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Reexamination of the Role of Nonhydrolyzable Guanosine 5′-Triphosphate Analogues in Tubulin Polymerization: Reaction Conditions Are a Critical Factor for Effective Interactions at the Exchangeable Nucleotide Site

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ABSTRACT: Recently it was proposed [O'Brien, E. T., & Erickson, H. P. (1989) *Biochemistry 28*, 1413-1422] that tubulin polymerization supported by guanosine 5′-(β,γ-imidotriphosphate) [p(NH)ppG], guanosine 5′-(β,γ-methylenetriphosphate) [p(CH_2)ppG], and ATP might be due to residual GTP in reaction mixtures and that these nucleotides would probably support only one cycle of assembly. Since we had observed polymerization with these three compounds, we decided to study these reactions in greater detail in two systems. The first contained purified tubulin and a high concentration of glycerol, the second tubulin and microtubule-associated proteins (MAPs). In both systems, reactions supported by nucleotides other than GTP were most vigorous at lower pH values. In the glycerol system, repeated cycles of polymerization *were* observed with ATP and p(CH_2)ppG, but not with p(NH)ppG. With p(NH)ppG, a single cycle of polymerization was observed, and this was caused by contaminating GTP. In the MAPs system, repeated cycles of polymerization were observed with both nonhydrolyzable GTP analogues, even without contaminating GTP, but ATP was not active at all in this system. Binding to tubulin of p(NH)ppG, p(CH_2)ppG, and, to a lesser extent, ATP was demonstrated indirectly, since high concentrations of the three nucleotides displaced radiolabeled GDP originally bound in the exchangeable site, with p(NH)ppG the most active of the three compounds in this displacement assay. The failure of GTP-free p(NH)ppG to support tubulin polymerization in our glycerol system even though it displaced GDP from the exchangeable site was further investigated by examining the effects of p(NH)ppG on polymerization and polymer-bound nucleotide with low concentrations of GTP. The two nucleotides appeared to act synergistically in supporting polymerization, so that a reaction occurred with a subthreshold GTP concentration if p(NH)ppG was also in the reaction mixture. Analysis of radiolabeled exchangeable-site nucleotide in polymers formed in reaction mixtures containing both GTP and p(NH)ppG demonstrated that p(NH)ppG which entered polymer did so primarily at the expense of GDP originally bound in the exchangeable site rather than at the expense of GTP. It appears that in the glycerol reaction condition, tubulin-p(NH)ppG cannot initiate tubulin polymerization but that it can participate in polymer elongation. ATP and p(CH_2)ppG also entered the exchangeable site during polymerization without GTP in glycerol, as demonstrated by displacement of radiolabeled GDP from polymer when these alternate nucleotides were used. Moreover, at pH 6.1 in the glycerol system, binding of alternate nucleotides to unpolymerized tubulin was demonstrated with radiolabeled p(NH)ppG and ATP and to polymer by high-performance liquid chromatographic analysis of acid extracts of pellets harvested by centrifugation.

Microtubule assembly normally is accompanied by the hydrolysis of stoichiometric amounts of GTP bound at the exchangeable nucleotide site of tubulin (Kobayashi, 1975; MacNeal & Purich, 1978; Hamel et al., 1986a; O'Brien et al., 1987). Nonetheless, many reports of polymerization not dependent on GTP (or GTP analogue) hydrolysis have appeared over the years. These have included reactions induced by non guanosine triphosphates (Penningroth & Kirschner, 1978; Zabrecky & Cole, 1980; Duanmu et al., 1986), by GDP

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analogues (Sandoval et al., 1978; Hamel et al., 1983a), by the antimitotic drug taxol in the absence of exogenous GTP (Schiff & Horwitz, 1981; Hamel et al., 1981), and, particularly, by the nonhydrolyzable GTP analogues guanosine 5′-(β,γ-imidotriphosphate) [p(NH)ppG],[1] p(CH_2)ppG, and guanosine 5′-*O*-(3-thiotriphosphate) (Arai et al., 1975; Arai & Kaziro, 1976; Sutherland, 1976; Penningroth & Kirschner, 1977, 1978; Weisenberg et al., 1976; Terry & Purich, 1980; David-Pfeuty & Huitorel, 1980; Kirsch & Yarbrough, 1981; Maccioni & Seeds, 1982; Hamel & Lin, 1984a; Roychowdhury & Gaskin, 1986; Nath et al., 1988).

However, not all workers observed polymerization reactions with nonhydrolyzable analogues under all conditions examined (Gaskin et al., 1974; Olmsted & Borisy, 1975; Maccioni & Seeds, 1982; Hamel & Lin, 1984a; O'Brien & Erickson, 1989). Most recently, O'Brien and Erickson (1989) described experiments with tubulin–GTP (tubulin bearing GTP in the exchangeable site) in a system containing a high concentration of glycerol. They observed that p(NH)ppG and p(CH_2)ppG (and ATP) enhanced an initial polymerization cycle which exhausted the GTP bound to the tubulin but that a second cycle did not occur unless GTP was added to the reaction mixture. O'Brien and Erickson (1989) suggested that microtubule assembly reactions reported by previous workers with nonhydrolyzable GTP analogues (and ATP) might be due to GTP contamination and postulated that these compounds would fail to support repeated cycles of polymerization and depolymerization. Because of our previous work with ATP-supported tubulin polymerization (Duanmu et al., 1986), and because we had observed microtubule assembly with both p(NH)ppG and p(CH_2)ppG (unpublished observations), we decided to investigate this issue raised by O'Brien and Erickson (1989). The question of polymerization reactions with nonhydrolyzable GTP analogues is of particular importance because of its implications for the mechanism of microtubule assembly.

Materials and Methods

Materials. Electrophoretically homogeneous, gel-filtered bovine brain tubulin bearing about 0.8 molar equiv of GTP in the nonexchangeable site and 0.8 molar equiv of GDP in the exchangeable site and heat-treated MAPs were prepared as described previously (Hamel & Lin, 1984b) as was an analogous tubulin preparation bearing about 0.8 molar equiv of [8-^{14}C]GDP in the exchangeable site (Duanmu et al., 1986). GTP, ATP, UTP, and CDP were obtained from Sigma, and [8-^{14}C]GTP was from Moravek Biochemicals, and they were repurified by ion-exchange chromatography (except for the CDP); [U-^{14}C]CDP, [4-^{14}C]UTP, and [U-^{14}C]ATP were from Amersham (the latter was repurified); glycerol kinase (from *Candida mycoderma*), p(NH)ppG, and p(CH_2)ppG were from Boehringer; [^{3}H]p(NH)ppG was from ICN; and maytansine was from the Natural Products Branch, National Cancer Institute.

Methods. Tublin polymerization was followed turbidimetrically (Gaskin et al., 1974) in Gilford Model 250 recording spectrophotometers equipped with Gilford Thermoset electronic temperature controllers. With these devices, temperature increases at the rate of approximately 0.5 °C/s and decreases at approximately 0.1 °C/s. Base lines were established at 0 °C, and zero time was defined as the time when the temperature was initially set at 37 °C on the temperature controller.

Polymerization was examined under two reaction conditions in the experiments described here. The "glycerol system" contained tubulin at 3.0 mg/mL, 5 mM $MgCl_2$, 3.4 M glycerol, 25 mM Mes, 1 mM EGTA, and nucleotides as indicated. All components except tubulin and nucleotides were mixed at double concentration, and the pH was adjusted with NaOH to 6.1, unless an alternate pH is indicated in the individual experiments. The "pH 6/MAPs system" contained tubulin at 2.0 mg/mL, heat-treated MAPs at 1.0 mg/mL, 0.5 mM $MgCl_2$, nucleotides as indicated, and 0.1 M Mes taken from a 1.0 M stock solution adjusted to pH 6.0 (or pH 7.0, as indicated).

Prior to use in experiments, as indicated, glycerol kinase (in an ammonium sulfate suspension) was diluted 10-fold to a final concentration of 0.5 mg/mL with a solution containing 10% (v/v) glycerol, 0.1 M Mes, and 0.5 mg/mL bovine serum albumin (adjusted to pH 6.1 with NaOH) and dialyzed against the same solution in a cold room. It was then either used immediately or stored frozen in liquid nitrogen.

