Inhibition of Tubulin Self-Assembly and Tubulin–Colchicine GTPase Activity by Guanosine 5′-(γ-Fluorotriphosphate)†

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ABSTRACT: The inhibitory effects of guanosine 5′-(γ-fluorotriphosphate) [GTP(γF)] on both the polymerization and the colchicine-dependent GTPase activity of calf brain tubulin have been studied. The results demonstrate that this analogue of GTP, with a fluorine atom on the γ-phosphate, is a reversible competitive dead-end inhibitor of the colchicine-induced GTPase activity with a K_I value of $(1.8 \pm 0.6) \times 10^{-4}$ M. GTP(γF) did not promote assembly of tubulin from which the E-site guanine nucleotide had been removed. It binds to the exchangeable nucleotide site competitively with respect to GTP, diminishing both the rate and extent of tubulin polymerization. Treatment in terms of the Oosawa–Kasai model of the inhibitory effect of GTP(γF) on the assembly led to a value of $K_{dis} = 1.1 \times 10^{-6}$ M for the complex GTP(γF)–tubulin. This analogue does not bind to the postulated third site. The growing of tubulin polymers at 37 °C was arrested by GTP(γF), and only limited depolymerization was induced by the addition of this analogue after assembly in the presence of GTP. This result confirms that the E-site is blocked in the polymer and that this analogue can bind only to the ends of the polymers. Sedimentation velocity and circular dichroism studies showed that the conformation of the tubulin–GTP(γF) complex is not identical with that of tubulin–GTP. This is caused by the replacement of the hydroxyl group in the γ-phosphate by the fluorine group, which have 2.20- and 1.35-Å van der Waals radii, respectively. Our results indicate that both negative charges on this terminal phosphate are required for the polymerization of tubulin but that only one is sufficient for its binding to the exchangeable nucleotide site on tubulin.

Tubulin is an asymmetric αβ dimer protein that self-assembles into microtubules (Timasheff & Grisham, 1980). Since its isolation from brain tissue by Weissenberg et al. (1968), it has been known that this protein contains two guanylyl nucleotide binding sites per 110 000 molecular weight dimer. One site has GTP tightly bound and is nonexchangeable (N-site). The other site, called the E-site, can exchange freely GTP and GDP. Photoaffinity labeling experiments with 8-azido-GTP (Gaehlen & Haley, 1977) have led to the conclusion that the exchangeable site is located on the β subunit. This site seems to be specific for guanine nucleotides, since it does not bind significantly ATP, UTP, or CTP (Jacobs et al., 1974; Arai et al., 1975). The polymerization of tubulin into microtubules is accompanied by GTP hydrolysis at one of these sites (Gaskin et al., 1974; Lee & Timasheff, 1977). This has been identified as the exchangeable site, the nonexchangeable GTP remaining intact (Kobayashi & Simizu, 1976; Weisenberg et al., 1976). When incorporated into microtubules, the E-site nucleotide becomes nonexchangeable (Weisenberg & Deery, 1976). The role of GTP hydrolysis at the exchangeable site in tubulin polymerization has been the subject of extensive investigations (Carlier, 1982). Hydrolysis of the GTP γ-phosphate is not required for polymerization since tubulin can assemble into microtubules in the presence of nonhydrolyzable GTP analogues (Weisenberg & Deery, 1976; Penningroth & Kirschner, 1978). The role of GTP hydrolysis seems to be related to the stabilization of microtubules (Bonne & Pantaloni, 1982). The GTPase activity on the tubulin dimer exchangeable site can also be promoted by the binding of colchicine, an inhibitor of microtubule assembly (Maccioni & Seeds, 1977; David-Pfeuty et al., 1979; Andreu & Timasheff, 1981). This activity seems to be induced by a conformational change in tubulin upon binding of colchicine (Andreu & Timasheff, 1982). It has also been shown that the GDP and GTP complexes of tubulin exist in different conformations (Howard & Timasheff, 1986).

While much has been learned about the function of the nucleotide at the E-site, its mechanism of action is still not well understood. Comparatively few studies have addressed the question of which parts of the nucleotide give the binding specificity to the exchangeable site and which are essential for polymerization. There appears to be a lack of absolute specificity with respect to the base and sugar moiety of the nucleotide (Kirsch & Yarbrough, 1981). The γ-phosphate moiety of the nucleotide appears to be essential for polymerization, because tubulin in the presence of GDP does not polymerize at protein concentrations accessible in the laboratory (Carlier & Pantaloni, 1978). Analogues of GTP with fairly bulky substituents on the γ-phosphate do not promote tubulin assembly at concentrations where the exchangeable nucleotide binding site should be more than 95% saturated (Yarbrough & Kirsch, 1981). GTP(γS) has been shown to be an effective nucleotide inhibitor of MAP[1]-dependent microtubule assembly and its associated GTP hydrolysis, but it

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[1] Abbreviations: MES, 4-morpholineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; GTP, guanosine triphosphate; GDP, guanosine diphosphate; GTP(γF), guanosine 5′-(γ-fluorotriphosphate); CD, circular dichroism; UV, ultraviolet; MAP(s), microtubule-associated protein(s); DEAE, diethylaminoethyl; GTP(γS), guanosine 5′-(γ-thiotriphosphate); P_i, inorganic phosphate.

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was effective in inducing the assembly of MAP-free tubulin, the rate of assembly being considerably greater than that found for tubulin containing MAPs.

In an attempt to define more precisely the role of the γ-phosphate moiety of GTP in the tubulin polymerization process, we have studied the behavior of GTP fluorinated on the γ-phosphate, GTP(γF), a nonhydrolyzable analogue of GTP, as an inhibitor of tubulin assembly and of the GTPase activity of tubulin, and the results of this study are presented in this paper.

Materials and Methods

Reagents. GTP (type IIS), alkaline phosphatase (type VII-S), MES, and GDP (type I, lot 71F-7005) were purchased from Sigma Chemical Co. Colchicine, triethylamine, and diphenyl chlorophosphate (lot 1123ME) were from Aldrich Chemical Co. Phosphorofluoridic acid disodium salt (lot 040981) and barium oxide were from Alfa Products. Tri-*n*-octylamine and tributylamine were from Pfaltz & Bauer Chemicals. Pyridine, EGTA, petroleum ether, absolute methanol, and dimethylformamide were from Baker Chemical Co. Pyridine was refluxed over BaO and distilled through a short column. Both pyridine and dimethylformamide were stored over Linde (Tonawanda, NY) type 4A $^1/_{16}$-in. pelleted molecular sieves for 1 week before using. Solvents were redistilled before using and stored under anhydrous conditions. Sodium phosphate, glycerol, and $MgCl_2$ were from Fisher. Guanidine hydrochloride was from Heico, Inc. Sephadex G-25, DEAE-Sephadex A-25, Sephacryl S300, and Blue Dextran were from Pharmacia. [8-^{3}H]GTP, 22 Ci/mmol, and [γ-^{32}P]GTP, 26 Ci/mmol, were from Amersham. Other chemicals were of reagent grade. Distilled, deionized water was used throughout.

