Deoxyguanosine Nucleotide Analogues: Potent Stimulators of Microtubule Nucleation with Reduced Affinity for the Exchangeable Nucleotide Site of Tubulin†

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ABSTRACT: Four analogues of guanosine 5′-triphosphate (GTP) (dGTP, 3′-deoxy-GTP, arabinosyl-GTP, and 2′,3′-dideoxy-GTP), which support more rapid and extensive microtubule assembly than GTP, were hydrolyzed more rapidly than GTP in reaction mixtures containing tubulin plus microtubule-associated proteins (MAPs). As with GTP, hydrolysis of the four analogues was initially closely coupled to the onset of polymerization and continued at a slower rate at the turbidity plateau. Relative to GTP, however, these analogues (and the cognate GDP analogues), particularly 3′-deoxy-GTP and 2′,3′-dideoxy-GTP, bound poorly to tubulin and had a reduced ability to displace bound radiolabeled GDP under nonpolymerizing reaction conditions. Despite their reduced binding to the tubulin dimer, if polymerization occurred, all four analogues were incorporated into microtubules (as the diphosphates) in stoichiometric amounts comparable to the incorporation of GTP (in the form of GDP) with displacement of the GDP initially present in the exchangeable site. Microtubule nucleation was specifically enhanced in the presence of the analogues. With MAPs the analogues initiated microtubule assembly at temperatures 10–15 °C below that required by the GTP-supported reaction, and the average microtubule length was significantly reduced. In addition, MAP-independent polymerization occurred only with 2′,3′-dideoxy-GTP with tubulin at 1.0 mg/mL, with the other three analogues at 2.0 mg/mL, and with GTP at 5.0 mg/mL. GTP inhibited analogue-supported polymerization at 20 °C with MAPs and at 37 °C without MAPs (tubulin, 3.5 mg/mL). Both 3′-deoxy-GTP and 2′,3′-dideoxy-GTP were poor inhibitors of GTP binding and hydrolysis, but GTP potently inhibited the more vigorous hydrolysis of these analogues. We conclude that alteration of the ribose moiety reduces the affinity of a guanine nucleotide for the exchangeable site of tubulin but that a nucleotide's affinity for this site is not the major factor in its ability to support the nucleation of tubulin polymerization.

Microtubules are largely composed of tubulin, a heterodimeric protein containing 2 molar equiv of guanosine nucleotide (Weisenberg et al., 1968; Bryan, 1972; Kobayashi, 1974; Hamel & Lin, 1981a). Removal of one of these nucleotides from the protein requires its denaturation, and the role of this "nonexchangeable GTP" remains entirely unknown. The second nucleotide, the "exchangeable GTP", binds tightly to tubulin, but it can be displaced by exogenous GDP or GTP and removed at least partially with retention of activity (Penningroth & Kirschner, 1977, 1978; Purich & MacNeal, 1978; Kirsch & Yarbrough, 1981). Tubulin polymerization is generally associated with hydrolysis of the exchangeably bound GTP (Kobayashi, 1975; Weisenberg et al., 1976; Penningroth & Kirschner, 1977; Arai & Kaziro, 1977; David-Pfeuty et al., 1977; MacNeal & Purich, 1978), but the resultant GDP in the microtubule is nonexchangeable until depolymerization occurs (Weisenberg et al., 1976; Arai & Kaziro, 1977; David-Pfeuty et al., 1977).

Our laboratory has been examining interactions of ribose-modified GDP and GTP analogues at the exchangeable site to define structural requirements for the nucleotide in greater detail. We initially studied the glutamate-induced polymerization of purified tubulin free of nucleoside diphosphate kinase and adenosinetriphosphatase (ATPase) contaminants (Hamel & Lin, 1981b; Lustbader & Hamel, 1982). More recently, having observed that heat treatment of microtubule-associated proteins (MAPs)[1] (Fellous et al., 1977; Cleveland et al., 1977) destroys these enzymes (Hamel et al., 1981) as well as a tubulin-independent GTPase activity (David-Pfeuty et al., 1978), we extended our studies to MAP-dependent polymerization in 0.1 M Mes–0.5 mM $MgCl_2$ (Hamel et al., 1983a,b; Hamel & Lin, 1984).

In most ways the analogues behaved similarly in the two systems. (1) Three deoxy-GTP analogues and ara-GTP were superior to GTP in supporting polymerization, while analogues with an open or substituted ribose ring were less active than GTP. The relative order of analogue activity differed little with MAPs or glutamate (Lustbader & Hamel, 1982; Hamel et al., 1983a). (2) No GDP analogue was as effective as GDP itself in inhibiting polymerization (Hamel & Lin, 1981b, 1984; Lustbader & Hamel, 1982). (3) The order of inhibitory activity of GDP analogues, although similar in the glutamate- and MAP-dependent reactions, differed greatly from the order of activity of the cognate GTP analogues in supporting polymerization (Hamel & Lin, 1981b, 1984). (4) One GDP analogue, ddGDP, had significant activity in supporting tubulin polymerization and was incorporated unaltered into the polymer (Hamel et al., 1983b).

We also found in 0.1 M Mes–0.5 mM $MgCl_2$ that a single analogue, ddGTP, supported polymerization of tubulin at 1.0 mg/mL without MAPs, with formation of a mixture of microtubules and sheets (Hamel et al., 1983a). This reaction

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[1] Abbreviations: MAPs, microtubule-associated proteins; ara-GDP and ara-GTP, 9-β-D-arabinofuranosylguanine 5′-di- and 5′-triphosphate; ddGDP and ddGTP, 2′,3′-dideoxyguanosine 5′-di- and 5′-triphosphate; 3′dGDP and 3′dGTP, 3′-deoxyguanosine 5′-di- and 5′-triphosphate; 2′OMeGTP, 2′-*O*-methylguanosine 5′-triphosphate; 3′OMeGTP, 3′-*O*-methylguanosine 5′-triphosphate; acyclo-GTP, the triphosphate derivative at the side-chain hydroxyl of 9-[(2-hydroxyethoxy)methyl]guanine (acycloguanosine); ox-red GTP, the dialcohol derivative of GTP obtained by periodate oxidation and borohydride reduction; Mes, 2-(*N*-morpholino)ethanesulfonate.

could be inhibited by both triphosphates (including GTP) and diphosphates. The relative inhibitory activity of the GTP analogues was comparable to that of the cognate GDP analogues, but very different from their relative activity in supporting polymerization (Hamel & Lin, 1984).

Further exploration of the interactions of these analogues with tubulin and MAPs required the preparation of radiolabeled compounds. In this paper we will focus on the highly active deoxyguanosine analogues (including ara-GTP). Although they were hydrolyzed more vigorously than GTP in the course of microtubule assembly, in keeping with the more extensive and rapid polymerization reactions which they support, all four analogues bound to tubulin less readily than GTP. Further studies demonstrated that these analogues enhanced the nucleation process. These findings lead to the conclusion that the ability of a nucleotide to support the nucleation of tubulin polymerization is not directly related to its affinity for the exchangeable GTP binding site of the protein.

Materials and Methods

Materials. Heat-treated MAPs and electrophoretically homogeneous calf brain tubulin free of unbound nucleotide and containing about 1 molar equiv each of bound GDP and GTP were prepared as described previously (Hamel & Lin, 1981b; Hamel et al., 1981). For calculations of tubulin–nucleotide stoichiometry a molecular weight of 100 000 was used for tubulin (Ponstingl et al., 1981; Krauhs et al., 1981). Neither tubulin nor MAPs had significant nucleoside diphosphate kinase or ATPase activity. GTP, GDP, [α-^{32}P]-GDP, [β-^{32}P]ddGDP, [β-^{32}P]ddGTP, ATP, and GDP and GTP analogues were prepared as described elsewhere (Hamel, 1975, 1977; Hamel & Lin, 1981b; Lustbader & Hamel, 1982; Hamel et al., 1983b). Mes (adjusted to pH 6.4 with NaOH), vinblastine, and yeast nucleoside diphosphate kinase were obtained from Sigma and [α-^{32}P]GTP and [^{32}P]P_i from Amersham; [8-^{14}C]GTP (57 mCi/mmol) was obtained from New England Nuclear.

Radiolabeled Nucleotides. The method of Glynn & Chappell (1964) was used to prepare γ-^{32}P-labeled ATP, dGTP, GTP, 3′dGTP, ddGTP, and ara-GTP. Preparation of β-^{32}P-labeled dGDP, 3′dGDP, ara-GDP, dGTP, 3′dGTP, and ara-GTP was as described previously for ddGDP and ddGTP (Hamel et al., 1983b).

Simultaneous Measurement of Tubulin Polymerization and Nucleotide Hydrolysis. In these experiments 1.3-mL reaction mixtures contained 1.0 mg/mL purified tubulin, 0.2 mg/mL heat-treated MAPs (if present), 0.1 M Mes, 0.5 mM $MgCl_2$, and the indicated nucleotide. Assays were performed in cuvettes with a 1-cm light path in a Gilford Model 250 spectrophotometer equipped with a Lauda circulating water bath. The water bath was initially at 0 °C. After base lines were established, the thermostat of the water bath was set at 37 °C. The point at which the water bath reached 37 °C was defined as zero time and is indicated in the figures by an arrow on the abscissa if a reaction occurred prior to zero time (see legend of Figure 2 for details of the temperature equilibration). Aliquots of the reaction mixtures were removed from the cuvettes when the water bath reached 10, 20, and 30 °C and at the indicated times at 37 °C for the quantitation of nucleotide hydrolysis (see below). Forty-five minutes after the water bath reached 37 °C 0.5 mL of each reaction mixture was centrifuged at 40 000 rpm in a Beckman Ti 50 rotor for 30 min at 25–30 °C. The protein concentration of the supernatant was compared to that of the total reaction mixture to quantitate the extent of polymerization, which was obtained by subtraction. It was assumed that the proportions of tubulin and MAPs were the same in the pellet and the total reaction mixture. The percent tubulin polymerized was converted to nanomoles per milliliter of reaction, and the turbidity reading at 45 min was placed at this value on the "P_i formed" scale of the appropriate figures, as indicated.

Thin-layer chromatography on poly(ethylenimine)–cellulose and autoradiography were used to measure nucleotide hydrolysis by following the formation of [^{32}P]P_i or ^{32}P-labeled diphosphates, as appropriate, from ^{32}P-labeled triphosphates (Hamel et al., 1983b), with data expressed as nanomoles of hydrolytic product formed per milliliter of reaction.