HPLC analyses of nucleotide preparations were performed as described previously (Batra et al., 1987), with minor modifications of the gradients as described in the individual experiments, on a Whatman Partisil SAX column (10 μm; 0.46 × 25 cm). Flow rate was 2.5 mL/min. An LKB HPLC system equipped with an integrating recorder was used. The effluent was monitored at 259 nm.

Binding of radiolabeled nucleotides to unpolymerized tubulin was measured as described elsewhere (Hamel & Lin, 1984a), with separation of bound from free nucleotide by centrifugal gel filtration chromatography on microcolumns prepared in tuberculin syringes, followed by quantitation of radioactivity and protein in the column filtrates.

Incorporation of radiolabeled nucleotide into glycerol-induced polymer, harvested by centrifugation, was determined as described in detail previously (Duanmu et al., 1986). Two types of reaction mixtures (0.25 mL) were employed. The first contained tubulin–[8-^{14}C]GDP at 3.0 mg/mL, nucleotides as indicated, and the other components of the glycerol system. The second contained tubulin–GDP, 10 μM [8-^{14}C]GTP, p(NH)ppG as indicated, and the other components of the glycerol system. In both cases, incubation prior to centrifugation was for 30 min at 37 °C. The first type of reaction mixture permitted estimation of the amount of GDP in the pellet derived from GDP originally bound in the exchangeable site of tubulin (Hamel et al., 1984, 1986b; Duanmu et al., 1986; Lin & Hamel, 1987). The second type of reaction mixture permitted estimation of the amount of GDP in the pellet derived from exogenously added GTP (Hamel et al., 1986b; Lin & Hamel, 1987). In the glycerol system, polymer pellets could be effectively harvested by short (5 min) or long centrifugations. Polymer-free reaction mixtures, however, had to be handled with more care, for we found that direct centrifugation for 5 min resulted in pellets containing small amounts of protein but little nucleotide, presumably representing denatured tubulin aggregates. Longer centrifugations of polymer-free reaction mixtures resulted in progressively larger pellets with substantial amounts of nucleotide, presumably representing active tublin oligomers. Thus, all centrifugations were for 5 min at 30000 rpm (81000*g*, maximum) as described before (Duanmu et al., 1986). Pellets were washed 3 times and dissolved in 0.5 mL of 8 M urea. Protein

[1] Abbreviations: p(NH)ppG, guanosine 5′-(β,γ-imidotriphosphate); p(CH_2)ppG, guanosine 5′-(β,γ-methylenetriphosphate); MAPs, microtubule-associated proteins; Mes, 4-morpholineethanesulfonate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetate; HPLC, high-performance liquid chromatography.

and radioactivity content of the urea solution was determined to establish nucleotide–tubulin stoichiometry.

Four experiments of each type were performed, and the nucleotide to protein ratio for a given experimental condition was found to be quite reproducible (see Results), while more variability was observed in the total protein recovered. In the experiments containing tubulin–[8-^{14}C]GDP and nonradiolabeled nucleotides, three types of control reaction mixtures were always included for evaluation of the experiment. In the first control, the reaction mixture contained no exogenous nucleotide, and no significant turbidity development occurred in such reaction mixtures (see below). The protein in the pellets averaged 41 μg, and the radioactivity 230 cpm. The specific values in the individual experiments were used to correct the data for calculation of nucleotide to tubulin stoichiometry. In the second control, the reaction mixture contained 1 mM UTP and 5 units of yeast nucleoside-diphosphate kinase (from Sigma) to convert the [8-^{14}C]GDP bound in the exchangeable site to GTP and give an estimate of the maximum exchangeable nucleotide that we could expect to recover in the polymer pellet. With these reaction mixtures, the protein in the pellets averaged 345 μg and the radioactivity 96 300 cpm. Nucleotide stoichiometry in the UTP + nucleoside-diphosphate kinase controls averaged 0.86 (±0.046, standard error). In the third control, the reaction mixture contained 2 mM GTP to verify that high concentrations of GTP would displace most of the radiolabeled GDP from the exchangeable site prior to polymerization. These pellets averaged 394 μg of protein with 4200 cpm, representing a corrected stoichiometry of 0.030 (±0.005, standard error).

For the experiments with tubulin–GDP and [8-^{14}C]GTP, somewhat different controls were required. Reaction mixtures without nucleotide provided estimates of the background protein in the pellets (average, 18 μg). To obtain an estimate of background radioactivity, reaction mixtures were prepared containing tubulin–GDP, 10 μM nonradiolabeled GTP, and 10 μM [U-^{14}C]CDP of the same specific activity as the [8-^{14}C]GTP (102 cpm/pmol). Pellets derived from these reaction mixtures contained an average of 238 μg of protein and 530 cpm. For comparison, the experimental reaction mixtures containing tubulin–GDP and 10 μM [8-^{14}C]GTP yielded pellets containing an average of 229 μg of protein and 38 400 cpm. Trapped radioactivity was therefore at most 1–2% of the total in pellets with exogenously added [8-^{14}C]GTP.

Incorporation of nonradiolabeled nucleotide into glycerol-induced polymer could not be measured by direct centrifugation of the reaction mixtures, as described above, since significant amounts of unbound nucleotide appeared to be trapped in the pellets based on studies with millimolar concentrations of radiolabeled nucleotides (unpublished observations). Instead, 2-mL reaction mixtures were prepared containing nucleotides as indicated, 3.0 mg/mL tubulin, and the components of the glycerol system. After 30 min at 37 °C, the sample was diluted with 18 mL of a 37 °C solution containing the components of the glycerol system and 45% (w/v) sucrose (Terry & Purich, 1980; Hamel et al., 1986b). The sample was divided into four 5-mL aliquots which were centrifuged for 2 h at 40 000 rpm (145000*g*, maximum) in a Ti 50 rotor (rotor warmed to 37 °C; centrifuge refrigeration turned off). The supernatants were discarded, the pellets washed 3 times, nucleotide was extracted from the protein with 10% (w/v) trichloroacetic acid, and the acid extracts were processed as described by Khym (1975). The deacidified nucleotide solutions were examined by HPLC as described above.

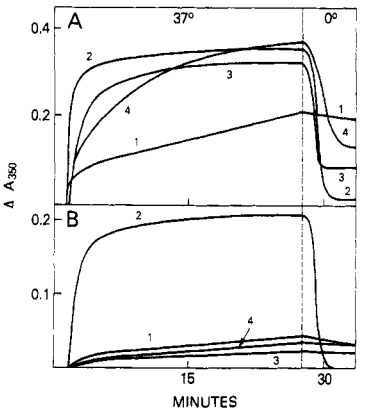

FIGURE 1: Effect of reaction pH on MAP-dependent microtubule assembly with nonhydrolyzable GTP analogues. Each 0.25-mL reaction mixture contained 2.0 mg/mL tubulin (with GDP initially in the exchangeable site), 1.0 mg/mL heat-treated MAPs, 0.1 M Mes [1 M stock solutions adjusted with NaOH to either pH 6.0 (panel A) or pH 7.0 (panel B)], 0.5 mM $MgCl_2$, and nucleotides as follows: curves 1, none; curves 2, 2 mM GTP; curves 3, 2 mM p(NH)ppG; and curves 4, 2 mM p(CH_2)ppG. At zero time, the temperature controller was set at 37 °C, and, at the time indicated by the vertical dashed line, the controller was set at 0 °C.