Calf Brain Tubulin Purification. Calf brains, for tubulin preparation, were dissected from freshly slaughtered animals, kept on ice, and used within 1 h of slaughter. Calf brain tubulin was purified by the method of Weisenberg et al. (Weisenberg et al., 1968; Weisenberg & Timasheff, 1970) as modified by Lee et al. (1973). Since the stock protein was stored in the presence of 1 M sucrose (Frigon & Lee, 1972), the experimental samples were prepared by batch equilibration with 5–10 volumes of dry-packed Sephadex G25 fine equilibrated in the experimental buffer at 4 °C, followed by filtration at 10 °C through a Sephadex G-25 column preequilibrated with the experimental buffer.

Synthesis of Guanosine 5′-(γ-Fluorotriphosphate) [GTP-(γ-F)]. Diphenyl fluoropyrophosphate was synthesized essentially according to the procedure of Haley and Yount (1972). Prior to the coupling reaction, GDP (disodium salt) was converted into its tri-*n*-octylammonium salt according to the method of Eckstein et al. (1975). The coupling reaction was carried out according to the method of Haley and Yount (1972). The product was purified by chromatography on a DEAE-Sephadex A-25 column. The eluate containing GTP-(γF) was treated with alkaline phosphatase to eliminate contamination with GTP or GDP: 0.65 mmol of product was treated for 2 h at 35 °C with 350 units of alkaline phosphatase in 0.03 mM $ZnCl_2$ and 45 mM $NaHCO_3$, pH 8.3. The product was chromatographed on a DEAE-Sephadex column and eluted with a gradient (0–1 M) of triethylammonium bicarbonate, pH 7.5. The elution started at 0.3 M. The yield was 12.3% of a free-flowing white powder which was identified as GTP(γF). This product was found to be pure by chromatography on DE-81 Whatman paper. The ultraviolet adsorption spectrum of GTP(γF) was essentially identical with that of GTP. Acid-labile phosphate (1 M HCl, 100 °C, 15 min) and total phosphate (Ames & Dubin, 1960) were determined by the Malachite Green method (Lanzetta et al., 1979). The ratio of guanosine to acid-labile phosphate to total phosphate ratio was 1.0:2.1:2.9, while theory requires 1.0:2.0:3.0. Elemental analyses performed by Galbraith Laboratories, Inc., Knoxville, TN, gave the results: Anal. Calcd for $C_{28}H_{62}N_8O_{14}P_3FNa$: N, 12.9; O, 25.76; P, 10.69; F, 2.19. Found: N, 10.85; O, 25.9; P, 12.86; F, 2.17. The fluorine 254.1-MHz ^{19}F NMR spectrum was a doublet arising from the spin–spin coupling of fluorine to the γ-phosphorus with no evidence of further splitting by the γ-phosphorus atom. The coupling constants (J_{FP}) of fluorine on the γ-phosphate of GTP and of Na_2PO_3F were 935 ± 1.3 and 868.8 ± 5 Hz, respectively, with corresponding chemical shifts of 0.53 (lower field peak) and 0.79 ppm. Mildvan et al. (1967) found a value of 868 ± 5 Hz for the coupling constant of Na_2PO_3F and Haley and Yount (1972) a value of 934 Hz for the coupling constant of adenosine 5′-(γ-fluorotriphosphate) [ATP(γF)], values essentially identical with our results.

Sedimentation Velocity Experiments. Sedimentation velocity experiments were performed in a Beckman Model E analytical ultracentrifuge equipped with an electronic speed control and RTIC temperature control. Samples were run in an An-D rotor in 12-mm double-sector cells with aluminum-filled Epon centerpieces and quartz windows. Sedimentation profiles were recorded on Kodak metallographic plates. Rates of sedimentation were measured with a Nikon Model 6C microcomparator. The measured sedimentation coefficients were corrected to water at 20 °C, with a value of $\bar{v}$ = 0.736 mL/mg for tubulin (Lee & Timasheff, 1974) used throughout.

GTPase Activity. Hydrolysis of the γ-phosphate of GTP was assayed essentially as described by Andreu and Timasheff (1981), after separation of tubulin from colchicine-independent Mg^{2+}-GTPase by chromatography on a Sephacryl S300 column equilibrated with 10 mM phosphate and the appropriate concentration of GTP at pH 7.0, 10 °C. ^{32}P-Labeled inorganic phosphate was determined after adsorption of nucleotides to activated charcoal as described by Nieto et al. (1975).

Circular Dichroism. CD spectra were recorded on a Jobin Yvon Mark V autodichrograph under nitrogen atmosphere. The measurements were carried out by using cylindrical fused quartz cells with a path length of 2 cm at 20 ± 0.3 °C. The calibration of the instrument was based on values of $[\theta]_{290.5}$ = 7600 and $[\theta]_{192.5}$ = −14600 deg cm^2 dmol^{-1} for a purified sample of *d*-10-camphorsulfonic acid (Chen & Yang, 1977; Gillen & Williams, 1975). Samples were prepared by diluting the protein with a buffer devoid of free nucleotides to a final concentration of 1.2 mg/mL. The CD data reported represent the average of three recordings. The ellipticity values (θ) were calculated by using a mean residue weight of tubulin of 110. Spectra were recorded from 350 to 250 nm at a sensitivity of 2×10^{-6} mdeg/cm with an instrumental time constant of 2 s. Far-UV CD spectra of 1.83 mg/mL tubulin samples were recorded from 250 to 200 nm, in cylindrical fused quartz cells (path length 0.01 cm) under the same experimental conditions.

Polymerization of Tubulin. The self-assembly of tubulin into microtubules was monitored by turbidity measurements (Gaskin et al., 1974). The assembly buffers consisted either of 10^{-2} M sodium phosphate, variable concentrations of $MgCl_2$ as indicated, 3.4 M glycerol, 10^{-3} M EGTA, and 10^{-4} M GTP at pH 7.0 or of 0.02 M MES, variable concentrations of $MgCl_2$ as indicated, 0.1 M glutamic acid, and 3.4 M glycerol at pH 6.8 (buffer M). Turbidity was monitored at 350 nm on Cary 14 or 118 recording spectrophotometers. The protein solutions were incubated at 37 °C in a water-jacketed cuvette, which

was regulated thermostatically by a Haake KT33 or Nestat RTE-4 circulator.

Other Methods. Protein concentrations were determined by diluting aliquots of the samples by volume into a 10-fold quantity of 6 M guanidine hydrochloride and measuring the absorbance of the solutions at 275 nm, using an absorptivity value of 1.03 L g^{-1} cm^{-1} (Na & Timasheff, 1981). The nucleotide at the E-site was exchanged by passing the tubulin through one cycle of assembly in a nucleotide-free assembly buffer. The tubulin was then incubated at 0 °C with 0.5 mM of either GTP or GTP(γF) for 30 min. Free nucleotide was removed by filtration through a G-25 Sephadex column preequilibrated with buffer M.

