Ernst, R. R. (1966) *Adv. Magn. Reson. 2*, 60.
Faulstich, H., Wieland, Th., & Jochum, Ch. (1968) *Liebigs Ann. Chem. 713*, 186.
Gibbons, W. A., Beyer, C. F., Dadok, J., Speicher, R. F., & Wyssbrod, H. R. (1975) *Biochemistry 14*, 420.
Kabsch, W. (1976) *Acta Crystallogr., Sect. A A32*, 922.
Kabsch, W. (1978) *Acta Crystallogr., Sect. A A34*, 827.
Kopple, K. D., Wiley, G. R., & Tauke, R. (1973) *Biopolymers 12*, 627.
Kostansek, E. C., Lipscomb, W. N., Yocum, R. R., & Thiessen, W. E. (1977) *J. Am. Chem. Soc. 99*, 1273.
Kostansek, E. C., Lipscomb, W. N., Yocum, R. R., & Thiessen, W. E. (1978) *Biochemistry 17*, 3790.
Main, P., Hull, S. E., Lessinger, L., Germain, G., Declercq, J. P., & Woolfson, H. M. (1978) MULTAN-78: *A System of Computer Programs for the Automatic Solution of Crystal Structures from X-Ray Diffraction Data*, Universities of York and Louvain.
McConnell, H. (1957) *J. Chem. Phys. 27*, 226.
Nuzzo, R. W., Simon, M. J., & San Filippo, J. H. (1977) *J. Org. Chem. 42*, 568.
Richarz, R., & Wüthrich, K. (1978) *J. Magn. Reson. 30*, 147.
Sheldrick, G. (1975) *Programs for Crystal Structure Determination*, Cambridge University Press, Cambridge, England.
Tonelli, A. E., Patel, D. J., Wieland, Th., & Faulstich, H. (1978) *Biopolymers 17*, 1973.
Wagner, G., & Wüthrich, K. (1979) *J. Magn. Reson. 33*, 657.
Wieland, H., Hallermayer, R., & Zilg, W. (1941) *Liebigs Ann. Chem. 548*, 1.
Wieland, Th., & Boehringer, W. (1960) *Liebigs Ann. Chem. 635*, 178.
Wieland, Th., & Faulstich, H. (1978) *CRC Crit. Rev. Biochem. 5*, 185.
Wieland, Th., Wirth, L., & Fischer, E. (1949) *Liebigs Ann. Chem. 564*, 152.
Wieland, Th., Götzendörfer, Ch., Zanotti, G., & Vaisius, A. C. (1981) *Eur. J. Biochem. 117*, 161.

Modulation of Tubulin–Nucleotide Interactions by Microtubule-Associated Proteins: Polymerization with Ribose-Modified Analogues of Guanosine 5′-Triphosphate†