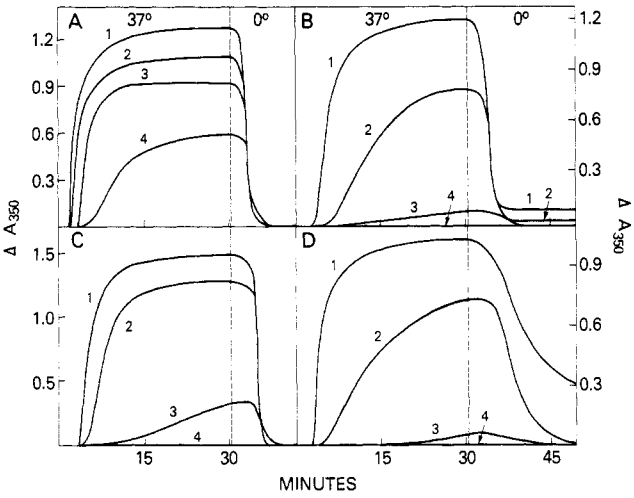

FIGURE 2: Effect of reaction pH on glycerol-dependent tubulin polymerization with nonhydrolyzable GTP analogues and ATP. Each 0.25-mL reaction mixture contained the components described in the text for the glycerol system and nucleotides as follows: panel A, 2 mM GTP; panel B, 4 mM ATP; panel C, 2 mM p(NH)ppG; panel D, 2 mM p(CH_2)ppG. Reaction pH was as follows: curves 1, pH 6.1; curves 2, pH 6.3; curves 3, pH 6.6; curves 4, pH 6.9. Temperature changes as in Figure 1.

Results

At first we thought that the failure of O'Brien and Erickson (1989) to obtain tubulin polymerization in glycerol with p(NH)ppG, p(CH_2)ppG, and ATP resulted from their reaction conditions, specifically the pH (i.e., pH 6.6). We have observed negligible microtubule assembly in reaction mixtures containing purified tubulin and heat-treated MAPs with millimolar concentrations of p(NH)ppG and p(CH_2)ppG at pH 7.0 (Figure 1B) but relatively vigorous reactions at pH 6.0 (Figure 1A). (Rapid and extensive assembly reactions occurred at both pH values with 20 μM GTP.) Similar effects were also obtained in a glycerol system (Figure 2). With GTP (Figure 2A), polymerization occurred at all pH values, although the reaction rate and extent declined as the pH increased (comparable to results obtained with MAPs; Hamel et al., 1986a). With ATP (Figure 2B), p(NH)ppG (Figure 2C), and p(CH_2)ppG (Figure 2D), significant reactions oc-

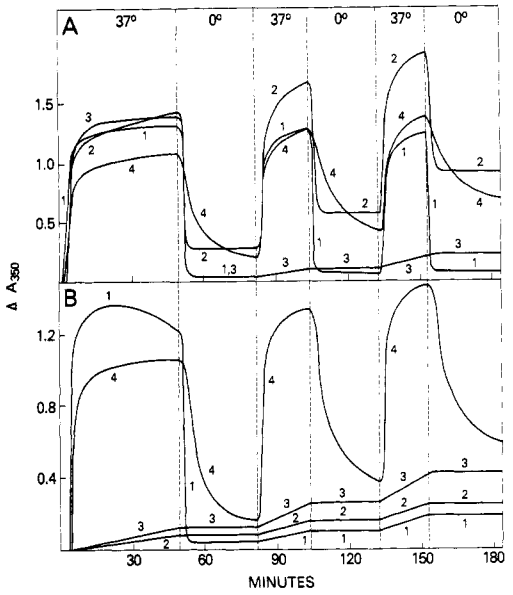

FIGURE 3: Multiple cycles of glycerol-dependent tubulin polymerization at pH 6.1. (A) Multiple cycles occur with GTP, ATP, and p(CH$_2$)ppG but not with p(NH)ppG. Each 0.25-mL reaction mixture contained the components described in the text for the glycerol system and nucleotides as follows: curve 1, 2 mM GTP; curve 2, 4 mM ATP; curve 3, 2 mM p(NH)ppG; and curve 4, 2 mM p(CH$_2$)ppG. At the times indicated, the temperature controller was set at 37 or 0 °C as indicated, for a total of three cycles of temperature-dependent assembly and disassembly. (B) Effect of preincubation of nonhydrolyzable analogues and of GTP in glycerol with glycerol kinase on subsequent glycerol-induced tubulin polymerization. Each reaction mixture, in an initial volume of 0.234 mL, contained the components described in the text for the glycerol system except for tubulin, and the following (nucleotide concentrations refer to the final concentration in a 0.25-mL reaction mixture): curve 1, 100 μM GTP and 50 μL of the solution used to dialyze the glycerol kinase; curve 2, 100 μM GTP and 25 μg of dialyzed glycerol kinase in 50 μL of the solution described in the text; curve 3, 2 mM p(NH)ppG and glycerol kinase as described for curve 2; curve 4, 2 mM p(CH$_2$)ppG and glycerol kinase as described for curve 2. (The 50 μL of glycerol kinase or dialysis solution results in 25 μg of albumin and an additional 2% glycerol and 20 mM Mes in the final polymerization reaction.) The reaction mixtures were incubated at 37 °C (in a water bath) for 30 min and chilled on ice. Tubulin (final concentration, 3.0 mg/mL) in a 16-μL volume was added to each reaction mixture, and they were transferred to cuvettes at 0 °C in the spectrophotometer. Base lines were established, and at zero time, the temperature controller was set at 37 °C. A total of three cycles of assembly and disassembly were performed, with temperature changes at the times indicated in the figure.

curred only at reaction pH values of 6.1 and 6.3, and pH 6.1 was used subsequently.[2]

Nonetheless, the experiment proposed by O'Brien and Erickson (1989) is simple. Would multiple cycles of tubulin polymerization occur with p(NH)ppG, p(CH$_2$)ppG, and ATP in a system in which these nucleotides were active? The three nucleotides yielded different results (Figure 3A). With p(NH)ppG, only one polymerization cycle occurred (curve 3), while with p(CH$_2$)ppG (curve 4) and ATP (curve 2) the tubulin did cycle repeatedly, as occurred with GTP (curve 1). Unlike the GTP reaction, which almost returned to the base line with each cold-induced depolymerization, the base line rose with each depolymerization in the ATP- and p(CH$_2$)ppG-supported reactions. The p(CH$_2$)ppG-induced polymer was also more cold-stable than those formed with the other nucleotides. Nath et al. (1988) have similarly described multiple cycles of polymerization supported by p(CH$_2$)ppG in a system requiring alkaline phosphatase treated tubulin and dimethyl sulfoxide.

The result with p(NH)ppG led us to determine the minimum nucleotide concentrations required for polymerization in the glycerol system. We observed a polymerization reaction with 7, but not 6, μM GTP; with 1.0, but not 0.5, mM p(NH)ppG; and with 0.5, but not 0.25, mM p(CH$_2$)ppG. Thus, contamination of the nonhydrolyzable analogues with as little as 1–2% GTP could explain the polymerization reactions observed with 2 mM p(CH$_2$)ppG or p(NH)ppG. Moreover, nonexchangeable nucleotide derived from denatured tubulin could serve as a source of GTP.

Glycerol kinase was found to be highly effective in degrading small amounts of GTP (50 μM GTP was 90% degraded to GDP within 1 min). Inhibition by either p(NH)ppG or p(CH$_2$)ppG was negligible, and tubulin provided only minimal protection of 50 μM GTP from degradation by glycerol kinase. No significant breakdown of [^{3}H]p(NH)ppG by glycerol kinase occurred, but the radiolabeled p(NH)ppG was degraded by bacterial alkaline phosphatase (Yount et al., 1971) (data not presented).

Glycerol kinase was preincubated with p(NH)ppG prior to addition of tubulin to the reaction mixture, and this abolished p(NH)ppG-induced polymerization (Figure 3B, curve 3). Preincubation of p(CH$_2$)ppG with the enzyme, however, had no effect on subsequent polymerization (Figure 3B, curve 4). [In this experiment, 100 μM GTP (curve 1) only supported one cycle of polymerization.] Nor did preincubation of tubulin with glycerol kinase (to degrade GTP theoretically released from the nonexchangeable site) affect subsequent p(CH$_2$)ppG-induced polymerization (data not presented).