Removal of GTP from the Exchangeable Nucleotide Site of Tubulin. Purich and MacNeal (1978) have shown that alkaline phosphatase can be used to remove GDP from the exchangeable site. To accomplish this, our tubulin was passed through one cycle of polymerization by incubating it for 45–50 min at 37 °C in a buffer consisting of 20 mM MES, 100 mM glutamic acid, 5 mM $MgCl_2$, and 3.4 M glycerol at pH 6.8. The polymers were disassembled by standing for 20 min at 0 °C. Alkaline phosphatase (3.5 units/mg of tubulin) and 0.03 mM $ZnCl_2$ were added to the reaction mixture, the pH was readjusted to 6.8 with KOH, and the reaction was continued for 20 min at 0 °C. The reaction was stopped by adding ethylenediaminetetraacetic acid (EDTA) to a final concentration of 0.01 mM. The tubulin was then equilibrated with the working buffer by filtration through Sephadex G-25.

Nucleotide Concentration. The total concentration of guanine nucleotides was measured by ultraviolet absorption at 256 nm by using a molar extinction coefficient of 12 350 M^{-1} cm^{-1} at pH 1 and of 13 700 mol L^{-1} cm^{-1} at neutral pH (Dawson et al., 1969). Tubulin samples were adjusted to 0.5 N perchloric acid and then kept for 15 min at 0 °C. The precipitate was removed by centrifugation of the samples at ca. 14000*g* for 10 min. Extraction of nucleotides at room temperature resulted usually in higher values for the nucleotide to tubulin ratio. The UV spectra of these extracts showed a maximum at 252 nm and a shoulder around 280 nm. The spectra of samples treated with charcoal showed a peak with a maximum at 275 nm. These results indicate that the temperature of nucleotide extraction is a crucial variable in nucleotide determination.

RESULTS

Inhibition of Microtubule Assembly by GTP(γF). The effect of GTP(γF) on the polymerization of tubulin that had been treated with alkaline phosphatase in order to remove GTP from the exchangeable site is shown in Figure 1A. In these experiments the E-site nucleotide-free tubulin was preincubated with the analogue at 0 °C, and GTP at the desired concentration was added 5 min prior to the start of the assembly. As seen in tracing 4, GTP(γF), at a level of 0.3 mM, does not stimulate the assembly of tubulin. Rather, this analogue has a reversible inhibitory effect on the assembly of tubulin, which is a function of its concentration relative to GTP. Thus, when GTP was added at a level of 0.4 mM, in the presence of 0.3 mM GTP(γF), the turbidity attained only 15% of the control value after 30 min of heating (Figure 1A, tracing 3). Since these experiments were done in succession with the same preparation of tubulin over a time span of approximately 5 h, the stability of tubulin without GTP at the exchangeable site was tested by keeping the protein at 0 °C and adding the GTP at various times over a total time span of 3 h. The results, shown in Figure 1B, indicate a progressive decrease in the ability of the protein to polymerize. While the inactivation

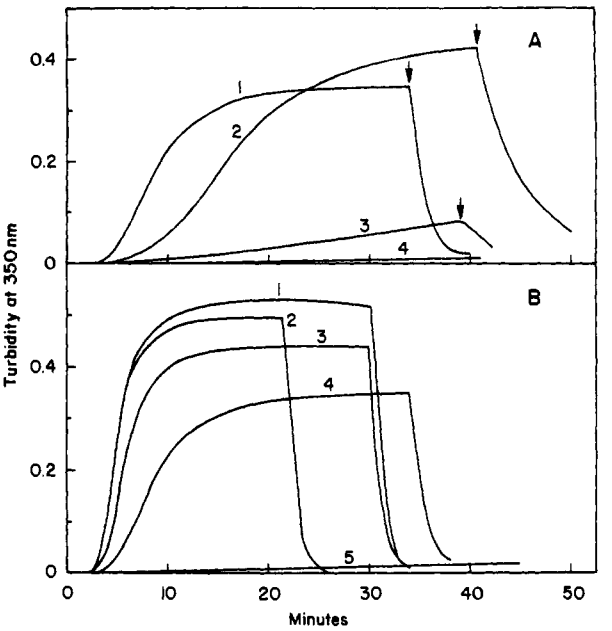


FIGURE 1: (A) Inhibition of tubulin polymerization by GTP(γF). The composition of the reaction mixture was 2.35 mg/mL tubulin, 100 mM glutamic acid, 5 mM $MgCl_2$, 3.4 × 10^3 mM glycerol, and 20 mM MES at a final pH of 6.8. In the experiments in the presence of GTP(γF), the tubulin was preincubated with the analogue at 0 °C and GTP at the desired concentration was added to the reaction mixture 5 min prior to the start of assembly. The final nucleotide concentrations were 0.4 mM GTP, curve 1; 0.34 mM GTP(γF) + 5 mM GTP, curve 2; 0.34 mM GTP(γF) + 0.4 mM GTP, curve 3; and 0.34 mM GTP(γF), curve 4. At the time indicated by the arrows, the temperature was lowered to 10 °C. Time zero corresponds to a jump in temperature from 10 to 37 °C. (B) Polymerization of tubulin after different times in the absence of nucleotide at the exchangeable site. The composition of the reaction mixture was 2.4 mg/mL tubulin, 100 mM glutamic acid, 5 mM $MgCl_2$, 3.4 × 10^3 mM glycerol, and 20 mM MES at a final pH of 6.8. GTP (0.42 mM) was added and the mixture was warmed to 37 °C at 0 min, curve 1; 45 min, curve 2; 100 min, curve 3; 160 min, curve 4; no nucleotide added, curve 5.

of unliganded tubulin precludes a quantitative interpretation of these results, qualitative conclusions can be nevertheless drawn. First, the presence of the analogue increases the lag period of assembly, as seen in tracings 2 and 3 of Figure 1A, which were obtained in the presence of 5 and 0.4 mM GTP and 0.3 mM GTP(γF). This indicates that the analogue binds to the tubulin dimer, slowing down the assembly process. Second, the speed of disassembly on cooling to 10 °C is also slower in the presence of the analogue, indicating that either the polymers formed are stabilized by the analogue or that the number of microtubules is smaller in the presence of the analogue than in its absence. The last possibility would mean that the analogue is acting already on the nucleation stage of the polymerization. Third, the turbidity attained in the presence of this analogue and 5 mM GTP is greater than the plateau value in 0.4 mM GTP, suggesting that a high concentration of GTP can reactivate tubulin molecules that were not able to enter into the polymerization reaction in 0.4 mM GTP. This may reflect also the kinetics of the reactivation, which may be a slow reaction.