Nucleotide Binding to Tubulin and Incorporation of Radioactive Nucleotides into Polymer. The determination of the amount of nucleotide in polymer recovered by centrifugation was performed as described elsewhere (Hamel et al., 1983b). The binding of nucleotides to tubulin and the displacement of radiolabeled GDP from tubulin were measured by the centrifugal gel filtration method of Penefsky (1977), except that Sephadex G-50 (superfine) was used. The protein concentration and radioactivity of the filtrates were determined, with the data expressed as picomoles of nucleotide bound per picomole of tubulin. Without tubulin, no radioactivity was found in the filtrates.

Tubulin Polymerization. Polymerization was followed turbidimetrically (Gaskin et al., 1974) at 350 nm in Gilford Model 250 and 2400-S recording spectrophotometers equipped with Gilford "Thermoset" electronic temperature controllers. After base lines were established at 0 °C, the desired temperature was set on the temperature controller. If 37 °C was set on the instrument, the temperature readout reached 37 °C at 70 s. At this point the temperature of the contents of the cuvettes was at 34 °C, and within 60 s temperature equilibration at 37 °C was complete.

Microtubule Length Determinations. Reaction mixtures (0.25 mL) containing 1.0 mg/mL tubulin, 0.33 mg/mL heat-treated MAPs, 0.1 M Mes, 0.5 mM $MgCl_2$, and 0.1 mM nucleotide were incubated for 30 min at 37 °C. Aliquots were then diluted 4-fold with an isothermic solution of 50% sucrose containing 0.1 M Mes and 0.5 mM $MgCl_2$ (Terry & Purich, 1980). Negatively stained specimens were prepared and examined in the electron microscope as described previously (Hamel et al., 1981, 1983b). High-power micrographs confirmed the formation of microtubules, and length measurements were made on micrographs with a magnification of 5250 by using a Zeiss Videoplan 2 image analyzer.

Preparation and Analysis of Tubulin Bearing [8-^{14}C]GDP in the Exchangeable Site. After 250 μCi of [8-^{14}C]GTP was dried in vacuo, a 6.0-mL solution of 10 mM [8-^{14}C]GTP was prepared by adding nonradioactive nucleotide (the final preparation had 8.95 cpm/pmol). A 19-mL solution was prepared containing 1.0 M glutamate, 750 mg of purified tubulin freed of unbound nucleotide by gel filtration chromatography (Hamel & Lin, 1981a), and 1.0 mM [8-^{14}C]GTP. The mixture was incubated for 30 min at 37 °C and centrifuged in a prewarmed Beckman Ti 45 rotor at 40 000 rpm for 30 min with the centrifuge refrigeration system turned off (warm centrifugation). The pellet was homogenized in 12 mL of cold 1.0 M glutamate. The suspension was centrifuged in a prechilled Beckman Ti 45 rotor at 0 °C at 40 000 rpm for 30 min (cold centrifugation), and the supernatant was removed.

One milliliter of this first cold supernatant was chromatographed on a 1.5 × 11 cm column of Sephadex G-50 (superfine) to remove unbound nucleotide. Solid urea for a final concentration of 8 M was added to the protein-containing

Table I: Incorporation of [8-^{14}C]GDP into the Exchangeable Nucleotide Binding Site of Tubulin[a]

polymerization cycle[b]	sp act. of tubulin-bound GDP relative to GTP supporting polymerization[c]
first	0.48
second	0.73
third	0.90

[a] Polymerization was induced with 1.0 M glutamate, with tubulin at 30–40 mg/mL and [8-^{14}C]GTP at 1.0 mM. See text for further details. [b] Nucleotide analyzed was obtained, as described in the text, from protein that had undergone an initial gel filtration chromatography step in 1.0 M glutamate subsequent to clarification by centrifugation in the cold of the appropriate polymer homogenate. [c] The specific activity of [8-^{14}C]GTP was 8.95 cpm/pmol. The specific activity of the peak GDP fractions, obtained following DEAE-Sephadex chromatography of tubulin-bound nucleotide, was determined as described in the text.

fractions, and denatured tubulin and nucleotides were separated by chromatography on Sephadex G-50 (superfine). The nucleotide peak was pooled, GDP and GTP were separated by chromatography on DEAE-Sephadex A-25, and the specific activity of the peak GDP fractions was determined (see Table I). Approximately equimolar amounts of GDP and GTP were bound to the tubulin, and minimal radioactivity was found in the peak GTP fractions (see Figure 1C).

The remainder of the first cold supernatant was used to prepare a solution containing 1.0 M glutamate and 1.0 mM [8-^{14}C]GTP in a 19-mL volume. Incubation, warm and cold centrifugations, and determination of the specific activity of the tubulin-bound [8-^{14}C]GDP (see Table I) were performed as described above. Again, approximately equimolar amounts of radiolabeled GDP and nonradioactive GTP were bound to the tubulin.

The remainder of the second cold supernatant was used to prepare a solution containing 1.0 M glutamate and 1.0 mM [8-^{14}C]GTP in a 19-mL volume. Incubation and warm and cold centrifugations were performed as described above. The third cold supernatant was chromatographed in 1.0 M glutamate on a 5 × 25 cm column of Sephadex G-50 (superfine) to separate tubulin from unbound nucleotide. The protein-containing fractions were precipitated by the addition of solid monosodium glutamate to a final concentration of 2.5 M. The tubulin was harvested by a cold centrifugation. A slurry was formed by adding 3 mL of cold water to the tubulin pellet, and this was dialyzed against 1.0 M glutamate to redissolve the tubulin. A small amount of aggregated protein was removed by a final cold centrifugation. The supernatant (6 mL, containing 40 mg/mL protein) was stored frozen in liquid nitrogen.

To verify that the above procedure for concentrating the tubulin did not dislodge protein-bound nucleotide, an aliquot of the final preparation was applied to a small Sephadex G-50 (superfine) column equilibrated and developed with 1.0 M glutamate (Figure 1A). There was no apparent unbound nucleotide peak detected by measuring either the radioactivity or the absorbance of the fractions. A large amount of ^{14}C-labeled nucleotide was, however, present in the protein-containing fractions.

Addition of urea to a final concentration of 8 M, followed by gel filtration chromatography in 8 M urea, quantitatively released the ^{14}C-labeled nucleotide from the protein, and the radioactivity was associated with a peak having a ratio of $A_{252.5}$:A_{280} expected for guanine nucleotides (Figure 1B). Quantitation of the protein and the nucleotide peaks yielded a molar ratio of 1.8 nucleotides per tubulin dimer for this preparation.

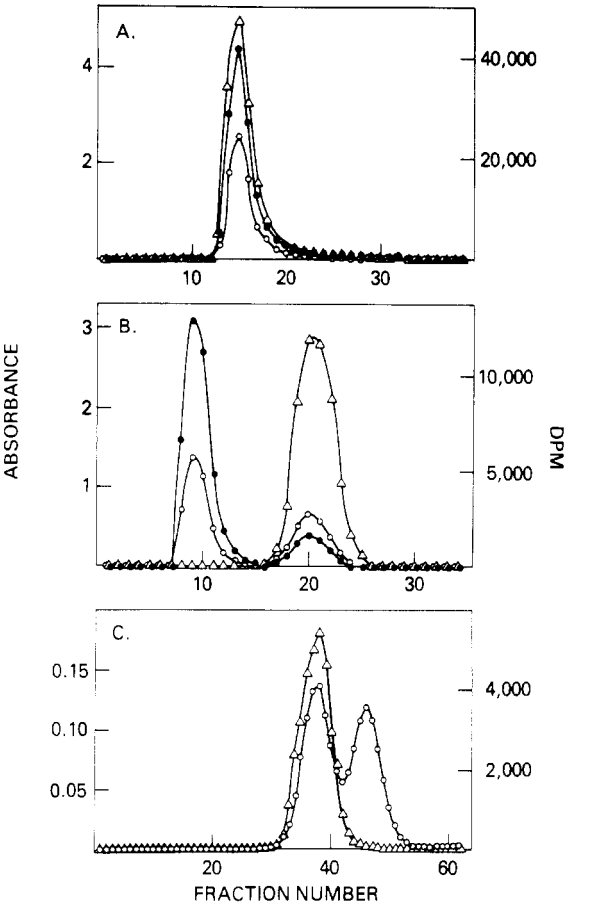

FIGURE 1: Characterization and quantitation of the nucleotide bound to tubulin after three cycles of glutamate-induced polymerization supported by [8-^{14}C]GTP. Symbols (all panels): ●, A_{280}; ○, $A_{252.5}$; △, decompositions per minute (dpm). (A) Gel filtration in 1.0 M glutamate. One-fourth milliliter of the stock solution of radiolabeled tubulin (concentration 40 mg/mL) was applied to a 1.5 × 8 cm column of Sephadex G-50 (superfine) equilibrated and developed with 1.0 M glutamate. The fraction size was 0.5 mL, and 0.2 mL of each fraction was counted. (B) Gel filtration in 8 M urea. Solid urea for a final concentration of about 8 M was added to 1.25 mL of the stock solution of radiolabeled tubulin. The resulting solution was applied to a 2.5 × 8 cm column of Sephadex G-50 (superfine) equilibrated and developed with 8 M urea. The fraction size was 2 mL, and 50 μL of each fraction was counted. (C) Ion-exchange chromatography of nucleotide released from tubulin in 8 M urea. The nucleotide-containing fractions from the experiment described in panel B were pooled, and approximately half was applied to a 1.5 × 20 cm column of DEAE-Sephadex A-25 equilibrated with water. The column was then washed with about 50 mL of 0.1 M NaH_2PO_4 (pH unadjusted) and developed with a 140-mL gradient from 0.1 to 1.5 M NaH_2PO_4 (pH unadjusted). The fraction size was 2 mL, and 50 μL of each fraction was counted.

When the nucleotide peak was applied to DEAE-Sephadex A-25 and the column developed by gradient chromatography (Figure 1C), approximately equimolar amounts of GDP and GTP were recovered (in this case, approximately 55% GDP, 45% GTP). The specific activity of the peak GDP fractions was determined (Table I).