We concluded that p(NH)ppG was unable to support tubulin polymerization in this glycerol system but that p(CH$_2$)ppG did support the reaction. Since the data were consistent with a single cycle of polymerization with p(NH)ppG caused by GTP contamination, the nucleotide preparations used in the studies described above were analyzed by HPLC under conditions where 0.1% contaminants can be detected (Batra et al., 1987). The p(NH)ppG preparation used in the experiments presented above was only 69% pure *and* contained 0.9% GTP. Thus, with 2 mM "p(NH)ppG" (Figure 3A, curve 3), the reaction contained 18 μM GTP, enough for a significant polymerization reaction (see below). Neither p(CH$_2$)ppG (98% pure) nor ATP (99% pure) contained detectable GTP.

An attempt to purify p(NH)ppG by column chromatography, removing the GTP contaminant, was unsuccessful. The next preparation of p(NH)ppG (92% pure) we purchased did not support tubulin polymerization in glycerol (see below) and, further, contained no GTP on HPLC analysis.

Although 2 mM p(NH)ppG (GTP-free) will not support tubulin polymerization in the glycerol system, this is not the case in the pH 6/MAPs system: both p(NH)ppG and p(CH$_2$)ppG supported multiple rounds of polymerization. Figure 4 compares the results obtained with GTP to those with p(CH$_2$)ppG and both batches of p(NH)ppG. The original p(NH)ppG (curve 4) supported a substantially more vigorous assembly reaction in the first cycle as compared to the second preparation of p(NH)ppG (curve 5), while in the later cycles there was little difference in the reactions with the two batches of p(NH)ppG. Undoubtedly, the more vigorous first cycle reaction was due to the GTP contaminant. The polymers formed with p(NH)ppG and p(CH$_2$)ppG depolymerized more

[2] It should be noted that with both ATP and GTP the polymer consists of a mixture of microtubules and open sheets (Duanmu et al., 1986). We have not examined polymer formed with p(CH$_2$)ppG or p(NH)ppG in the electron microscope, but the high turbidity readings obtained with both analogues are similar to those obtained with ATP and GTP, suggesting similar mixed polymer morphology.

Table I: Displacement of [8-^{14}C]GDP from Tubulin by p(NH)ppG, p(CH_2)ppG, and ATP

nucleotide added	pmol of [8-^{14}C]GDP bound/pmol of tubulin ± SE[c] (% of control)	
	glycerol system[a]	pH 6/MAPs system[b]
none	0.68 ± 0.015 (100)	0.69 ± 0.012 (100)
p(CH_2)ppG	0.47 ± 0.011 (69)	0.40 ± 0.009 (58)
p(NH)ppG	0.42 ± 0.009 (62)	0.32 ± 0.011 (46)
ATP	0.52 ± 0.025 (76)	0.62 ± 0.009 (90)
UTP	0.68 ± 0.020 (100)	0.67 ± 0.015 (97)
GTP	0.015 ± 0.001 (2.2)	0.011 ± 0.001 (1.6)
GDP	0.028 ± 0.002 (4.1)	0.016 ± 0.001 (2.3)

[a] Each reaction mixture contained the components described in the text for the glycerol system, except that the tubulin contained [8-^{14}C]-GDP bound in the exchangeable site, and the following nucleotides, as indicated: 2 mM p(CH_2)ppG, 2 mM p(NH)ppG, 5 mM ATP, 5 mM UTP, 2 mM GTP, or 2 mM GDP. Reaction mixtures were incubated at room temperature for 20 min. Prior to centrifugal gel filtration, 20 μM maytansine was added to minimize loss of nucleotide from the exchangeable site (Huang et al., 1985; Lin & Hamel, 1987). Aliquots of each reaction mixture were applied to microcolumns of Sephadex G-50 (superfine) equilibrated with 3.4 M glycerol, 5 mM $MgCl_2$, 25 mM Mes, 1 mM EGTA, and 20 μM maytansine (pH adjusted to 6.1 with NaOH) and processed as described in the text. The experiment was performed at least 3 times with all nucleotides (except GDP), and average values are presented in the table. [b] Each reaction mixture contained 3.0 mg/mL tubulin bearing [8-^{14}C]GDP in the exchangeable site, 1.5 mg/mL heat-treated MAPs, 0.1 M Mes (from a 1.0 M stock solution adjusted to pH 6.0 with NaOH), 0.5 mM $MgCl_2$, and the following nucleotides, as indicated: 2 mM p(CH_2)ppG, 2 mM p(NH)ppG, 5 mM ATP, 5 mM UTP, 2 mM GTP, or 2 mM GDP. Reaction mixtures were incubated at room temperature for 20 min. Prior to centrifugal gel filtration, 20 μM maytansine was added to minimize loss of nucleotide from the exchangeable site. Aliquots of each reaction mixture were applied to microcolumns of Sephadex G-50 (superfine) equilibrated with 0.1 M Mes (pH 6.0), 0.5 mM $MgCl_2$, and 20 μM maytansine and processed as described in the text. The experiment was performed at least 3 times with all nucleotides (except GDP), and average values are presented in the table. For the calculations of the stoichiometry of nucleotide retained by tubulin, it was assumed that the concentrations of tubulin and MAPs in the column filtrates were identical with those in the reaction mixtures. This assumption was validated in a comparison of the polyacrylamide gel patterns obtained from a control reaction mixture with that obtained from the filtrate derived from the same sample. [c] SE, standard error. Besides the standard error analysis presented in the table, data for the control values and the ATP samples in both systems were compared by *t*-test difference analysis. This yielded two-tailed *p* values of less than 0.01 for the differences between the mean values of the control and ATP samples. In addition, Student's *t* test analysis of the mean values indicated no overlap would occur between the control and ATP means with *p* values of less than 0.05 in both systems.

slowly than the GTP polymer. ATP, too, was examined for potential activity in supporting polymerization with MAPs at pH 6.0, but no reaction occurred at nucleotide concentrations as high as 5 mM.

In summary, in the glycerol system (pH 6.1), no tubulin polymerization occurs with p(NH)ppG in the absence of contaminating GTP, but polymerization occurs with high concentrations of ATP or p(CH_2)ppG. In the pH 6/MAPs system, polymerization occurs with the nonhydrolyzable GTP analogues, but not with ATP. If polymerization occurs with an alternate nucleotide, multiple cycles are easily demonstrated.

There are at least two explanations for the failure of p(NH)ppG and ATP to support polymerization, respectively, in the glycerol and pH 6/MAPs systems. The nucleotide could fail to bind to tubulin, or it could bind in a nonproductive manner (essentially as a GDP, as opposed to a GTP, analogue). From earlier studies (Hamel & Lin, 1984a; Duanmu et al., 1986; unpublished observations), we knew that p(NH)ppG, p(CH_2)ppG, and ATP had only weak effects on the binding

Table II: Binding of Radiolabeled Nucleotides to Tubulin[a]

radiolabeled nucleotide added	pmol of nucleotide bound/pmol of tubulin ± SE[b]
GTP	0.77 ± 0.017
p(NH)ppG	0.24 ± 0.016
ATP	0.078 ± 0.003
UTP	0.021 ± 0.001

[a] Each reaction mixture contained the components described in the text for the glycerol system and the indicated nucleotides at 2 mM. Nucleotide additions were of mixtures of nonradiolabeled and radiolabeled nucleotides to the following final specific activities: for GTP, 26 cpm/pmol with [8-^{14}C]GTP; for p(NH)ppG, 18 cpm/pmol with [^{3}H]p(NH)ppG; for ATP, 6 cpm/pmol with [U-^{14}C]ATP; and for UTP, 4 cpm/pmol with [4-^{14}C]UTP. Reaction mixtures were incubated at room temperature for 20 min. Samples were processed by centrifugal gel filtration as described in Table I for the glycerol system. The experiment was performed twice, with nearly identical results, and average values are presented in the table. [b] SE, standard error.

of radiolabeled GTP to tubulin [also see Arai et al. (1975)]. Similarly, Yarbrough and Kirsch (1981) described only feeble displacement in glycerol of a fluorescent GTP analogue, bound in the exchangeable site, by p(CH_2)ppG as compared to GDP and GTP. We therefore decided to evaluate displacement of radiolabeled GDP from the exchangeable site by high concentrations of p(NH)ppG (GTP-free), p(CH_2)ppG, or ATP, with UTP as a control since UTP has no activity in supporting polymerization in either the glycerol (Duanmu et al., 1986) or the pH 6/MAPs (data not presented) system (Table I).