Figure 2 shows the inhibition of assembly of microtubules as a function of GTP(γF) concentration in the presence of 0.1 mM free GTP. The extent of polymerization was measured from the plateau value of the turbidity at 350 nm. In these experiments tubulin was equilibrated with the GTP-containing buffer and then incubated at 0 °C in the presence of GTP(γF) for 5 min before the assay was started. This assured equilibration with the analogue, since the exchange is fast between

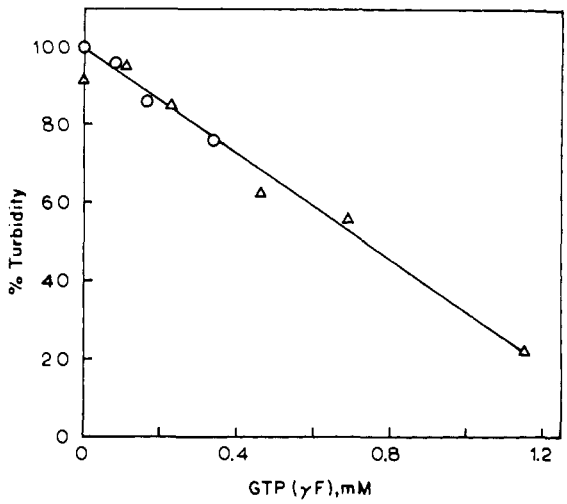


FIGURE 2: Inhibition of tubulin polymerization by GTP(γF). The composition of the reaction mixture was either 20 mM MES, 100 mM glutamic acid, 0.1 mM GTP, 5 mM $MgCl_2$, and 3.4×10^3 mM glycerol at pH 6.8 (O) or 10 mM sodium phosphate, 1 mM EGTA, 12 mM $MgCl_2$, 0.1 mM GTP, and 3.4×10^3 mM glycerol, at pH 7.0 (Δ). Analogue at the proper level was added to an aliquot of the reaction mixture 5 min prior to the temperature jump from 0 to 37 °C to assure equilibration with free tubulin prior to the initiation of polymerization.

GTP–tubulin and analogue (Monasterio, 1987). In the range of concentrations studied, the inhibition was linear. In order to explain the linearity, we must recall that the turbidity value represents the total mass of polymerized protein and, thus, the number concentration of polymers at the steady state (Berne, 1973). These data can be represented then by a simple model for the pseudoequilibrium, in which GTP and GTP(γF) compete for the same binding site on tubulin, the binding of GTP leading to microtubule elongation and that of GTP(γF) resulting in a dead-end complex. The corresponding reaction scheme is

$$\begin{array}{ccccccccc} TI & \overset{K_5}{\rightleftharpoons} & T + M_{n-1} + G + I & \overset{K_1}{\rightleftharpoons} & TG + M_{n-1} \\ & & \Big\uparrow K_3 \quad \Big\downarrow K_2 & & \\ & & M_n + G & \overset{K_4}{\rightleftharpoons} & M_nG \end{array} \qquad (1)$$

where TI is the dead-end complex formed by tubulin and GTP(γF), T, M_{n-1}, G, and I are tubulin, microtubule, GTP, and GTP(γF), respectively, and M_n and M_nG are microtubules extended by one additional molecule of tubulin, without or with GTP in the exchangeable site.

Since it is known that the binding of GTP to dimeric tubulin is required for addition to a growing microtubule, the ligand-facilitated pathway (K_3K_4) can be neglected. Then, considering only the ligand-mediated pathway (K_1K_2), we have

$$K_1K_2K_5 = [TI][M_n]/[T]^2[I][G][M_{n-1}] \qquad (2)$$

The total concentration of tubulin, T_{tot}, expressed in moles of α–β dimer, is

$$T_{tot} = [T] + [TG] + [TI] + [M] \qquad (3)$$

where [M] is the number of moles of tubulin dimers present in the form of microtubules.

Recalling that the critical concentration C_r in the absence of inhibitor is given by

$$C_r = [T] + [TG] \qquad (4)$$

and that, in the absence of inhibitor, the amount of tubulin

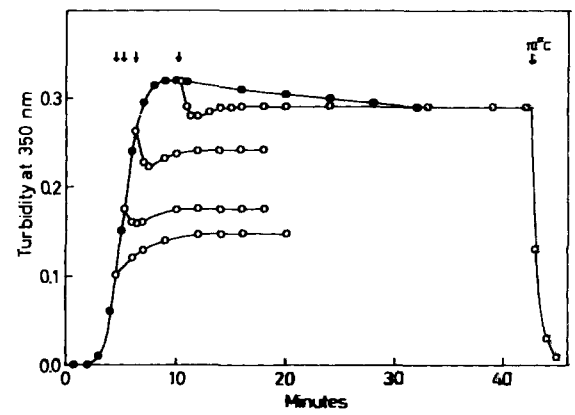


FIGURE 3: Inhibition of polymer elongation by GTP(γF). Polymerization was initiated by increasing the temperature to 37 °C, and polymer formation was followed by the increase in turbidity at 350 nm. The composition of the reaction mixture was 2.1 mg/mL tubulin, 10 mM sodium phosphate, 1 mM EGTA, 12 mM $MgCl_2$, 3.4×10^3 mM glycerol, and 0.1 mM GTP at a final pH of 7.0. The arrows indicate the time of addition of 1.2 mM GTP(γF).

polymerized is $T_{tot} - C_r$, we find that the fraction of turbidity in the presence of inhibitor corresponds to

$$\text{fract} = [M]/(T_{tot} - C_r) \qquad (5)$$

Combining eq 2–5, we have

$$\text{fract} = \frac{T_{tot} - C_r[1 + K_5[I]/(1 + K_1[G])]}{T_{tot} - C_r} \qquad (6)$$

Then, at any given concentration of GTP, we can express the assembly inhibition as a linear function of the GTP(γF) concentration:

$$\text{fract} = [T_{tot} - C_r(1 + K_{i,app}[I])]/(T_{tot} - C_r) \qquad (7)$$

where $K_{i,app}$ is the apparent binding constant of GTP(γF) to tubulin. Application of eq 7 to the data of Figure 2 resulted in a value of $K_{i,app}$ of 200 M^{-1} in the presence of 0.1 mM GTP.

In the experiments of Figure 2, an excess of free GTP was present in the assembly buffer to assure stability of the tubulin molecules. Addition of analogue 5 min prior to the start of the assembly led to the turbidity decreases of Figure 2. For example, in 0.34 mM GTP(γF), the steady-state turbidity was decreased by 24%. On the other hand, incubation of tubulin with the same concentration of the analogue and in the absence of exchangeable nucleotide on the E-site did not generate any turbidity (Figure 1A, tracing 4). Furthermore, when tubulin, depleted of nucleotides at the exchangeable site, was first preincubated at 0 °C with 0.34 mM GTP(γF) and 0.4 mM GTP was then added to the mixture 5 min before the start of the heating, the value of the turbidity reached was only 15% of that without inhibitor after approximately 40 min (Figure 1A). These results indicate that the kinetics of the polymerization of tubulin depend on the order of addition of GTP and GTP(γF), suggesting that the binding of GTP(γF) produces a slowly reversible conformational change in tubulin. The exchange of nucleotide from the E-site in this conformer should be slower than that in the tubulin–GTP complex.