These observations demonstrate that our preparations of tubulin contain GTP bound largely at the nonexchangeable site and GDP bound primarily at the exchangeable site. Furthermore, after addition of exogenous GTP, in glutamate at high tubulin concentrations, it appears that significant amounts of tubulin still bearing GDP copolymerize with tubulin bearing GTP in the exchangeable site, for the specific activity of the tubulin-bound GDP rises significantly with each cycle of polymerization (Table I). A similar preparative procedure with tubulin at 5 mg/mL and 1.0 mM GTP resulted

Table II: Effects of MAPs and Vinblastine on Tubulin-Dependent Hydrolysis of GTP and GTP Analogues[a]

nucleotide added	nmol of P_i formed				
	MAPs only	tubulin only	tubulin + vinblastine	tubulin + MAPs	tubulin + MAPs + vinblastine
GTP	0	0	0	32.5	0
ddGTP	0	37.0	0	31.8	0.3
dGTP	0	2.5	0	44.1	0
3′dGTP	0	9.0	0	38.6	0
ara-GTP	0	4.3	0	43.1	0

[a] Each 50-μL reaction mixture was incubated at 37 °C for 45 min and contained 0.1 M Mes, 0.5 mM $MgCl_2$, and, if indicated, 1.0 mg/mL tubulin, 0.2 mg/mL heat-treated MAPs, 10 μM vinblastine, and the indicated γ-^{32}P-labeled nucleotide at 50 μM. At zero time reaction mixtures were transferred from ice to a 37 °C water bath. The relative amounts of P_i formed with tubulin and tubulin + MAPs with the different nucleotides were similar in three different experiments.

in a significantly higher specific activity for the GDP bound to the protein after one cycle of polymerization (data not presented).

Results

Nucleotide Hydrolysis. Eight ribose-modified analogues of GTP (ddGTP, dGTP, 3′dGTP, ara-GTP, 2′OMeGTP, 3′OMeGTP, acyclo-GTP, and ox-red GTP) display a wide range of activity in supporting MAP-dependent tubulin polymerization in 0.1 M Mes–0.5 mM $MgCl_2$, while only ddGTP is active without MAPs (at 1.0 mg/mL tubulin) (Hamel et al., 1983a). Table II presents a preliminary experiment in which the hydrolysis at 37 °C of GTP, ddGTP, 3′dGTP, dGTP, and ara-GTP was examined at a single time point (45 min). Except for ddGTP, maximum hydrolysis of all compounds required both heat-treated MAPs and tubulin. The hydrolysis of ddGTP was greater without MAPs than with MAPs (an observation reproduced three times, but also see Figure 3). The MAPs did not hydrolyze any nucleotide, but with tubulin alone there was sluggish hydrolysis of dGTP, ara-GTP, and 3′dGTP as well as the substantial hydrolysis of ddGTP. In all cases hydrolysis was inhibited by vinblastine, indicating that tubulin was the hydrolytic factor (David-Pfeuty et al., 1979; Lin & Hamel, 1981).

We next examined the relationship of nucleotide hydrolysis to polymerization. Figure 2 presents a study comparing the hydrolytic and polymerization reactions with GTP to those with dGTP, 3′dGTP, and ara-GTP. With these three analogues, as well as ddGTP, not only does microtubule assembly require a lower nucleotide concentration than with GTP but also the analogue-supported MAP-dependent polymerization reactions begin earlier and are more extensive[2] than the GTP-supported reaction (Hamel et al., 1983a). These features of the assembly reaction are shown in Figure 2B in an experiment performed with 0.1 mM nucleotide. Simultaneously obtained hydrolytic data are presented in Figure 2A. Parts C (GTP), D (ara-GTP), E (3′dGTP), and F (dGTP) of Figure 2 correlate the hydrolysis and turbidity data for each nucleotide. In Figure 2C–F polymerization and nucleotide hydrolysis are set on the same nanomolar scale, with the extent of polymerization determined by removal of polymer by high-speed centrifugation.

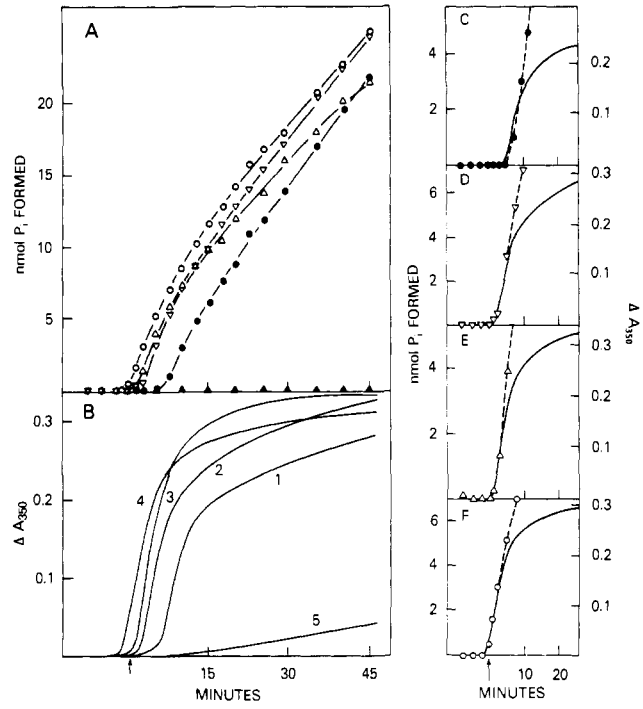

FIGURE 2: Simultaneous nucleotide hydrolysis and tubulin polymerization with GTP, dGTP, 3′dGTP, and ara-GTP. Each reaction mixture contained 1.0 mg/mL tubulin, 0.2 mg/mL heat-treated MAPs, 0.1 M Mes, 0.5 mM $MgCl_2$, and the indicated nucleotide at 0.1 mM. (In this experiment the temperature was controlled with the circulating water bath. When the temperature of the water bath reached 37 °C, defined as zero time, the cuvette contents were at 14 °C. The cuvette contents were at 21 °C at 5 min, at 27 °C at 10 min, at 30.5 °C at 15 min, and at 32.5 °C at 20 min and finally equilibrated at 35 °C at 30–35 min.) (A) Hydrolytic data: ●, [γ-^{32}P]GTP; ▽, [γ-^{32}P]ara-GTP; △, [γ-^{32}P]3′dGTP; ○, [γ-^{32}P]dGTP; ▲, [γ-^{32}P]ATP. (B) Simultaneously obtained turbidity data: curve 1, [γ-^{32}P]GTP; curve 2, [γ-^{32}P]ara-GTP; curve 3, [γ-^{32}P]3′dGTP; curve 4, [γ-^{32}P]dGTP; curve 5, [γ-^{32}P]ATP. (C, D, E, and F) The hydrolytic and turbidity data plotted on the same nanomolar scale, as described in the text, for GTP, ara-GTP, 3′dGTP, and dGTP, respectively. In this experiment 52% polymerization occurred with GTP and 3′dGTP (5.2 nmol of tubulin polymerized/mL of reaction), 69% with dGTP (6.9 nmol/mL), and 75% with ara-GTP (7.5 nmol/mL). [The final turbidity readings at 45 min were placed at these nanomole values in expanded figures, the hydrolytic data were plotted, and the initial portion of each reaction was excerpted for panels C–F. We have assumed that turbidity and nanomoles of tubulin polymerized are related linearly during the entire course of the reaction (Gaskin et al., 1974).]

[2] Extent of reaction is arbitrarily defined as the percent of total protein removed by high-speed centrifugation at 45 min. It should be noted that the turbidity curves in most of the MAP-dependent reactions are still slowly rising at this time. This does not represent continuing, slow microtubule assembly but rather aggregation of tubulin resulting from at least three factors: storage of tubulin without exogenous nucleotide, the reaction temperature, and the relatively low pH of the reaction. In agreement with this conclusion, the proportion of the total turbidity that is cold reversible decreases with time—it is less after a 2-h incubation than after 1 h and less after 1 h than after 30 min. It is unknown to what extent this aggregation affects the amount of protein in the pellets recovered by centrifugation, but it would lead to an overestimate of the amount of polymer formed and, hence, if a correction could be made for aggregation, a "shift to the left" of the hydrolytic curves relative to the turbidity curves in Figures 2C–F and 3. It must be emphasized, moreover, that the most important aspects of the experiments presented in Figures 2 and 3 are the close temporal correlation of polymerization and nucleotide hydrolysis in all MAP-dependent reactions and the basic similarity between the GTP-supported reaction and the analogue-supported reactions except for the time of onset.

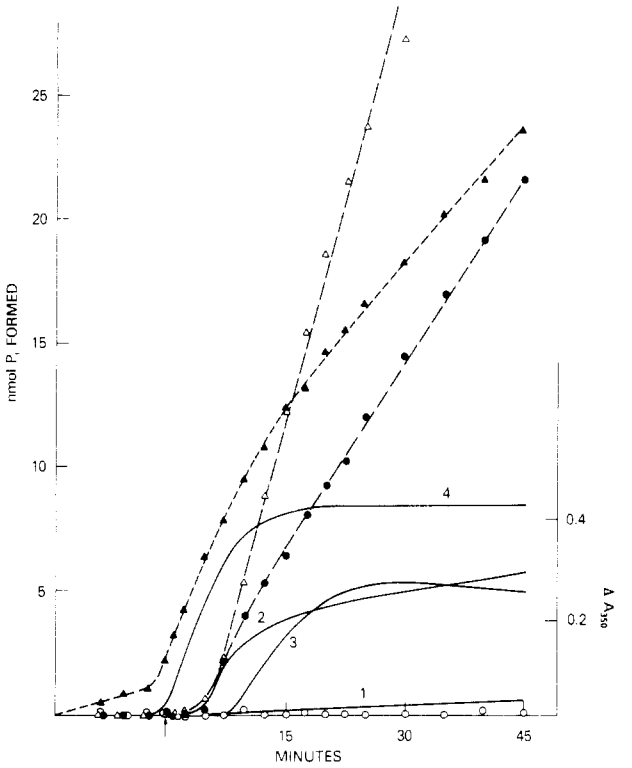

FIGURE 3: Effect of MAPs on nucleotide hydrolysis and polymerization with GTP and ddGTP. Each reaction mixture contained 1.0 mg/mL tubulin, 0.1 M Mes, 0.5 mM $MgCl_2$, 0.2 mg/mL heat-treated MAPs, as indicated, and the indicated nucleotide at 0.1 mM. Hydrolytic (symbols) and turbidity (solid curves) data were obtained simultaneously. (In this experiment the temperature was controlled with the circulating water bath. See Figure 2 for details of the temperature equilibration.) Curve 1 and the symbol O represent the reaction with [γ-^{32}P]GTP but no MAPs, curve 2 and the symbol ● represent the reaction with [γ-^{32}P]GTP with MAPs, curve 3 and the symbol △ represent the reaction with [γ-^{32}P]ddGTP but no MAPs, and curve 4 and the symbol ▲ represent the reaction with [γ-^{32}P]ddGTP with MAPs. The hydrolytic and turbidity data for the reactions with MAPs are plotted on the same nanomolar scale, as described in the text and in Figure 2. In this experiment 57% polymerization occurred with GTP and MAPs (5.7 nmol of tubulin polymerized/mL of reaction), 85% with ddGTP and MAPs (8.5 nmol/mL), and 41% with ddGTP without MAPs (4.1 nmol/mL).