The left-hand column of Table I summarizes data in which displacement of [8-^{14}C]GDP from tubulin was measured in the glycerol system (pH 6.1). UTP had no effect, while GTP or nonradiolabeled GDP displaced virtually all the radiolabeled GDP originally bound to the tubulin. Reduced displacement occurred with p(NH)ppG, p(CH_2)ppG, and ATP. In these experiments, ATP was the least and p(NH)ppG the most effective of the three compounds in displacing GDP from tubulin. Thus, p(NH)ppG fails to support polymerization in the glycerol system used here not because it fails to bind to tubulin [cf. to ATP and p(CH_2)ppG] but because it binds nonproductively.

The right-hand column of Table I summarizes data in which displacement of [8-^{14}C]GDP from tubulin was measured in the pH 6/MAPs system. Again UTP had no effect, while GTP or nonradiolabeled GDP displaced virtually all the radiolabeled GDP originally bound to the tubulin. As in glycerol, p(NH)ppG was somewhat more effective than p(CH_2)ppG in displacing radiolabeled GDP from tubulin, and both nucleotides were more effective in displacing the radiolabeled GDP in this reaction condition than they had been in glycerol. ATP, however, was less effective in the pH 6/MAPs system than it had been in the glycerol system. The difference from the control value, although small (10%), does appear to be significant (see legend, Table I), but we still conclude that ATP is inactive in the pH 6/MAPs system primarily because it fails to bind to tubulin (see below).

We were also able to demonstrate apparent binding of radiolabeled p(NH)ppG and ATP to tubulin in the glycerol system (Table II), although we have not excluded trace contamination of the radiolabeled nucleotide preparations with GDP or GTP (no radiolabel entered the filtrates without tubulin). The molar quantities of p(NH)ppG and ATP bound were about one-third and one-tenth, respectively, that of the [8-^{14}C]GTP bound to tubulin, and the values obtained are in reasonable agreement with the displacement results summarized in Table I. Little UTP was bound to tubulin, consistent

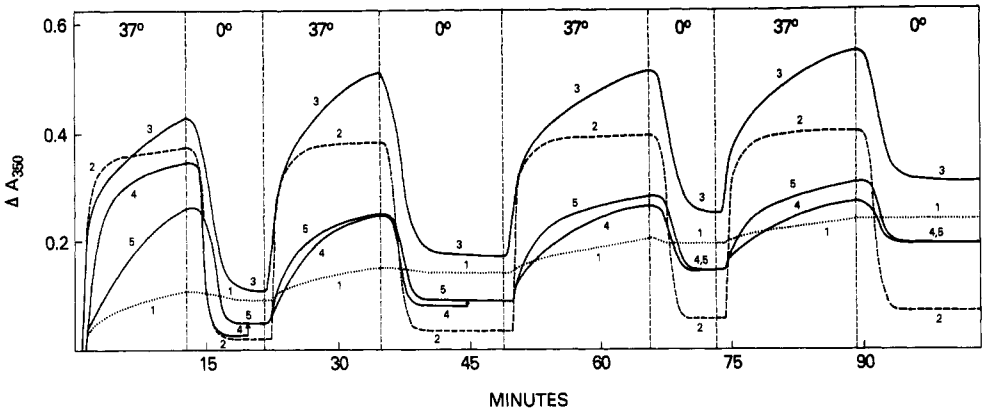

FIGURE 4: Comparison of GTP, p(CH_2)ppG, GTP-contaminated p(NH)ppG, and GTP-free p(NH)ppG in multiple cycles of microtubule assembly in the pH 6/MAPs system. Each 0.25-mL reaction mixture contained components as described for Figure 1A, with nucleotides as follows: curve 1 (dotted line), none; curve 2 (dashed line), 2 mM GTP; curve 3, 2 mM p(CH_2)ppG; curve 4, first preparation (GTP-contaminated) of p(NH)ppG at 2 mM; curve 5, second preparation (GTP-free) of p(NH)ppG at 2 mM. A total of four cycles of assembly and disassembly were performed, with temperature changes as indicated in the figure. After depolymerization was complete in the first two cycles, curve 4 was adjusted to the same position as curve 5 so that the subsequent polymerization reactions could be more readily compared.

with its failure to displace [8-^{14}C]GDP from the exchangeable site. (It should be emphasized that only 0.03% of the [4-^{14}C]UTP and 0.12% of the [U-^{14}C]ATP added to the reaction mixtures were recovered in the filtrates, relative to the tubulin. Since such levels of GDP/GTP contamination are feasible, the GDP displacement data of Table I are a better indicator of alternate nucleotide binding to tubulin.)

The low affinities of the nonhydrolyzable GTP analogues and ATP for tubulin make accurate determination of their binding parameters difficult. To obtain a more quantitative estimate of their relative affinities for tubulin, we further examined GDP and GTP to determine what concentrations would displace amounts of [8-^{14}C]GDP from the exchangeable site equivalent to the amounts displaced by p(NH)ppG, p(CH_2)ppG, and ATP. Both the glycerol system (Figure 5A) and the pH 6/MAPs system (Figure 5B) were evaluated, using 30 μM tubulin–[8-^{14}C]GDP in the reaction mixtures (cf. Table I). In the glycerol system, 50% of the bound radiolabeled GDP was displaced (for purposes of this discussion, a "K_E" value) by 28 μM nonradiolabeled GDP and by 13 μM GTP. In the pH 6/MAPs systems, the K_E values for GDP and GTP were 26 and 15 μM, respectively. Assuming that the displacement values reported in Table I can be directly compared to extrapolated values for GDP and GTP obtained from Figure 5, then K_E values for p(NH)ppG, p(CH_2)ppG, and ATP can be derived. In the glycerol system, the K_E values relative to that of GDP are, respectively, 3.2, 4.2, and 14 mM. With GTP as the standard, K_E values derived for the three nucleotides are somewhat lower (2.7, 3.3, and 9.8 mM). In the pH 6/MAPs system, the K_E values relative to that of GDP for p(NH)ppG, p(CH_2)ppG, and ATP are 1.7, 2.9, and 34 mM; and relative to that of GTP, 1.8, 2.8, and 29 mM. We assume that the relationships among these K_E values are closely related to those of the K_D values for the different nucleotides, but K_D values are not available for either GDP or GTP in any of the reaction conditions examined here. The substantially higher K_D value implied for ATP in the pH 6/MAPs as opposed to the glycerol system probably explains the inability of ATP to support polymerization in the former reaction condition, and the low affinities for tubulin of all three nucleotides as compared to GTP explains why polymerization, when it occurs, requires such high nucleotide concentrations.

Since a tubulin–p(NH)ppG complex forms in the glycerol system, what explains its failure to polymerize? Perhaps this complex is simply unable to support polymerization, and it may even act as an inhibitor of the reaction. Alternatively, the

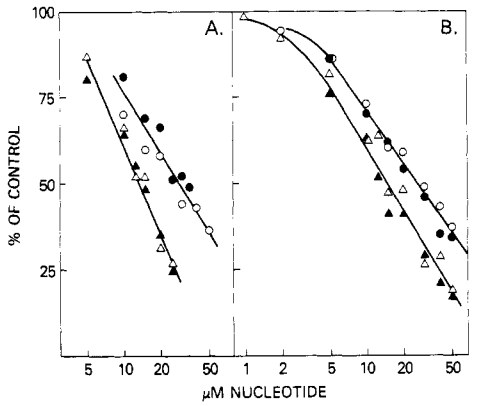

FIGURE 5: Displacement of radiolabeled GDP from tubulin by lower concentrations of GDP and GTP in the glycerol system (panel A) and the pH 6/MAPs system (panel B). These experiments were performed as described for both reaction conditions in the legend of Table I, with reaction mixtures containing the indicated concentrations of GDP (circles) or GTP (triangles). Each experiment was performed twice, as indicated by either open or closed symbols.

tubulin–p(NH)ppG complex could support elongation but not nucleation of tubulin polymers. This actually appears to be the case with tubulin–GDP in a number of polymerization systems: tubulin–GDP will elongate microtubule seeds (Carlier & Pantaloni, 1978; Karr et al., 1979; Zackroff et al., 1980), and in MAP-dependent polymerization, we have found that tubulin–GDP is incorporated into microtubules with reasonable efficiency, roughly in proportion to its concentration relative to tubulin–GTP in the reaction mixture, implying that the main inhibitory effect of tubulin–GDP is at the level of polymer initiation (Hamel et al., 1986b; Lin & Hamel, 1987).