Ernest Hamel,* Anthony A. del Campo, Jay Lustbader, and Chii M. Lin

ABSTRACT: We have examined the ability of a series of ribose-modified analogues of GTP to support microtubule-associated protein (MAP)-dependent polymerization of purified tubulin in 0.1 M 2-(*N*-morpholino)ethanesulfonate–0.5 mM $MgCl_2$. Both the tubulin and the MAPs were free of nucleosidediphosphate kinase activity. Deoxyguanosine 5′-triphosphate analogues and arabinosylguanosine 5′-triphosphate all supported polymerization reactions in which microtubules were formed and which began earlier, were more extensive, and required a lower nucleotide concentration than the GTP-supported reaction. One of these nucleotides, 2′,3′-dideoxyguanosine 5′-triphosphate (ddGTP), was unique in also supporting polymerization without MAPs, although a higher nucleotide concentration was required and the polymer was a mixture of microtubules and open sheets. The microtubules formed with ddGTP plus MAPs were significantly more cold stable than microtubules formed with GTP plus MAPs, while the polymer formed with ddGTP minus MAPs had similar cold lability to the GTP-dependent tubules. Analogues with an open ribose ring or bearing a methyl or phosphate group at the 2′- or 3′-hydroxyl were also examined. While 2′-*O*-methylguanosine 5′-triphosphate (2′OMeGTP) seemed comparable to GTP in its ability to support MAP-dependent polymerization as a function of nucleotide concentration, progressively decreasing activity was observed with acycloguanosine 5′-triphosphate (acyclo-GTP), 3′-*O*-methylguanosine 5′-triphosphate (3′OMeGTP), and the periodate-oxidized, borohydride-reduced derivative of GTP (ox–red-GTP). The guanosine tetraphosphates had little or no ability to support tubulin polymerization with MAPs. Microtubules were formed only with acyclo-GTP, while the polymer formed with 3′-OMeGTP was a mixture of microtubules and open sheets. The structures formed with 2′OMeGTP and ox–red-GTP were thick-walled, relatively short, enlarged tubules about 82 nm in diameter. No definite protofilaments could be distinguished in these bizarre structures. If nucleosidediphosphate kinase was included in the reaction mixture, however, microtubules became the predominant product with ox–red-GTP. Moreover, 2′OMeGTP and ox–red-GTP, as well as GTP, supported microtubule formation from purified tubulin (in the absence of MAPs) in the presence of 3.4 M glycerol–6 mM $MgCl_2$.

Guanosine nucleotides are intimately involved in the structure and function of tubulin, the major component of the mitotic spindle. The protein binds 2 molar equiv of nucleotide (Weisenberg et al., 1968; Bryan, 1972; Kobayashi, 1974; Hamel & Lin, 1981a). Half cannot be exchanged with free GTP and can only be removed from the protein by its denaturation (the nonexchangeable GTP). The other half can be exchanged with free GDP or GTP and can also be removed from tubulin with difficulty (the exchangeable GTP). Tubulin polymerization is usually associated with hydrolysis of exchangeably bound GTP (Kobayashi, 1975; Weisenberg et al., 1976; Penningroth & Kirschner, 1977;, Arai & Kaziro, 1977;

† From the Laboratory of Medicinal Chemistry and Biology (E.H., J.L., and C.M.L.) and the Laboratory of Experimental Therapeutics and Metabolism (A.A.d.C.), Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205. *Received July 23, 1982; revised manuscript received October 5, 1982.*

David-Pfeuty et al., 1977; MacNeal & Purich, 1978). Although the resultant GDP is not exchangeable in the microtubule, exchangeability is restored after the polymer is disassembled (Weisenberg et al., 1976; Arai & Kaziro, 1977; David-Pfeuty et al., 1977). The functions of the nonexchangeable GTP remain unknown, but it is not hydrolyzed in the assembly–disassembly process.

Since understanding the role of nucleotides in tubulin structure and function in greater detail may aid in the rational design of effective antineoplastic agents, we have been studying the effects of GDP and GTP analogues. In our initial studies, we examined how a series of ribose-modified analogues of GDP and GTP (Hamel & Lin, 1981b; Lustbader & Hamel, 1982) affected glutamate-induced polymerization of purified tubulin which we had demonstrated to be free of both nucleosidediphosphate kinase and ATPase activities (Hamel & Lin, 1981a). While the glutamate system did permit study of direct effects of nucleotides on tubulin, a major consideration in our choosing it was the well-described contamination of both microtubule protein and microtubule-associated proteins (MAPs),[1] as well as some preparations of electrophoretically homogeneous tubulin, by these enzymes (Gaskin et al., 1974; Kobayashi, 1974; Weisenberg et al., 1976; Penningroth & Kirschner, 1977; David-Pfeuty et al., 1978; Jacobs & Huitorel, 1979; Terry & Purich, 1979; Hamel et al., 1981). We have recently observed, however, that heat treatment of MAPs, which has little effect on their ability to promote tubulin polymerization (Fellous et al., 1977; Cleveland et al., 1977), destroys the nucleosidediphosphate kinase and ATPase contaminants (Hamel et al., 1981) as well as a tubulin-independent GTPase activity (David-Pfeuty et al., 1978). We therefore decided to use the ribose-modified analogues to explore whether the MAPs altered tubulin–nucleotide interactions.