Like the polymerization reactions they supported (Figure 2B), hydrolysis of dGTP, 3′dGTP, and ara-GTP began earlier than GTP hydrolysis (Figure 2A). With all four nucleotides an initial burst of hydrolysis was followed by sustained reactions that far exceeded polymerization on a molar basis. The four hydrolytic reactions were similar in magnitude, differing primarily in the time of onset (caused in part by different temperature thresholds for each reaction, as described below). As shown in Figure 2C–F, with each nucleotide hydrolysis and polymerization were simultaneous in onset, and the initial bursts of hydrolysis were essentially stoichiometric with the amount of tubulin polymerized.

Figure 2 also demonstrates that ATP neither was hydrolyzed nor supported polymerization of tubulin with heat-treated MAPs.

One additional ribose-modified GTP analogue, ddGTP, is superior to GTP in supporting tubulin polymerization with MAPs, and in addition only ddGTP can support the polymerization of tubulin at 1.0 mg/mL without MAPs in 0.1 M Mes–0.5 mM $MgCl_2$ (Hamel et al., 1983a). Figure 3 presents a study comparing the hydrolytic and polymerization reactions with ddGTP and GTP, both in the presence and in the absence of MAPs. (In this figure polymerization and hydrolysis are plotted on the same nanomolar scale for the reactions with

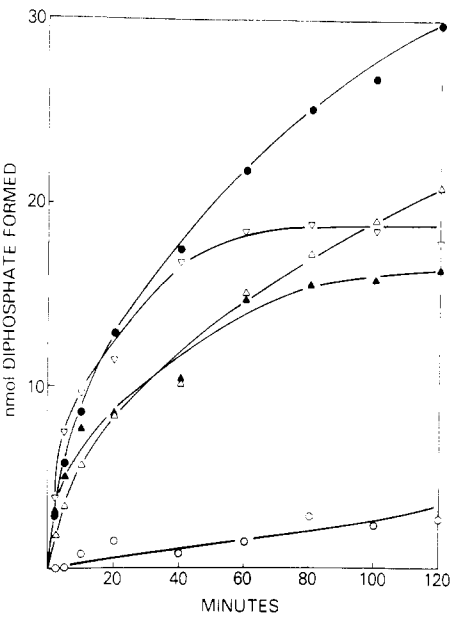

FIGURE 4: Nucleotide hydrolysis at 20 °C. Each 0.25-mL reaction mixture contained 1.0 mg/mL tubulin, 0.2 mg/mL heat-treated MAPs, 0.1 M Mes, 0.5 mM $MgCl_2$, and the indicated nucleotide at 50 μM: O, [α-^{32}P]GTP; ●, [β-^{32}P]dGTP; ▽, [β-^{32}P]ddGTP; △, [β-^{32}P]ara-GTP; ▲, [β-^{32}P]3′dGTP. The reaction mixtures were incubated at 20 °C in a water bath, and aliquots were analyzed for nucleotide hydrolysis at the indicated times.

GTP + MAPs and ddGTP + MAPs.) Again GTP hydrolysis and GTP-supported polymerization with MAPs began simultaneously and were initially equimolar. Without MAPs there was neither significant polymerization with GTP nor GTP hydrolysis.

With ddGTP plus MAPs a sluggish hydrolysis of the nucleotide occurred prior to the onset of elongation, perhaps associated with nucleation events. There was a sudden increase in the rate of ddGTP hydrolysis, which began about the same time as a comparable rise in the rate of polymerization. The maximum rate of ddGTP hydrolysis with MAPs was comparable to the maximum rate of polymerization (cf. the slopes of the two reactions). As with GTP, dGTP, 3′dGTP, and ara-GTP, the rate of ddGTP hydrolysis slowed as the turbidity approached its plateau, but only after hydrolysis had substantially exceeded polymerization on a molar basis.[3]

Without MAPs ddGTP hydrolysis began about 5 min prior to the onset of the elongation phase of polymerization. Without MAPs, therefore, ddGTP hydrolysis occurred during the nucleation phase or, alternatively, was completely uncoupled from the polymerizaton reaction supported by the nucleotide. Once initiated this reaction was extremely rapid, and within 10 min ddGTP hydrolysis without MAPs reached and then surpassed ddGTP hydrolysis with MAPs.

The previous studies clearly demonstrate that hydrolysis of dGTP, 3′dGTP, ara-GTP, and ddGTP has an earlier onset than hydrolysis of GTP by tubulin with MAPs, a property

[3] In the experiment presented in Figure 3 the maximum, initial rate of hydrolysis with ddGTP + MAPs (derived from the values at zero time and 5 min) was 0.83 nmol/(min·mg of tubulin), while the average rate from 25 through 45 min was 0.35 nmol/min and from 35 through 45 min was 0.34 nmol/min. With GTP + MAPs the maximum, initial rate (derived from the values at 5 and 10 min) was 0.75 nmol/(min·mg of tubulin), while the average rate from 25 through 45 min was 0.48 nmol/min and from 35 through 45 min was 0.46 nmol/min. (The values derived from Figure 2 for GTP are almost identical.) A comparison of the initial rates is limited, however, by the fact that temperature equilibration had not yet occurred (see Figure 2 legend).

Table III: Binding of Nucleotides to Tubulin[a]

nucleotide added	pmol of nucleotide/pmol of tubulin: 10 μM nucleotide	100 μM nucleotide
[α-^{32}P]GTP	0.52	0.62
[α-^{32}P]GDP	0.25	0.34
[β-^{32}P]dGTP	0.29	0.42
[β-^{32}P]dGDP	0.07	0.17
[β-^{32}P]ara-GTP	0.21	0.42
[β-^{32}P]ara-GDP	0.02	0.06
[β-^{32}P]ddGTP	0.01	0.12
[β-^{32}P]ddGDP	0.002	0.03
[β-^{32}P]3′dGTP	0.02	0.05
[β-^{32}P]3′dGDP	0.002	0.01

[a] Each 0.35-mL reaction mixture contained the indicated concentration of the indicated nucleotide, 1.0 mg/mL tubulin, 0.1 M Mes, and 0.5 mM $MgCl_2$ and was incubated 15 min at 0 °C. Triplicate 0.1-mL aliquots of each reaction mixture were placed on 1.0-mL columns of Sephadex G-50 (superfine) and centrifuged in the cold as described by Penefsky (1977). The radioactivity and protein content of the filtrates were determined, and the average values obtained (picomoles of nucleotide per picomole of tubulin) are presented in the table. Triplicate values were within 20% of each other, but the relative binding of different nucleotides presented in the table was observed in all experiments performed.

Table IV: Displacement of [8-^{14}C]GDP Bound in the Exchangeable Site by Exogenous Nucleotide[a]

nucleotide added	pmol of [8-^{14}C]GDP/pmol of tubulin: no exogenous nucleotide	10 μM exogenous nucleotide	100 μM exogenous nucleotide
none	0.40		
GTP		0.08	0.01
GDP		0.22	0.04
dGTP		0.19	0.02
dGDP		0.30	0.08
ara-GTP		0.22	0.05
ara-GDP		0.37	0.18
ddGTP		0.41	0.24
ddGDP		0.42	0.30
3′dGTP		0.36	0.12
3′dGDP		0.40	0.20

[a] Each 0.65-mL reaction mixture contained 1.0 mg/mL tubulin with bound [8-^{14}C]GDP (see text for details of its preparation), the indicated concentration of the indicated nucleotide, 0.1 M Mes, and 0.5 mM $MgCl_2$ and was incubated 15 min at 0 °C. Triplicate 0.2-mL aliquots of each reaction mixture were placed on 1.0-mL columns of Sephadex G-50 (superfine) and centrifuged in the cold as described by Penefsky (1977). The radioactivity and protein content of the filtrates were determined, and the average values obtained (picomoles of [8-^{14}C]GDP per picomole of tubulin) are presented in the table. Triplicate values were within 20% of each other.

consistent with the superiority of these nucleotides in supporting polymerization. We have also observed that the superiority of the analogues in both polymerization (see below) and hydrolysis is magnified at lower reaction temperatures. Figure 4 presents a comparison of MAP-dependent hydrolysis of GTP to hydrolysis of the four analogues at 20 °C. At this temperature hydrolysis of GTP was minimal, while that of the other four compounds was substantial.

We have also examined the hydrolysis of γ-^{32}P-labeled 3′OMeGTP, acyclo-GTP, 2′OMeGTP, and ox-red GTP relative to the MAP-dependent polymerization reactions they supported (data not presented). The hydrolysis of all four analogues was significantly reduced compared to that of GTP. With 3′OMeGTP and acyclo-GTP the patterns observed were analogous to those described above: simultaneous and apparently stoichiometric onset of polymerization and hydrolysis, with hydrolysis continuing at the turbidity plateau. With 2′OMeGTP and ox-red GTP, both of which lead to the formation of 80-nm tubules rather than microtubules (Hamel et al., 1983a), hydrolysis was delayed relative to turbidity development.

Binding of GDP and GTP Analogues to Tubulin. Since the ribose-modified GTP analogues display a wide range of activity in supporting tubulin polymerization, we had assumed that a nucleotide's activity in supporting polymerization would reflect its affinity for the exchangeable binding site (Hamel & Lin, 1981b; Hamel et al., 1983a). To test this hypothesis, we examined the binding of γ-^{32}P-labeled compounds to tubulin at 0 °C, using centrifugal separation by gel filtration of bound and free nucleotide (Penefsky, 1977; Caplow & Zeeberg, 1980). In these preliminary experiments we found substantial binding of GTP and moderate binding of dGTP and ara-GTP but poor to negligible binding of ddGTP, 3′dGTP, 2′OMeGTP, 3′OMeGTP, acyclo-GTP, and ox-red GTP (data not presented). These were surprising results since ddGTP, 3′dGTP, ara-GTP, and dGTP are all more vigorously hydrolyzed than GTP, and all support polymerization better than GTP under all conditions we have examined. It therefore seemed important to follow the fate of these nucleotides in further detail, so β-^{32}P-labeled ddGTP, ddGDP, 3′dGTP, 3′dGDP, dGTP, dGDP, ara-GTP, and ara-GDP were prepared.