To explore these possibilities, we examined the effects of the GTP-free p(NH)ppG on polymerization in the glycerol system at low concentrations of GTP. Figure 6A presents an experiment in which all reaction mixtures contained 10 μM GTP. Compared to the reaction without p(NH)ppG (curve 1), stimulation of initiation, rate, and extent was observed with 1 mM p(NH)ppG (curve 2), while stimulation only of rate and extent was observed with 2 mM p(NH)ppG (curve 3). With 5 mM p(NH)ppG (curve 4), inhibition of nucleation and rate was observed, while the extent of the reaction was little changed.

This experiment suggested that both inhibition and stimulation of the GTP-dependent reaction can occur with p(NH)ppG. [GDP, in contrast, only inhibits polymerization,

Table III: Exchangeable-Site Nucleotide Content of Glycerol-Induced Tubulin in Polymers[a]

nucleotides added	pmol of GDP/pmol of tubulin ± SE[e] (% of control)			calculated pmol of p(NH)ppG/pmol of tubulin[d]
	derived from tubulin–[8-^{14}C]GDP[b]	derived from [8-^{14}C]GTP[c]	total	
10 μM GTP	0.48 ± 0.022 (100)	0.35 ± 0.016 (100)	0.83	0
10 μM GTP + 1 mM p(NH)ppG	0.30 ± 0.009 (63)	0.31 ± 0.014 (89)	0.61	0.22
10 μM GTP + 2 mM p(NH)ppG	0.26 ± 0.012 (54)	0.30 ± 0.013 (86)	0.56	0.27
10 μM GTP + 5 mM p(NH)ppG	0.15 ± 0.012 (31)	0.26 ± 0.015 (74)	0.41	0.42

[a] Reaction mixtures contained the components described for the glycerol system, 3.0 mg/mL tubulin, and nucleotides as indicated. Incubation, centrifugation, and analysis were described in detail in the text. The second preparation of p(NH)ppG (inactive in supporting polymerization) was used in these experiments. [b] Reaction mixtures contained tubulin–[8-^{14}C]GDP and nonradiolabeled GTP. [c] Reaction mixtures contained nonradiolabeled tubulin–GDP and [8-^{14}C]GTP. [d] Obtained by subtracting the total picomoles of GDP per picomoles of tubulin from 0.83 [i.e., the total obtained from the control reaction mixtures—those lacking p(NH)ppG]. [e] SE, standard error.

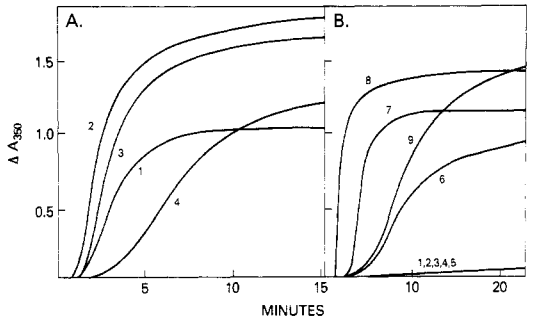

FIGURE 6: Effects of mixing GTP-free p(NH)ppG with low concentrations of GTP on polymerization in the glycerol system. (A) Effects of increasing p(NH)ppG concentration on polymerization supported by 10 μM GTP. Each 0.25-mL reaction mixture contained the components described in the text for the glycerol system, 10 μM GTP, and the following concentrations of p(NH)ppG: curve 1, none; curve 2, 1 mM; curve 3, 2 mM; curve 4, 5 mM. (B) Effect of p(NH)ppG on polymerization below the minimum GTP concentration required for polymerization. Each 0.25-mL reaction mixture contained the components described in the text for the glycerol system and nucleotides as follows: curve 1, none; curve 2, 6 μM GTP; curve 3, 1 mM p(NH)ppG; curve 4, 2 mM p(NH)ppG; curve 5, 5 mM p-(NH)ppG; curve 6, 8 μM GTP; curve 7, 10 μM GTP; curve 8, 1 mM GTP; curve 9, 6 μM GTP + 1 mM p(NH)ppG.

even though some tubulin–GDP is incorporated into microtubules [see Hamel et al. (1986b), Duanmu et al. (1986), and Lin and Hamel (1987)].] If tubulin–p(NH)ppG can elongate but not initiate tubulin polymerization, then p(NH)ppG might act synergistically with GTP at concentrations below the threshold required for a GTP-supported polymerization reaction. With tubulin at 30 μM, no difference was ever obtained between a reaction mixture without GTP (Figure 6B, curve 1) and one with 6 μM GTP (curve 2). Figure 6B also documents the failure of the second p(NH)ppG preparation to support polymerization at 1, 2, and 5 mM (curves 3, 4, and 5) and presents for comparison the reactions supported by 8 μM, 10 μM, and 1 mM GTP (curves 6, 7, and 8). Mixing 6 μM GTP with 1 mM p(NH)ppG (Figure 6B, curve 9) resulted in a substantial polymerization reaction which began about the same time as the reaction with 8 μM GTP, but later than the reactions with 10 μM or 1 mM GTP. Reaction extent was similar to that obtained with 1 mM GTP.

This result supports the idea that tubulin–p(NH)ppG is deficient in initiation as opposed to propagation of tubulin polymers in glycerol. The apparent synergy of 6 μM GTP and 1 mM p(NH)ppG must, however, be considered further. GTP and ATP appear to act synergistically on tubulin polymerization when combined at concentrations just below their individual thresholds (Duanmu et al., 1986). However, we concluded that this did not represent synergy because doubleing the concentration *either* of ATP or of GTP resulted in more vigorous polymerization than did the apparently synergistic combination. Since the addition of 1 mM p(NH)ppG to 6 μM GTP only produced a polymerization reaction similar to that obtained with 8–9 μM GTP, the equivalent of an additional 2–3 μM GTP, we were uncomfortable concluding that this represented synergy. However, as little as 0.25 mM p(NH)ppG added to 6 μM GTP led to some tubulin polymerization, while p(NH)ppG concentrations as high as 5 mM were inert. Thus, even though the polymerization reaction with p(NH)ppG and a subthreshold concentration of GTP is weaker than that observed with higher concentrations of GTP, it does appear to represent a synergistic effect of p(NH)ppG with GTP on tubulin polymerization. This is consistent with the idea that tubulin–p(NH)ppG has negligible activity as a initiating species but some activity in elongation.