Figure 3 shows the effects of the addition of 1.2 mM GTP(γF) at different times during the course of the polymerization of tubulin. When the inhibitor was added at time zero, no polymerization occurred. Addition of GTP(γF) at all the other times indicated by the arrows resulted in inhibition of the further growth of the polymers. At 4.5 min, its addition produced a change in the speed of assembly and a new steady-state was reached. At 5.5 min and longer times, addition first decreased the turbidity during the first minute,

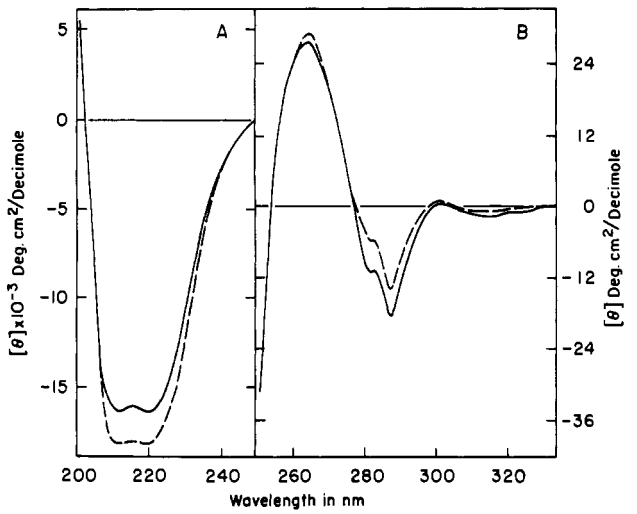


FIGURE 4: Circular dichroism spectra of tubulin with GTP or GTP(γF) at the exchangeable site at 20 ± 0.3 °C in buffer M. Protein concentration was 1.2 mg/mL: (---) GTP; (—) GTP(γF).

followed by an increase essentially back to the turbidity value at which addition took place, the system then remaining in this new steady state. This behavior can possibly be explained by a redistribution of the shapes of the polymers in the solution rather than a disassembly and reassembly of the polymers. In all cases a decrease in the temperature to 10 °C produced a complete disassembly of the polymers with identical velocity.

This result confirms that the exchangeable site in the polymer is blocked (Jacobs et al., 1974; Weisenberg et al., 1976). Addition of GTP(γF) arrests the polymerization process at a plateau level corresponding to the extent of polymerization attained. It does not, however, depolymerize existing polymers. This analogue behaves in a manner similar to GDP, and the conclusion reached by Weisenberg et al. (1976) that the polymerization of tubulin is not a reversible reaction with respect to the nucleotide interaction is supported by these results.

Characterization of the GTP(γF)–Tubulin Interaction. The inhibition studies on tubulin polymerization have shown that GTP(γF) interacts principally with free tubulin. In order to understand the mechanism of action of this analogue on the conformational and self-association states of tubulin, we have carried out a characterization of the binding of this analogue to tubulin by circular dichroism, sedimentation velocity, and GTPase activity in the presence of colchicine.

Circular Dichroism. The results of the circular dichroism experiments are shown in Figure 4. In the far-UV region, the negative ellipticity diminished by about 10% between 207 and 240 nm in the presence of GTP(γF). The near-UV CD spectra of tubulin with either GTP or GTP(γF) bound to the exchangeable site were quite similar. Both showed two negative bands at 281 and 287 nm, a weak positive band at 301 nm, and a stronger one at 264 nm. These results are in good agreement with the published spectra (Lee et al., 1978; Clark et al., 1981). These results also show that the composition of the buffer has no effect on the CD spectrum of tubulin, if we compare the composition of buffer M with the PG buffer (10 mM phosphate and 0.1 mM GTP at the same pH) used by Lee and Timasheff (1977). Comparison of the near-UV CD spectra of GTP and GTP(γF) tubulins revealed an increase in negative ellipticity in the region between 278 and 298 nm upon binding of GTP(γF) to tubulin. A similar difference between the spectra was obtained when the free concentrations of the nucleotides were around 0.9 mM. In this region the bands originate from tyrosine and tryptophan transitions (Kahn, 1979; Timasheff, 1970; Townend et al., 1967).

Sedimentation Velocity. The effect of GTP(γF) on the state of tubulin dimers and the equilibrium between rings and dimers was examined in sedimentation velocity experiments. Native tubulin in the presence of either GTP or GTP(γF) and 5 or 16 mM $MgCl_2$ was sedimented at 52 000 and 48 000 rpm, respectively. In the presence of GTP, formation of rings depends on both tubulin and magnesium concentrations. Thus, in 16 mM Mg^{2+} the ultracentrifuge pattern becomes bimodal at 5 mg/mL tubulin, while in 5 mM Mg^{2+} no inflections appear in the pattern up to 22 mg/mL protein (Frigon & Timasheff, 1975). Sedimentation experiments of tubulin at 8.1 mg/mL in 5 mM $MgCl_2$ and 0.52 mM either GTP or GTP(γF) resulted in patterns in which >95% of the protein sedimented under an essentially symmetrical peak. The rest of the material was found under a very rapid minor peak that disappeared during the first 3 min of sedimentation. The sedimentation coefficient $s_{20,w}$ of tubulin at 8.1 mg/mL was 7.25 S in the presence of GTP and 7.96 S in the presence of GTP(γF). Within experimental error, the value in the presence of GTP is similar to that obtained by Frigon and Timasheff (1975) in 0.01 M sodium phosphate, 10^{-4} M GTP, and 5 mM $MgCl_2$ (pH 7.0), 20 °C, indicating that the nature of the buffer has no effect on the hydrodynamic properties of tubulin under these conditions. The value of 7.96 S in the presence of GTP(γF) could indicate that this compound stimulates the formation of aggregates. In order to check this possibility, the concentration of $MgCl_2$ was increased to 16 mM, which favors the formation of rings. The resulting sedimentation patterns were bimodal, the $s_{20,w}$ value of the slow peak being 9.62 and 8.80 S in the presence of GTP(γF) and GTP, respectively. The faster peak had $s_{20,w}$ values of 22.37 and 23.37 S in the presence of GTP(γF) and GTP, respectively. Comparison of the areas under the peaks showed a slight decrease in the fraction of protein under the fast peak in GTP(γF) tubulin, suggesting that GTP(γF) can induce the formation of large aggregates in a manner similar to GTP, the aggregation possibly being slightly weaker. The higher value for the sedimentation coefficient of the slow peak in the presence of GTP(γF) can be explained either by a decrease in the frictional coefficient of tubulin as a result of a conformational change or by the facilitation by GTP(γF) of a progressive self-association of tubulin.