The binding studies were repeated with these β-^{32}P-labeled nucleotides, and they were compared to [α-^{32}P]GTP and [α-^{32}P]GDP (Table III). Results similar to those with the γ-^{32}P-labeled nucleotides were obtained. The experiments presented in Table III were performed with a 15-min incubation at 0 °C. Like Caplow & Zeeberg (1980), we found no increase in the amount of GTP or of any triphosphate bound after longer incubation times at 0 °C. The data for GDP presented in Table III refer only to the exogenously added [α-^{32}P]GDP with no correction made for the unlabeled GDP initially present in the exchangeable site. Since the tubulin concentration was 10 μM (1.0 mg/mL), these initial studies suggested that the GDP in the exchangeable site was not completely exchangeable with free GDP (however, see below).

At 10 μM nucleotide the tubulin was 52% saturated with GTP, and this value rose to 62% at 0.1 mM nucleotide. Significantly lower levels of binding were observed with dGTP and ara-GTP at both nucleotide concentrations. Binding was essentially undetectable with 10 μM ddGTP and 3′dGTP and sharply reduced relative to GTP at 0.1 mM nucleotide.[4]

In all cases the diphosphates bound significantly less well than the cognate triphosphates, although exogenously added GDP was bound in substantially greater quantities than any of the GDP analogues. At both nucleotide concentrations, moreover, the ratio of bound GDP to bound dGDP or ara-GDP was significantly greater than the comparable ratios of bound GTP to bound dGTP or ara-GTP. Finally, the relative binding of the series of diphosphates (GDP > dGDP > ara-GDP > ddGDP > 3′dGDP) is exactly the same as the relative binding of the triphosphates (GTP > dGTP > ara-GTP > ddGTP > 3′dGTP).

To confirm the feeble binding of the active GTP analogues, we prepared tubulin containing approximately 1 molar equiv of [8-^{14}C]GDP in the exchangeable site and examined displacement of this GDP by unlabeled nucleotides (Table IV).

[4] There was sufficient radioactivity bound to tubulin to verify the identity of the nucleotide by thin-layer chromatography when [α-^{32}P]-GTP, [β-^{32}P]dGTP, [β-^{32}P]ara-GTP, and [β-^{32}P]ddGTP were used. There was little, if any, nucleotide hydrolysis since the radioactivity in the filtrates comigrating with nonradioactive standards was respectively 98% GTP, 98% dGTP, 97% ara-GTP, and 93% ddGTP.

Before describing the findings with the analogues, the results without exogenous nucleotide and with exogenous GDP require comment.

Although gel filtration chromatography in 1.0 M glutamate demonstrated that virtually all the nucleotide in the stock solution of tubulin was protein bound (Figure 1A), and analysis of bound nucleotide demonstrated almost 1 mol of GDP per mole of tubulin (Figure 1B,C), only about 40% of the recovered tubulin still contained GDP following dilution of the tubulin into the reaction mixture, incubation on ice, and analysis by the gel filtration method of Penefsky (1977). In experiments to explore further this dissociation of exchangeably bound GDP, we applied samples of tubulin with bound [8-^{14}C]GDP at 40 mg/mL (the stock solution) and 1.0 mg/mL (the concentration used in the binding experiments) to Sephadex G-50 (superfine) columns equilibrated and developed with either 1.0 M glutamate or 0.1 M Mes–0.5 mM $MgCl_2$. At the higher protein concentration 95% of the GDP remained protein bound in glutamate (as in Figure 1A) and 80% in Mes–$MgCl_2$. At the lower tubulin concentration 65% of the GDP remained protein bound in glutamate, but only 7% remained associated with the tubulin in Mes–$MgCl_2$. In conclusion, these studies demonstrate that nucleotide cofiltering with tubulin in the centrifugal filtration method must reflect not only the amount of nucleotide bound to the protein in the reaction mixture but also subsequent dissociation in the syringe column.

When the effect of adding exogenous nonradioactive GDP on the amount of [8-^{14}C]GDP remaining bound to tubulin is considered (Table IV), it is clear that the GDP bound in the exchangeable site is actually completely exchangeable. Adding 10 μM GDP to 10 μM tubulin (initially containing about 1 molar equiv of bound GDP) reduces the bound [8-^{14}C]GDP by half, while adding 100 μM GDP reduced the amount of bound radioactive nucleotide 10-fold. The apparent partial nonexchangeability noted above when the binding of [α-^{32}P]GDP was measured thus results from the partial dissociation of GDP from tubulin in the reaction mixture and/or during centrifugal gel filtration.

When the triphosphates were examined for their ability to displace [8-^{14}C]GDP from tubulin, GTP was most effective, followed in order by dGTP, ara-GTP, 3′dGTP, and ddGTP. This relative activity replicates the order of the radiolabeled nucleotides in binding to tubulin (Table III), except that 3′dGTP displaces more GDP than does ddGTP, while larger amounts of the latter nucleotide were bound. This difference probably results from 3′dGTP binding to tubulin more readily than ddGTP but also dissociating more rapidly either in the reaction mixture or during gel filtration.

The ability of the diphosphates to displace [8-^{14}C]GDP from tubulin was in every case less than that of the cognate triphosphate (Table IV), in agreement with their reduced binding when measured directly (Table III). The relative activity of the diphosphates in displacing [8-^{14}C]GDP was, however, identical with that of the cognate triphosphates. Thus ddGDP displaces [8-^{14}C]GDP less well than does 3′dGDP, even though more tubulin-bound [β-^{32}P]ddGDP than [β-^{32}P]3′dGDP was recovered by centrifugal gel filtration.

Incorporation of Nucleotide into Polymer. The poor binding of dGTP, ara-GTP, and especially ddGTP and 3′dGTP to tubulin was unexpected since these nucleotides are so readily hydrolyzed and support vigorous polymerization reactions. A number of experiments were performed under different nonpolymerizing reaction conditions in attempts to enhance these binding reactions,[5] but without polymerization no significant change in relative nucleotide binding occurred. Under polymerizing conditions, however, there was a substantial rise in the binding of ddGTP, 3′dGTP, dGTP, and ara-GTP. To document that this increase reflected incorporation into polymer, reaction mixtures were centrifuged, and the radioactive nucleotide content of the pellets was determined (Table V). Similar amounts of nucleotide were found in the pellet whether GTP, dGTP, ara-GTP, 3′dGTP, or ddGTP was used to support polymerization. With ddGTP, substantial

Table V: Incorporation of Nucleotide into Polymerized Tubulin[a]

nucleotide added	pmol of nucleotide/pmol of tubulin in pellet
0.1 mM [α-^{32}P]GTP	1.02
0.1 mM [β-^{32}P]dGTP	1.06
0.1 mM [β-^{32}P]ara-GTP	1.06
0.1 mM [β-^{32}P]3′dGTP	1.09
0.1 mM [β-^{32}P]ddGTP	1.16
0.2 mM [β-^{32}P]ddGTP – MAPs	0.77

[a] Each 0.25-mL reaction mixture contained 0.1 M Mes, 0.5 mM $MgCl_2$, 0.2 mg/mL heat-treated MAPs except where indicated, the indicated nucleotide, and tubulin at 1.0 mg/mL, except in the reaction with ddGTP without MAPs in which 2.0 mg/mL tubulin was used, and was incubated 30 min at 37 °C. Polymer was recovered by centrifugation, and nucleotide content of the pellet was determined, as described elsewhere (Hamel et al., 1983b). The calculation of picomoles of tubulin was made by assuming that the proportions of tubulin and MAPs in the pellets were the same as in the initial reaction mixtures. Although the data in the table have not been corrected for nonspecific entrapment of nucleotide in the polymer pellet, experiments with [γ-^{32}P]ATP indicated such entrapment would be 0.06–0.12 pmol of nucleotide/pmol of tubulin.

Table VI: Displacement of [8-^{14}C]GDP from Tubulin during Polymerization with GTP and GTP Analogues[a]

nucleotide added	pmol of [8-^{14}C]GDP/pmol of tubulin in pellet		
	0.1 mM nucleotide	0.2 mM nucleotide	1.0 mM nucleotide
UTP + NDK[b]			0.84
GTP	0.04		0.01
dGTP	0.04		0.01
ara-GTP	0.05		0.03
3′dGTP	0.08		0.03
ddGTP	0.15		0.08
ddGTP – MAPs		0.09	0.07

[a] Each 0.25-mL reaction mixture contained 0.1 M Mes, 0.5 mM $MgCl_2$, 0.25 mg/mL heat-treated MAPs except where indicated, the indicated nucleotide at the indicated concentration, and tubulin with bound [8-^{14}C]GDP (see text for details of its preparation) at 1.0 mg/mL, except in the reactions with ddGTP without MAPs in which 2.0 mg/mL of tubulin was used, and was incubated 30 min at 37 °C. Polymer was recovered by centrifugation, and nucleotide content of the pellet was determined, as described elsewhere (Hamel et al., 1983b). The calculation of picomoles of tubulin was made by assuming that the proportions of tubulin and MAPs in the pellets were the same as in the initial reaction mixtures. [b] Nucleoside diphosphate kinase; 5 units was added to the reaction mixture.