To obtain further support for this hypothesis, we examined, in the glycerol system, the effects of 1, 2, and 5 mM p-(NH)ppG (GTP-free) on nucleotide in polymer derived from GDP originally bound in the exchangeable site and from 10 μM GTP added to induce polymerization (Table III). In these experiments, parallel reaction mixtures were prepared containing either tubulin–[8-^{14}C]GDP + nonradiolabeled GTP or tubulin–GDP + [8-^{14}C]GTP. When the exchangeable nucleotide contents of pellets derived from the reaction mixtures without p(NH)ppG were summed, the total GDP (0.83 mol/mol of tubulin) is almost identical with that obtained in the nucleoside-diphosphate kinase + UTP controls (0.86 mol/mol; see Methods). This value can thus be used as an estimate of the total exchangeable guanine nucleotide in polymer pellets in order to derive the amount of p(NH)ppG they would contain in the exchangeable site.[3]

The data presented in Table III indicate a progressive increase in the amount of p(NH)ppG in polymer as the concentration of the analogue added to the reaction mixture increases. This rise in p(NH)ppG content occurs at the expense primarily of GDP originally bound in the exchangeable site rather than of the GTP required for polymerization. At the extreme condition studied [10 μM GTP + 5 mM p(NH)ppG], the polymer exchangeable site GDP derived from tubulin was only 31% of the control value, while polymer GDP derived from GTP was 74% of the control value. This finding is consistent with a minimum concentration of GTP being required for initiation of polymerization, while both tubulin–

[3] In previous experiments with radiolabeled ATP, we found millimolar nucleotide concentrations in the reaction mixture led to high levels of nonspecific entrapment in the pellet, so no attempt was made to estimate the p(NH)ppG content of the pellet using [^{3}H]p(NH)ppG. We also determined residual exchangeable site GDP in polymer pellets formed with 4 mM ATP and 2 mM p(CH_2)ppG. In the former case, there was 0.33 mol of GDP/mol of tubulin in the pellets, implying 0.50 mol of ADP/mol of tubulin in the pellet [in our earlier study (Duanmu et al., 1986), we documented that ATP hydrolysis probably occurred during ATP-supported polymerization]. In the latter case, there was 0.22 mol of GDP/mol of tubulin in the pellets, implying 0.61 mol of p(CH_2)-ppG/mol of tubulin.

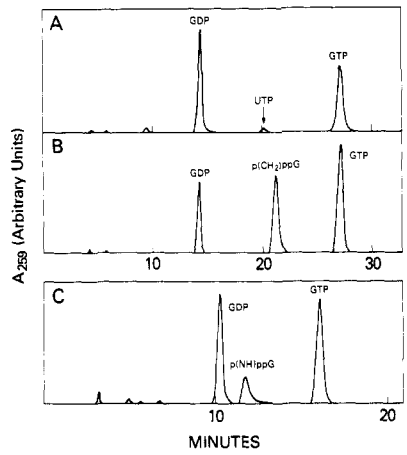

FIGURE 7: HPLC analysis of nonradiolabeled nucleotides derived from polymer formed in the glycerol system. Reaction mixtures were prepared as described in the text and contained the following nucleotides: panel A, 2 mM UTP and 100 units/mL yeast nucleoside-diphosphate kinase; panel B, 2 mM p(CH$_2$)ppG; panel C, 10 μM GTP and 2 mM p(NH)ppG. In the experiments presented in panels A and B, the gradient used was from zero to 0.8 M ammonium phosphate (pH adjusted to 3.8 with H_3PO_4) over 40 min. In the experiment presented in panel C, the gradient used was from zero to 0.5 M ammonium phosphate (pH adjusted to 6.5 with NH_4OH) over 25 min. At pH 3.8, the elution time of p(NH)ppG is similar to that of GTP; at pH 6.5, the elution time of p(CH$_2$)ppG is similar to that of GDP.

GDP and tubulin–p(NH)ppG can participate only in polymer propagation.

Finally, we attempted to document alternate nucleotide incorporation into polymer in the glycerol system by HPLC analysis of nucleotides extracted from polymer pellets formed with nonradiolabeled nucleotides. Polymer formed with 2 mM UTP and nucleoside-diphosphate kinase contained only a trace of UTP (Figure 7A), demonstrating that nonspecific entrapmment of nucleotide was not a significant problem. In this experiment, the areas of the GDP and GTP peaks were equal and presumably represent exchangeable and nonexchangeable nucleotide, respectively.

Figure 7B presents a similar experiment in which 2 mM p(CH$_2$)ppG supported polymerization. In this experiment at pH 6.1, in contrast to the result reported by O'Brien and Erickson (1989) at pH 6.6, a large proportion of the polymer-bound nucleotide was p(CH$_2$)ppG. Excluding the minor early peaks, the polymer contained 19% GDP, 35% p(CH$_2$)-ppG, and 46% GTP. This result does not differ greatly from the results obtained in experiments[3] in which p(CH$_2$)ppG was examined for its ability to displace radiolabeled GDP from polymer. The latter experiment implied a ratio of p(CH$_2$)ppG to GDP of 2.8 in the pellet, as opposed to the ratio of 1.8 found in the experiment of Figure 7B. Presumably the difference results from the very different centrifugation techniques used in the two types of experiments, but its cause has not been investigated.

We were also able to detect nonradiolabeled p(NH)ppG in a polymer pellet obtained in an experiment in which polymerization was supported by 10 μM GTP + 2 mM p(NH)ppG (GTP-free) (Figure 7C). Again ignoring minor peaks, the proportions of the nucleotides recovered were 37% GDP, 16% p(NH)ppG, and 47% GTP. If the GTP represents nonexchangeable nucleotide, this result is in agreement with the value for polymer-bound p(NH)ppG calculated from the amounts of exchangeable-site GDP derived from endogenous exchangeable-site [8-^{14}C]GDP and exogenously added [8-^{14}C]GTP (Table III). The p(NH)ppG to GDP ratio derived from the data of Table III is 0.48, and from Figure 7C, 0.43.

With ATP, a somewhat different result was obtained. Consistent with the displacement data summarized in footnote 3, the polymer pellet formed with 4 mM ATP contained much less GDP than GTP. There was, however, only a minimal amount of ADP found in the pellet, suggesting its loss following dilution and/or during centrifugation (data not presented).

Discussion

The effects of nonhydrolyzable GTP analogues and ATP on tubulin polymerization have proven to be unexpectedly complex and greatly affected by reaction conditions. The variable findings reported in the literature may entirely result from the differing conditions chosen by each group of investigators.

One major factor is reaction pH. In both the glycerol and the MAP systems, nucleotide specificity for polymerization is much more stringent at higher than at lower pH values. In the glycerol system, polymerization readily occurred with ATP, p(CH$_2$)ppG, and GTP-contaminated p(NH)ppG at pH 6.1, but only with GTP at pH 6.9. In the MAPs system, both GTP-free p(NH)ppG and p(CH$_2$)ppG supported polymerization at pH 6.0, while the nonhydrolyzable analogues had little activity at pH 7.0.[4] The GDP displacement experiments (Table I) nevertheless make it clear that p(NH)ppG, p-(CH$_2$)ppG, and ATP have only low affinities for the exchangeable site of tubulin even at low reaction pH values. Despite these low affinities, we were also able to demonstrate apparent binding of substoichiometric amounts of radiolabeled p(NH)ppG and ATP to tubulin in the glycerol system at pH 6.1 (Table II). Our results, obtained by centrifugal gel filtration, should be contrasted to those of Zabrecky and Cole (1982), who were only able to demonstrate binding of ATP to tubulin by the Hummel–Dreyer equilibrium technique (at pH 7.0),[5] and of O'Brien and Erickson (1989), who failed to detect any binding of nonradiolabeled p(NH)ppG to tubulin (at pH 6.6) by HPLC analysis of nucleotide extracted from protein isolated by column gel filtration.

A possible explanation both for the ability of p(NH)ppG, p(CH$_2$)ppG, and ATP to support tubulin polymerization at lower reaction pH and for our ready observation, both directly and indirectly (by displacement of GDP), of the binding of these nucleotides at the exchangeable site is our earlier finding that GDP dissociates from the exchangeable site 50 times more readily at pH 6 than at pH 7 (in 0.1 M Mes–0.5 mM $MgCl_2$ without MAPs) (Hamel et al., 1986a). At lower pH values, a vacant exchangeable site should occur more frequently, allowing a low-affinity nucleotide more opportunity to bind in the site and thereby have the potential to initiate a polymerization reaction. We have, however, been unable to document a significant difference in the relative abilities of p-(NH)ppG and p(CH$_2$)ppG to displace radiolabeled GDP from tubulin at higher as compared to lower pH values in either the glycerol or the MAP systems (data not presented). Thus, binding of alternate nucleotides must occur at higher reaction pH values, and the tighter binding of GDP to tubulin at such

[4] We have found that a number of hydrolyzable GTP analogues (Hamel et al., 1983b) and the GDP analogue 2',3'-dideoxyguanosine 5'-triphosphate (Hamel et al., 1983a) active with MAPs at pH 6.4 retain good activity at pH 7.0. The GDP analogue also supports tubulin polymerization in the glycerol system after preincubation with glycerol kinase, and it will support a second cycle of assembly both in glycerol and with MAPs (unpublished observations).