Experimental Procedures

Materials. Electrophoretically homogeneous calf brain tubulin free of unbound nucleotide and containing about 1 molar equiv each of bound GDP and GTP was prepared as described previously (Hamel & Lin, 1981a; Hamel et al., 1981), as were nucleotides (Hamel & Lin, 1981b; Lustbader & Hamel, 1982). Heat-treated MAPs, with the DEAE-cellulose chromatography step preceeding heat treatment, were prepared as described previously (Hamel et al., 1981), and their electrophoretic appearance was similar to that described earlier (Hamel et al., 1981). Mes and yeast nucleosidediphosphate kinase were obtained from Sigma, pp(CH_2)pG was from ICN, and Quetol 651 was from Polysciences.

Polymerization Assays. Tubulin polymerization was followed turbidimetrically (Gaskin et al., 1974) in a Gilford Model 250 recording spectrophotometer equipped with a Lauda thermostatically controlled water bath. After base lines were established with the water bath at 0 °C, the thermostat was set at 37 °C. The point at which the water bath reached 37 °C was defined as zero time. In some experiments, turbidity began to rise prior to this point, which is indicated in the appropriate figures by an arrow.

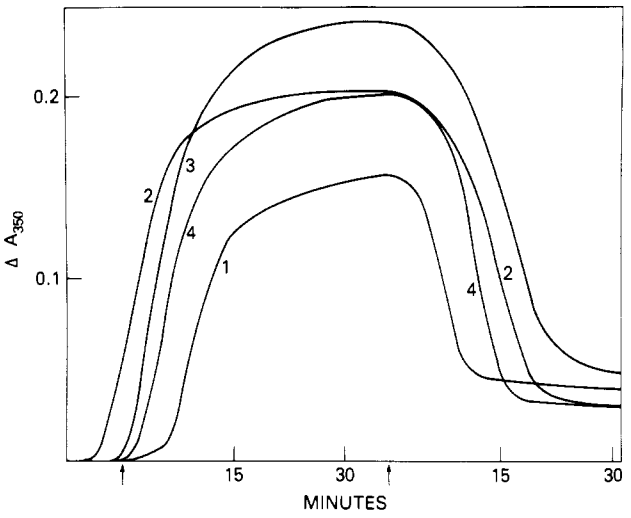

FIGURE 1: MAP-dependent polymerization of tubulin supported by dGTP, 3′dGTP, and ara-GTP. Each reaction mixture contained tubulin, heat-treated MAPs, Mes, and $MgCl_2$ as described in the text and the indicated nucleotide at 50 μM: curve 1, GTP; curve 2, dGTP; curve 3, 3′dGTP; curve 4, ara-GTP. The first arrow on the abscissa indicates the point at which the circulating water bath reached 37 °C and the second arrow the point at which it was again 0 °C (see text for further details).

This method was chosen, rather than adding nucleotides to preincubated tubulin–MAP solutions, to maximize the probability of equilibration of the nucleotides with the proteins. Equally important, if the tubulin–MAP solutions were at 30 °C or higher, polymerization with GTP and the more active analogues began before the reaction mixtures could be completely mixed, but little polymerization occurred with lower concentrations of the less active analogues at lower temperatures. Temperature equilibration in different runs was very similar, and the four cuvettes were within 1.5 °C of each other. The cuvette contents were at 14 °C when the water bath initially reached 37 °C (zero time), at 21 °C at 5 min, at 27 °C at 10 min, at 30.5 °C at 15 min, at 32.5 °C at 20 min, and finally equilibrated at 35 °C by 30–35 min.

When cold reversibility was examined, the thermostat was reset at 0 °C while ice was rapidly added to the circulating water bath. An arrow in the appropriate figures indicates the point at which the water bath was again at 0 °C.