Exchange between GTP and GTP(γF) in Tubulin Dimers. To determine if GTP(γF) replaces GTP at the exchangeable site, 8.52 mg of GDP–tubulin in 1 mL was incubated without free GTP and without free Mg^{2+} in 0.5 μCi of [8-^{3}H]GTP of specific activity 22 Ci/mmol at 0 °C for 30 min. The excess nucleotide was removed on a Sephadex G-25 column, leaving the radioactive complex, [8-^{3}H]GTP. The sample was divided into two halves. To one, GTP(γF) was added to a final concentration of 0.9 mM. To the other, the same amount of GTP(γF)-free solvent was added. Figure 5 shows the elution patterns of tubulin–GTP in the presence and in the absence of GTP(γF). The elution volumes of the faster peaks were coincident with the elution of tubulin and the void volume of the column (8.1 mL). The other peaks eluted at the elution volume of GTP. The amount of GTP bound to the exchangeable site of tubulin decreased by 31.5% in the presence of GTP(γF), indicating exchange between the nucleotides. The difference was found in the peak eluting in the nucleotide region. The low level of exchange obtained can possibly reflect the fact that normally only ca. 60% of the tubulin exchanges nucleotides at the exchangeable site (Croom et al., 1985;

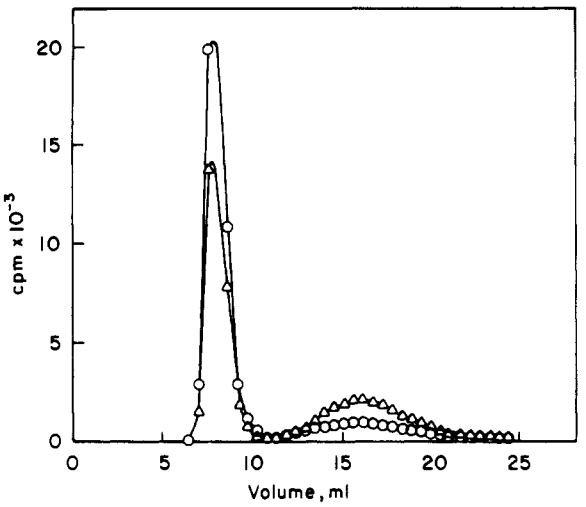


FIGURE 5: Chromatography of [8-^{3}H]GTP–tubulin complex (8.52 mg/mL protein) with 0.9 mM GTP(γF) (△) or the same volume of GTP(γF)-free solvent (○). Mixtures were chromatographed at 10 °C on a 0.9 × 25.5 cm Sephadex G-25 column equilibrated with the same buffer (50 mM MES and 3.4 × 10^3 mM glycerol at pH 7.0) used to exchange successively GTP by [8-^{3}H]GTP and this by GTP(γF) on the exchangeable site of tubulin.

Correia & Williams, 1983; Howard & Timasheff, 1986; Monasterio and Timasheff, unpublished results). This could also reflect the relative binding constants of the two nucleotides for tubulin, namely, 4.55 × 10^7 M^{-1} for GTP (Zeeberg & Caplow, 1979) and 9.13 × 10^5 M^{-1} for GTP(γF) (see below), and the fact that the binding kinetics are slow (Engelborghs & Eccleston, 1982).

Effect of GTP(γF) on Tubulin–Colchicine GTPase Activity. The interaction of GTP(γF) with the exchangeable site of tubulin was further probed through its effect on the colchicine-dependent GTPase activity of tubulin. The results are presented in Figure 6, where the data are plotted according to the procedure of Cornish-Bowden (1974) for reversible dead-end linear inhibitors. The GTP concentration was varied from $1.2K_m$ to $4.8K_m$. In the lower plot the lines intersect at a point to the left of the ordinate over the abscissa at [I] = −0.6 ± 0.2 mM. This indicates a competitive inhibition with an apparent inhibition constant of (6 ± 2) × 10^{-4} M. The upper plot, where the lines are parallel, confirms this type of inhibition. Parallel lines mean that the dissociation constant for the uncompetitive component of this inhibition is infinite. These results indicate that both GTP(γF) and GTP bind to the same site on tubulin, which can exchange nucleotide and on which GTP hydrolysis is induced by colchicine.

In our studies, the kinetics were examined by measuring initial velocities, since the velocity of phosphate release was constant over at least 30 min. The initial steady-state velocity was found to be proportional to the tubulin–colchicine complex concentration used in our experiments (Andreu & Timasheff, 1981), which permitted the use of the Michaelis–Menten steady-state analysis (Wong, 1965). When the concentrations of substrate and enzyme are of the same order of magnitude, the determination of the true value of K_m requires that the concentration of products be taken into account. The proper equation is

$$v_i = [V_{max}(s_0 - p)]/[K_m + e_0 + (s_0 - p)] \quad (8)$$

where v_i is the initial velocity, V_{max} is the maximal velocity under substrate saturating conditions, K_m is the Michaelis–Menten constant, e_0 is the total concentration of enzyme, s_0 is the initial concentration of substrate (GTP), and p is the concentration of product (GDP). With this equation, the

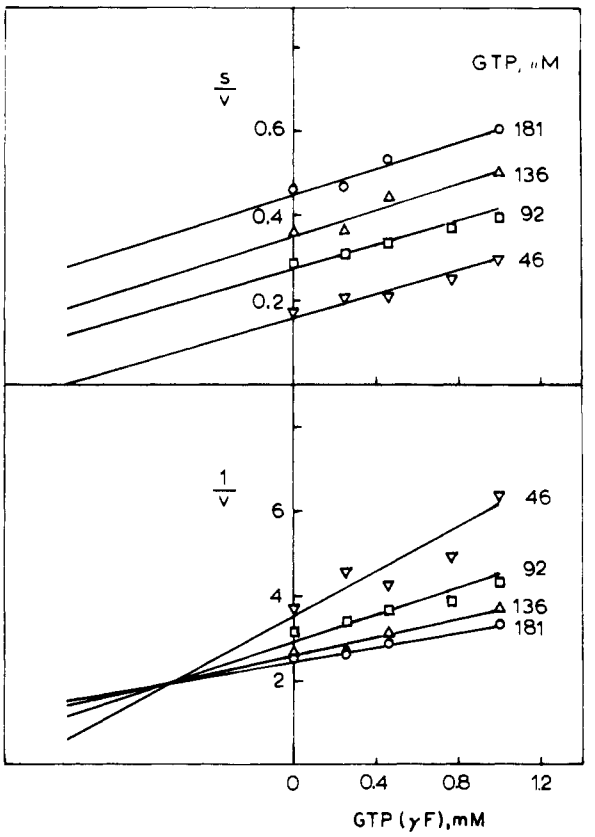


FIGURE 6: Inhibition of the colchicine-dependent GTPase activity of tubulin by GTP(γF). The composition of the reaction mixture was 21.6 ± 1.0 μM tubulin and 0.56 mM colchicine (preincubated for 30 min at 0 °C before the experiments were started), 10 mM sodium phosphate, 1 mM $MgCl_2$, and [γ-^{32}P]GTP and GTP(γF) as indicated in the figure. The time course of the reaction was followed at 37 °C, taking samples of 0.25 mL every 5 min during 20 min. [^{32}P]P_i was determined as described under Materials and Methods. Velocity is expressed in nanomoles of [^{32}P]P_i hydrolyzed per minute per 1.5 mL.

values of K_m and of the turnover number were found to be 9 μM and 0.014 min^{-1}, respectively. Similar values had been obtained by Andreu and Timasheff (1982). This value of K_m is surprisingly equal to that (10 μM) reported by Saltarelli and Pantaloni (1982) for tubulin obtained from a different source and purified by a different method.