[5] A total of 28 different experiments were performed under the following reaction conditions with γ-^{32}P- and/or β-^{32}P-labeled nucleotides, all with tubulin at 1.0 mg/mL: (i) 0 °C; 0.1 M Mes, 0.5 mM $MgCl_2$, and 3, 10, or 100 μM nucleotide; incubation times varying from 10 min to 1 h plus one experiment with an overnight incubation; (ii) 0 °C; 0.1 M Mes, 0.5 mM $MgCl_2$, 0.2 mg/mL heat-treated MAPs, and 10 and 100 μM nucleotide; (iii) 0 °C; 1.0 M glutamate, 1.0 mM $MgCl_2$, and 10 and 100 μM nucleotide; (iv) 0 °C; 0.1 M Mes and 100 μM nucleotide; (v) 0 °C; 0.1 M Mes, 0.2 mg/mL heat-treated MAPs, and 100 μM nucleotide; (vi) 22 °C; 0.1 M Mes, 0.5 mM $MgCl_2$, and 10 μM nucleotide, with no further addition, with 1 mM $CaCl_2$, or with 1 μM vinblastine. In these 28 experiments the maximum ratios of dGTP and ddGTP to GTP bound were respectively 0.76 and 0.31.

amounts of nucleotide were in the pellet whether or not MAPs were included in the polymerization reaction. Thin-layer chromatography was performed on all specimens described in Table V, and in all cases the diphosphate was the predominant form found in the polymer.[6]

Tubulin with [8-^{14}C]GDP in the exchangeable site was used to confirm that polymerization with the active analogues also resulted in displacement of GDP from the polymer (Table VI). As a positive control, when this tubulin supplemented with heat-treated MAPs was polymerized in the presence of UTP and nucleoside diphosphate kinase, 0.84 pmol of [8-^{14}C]GDP per picomole of tubulin was recovered in the pellet. With 0.1 mM GTP supporting polymerization with MAPs (reaction conditions comparable to those used in the experiments of Table V) a small amount of [8-^{14}C]GDP was recovered in the pellet (0.04 pmol/pmol of tubulin), and this was reduced still further with 1.0 mM GTP (to 0.01 pmol/pmol of tubulin). Comparable results were obtained with dGTP and ara-GTP. With 0.1 mM 3′dGTP slightly more [8-^{14}C]GDP remained in the pellet (0.08 pmol/pmol of tubulin), and this, too, was displaced when 1.0 mM 3′dGTP was used to support polymerization. With ddGTP, both with and without MAPs, somewhat more [8-^{14}C]GDP remained in the pellet than with the other nucleotides. With MAPs 0.15 pmol of [8-^{14}C]GDP per picomole of tubulin was in the pellet with 0.1 mM ddGTP, and this was reduced to 0.08 pmol/pmol of tubulin with 1.0 mM ddGTP. Without MAPs 0.09 pmol/pmol of tubulin was in the pellet with 0.2 mM ddGTP and 0.07 pmol/pmol of tubulin with 1.0 mM ddGTP. Thus, compared to the other nucleotides, ddGTP has a reduced ability to displace [8-^{14}C]GDP from the exchangeable site in the course of polymerization. Nevertheless, 90% of the [8-^{14}C]GDP could be displaced by 1.0 mM ddGTP, and significantly more [β-^{32}P]ddGTP than [8-^{14}C]GDP was recovered in the pellet under comparable reaction conditions (cf. Tables V and VI).

Inhibition of Nucleotide Hydrolysis and Binding. The difference between the vigorous hydrolysis of ddGTP and 3′dGTP and the weak binding of these analogues was striking. The same discrepancy exists, to a lesser extent, with dGTP and ara-GTP. Furthermore, the relative order of binding of the five sets of diphosphates and triphosphates more closely paralleled the inhibition of polymerization by the diphosphates than the activity of the triphosphates in supporting the reaction.

Do these findings indicate that nucleotide binding and hydrolysis occur at separate sites on tubulin? To explore this possiblity, we examined whether ddGTP and 3′dGTP would inhibit GTP hydrolysis and binding and, conversely, whether GTP would inhibit ddGTP and 3′dGTP hydrolysis (Figure 5).

As would be predicted from the binding studies with the individual nucleotides, neither ddGTP nor 3′dGTP had a significant effect on GTP binding at up to a 10-fold molar excess over GTP (Figure 5A).

The effects of these two analogues on GTP hydrolysis were more complex (Figure 5A). At 37 °C, lower molar ratios of ddGTP to GTP (5:1 and less) resulted in stimulation of GTP hydrolysis, while essentially no effect was observed at higher ddGTP to GTP ratios. With molar ratios of 3′dGTP to GTP up to 5:1 no significant effect on GTP hydrolysis was observed, with mild inhibition occurring at higher 3′dGTP concentrations. In a similar experiment performed at 20 °C instead of 37 °C, a reaction condition under which little GTP hydrolysis occurs (Figure 4), a 10-fold molar excess of ddGTP and 3′dGTP resulted in marked stimulation of GTP hydrolysis (Table VII), and with dGTP and ara-GTP a weaker stimulation resulted.

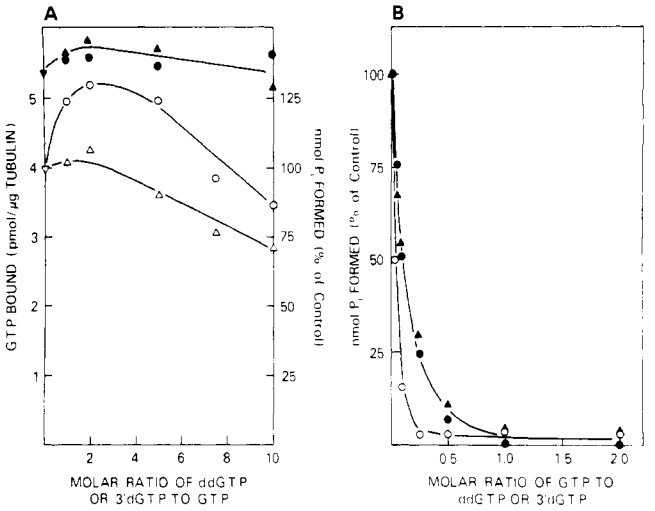

FIGURE 5: Effects of ddGTP and 3′dGTP on GTP binding and hydrolysis and of GTP on ddGTP and 3′dGTP hydrolysis. In all experiments the nucleotides were mixed prior to the addition of the proteins. (A) Minimum inhibitory effects of ddGTP and 3′dGTP on GTP binding and hydrolysis. The binding experiment, represented by the solid symbols, was performed at 0 °C as described in Table III, except that the data are expressed as picomoles of GTP bound per microgram of tubulin. Each reaction mixture contained 1.0 mg/mL tubulin, 0.1 M Mes, 0.5 mM $MgCl_2$, 50 μM [α-^{32}P]GTP, and either no further addition (▼), ddGTP (●) at 50, 100, 250, or 500 μM, or 3′dGTP (▲) at 50, 100, 250, or 500 μM. The GTPase experiment, represented by the open symbols, was performed at 37 °C in a water bath with a 20-min incubation. Each 50-μL reaction mixture contained tubulin, MAPs, Mes, and $MgCl_2$ as described above, 0.1 mM [γ-^{32}P]GTP, and either no further addition (▽), ddGTP (○) at 100, 200, 500, 750, or 1000 μM, or 3′dGTP (△) at 100, 200, 500, 750, or 1000 μM. (B) Potent inhibition of ddGTP and 3′dGTP hydrolysis by GTP. Each 50-μL reaction mixture contained tubulin, Mes, and $MgCl_2$ as described above, GTP at 0, 5, 10, 25, 50, 100, or 200 μM, and one of the following: 100 μM [γ-^{32}P]ddGTP but no MAPs (○), 100 μM [γ-^{32}P]ddGTP and 0.2 mg/mL heat-treated MAPs (●), or 100 μM [γ-^{32}P]3′dGTP and 0.2 mg/mL heat-treated MAPs (▲). Reaction mixtures were incubated for 20 min at 37 °C in a water bath.

Table VII: Analogue Stimulation of GTP Hydrolysis at 20 °C[a]

analogue added	nmol of GDP formed
none	0.6
ddGTP	14.9
3′dGTP	7.9
dGTP	3.0
ara-GTP	4.1

[a] Each 50-μL reaction mixture was incubated at 20 °C for 60 min and contained 0.1 M Mes, 0.5 mM $MgCl_2$, 1.0 mg/mL tubulin, 0.2 mg/mL heat-treated MAPs, 50 μM [α-^{32}P]GTP, and the indicated GTP analogue at 500 μM. The nucleotides were mixed prior to the addition of the proteins.

Very different results were obtained when we examined the effect of GTP on 3′dGTP hydrolysis (with MAPs) and ddGTP hydrolysis (both with and without MAPs) (Figure 5B). These reactions were highly sensitive to inhibition by GTP. Hydrolysis of both 3′dGTP and ddGTP with MAPs was 50% inhibited at a GTP to analogue molar ratio of 1:10 and about 90% inhibited at a GTP to analogue molar ratio of 1:2. Hydrolysis of ddGTP in the absence of MAPs was even more sensitive to inhibition by GTP: 50% inhibition occurred when

[6] The radioactivity in the pellet was 92% GDP after polymerization with GTP + MAPs, 93% dGDP with dGTP + MAPs, 91% ara-GDP with ara-GTP + MAPs, 91% 3′dGDP with 3′dGTP + MAPs, 95% ddGDP with ddGTP + MAPs, and 80% ddGDP with ddGTP but without MAPs.

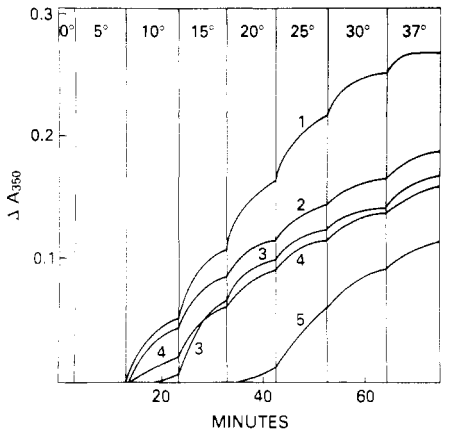

FIGURE 6: Reduced temperature requirement for MAP-dependent polymerization with ddGTP, 3′dGTP, ara-GTP, and dGTP. Each 0.25-mL reaction mixture contained 1.0 mg/mL tubulin, 0.33 mg/mL heat-treated MAPs, 0.1 M Mes, 0.5 mM $MgCl_2$, and the following nucleotides at 1.0 mM: curve 1, ddGTP; curve 2, 3′dGTP; curve 3, ara-GTP; curve 4, dGTP; curve 5, GTP. At the times indicated by the vertical lines, the indicated temperatures were set on the electronic temperature control unit (for these small changes temperature equilibration was complete within 15 s).

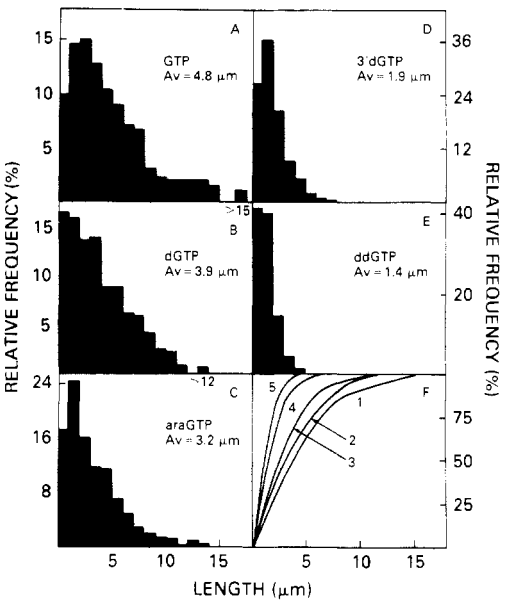

FIGURE 7: Lengths of microtubules in GTP- and analogue-supported polymerization reactions with MAPs. The methodology and reaction conditions are described in detail in the text. (A–E) Histograms of microtubules formed with the indicated nucleotide: (A) GTP, (B) dGTP, (C) ara-GTP, (D) 3′dGTP, and (E) ddGTP. The numbers of microtubules counted were as follows: A, 643; B, 716; C, 829; D, 986; E, 714. (F) Cumulative length distributions with each nucleotide: curve 1, GTP; curve 2, dGTP; curve 3, ara-GTP; curve 4, 3′dGTP; curve 5, ddGTP.

