microtubule formation and GTP hydrolysis were similar to those seen in Fig. 4, and a stoichiometry of 2.2 mol of GTP hydrolyzed per mol of tubulin dimer polymerized was determined from the reaction rates. In another study (not shown) tubulin isolated by four cycles of polymerization-depolymerization according to the procedure of Borisy *et al.* (19) was characterized for polymerization-dependent hydrolysis of GTP. Although the uncoupled GTPase activity was somewhat greater, a polymerization-dependent activity was detected and a stoichiometry of 2.08 mol of GTP hydrolyzed per mol of tubulin dimer incorporated into microtubules was calculated.

DISCUSSION

The studies presented here have shown that GTP is required for maximal rate and extent of microtubule formation with purified lamb brain tubulin, and that nucleotide analogs containing a nonhydrolyzable γ-phosphate are unable to support polymerization and are competitive inhibitors of GTP-promoted polymerization; furthermore, 2 mol of inorganic phosphate are released from GTP for every mole of tubulin dimer that is incorporated into microtubules. Although the dependence on GTP for polymerization in high concentrations of glycerol would appear contradictory to the results of Shelanski *et al.* (15), the tubulin preparations used in their studies probably contain GTP in the *E* site, since their later studies (7) showed a dependence on GTP when dialyzed tubulin was used. ATP has been shown to support tubulin polymerization when microtubule associated proteins, including a transphosphorylase, are present in the incubation (11, 16). The ability of ATP to promote limited polymerization with lamb brain tubulin (Fig. 1) probably reflects the presence of trace amounts of GDP and transphosphorylase (16) in the DE-MAP preparation, since tubulin isolated by affinity chromatography is lacking transphosphorylase activity (10).

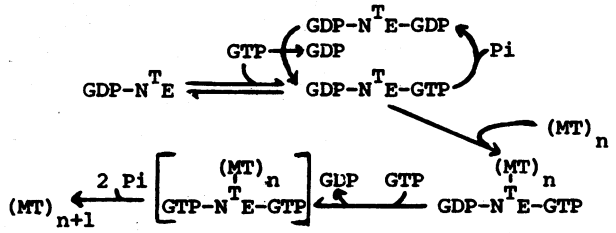
The inability of the GTP analogs, possessing a nonhydrolyzable γ-phosphate, to promote polymerization in the presence of DE-MAP or 5 M glycerol is in agreement with our earlier results (9) using purified tubulin and those of other laboratories (7, 8) that use different methods for tubulin isolation and polymerization. Olmsted and Borisy (8) have shown that GMP-P(CH_2)P inhibits tubulin polymerization when low concentrations of GTP are used; furthermore, Fig. 3 shows that GMP-P(NH)P and GDP are competitive inhibitors of GTP for microtubule formation. These results indicate that all three nucleotides, GDP, GMP-P(NH)P, and GMP-P(CH_2)P, interact with tubulin and prevent its polymerization, suggesting that hydrolysis of the γ-phosphate of GTP is a necessary event for tubulin polymerization. Although Arai *et al.* (17) have shown that the β,γ-imido analog can bind to tubulin, they found very little interaction between tubulin and GMP-P(CH_2)P. This is not too surprising since the methylene diphosphate bond angles are markedly different from those of pyrophosphate and imidodiphosphate, and GMP-P(CH_2)P is the weakest acid of the three nucleotides, with a last pK_a of approximately 8, or 1 unit higher than GTP (18). In addition, recent studies by Arai and Kaziro (20) have demonstrated that high concentrations of GMP-P(NH)P could promote polymerization of rat brain tubulin in glycerol-containing buffer; however, these microtubules were unique in that they were insensitive to Ca^{2+}. Also, a direct quantitative comparison of tubulin polymerization in GTP and GMP-P(NH)P was not presented. Although both Figs. 1 and 2 show that GMP-P(NH)P always gave an extent of polymerization slightly higher than the control samples and electron microscopic observations show the presence of an occasional microtubule in these preparations, we cannot readily explain this apparent conflict of results. However, one possible explanation is that when tubulin contains GTP in the nonexchangeable (*N*) site it can bind GMP-P(NH)P at the *E* site and enter into polymerization; whereas, tubulin preparations that have GDP in the *N* site, such as ours, cannot, and thus they require GTP for polymerization.

The high level of uncoupled GTPase activity (Figs. 4 and 5) does not appear physiological. However, this is not surprising since 80% of the tubulin is not polymerized, some of which may

be defective for polymerization but not for GTPase. Similar findings have been observed in the ribosome-dependent GTPase of protein biosynthesis (21).

Under conditions of tubulin polymerization the coupled GTPase activity displays a brief lag (Figs. 4B and 5B) similar to that seen for polymerization (Figs. 4A and 5A), suggesting that the initial events in microtubule formation, which may represent the formation of nucleation centers, are independent of specific GTPase activity, while elongation events require the coupled hydrolysis of GTP. Additional studies with [γ-^{32}P,^{3}H]GTP have shown that both ^{32}P and ^{3}H are associated with the small amount of material retained by the glass-fiber filter during the first minutes (0–1.5) of incubation; however, as polymerization proceeds there is a rapid loss of ^{32}P, and microtubules collected after 5 min contain only [^{3}H]nucleotide. These results suggest hydrolysis of the γ-phosphate from all bound nucleotides during microtubule formation. The delay in onset of GTPase activity also suggests that the lag is a true phenomenon and not an artifact of the turbidimetric and glass fiber filter assays for polymerization. Furthermore, we have demonstrated that the addition of DE-MAP fractions reduces the lag time for polymerization when purified tubulin is used at limiting concentrations, and the addition of rings or microtubule fragments obtained from the void volume of agarose 4B column chromatography of depolymerized tubules essentially abolishes the lag period in polymerization*, in agreement with the condensation mechanism of Johnson and Borisy (12).

A stoichiometry of approximately 2 mol of inorganic phosphate released per tubulin dimer polymerized has been obtained under various experimental conditions, including high concentrations of affinity chromatographically pure tubulin, low concentration of tubulin with added DE-MAP, and with tubulin preparations isolated by four cycles of polymerization–depolymerization. Furthermore, the competitive inhibition displayed by GDP and GMP-P(NH)P suggests that hydrolysis of GTP may be required for tubulin polymerization. Based on these findings we would like to propose the following model of GTPase activity in microtubule polymerization, where tubulin dimer with an exchangeable (E) and nonexchangeable (N) binding site for GTP is represented by $_{N}T_{E}$ and the growing microtubule is $(MT)_n$:



Under conditions where polymerization does not occur, only the uncoupled GTPase activity at the E site is observed. However, the model postulates that when nucleation centers $(MT)_n$ are present, polymerization is favored and the tubulin dimer associates with these centers, thus creating or activating an additional site for nucleotide interaction with tubulin. This site may represent a distinct GTPase or transphosphorylase activity that leads to an intermediate containing 2 mol of GTP. The subsequent hydrolysis of these 2 mol of bound GTP would liberate 2 mol of inorganic phosphate. Speculatively, this energy requirement may be necessary for stabilizing lateral interactions between adjacent tubulin dimers in the microtubule lattice. Portions of this model are very similar to that proposed by Jacobs (11).

The specific GTPase activity continues for several minutes after polymerization has essentially plateaued (Fig. 4). This observation may be explained by several possibilities, such that GTP hydrolysis may reflect a dynamic nature of tubulin subunits in the microtubules, or be required for maintenance of microtubule structure, especially in the absence of high concentration of glycerol or other stabilizing agents.

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