Discussion

The results of this study lead to the conclusion that GTP(γF) binds to the exchangeable nucleotide site on tubulin and that it is a reversible competitive dead-end inhibitor both of microtubule assembly and of the colchicine-induced GTPase activity. The kinetics of both assembly and disassembly in 5 mM GTP in the presence of the analogue were slower than the control in 0.4 mM GTP. A possible explanation for these results is that GTP(γF) binds to free tubulin, affecting both the nucleation and the elongation processes. If this slows down the nucleation rate, the initial number of nuclei formed in the solution should be small, and the resulting polymerization process should yield a small number of comparatively large microtubules. The resulting small number of microtubule ends would then diminish the rate of depolymerization, since that rate is proportional to the number of microtubule ends (David-Pfeuty et al., 1978).

Removal of nucleotide from the exchangeable site renders tubulin unstable, as shown in Figure 1. The inactive tubulin can be reactivated, at least in part, by high concentrations of GTP (5 mM) even in the presence of GTP(γF), indicating that the presence of GTP at the E-site is essential to keep the native,

microtubule-forming conformation of tubulin. The conformation of tubulin seems to be controlled by the phosphate moiety of the nucleotide. Thus, GTP–tubulin can self-assemble into microtubules, while GDP-tubulin cannot. This makes possible regulation of the assembly of microtubules, with GTP acting as an allosteric effector at the E-site (Pennigroth & Kirschner, 1978). In such a case, hydrolysis of GTP would be required for maintaining the polymerization–depolymerization cycle, in a manner similar to some other reversible associations of macromolecules regulated by GTP (Allende, 1982).

Replacement of the GTP γ-hydroxyl group with p$K \sim 6.8$ by a fluorine atom turns the nucleotide into a competitive reversible dead-end inhibitor of the polymerization of tubulin. Since this inhibition can be abolished by high concentrations of GTP, the possibility must be considered that GTP(γF) binds to the proposed third nucleotide-binding site on tubulin. This site is described as having low affinity for both ATP and GTP (Zabrecky & Cole, 1982a,b) and an inhibiting influence on microtubule assembly (Jameson & Caplow, 1980; Maccioni & Seeds, 1982; Zabrecky & Cole, 1982a,b). Binding of GTP(γF) to the third site on tubulin can be excluded by the following observations: (i) The inhibitory effect of GTP is produced at concentrations >3 mM (Jameson & Caplow, 1980); in our studies the highest concentration of GTP(γF) used was 1.2 mM, and this analogue showed lower affinity for tubulin than GTP. (ii) Under our experimental conditions 5 mM GTP did not inhibit polymerization. To the contrary, it enhanced the extent of assembly. This discrepancy with the results of Jameson and Caplow (1980) could be due to the presence of MAPs in their tubulin preparations. (iii) It has been proposed that the third site is exposed to solvent on the polymers (Jameson & Caplow, 1980) since high concentrations of GTP (>5 mM) or GDP (>2 mM) lead to depolymerization of microtubules. Our results, Figure 3, show that GTP(γF) does not depolymerize preexisting polymers and that it behaves like GDP at low concentrations (<1 mM), indicating that it binds to a site buried inside microtubules.

The treatment of our kinetics results according to the procedure of Cornish-Bowden (1974) and of the Laidler (1955) equation has yielded a value of K_m (9 μM) identical with those reported previously (Andreu & Timasheff, 1982; Saltarelli & Pantaloni, 1982) and an apparent inhibition constant of 0.6 ± 0.2 mM. For the case of competitive inhibition, eq 8 should be

$$v_i = [V_{max}(s_0 - p)]/[K_m(1 + [I]/K_I) + e_0 + (s_0 - p)] \quad (9)$$

where [I] is the concentration of GTP(γF) and K_I is the apparent dissociation constant for the GTP(γF)–tubulin complex. The intersection point of the $1/v$ vs. [I] plot (Figure 6) is then

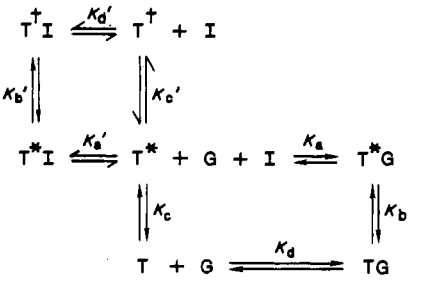
$$-[I] = K_I(K_m + e_0)/K_m \quad (9)$$

Introduction of $K_m = 9$ μM and e_0 (tubulin concentration) = 2.16×10^{-5} M results in $K_I = (1.8 \pm 0.6) \times 10^{-4}$ M.

Let us compare this result with the binding constant derived from microtubule inhibition. The slope of Figure 2 resulted in $K_{i,app} = 200$ M^{-1} in the presence of 0.1 mM GTP. According to the ligand-mediated pathway of the reaction scheme (eq 1), the equilibrium constant for the binding of inhibitor to the protein K_5 is related to the slope of Figure 2 by (see eq 6)

$$K_5 = K_{i,app}(1 + K_I[G]) \quad (10)$$

Introduction of the values of the binding constant of GTP to tubulin, $K_I = 4.55 \times 10^7$ (Zeeberg & Caplow, 1979), and of the GTP concentration, $[G] = 1 \times 10^{-4}$ M, gives $K_5 = 9.13 \times 10^5$ M^{-1}. Thus, the values of the apparent binding constants deduced from the inhibition of microtubule assembly and from the inhibition of the GTPase activity of the tubulin–colchicine complex differ by a factor of 1.6×10^2. It seems worth noting that, in the case of GDP–tubulin, this ratio attains a value of 10^3 (Saltarelli & Pantaloni, 1982; Zeeberg & Caplow, 1979). The reason for this discrepancy might reside in the fact that the bindings of the nucleotides and of colchicine to tubulin are accompanied by conformational changes of the protein. In fact, a more realistic representation of the processes described by K_1 and K_5 of eq 1 is



(11)

where G and I are GTP and GTP(γF), respectively. T* is the inactive tubulin in the absence of nucleotide at the exchangeable site; T† and T are inactive and active conformers of tubulin in the presence of GTP(γF) and in the absence of nucleotide at the E-site, respectively.