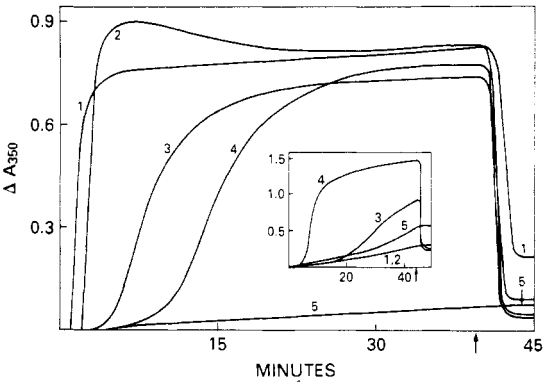

FIGURE 8: MAP-independent polymerization at higher tubulin concentrations. Each 0.25-mL reaction mixture contained 2.0 mg/mL tubulin, 0.1 M Mes, 0.5 mM $MgCl_2$, and the following nucleotides at 1.0 mM: curve 1, ddGTP; curve 2, 3′dGTP; curve 3, ara-GTP; curve 4, dGTP; curve 5, GTP. Inset: MAP-independent tubulin polymerization supported by GTP. Each 0.25-mL reaction mixture contained 0.1 M Mes, 0.5 mM $MgCl_2$, 1.0 mM GTP, and the following indicated amount of tubulin: curve 1, 4.0 mg/mL; curve 2, 4.5 mg/mL; curve 3, 5.0 mg/mL; curve 4, 5.5 mg/mL; curve 5, 5.5 mg/mL but no GTP. In these experiments the electronic temperature controller was set at 37 °C at zero time and at 0 °C at the time indicated by the arrows on the abscissas.

only $^1/_{20}$th as much GTP was present as ddGTP, and inhibition was virtually complete at a GTP to ddGTP molar ratio of 1:4.

To summarize these results, neither ddGTP nor 3′dGTP significantly inhibits either GTP binding to tubulin or the hydrolysis of GTP by tubulin with MAPs. At 20 °C, moreover, ddGTP and 3′dGTP stimulate GTP hydrolysis. Hydrolysis of the analogues, on the other hand, is potently inhibited by GTP, despite the apparent superiority of ddGTP and 3′dGTP over GTP in supporting polymerization and as hydrolytic substrates.

Enhanced Nucleation with Deoxyguanosine Analogues. The faster onset of polymerization with ddGTP, 3′dGTP, ara-GTP, and dGTP as compared to GTP (observed when a circulating water bath is used to warm the reaction mixtures) is partly due to differences in the minimum temperature required for polymerization. Our acquisition of electronic temperature controllers has permitted us to study in greater detail the effects of temperature changes on these reactions (Figure 6). At temperatures as low as 10 °C reactions occur with all four analogues, while a temperature of 20 °C was required for a minimal reaction with GTP. Turbidity development with ddGTP and 3′dGTP was similar to that with GTP at temperatures about 15 °C higher, while ara-GTP and dGTP were about 10 °C ahead of GTP.

If the lower temperature thresholds required for polymerization with the analogues result from enhanced nucleation, then microtubules formed with them should be shorter than those formed with GTP (Sandoval & Weber, 1980; Terry & Purich, 1980). When length distributions of microtubules formed at 37 °C were determined, this was found to be the case (Figure 7). Comparing microtubules formed with GTP, dGTP, ara-GTP, 3′dGTP, and ddGTP, we found that the average microtubule length progressively decreased from 4.8 μm with GTP to 1.4 μm with ddGTP. No long tubules at all (>10 μm) were observed with either ddGTP or 3′dGTP. In addition to histograms of the length distributions with each nucleotide, Figure 7 (panel F) presents a comparison of cumulative lengths, allowing the distributions obtained with all five nucleotides to be compared on the same scale.

A third approach to the relative effects of these analogues on nucleation is to examine polymerization without MAPs, since these proteins are believed to enhance the nucleation process (Sloboda et al., 1976). In our earlier studies (Hamel et al., 1983a) we had examined polymerization reactions in 0.1 M Mes–0.5 mM $MgCl_2$ with tubulin at 1.0 mg/mL. At this protein concentration a MAP-independent reaction was observed only with ddGTP. At 2.0 mg/mL tubulin, however, polymerization occurred as well with the three other analogues, but not with GTP (Figure 8). Polymerization with ddGTP began prior to temperature equilibration at 37 °C while the reactions with 3′dGTP, ara-GTP, and dGTP began after progressively longer lag periods at 37 °C. The order of activity with the four analogues (ddGTP > 3′dGTP > ara-GTP > dGTP) in this MAP-independent reaction is identical with their relative enhancement of nucleation in MAP-dependent

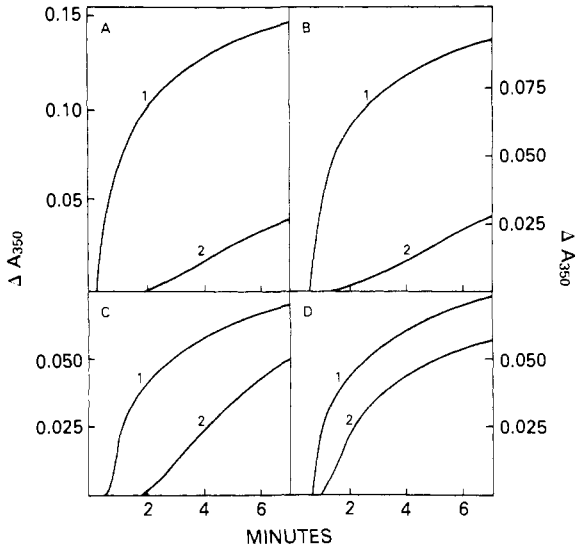

FIGURE 9: Inhibition by equimolar GTP of MAP-dependent, analogue-supported polymerization at 20 °C. Each 0.25-mL reaction mixture contained 1.0 mg/mL tubulin, 0.33 mg/mL heat-treated MAPs, 0.1 M Mes, 0.5 mM $MgCl_2$, the analogue indicated below at 0.5 mM, and either no further addition (curves 1) or 0.5 mM GTP (curves 2): (A) ddGTP, (B) 3′dGTP, (C) ara-GTP, and (D) dGTP. In these experiments the electronic temperature controller was set at 20 °C at zero time. The nucleotides were mixed prior to the addition of the proteins.

polymerization (based on microtubule length distributions).

With GTP there was no MAP-independent polymerization at tubulin concentrations as high as 4.5 mg/mL (Figure 8, inset). A sluggish reaction occurred at 5.0 mg/mL and a brisk one at 5.5 mg/mL. At 5.5 mg/mL polymerization still required exogenous nucleotide; although there was a slow rise in turbidity without GTP (curve 5), the reaction was not cold reversible and probably represents aggregation rather than polymerization.

The high turbidity readings observed in the MAP-independent reactions probably indicate the formation of aberrant structures rather than microtubules (Hamel et al., 1983a), but the morphology of these polymers has not yet been examined except with ddGTP.

Inhibition of Analogue-Supported Polymerization by GTP. The studies presented above with the radiolabeled nucleotides strongly suggest that dGTP, ara-GTP, and especially 3′dGTP and ddGTP have a lower affinity for tubulin than does GTP, despite the marked enhancement of nucleation with the analogues. It proved possible to strengthen this conclusion indirectly by examining inhibitory effects of GTP on analogue-supported polymerization under conditions in which GTP has little activity. In the experiments to be presented below, analogue-supported reactions were compared to those with analogue and equimolar GTP. Although there are three parameters of polymerization (lag period, rate of elongation, and final turbidity plateau) that could be considered, we shall limit this discussion to a comparison of the maximum rate of turbidity development with analogue alone to that with analogue and equimolar GTP. It seems reasonable to assume that if GTP has a greater affinity for tubulin than does the analogue, then equimolar GTP should inhibit the maximum rate of turbidity development with the analogue by at least 50%.

Figure 9 presents such studies of MAP-dependent, analogue-supported polymerization at 20 °C since the GTP-supported reaction at this temperature is minimal (Figure 6). The reactions with ddGTP (Figure 9A) and 3′dGTP (Figure 9B) were strongly inhibited by equimolar GTP, as the maximum rates of turbidity development with the analogues and

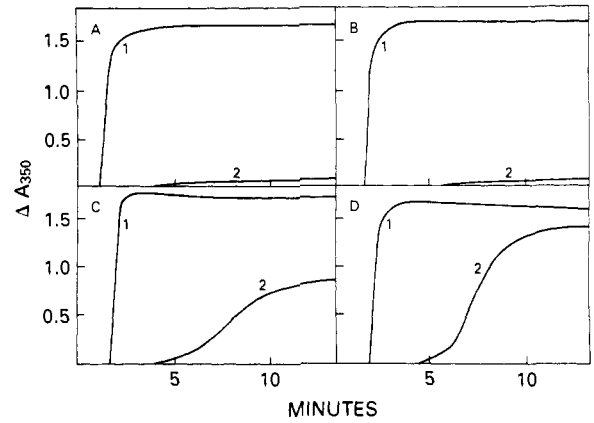

FIGURE 10: Inhibition by equimolar GTP of MAP-independent, analogue-supported polymerization. Each 0.25-mL reaction mixture contained 3.5 mg/mL tubulin, 0.1 M Mes, 0.5 mM $MgCl_2$, the analogue indicated below at 0.5 mM, and either no further addition (curves 1) or 0.5 mM GTP (curves 2): (A) ddGTP, (B) 3′dGTP, (C) ara-GTP, and (D) dGTP. In these experiments the electronic temperature controller was set at 37 °C at zero time. The nucleotides were mixed prior to the addition of tubulin.

GTP were less than 10% of the rates with the analogues alone. With ara-GTP the maximum rate of turbidity development with GTP was about 25% of that of the uninhibited reaction (Figure 9C), and with dGTP the rate of the inhibited reaction was 35% of that with dGTP alone (Figure 9D).