[5] Zabrecky and Cole (1981, 1982) postulated that ATP effects on tubulin polymerization are mediated through a third nucleotide binding site, while we believe these effects result from the interaction of ATP at the exchangeable GTP site (Duanmu et al., 1986).

pH values cannot explain the feeble polymerization reactions observed with these nucleotides.

A second factor that could play a role in the apparent differences in nucleotide activity as a function of pH is the lower critical concentration of tubulin (with MAPs) observed at lower pH values (Gaskin et al., 1974; Regula et al., 1981; Hamel et al., 1986a). We therefore determined the minimum tubulin concentrations required for polymerization in the glycerol system at both pH 6.1 and pH 6.9 with 4 mM ATP, 1 mM GTP, 2 mM p(CH_2)ppG, and 2 mM p(NH)ppG (GTP-free). In all cases, turbidity was a sigmoidal rather than linear function of tubulin concentration, so that it is more appropriate to speak of "threshold" rather than "critical" concentrations for polymerization.

No polymerization was observed with p(NH)ppG at either pH value with even 20 mg/mL tubulin. With GTP, the threshold concentrations for polymerization were 0.7 mg/mL at pH 6.1 and 1.5 mg/mL at pH 6.9 With tubulin at 20 mg/mL, a rapid polymerization reaction occurred at pH 6.9 with ATP and a sluggish one with p(CH_2)ppG. The threshold concentrations for polymerization with ATP were about 1.4 mg/mL at pH 6.1 and 4.5 mg/mL at pH 6.9. With p(CH_2)ppG, these values were 0.2 mg/mL (significantly lower than that obtained with GTP) at pH 6.1 and 5 mg/mL at pH 6.9. Although the basis for these differences in threshold concentration values as a function of both reaction pH and nucleotide is unknown at present, they clearly complicate comparison of contradictory results reported by different workers with nonhydrolyzable GTP analogues and non guanine nucleotides.

Another important, but trivial, factor is the contamination of other nucleotides with GTP. This occurred with the first preparation of p(NH)ppG and led to a spurious polymerization reaction in the glycerol system, for GTP-free p(NH)ppG [or p(NH)ppG preincubated with glycerol kinase] had no ability to support tubulin polymerization in our glycerol system even at pH 6.1. We should note that on other occasions we have found commercial nucleotide analogue preparations to be contaminated with compounds which led to spurious results (Hamel & Lin, 1984a; Batra et al., 1987). Since most workers did not document the quality of the analogue preparations they had used, this could easily account for some differences in reported results.

Although our studies were stimulated by the failure of O'Brien and Erickson (1989) to obtain a second polymerization cycle with ATP, p(NH)ppG, and p(CH_2)ppG, we do not believe there is an actual contradiction between our results and theirs. Besides very different reaction conditions, which themselves may mitigate against polymerization with alternate nucleotides, O'Brien and Erickson (1989) used an unfavorable pH value (pH 6.6) in their studies. Moreover, they used tubulin–GTP (which was presumably converted to tubulin–GDP after the first polymerization cycle) as opposed to the tubulin–GDP we used, and they purposefully chose conditions where an unstable turbidity plateau occurred with GTP and found that ATP, p(NH)ppG, and p(CH_2)ppG stabilized the plateau. In the studies presented here, we have not examined nucleotide mixtures in polymerization (except Figure 6), and we have chosen GTP concentrations which yield stable turbidity plateaus[6] to examine in detail. In the studies of Figure 6, where high concentrations of p(NH)ppG were mixed with low concentrations of GTP, our results are not greatly different from those of O'Brien and Erickson (1989), who added high concentrations of p(NH)ppG to tubulin-GTP—we, too, find enhancement of a polymerization reaction in which GTP is required.

We believe, however, that this effect of p(NH)ppG in our glycerol system is mediated through the exchangeable nucleotide site of tubulin rather than through an additional nucleotide binding site which occurs only in polymer (O'Brien & Erickson, 1989). Here we have demonstrated the displacement of radiolabeled GDP from unpolymerized tubulin and in polymer by p(NH)ppG, as well as by p(CH_2)ppG and ATP [Tables I and III; footnote 3; see also Duanmu et al. (1986)], and, conversely, the binding of alternate nucleotides to unpolymerized tubulin and polymer (Table II; Figure 7). Moreover, we have previously found that ATP is hydrolyzed during the polymerization reaction it supports, that ATP hydrolysis by tubulin is stimulated or inhibited by antimitotic drugs in parallel with GTP hydrolysis, and that the relative activity of cognate GTP and ATP analogues in tubulin polymerization is similar (Duanmu et al., 1986).

The most remarkable finding in the study presented here is the apparent synergy in the glycerol system of p(NH)ppG (GTP-free) and subthreshold concentrations of GTP. The effects of the nonhydrolyzable analogue, however, are complex, for, at the highest concentrations examined, p(NH)ppG inhibited the GTP-supported reaction. The amount of p(NH)ppG incorporated into polymer (determined indirectly, Table III) nonetheless rose steadily as its concentration in the reaction mixture increased. This is reminiscent of the effects of GDP on microtubule assembly (Hamel et al., 1986b; Lin & Hamel, 1987): increasing concentrations of GDP in reaction mixtures led to increased amounts of GDP incorporated directly into polymer (from GDP added to the reaction mixtures) without GTP hydrolysis. With GDP, however, we have never observed an enhanced polymerization reaction, either with MAPs (Hamel et al., 1986b) or in glycerol (Duanmu et al., 1983)—even as increased amounts of the nucleotide enter polymer, the reaction is progressively inhibited until it completely disappears. The inhibitory effect of GDP was most consistent with specific effects on initiation as opposed to elongation of microtubules (Lin & Hamel, 1987).

Although p(NH)ppG seems truly to act synergistically with low GTP concentrations in supporting polymerization in the glycerol system, the tubulin–p(NH)ppG complex cannot be viewed simply as an elongating species. We could not produce reactions as vigorous as those observed with higher GTP concentrations (1–2 mM) with low GTP + p(NH)ppG, and a fairly high ratio of GTP to tubulin had to be in the reaction mixture with p(NH)ppG to obtain a substantial polymerization reaction. In the study presented in Figure 6B, the GTP to tubulin ratio was 1:5. At lower GTP concentrations, 1 mM p(NH)ppG still converted an inert reaction mixture to one in which polymerization occurred, but the reactions became progressively delayed and less extensive as the GTP concentration decreased (data not presented). If initiation requires tubulin–GTP oligomers (Voter & Erickson, 1984; Spann et al., 1987), the presence of p(NH)ppG in the reaction mixture probably results in partial displacement of tubulin–GTP with tubulin–p(NH)ppG in these oligomers. In contrast to the case with tubulin–GDP, small amounts of tubulin–p(NH)ppG appear not to inhibit initiation and may even enhance the reaction [the case with 1 mM p(NH)ppG, Figure 6A], but larger amounts of tubulin–p(NH)ppG in oligomers must in-

[6] In the glycerol system studied here, we have observed stable turbidity plateaus with both very high (≥1 mM) and very low (≤10 μM) GTP concentrations. With intermediate concentrations (20–100 μM) of GTP, the turbidity plateau is unstable. The reason for the difference between low and intermediate GTP concentrations is unclear.

hibit initiation to account for the inhibition observed with 5 mM p(NH)ppG.

Registry No. p(NH)ppG, 34273-04-6; p(CH_2)ppG, 13912-93-1; ATP, 56-65-5; GTP, 86-01-1; GDP, 146-91-8; glycerol, 56-81-5.

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