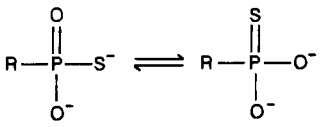
Tubulin in the presence of GTP(γF) has a different CD spectrum in the far-UV region from that of the GTP–tubulin complex, indicating that the conformation of the protein is a function of the state of liganding at the exchangeable site. A similar effect has been observed when the E-site GTP is replaced by GDP (Howard & Timasheff, 1986). As has been shown above, the binding of GTP(γF) to tubulin seems to include a slow step. Kinetics studies of the binding to tubulin of fluorescent GTP analogues (Engelborghs & Eccleston, 1982; Yarbrough & Kirsh, 1981) have shown that both a conformational change and a preequilibrium of tubulin may be involved in the binding of those nucleotides that support assembly, mainly via the facilitated pathway of the scheme shown above. The conformational change appears to be rate-limiting, and a slow phase (half-life = 5 min) is also seen in the binding process (Engelborghs & Eccleston, 1982). Thus, if inhibition of microtubule assembly and of the tubulin–colchicine complex GTPase activity involves different conformational states of tubulin, apparent binding constants deduced from the two phenomena must be different combinations of K_a', K_b', K_c', and K_d' as well as of the effect of colchicine binding on the values of these parameters. Our data, however, do not permit us to resolve this discrepancy quantitatively.

The effects of GTP(γF) on microtubules, reported above, confirm that the E-site inside the polymer is blocked to contact with solvent and that interactions of the ligand with both microtubule ends and free tubulin stop the addition of new tubulin molecules to the growing microtubule but do not induce depolymerization. It might appear that the model of Weisenberg et al. (1976) could account for these observations. This model cannot be used, however, since their studies were done in the presence of MAPs. On the other hand, the model proposed by Pantaloni and Carlier (1985) [see also Carlier (1982), Carlier et al. (1984), David-Pfeuty (1980), and Carlier and Pantaloni (1981)] in which GTP–tubulin at the end of microtubules prevents their depolymerization and free GTP exchanges with the E-site of tubulin at microtubule ends can give a simple explanation for our results. The E-site of tubulin at the end of a microtubule being exchangeable, it can bind

GTP(γF), which induces a conformational change in tubulin molecules situated in the "cap" of microtubules. This should prevent further growth of microtubules but avoid their depolymerization because of the presence of the cap at the microtubule ends. The reaction of GTP(γF) with tubulin at microtubule ends must depend, then, on the concentrations of free tubulin, free GTP, and free GTP(γF). As the polymer grows, the amount of free tubulin diminishes and the relative amount of GTP(γF) increases with respect to microtubule ends. This could explain the results of Figure 3 which indicate an enhancement of the inhibitory effect when the inhibitor is added at later times of polymerization during the growth phase. Binding of GTP(γF) does not prevent the cold depolymerization of microtubules, showing that it has no effect on the lateral interactions of protofilaments and cannot penetrate to the E-site of tubulin molecules incorporated into microtubules.

Since fluorination of the nucleotide modifies only one hydroxyl group in the γ-phosphate in the nucleotide, our results suggest that the negative charges of both hydroxyls of the terminal phosphate are required for promotion of polymerization by the nucleotide but that only one is needed for binding to the exchangeable nucleotide site on tubulin. It can be concluded, therefore, that the second negative charge of the γ-phosphate does not contribute greatly to the binding energy. Results obtained from other laboratories confirm this conclusion. Kirsch and Yarbrough (1981), using three GTP analogues with bulky substituents on the γ-phosphate, failed to promote assembly either of tubulin containing associated proteins or of tubulin from which these proteins and guanine nucleotide had been removed. They found also that GTP(γS) promoted the assembly of tubulin that contained associated proteins at a greatly reduced rate. On the other hand, GTP(γS) promoted the assembly of tubulin from which both associated proteins and guanine nucleotide had been removed at a rate more nearly that of GTP. These results have been contradicted in part by Hamel and Lin (1984), who found that this analogue was an inhibitor of both tubulin polymerization and GTP hydrolysis under conditions in which these reactions were dependent on MAPs.

A rigorous analysis of these observations must take into consideration the fact that the γS analogue exists in several isomers in solution and that the γ-phosphate can have at least two resonance structures:



Furthermore, the γ-phosphate at the E-site is part of a complex with Mg^{2+} (Monasterio, 1987). These facts invalidate the proposed attribution of the interactions of the γ-phosphate moiety of GTP(γS) to its lipophilicity and the extrapolation of this effect to a possible conformational change with reduction in the hydrophilicity of the tubulin exchangeable nucleotide binding site (Hamel & Lin, 1984). A more exhaustive analysis of the interaction of GTP(γF) with the exchangeable nucleotide site has been carried out by nuclear magnetic resonance and the results are described in the following paper (Monasterio, 1987).

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^{19}F Nuclear Magnetic Resonance Measurement of the Distance between the E-Site GTP and the High-Affinity Mg^{2+} in Tubulin†

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ABSTRACT: The distance separating the divalent metal ion high-affinity binding site and the exchangeable nucleotide binding site on tubulin was evaluated by using high-resolution ^{19}F NMR. The ^{31}P and ^{19}F NMR spectra of guanosine 5′-(γ-fluorotriphosphate) [GTP(γF)] were studied. Both the fluorine and the γ-phosphate were split into a doublet with a coupling constant of 936 Hz. Tubulin purified according to the method of Weisenberg [Weisenberg, R. C., & Timasheff, S. N. (1970) *Biochemistry 9*, 4110–4116] was incubated with 1 mM Mn^{2+}. After one cycle of assembly, Mn^{2+} replaced Mg^{2+} only partially, i.e., 60% at the high-affinity binding site. After colchicine treatment of tubulin to stabilize it, GTP(γF) was added, and the 254-MHz fluorine-19 relaxation rates were measured within the first 4 h. Longitudinal and transversal relaxation rates were determined at two concentrations of GTP(γF) and variable concentrations of colchicine–tubulin–Mn(II) (paramagnetic complex) or the ternary complex with magnesium (diamagnetic complex). The analysis of the relaxation data indicates that the rate of exchange of GTP(γF) from the exchangeable nucleotide site has a lower limit of 8.7×10^4 s^{-1} and the metal and exchangeable nucleotide binding sites are separated by an upper distance between 6 and 8 Å. These data confirm that the high-affinity divalent cation site is situated in the same locus as that of the exchangeable nucleotide, forming a metal–nucleotide complex.

The process of the self-assembly of microtubules from tubulin depends on magnesium ions and GTP. It has been well established that each tubulin α–β heterodimer contains one tightly bound Mg^{2+} ion and that extra Mg^{2+} ions are required for the polymerization process (Olmsted & Borisy, 1975; Lee & Timasheff, 1975; Himes et al., 1977; Williams & Detrich, 1979). When other divalent cations are used in place of Mg^{2+}, aberrant structures are formed. Zn^{2+} (Larsson et al., 1976; Gaskin & Kress, 1977; Eagle et al., 1983) and Co^{2+} (Wallin et al., 1977) promote the assembly of sheets consisting of as many as 50–60 protofilaments aligned in parallel. It has been reported, however, that microtubules can be formed over a very narrow range of Zn^{2+} and Co^{2+} concentrations (Haskins et

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