MAP-independent polymerization (tubulin at 3.5 mg/mL) supported by the analogues was even more sensitive to inhibition by GTP (Figure 10). The reactions with ddGTP (Figure 10A) and 3′dGTP (Figure 10B) were totally inhibited by equimolar GTP (incubation times up to 30 min were examined). With ara-GTP the maximum rate of tubidity development with GTP was 5% of that of the uninhibited reaction (Figure 10C), and with dGTP the rate with equimolar GTP was 20% of that with dGTP alone (Figure 10D).

Discussion

Our studies with ribose-modified GDP and GTP analogues have demonstrated notable discrepancies in different aspects of nucleotide interactions at the exchangeable GTP site of tubulin. We have previously found no relationship between the ability of GTP analogues to support polymerization and the ability of cognate GDP analogues to inhibit polymerization, or of the triphosphates' own ability to inhibit MAP-independent, ddGTP-supported polymerization (Hamel et al., 1983a; Hamel & Lin, 1984). In this paper we have extended this observaton to GTP hydrolysis and binding: ddGTP and 3′GTP, two analogues that are highly active in polymerization, have negligible inhibitory activity on the binding of GTP to tubulin and little inhibitory effect on GTP hydrolysis. We have also observed no relationship between the hydrolysis of a nucleotide during polymerization and its binding to tubulin, whether measured by direct means or by displacement of radiolabeled GDP from tubulin.

On the basis of the marked discrepancies between the relative activity of the GTP analogues in supporting polymerization and of the GDP analogues in inhibiting the reaction, we had suggested that the exchangeable site might actually consist of two mutually exclusive sites whose occupancy renders tubulin either polymerizable or nonpolymerizable (Hamel & Lin, 1981b). The finding that ddGDP supported rather than inhibited tubulin polymerization (Hamel et al., 1983b) and the substantial differences between nucleotide binding and nucleotide hydrolysis reported here also suggest two distinct

exchangeable sites on tubulin.

At present, however, we believe a single exchangeable site is more likely. All polymerization reactions supported by ddGDP and ddGTP are significantly more sensitive to inhibition by GDP than is GTP-supported polymerization (Hamel et al., 1983b; Hamel & Lin, 1984), and cognate diphosphates and triphosphates had comparable relative inhibitory effects on MAP-independent, ddGTP-supported polymerization (Hamel & Lin, 1984). Moreover, the studies on the inhibition of nucleotide hydrolysis presented in Figure 5 favor a single exchangeable site. In the presence of MAPs both ddGTP and 3′dGTP are hydrolyzed more rapidly than GTP, but their hydrolysis is potently inhibited by GTP. The more sluggish hydrolysis of GTP, on the other hand, is not greatly affected by ddGTP and 3′dGTP at 37 °C and is even stimulated by the analogues at 20 °C. If given a choice then, tubulin (with MAPs) clearly will hydrolyze GTP in preference to ddGTP or 3′dGTP. (The stimulation of GTP hydrolysis by analogues at 20 °C required a substantial analogue molar excess. Most likely the analogues enhanced nucleation reactions, thereby stimulating GTP hydrolysis associated with elongation and/or the turbidity plateau.) In the absence of MAPs, tubulin at 1.0 mg/mL vigorously hydrolyzes ddGTP, while GTP hydrolysis is minimal. This ddGTPase activity without MAPs is particularly sensitive to GTP inhibition, indicating that tubulin binds GTP in preference to hydrolyzing ddGTP. Finally, under conditions in which it does not support polymerization, GTP has substantial inhibitory effects on both MAP-dependent (Figure 9) and MAP-independent (Figure 10) polymerization supported by ddGTP, 3′dGTP, ara-GTP, and dGTP.

These seemingly contradictory effects probably derive from the potent enhancement of nucleation that occurs with these analogues relative to the reaction with GTP. These superior nucleation reactions manifest themselves by producing larger amounts (i.e., more extensive polymerization; Hamel et al., 1983a) of shorter microtubules (Figure 7), by permitting MAP-dependent assembly at substantially lower reaction temperatures than with GTP (Figure 6), and by permitting MAP-independent polymerization at much lower tubulin concentrations than with GTP (Figure 8).

These findings lead us to conclude that the ability of a guanine nucleotide to support tubulin nucleation and subsequent polymerization, with the nucleotide's associated hydrolysis, is a property distinct from its affinity for the exchangeable site. The relative inhibitory effects of both diphosphates and triphosphates, on the other hand, are comparable to their relative binding to tubulin and thus reflect their affinity for the protein.

We cannot exclude the possibility that a significant increase in affinity of the active triphosphate analogues for tubulin may occur under polymerizing conditions (e.g., +MAPs at 37 °C). At this time, however, we have no means to reliably evaluate dimer-bound nucleotide when polymerization has occurred.

We have thus far not attempted to derive association constants for the binding of these analogues to tubulin, for it is clear from the studies presented above that the centrifugal gel filtration method does not measure a true equilibrium. Nevertheless, the data of Tables III and IV unequivocally demonstrate that the binding constants for ddGTP and 3′dGTP must be at least an order of magnitude higher than that of GTP: not only do smaller amounts of both analogues bind at 100 μM than does GTP at 10 μM but also less [8-^{14}C]GDP was displaced from tubulin by 100 μM ddGTP or 3′dGTP than by 10 μM GTP. The binding constants of ara-GTP and dGTP are probably less than an order of magnitude higher than that of GTP, since these nucleotides at 100 μM displaced more [8-^{14}C]GDP from tubulin than did 10 μM GTP (Table IV).

Except with 10 μM ddGTP, we have detected some binding of active deoxyguanosine analogues under all conditions in which polymerization occurs. Evidently nucleation can begin with these analogues even though they do not saturate the exchangeable site. Further, the analogue studies demonstrate that nucleotide binding must play no role as the rate-limiting step in nucleation, consistent with the well-described rapid equilibration of GTP and GDP at the exchangeable site (Weisenberg et al., 1968, 1976; Bryan, 1972; Berry & Shelanski, 1972; Jacobs et al., 1974; Levi et al., 1974; Penningroth & Kirschner, 1977; MacNeal & Purich, 1978; Caplow & Zeeberg, 1980).

Nucleotide hydrolysis, unlike binding, is more closely related to a GTP analogue's ability to support polymerization, although polymerization with ddGDP occurs without nucleotide hydrolysis (Hamel et al., 1983b). A characteristic pattern of hydrolysis was observed with GTP and the deoxyguanosine analogues when MAPs were present. An initial burst of hydrolysis associated with the onset of elongation was followed by a sustained hydrolytic reaction at the turbidity plateau. With ddGTP in the absence of MAPs rapid nucleotide hydrolysis began about 5 min prior to the onset of polymerization, and the rate of hydrolysis was always significantly greater than the rate of polymerization. The significance of this uncoupled ddGTP hydrolysis is unclear, but it may represent abortive nucleation or elongation events.

Our finding of initially simultaneous and stoichiometric polymerization and hydrolysis of GTP and a number of GTP analogues with tubulin + heat-treated MAPs should be compared to reports of other workers using microtubule protein (unresolved tubulin and MAPs). MacNeal & Purich (1978) found that when GTP was prebound to the tubulin in a microtubule protein preparation (as well as in a system reconstituted with tubulin and MAPs prepared on phosphocellulose), GTP hydrolysis and polymerization were simultaneous and stoichiometric. On the other hand, Sandoval & Weber (1980) reported a lag in hydrolysis compared to polymerization using microtubule protein and [^{3}H]GTP and an even more impressive lag in hydrolysis when [^{3}H]guanosine 5′-(α,β-methylenetriphosphate) was used to support polymerization. They were, however, unable to demonstrate the presence of significant amounts of radioactive GTP or guanosine 5′-(α,-β-methylenetriphosphate) in the polymer. Carlier & Pantaloni (1982) similarly reported that GTP hydrolysis lagged behind polymerization with microtubule protein when adenosine 5′-(β,γ-imidotriphosphate) was used to inhibit nonspecific GTP hydrolysis. This conclusion was reached in an elegant kinetic analysis in which the hydrolytic reaction occurring at the turbidity plateau was extrapolated into the elongation phase of polymerization and the value obtained subtracted from hydrolysis occurring during elongation. These workers, too, found only [^{3}H]GDP in the polymer, and they reported only 0.55 mol of [^{3}H]GDP per mole of tubulin. In contrast, we have found essentially stoichiometric amounts of [α-^{32}P]GDP in the microtubule pellet (Table V). The most reasonable reconciliation of these differing results is that, depending on either experimental conditions or precise composition of the MAPs, variable amounts of tubulin bearing GDP in the exchangeable site copolymerize with tubulin bearing GTP. Such copolymerization apparently occurs in glutamate-induced polymerization of high concentrations of tubulin (Figure 1;

Table I). Preliminary experiments have indicated that at lower tubulin concentrations significantly less copolymerization occurs in glutamate (data not presented).

Although the variety of effects we have observed with the ribose-modified analogues limits a structure–function analysis, several conclusions are possible. On the basis of the studies of nucleotide binding and inhibition of polymerization, the ribose moiety is an important feature in the affinity of nucleotides for the exchangeable site of tubulin. Any modification results in reduced binding and decreased inhibitory activity. It also appears that the 3′-hydroxyl group is of greater importance than the 2′-hydroxyl in the affinity of nucleotides for tubulin. More extensive binding occurs with dGTP and ara-GTP than with 3′dGTP and ddGTP, as well as with dGDP and ara-GDP than with 3′dGDP and ddGDP. Similarly, dGDP, ara-GDP, and 2′OMeGDP inhibit polymerization to a greater extent than do 3′dGDP, ddGDP, and 3′OMeGDP.

These structural strictures, however, are not applicable to the ability of ribose-modified GTP analogues to support tubulin nucleation and subsequent polymerization and to act as hydrolytic substrates. The key feature in these reactions is an intact and unsubstituted pentose ring, provided minimal exogenous GDP or GTP is present. Removal of one hydroxyl group (or changing its configuration, as in ara-GTP) enhances the activity of a guanine triphosphate nucleotide, and removal of both hydroxyl groups even minimizes the requirement either for MAPs in polymerization and hydrolysis (polymerization with ddGTP – MAPs with 1.0 mg/mL tubulin) or for the γ-phosphate group and the hydrolytic event (polymerization with ddGDP + MAPs).

Registry No. GTP, 86-01-1; GDP, 146-91-8; dGTP, 2564-35-4; dGDP, 3493-09-2; ara-GTP, 72490-81-4; ara-GDP, 84368-38-7; ddGTP, 68726-28-3; ddGDP, 84328-12-1; 3′-dGTP, 55968-37-1; 3′-dGDP, 60814-15-5.

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