Reaction volume was 0.2 mL, and microcuvettes with a 1.0-cm light path were used. Unless otherwise indicated, reaction mixtures contained 1.0 mg/mL tubulin, 0.2 mg/mL heat-treated MAPs, 0.1 M Mes (adjusted to pH 6.4 with NaOH), 0.5 mM $MgCl_2$, and nucleotides as indicated. Since both tubulin and MAPs were stored in glutamate (Hamel & Lin, 1981a; Hamel et al., 1981), reaction mixtures contained 0.028 M glutamate added with the proteins.

Electron Microscopy. Thin sections and negatively stained specimens were prepared as described previously (Hamel et al., 1981), but in some cases, the tannic acid procedure of Tilney et al. (1973) as modified by Kim et al. (1979) was used.

Results

Deoxy-GTP Analogues. In glutamate-induced polymerization of purified tubulin, we had observed that four GTP analogues, dGTP, 3′dGTP, ddGTP, and ara-GTP, were superior to GTP itself in supporting tubulin polymerization (Hamel & Lin, 1981b; Lustbader & Hamel, 1982). Figures

[1] Abbreviations: MAPs, microtubule-associated proteins; acyclo-GTP, triphosphate derivative at the side-chain hydroxyl of 9-[(2-hydroxyethoxy)methyl]guanine (acycloguanosine); ox-red-GTP, dialcohol derivative of GTP obtained by periodate oxidation and borohydride reduction; ara-GTP, 9-β-D-arabinofuranosylguanine 5′-triphosphate; ddGTP, 2′,3′-dideoxyguanosine 5′-triphosphate; 3′dGTP, 3′-deoxyguanosine 5′-triphosphate; 2′OMeGTP, 2′-*O*-methylguanosine 5′-triphosphate; 3′OMeGTP, 3′-*O*-methylguanosine 5′-triphosphate; 2′PGTP, guanosine 2′-monophosphate 5′-triphosphate; 3′PGTP, guanosine 3′-monophosphate 5′-triphosphate; pp(CH_2)pG, guanosine 5′-(α,β-methylenetriphosphate); Mes, 2-(*N*-morpholino)ethanesulfonate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

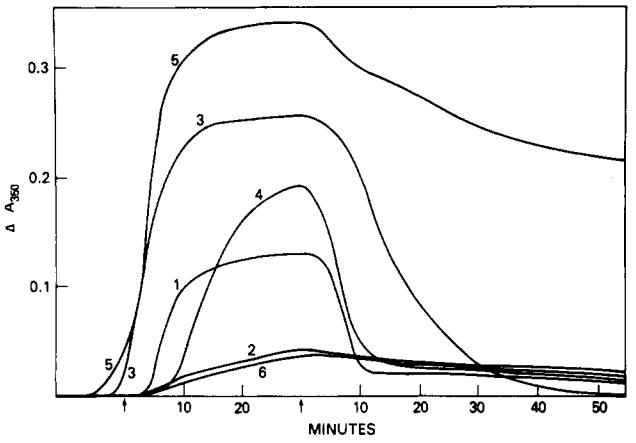

FIGURE 2: Lack of an absolute requirement for MAPs in ddGTP-supported tubulin polymerization. Each reaction mixture contained tubulin, Mes, and $MgCl_2$ as described in the text, 0.2 mg/mL heat-treated MAPs if indicated, and the indicated nucleotide at 0.1 mM: curve 1, MAPs and GTP; curve 2, GTP only; curve 3, MAPs and ddGTP; curve 4, ddGTP only; curve 5, MAPs and $pp(CH_2)pG$; curve 6, $pp(CH_2)pG$ only. The arrows on the abscissa have the same significance as those in Figure 1.

Table I: Proportion of Protein Polymerized As Determined by Ultracentrifugation[a]

nucleotide added	% protein polymerized[b]
0.1 mM GTP	51 (43–61; 8)
0.1 mM dGTP	71 (64–83; 6)
0.1 mM 3′dGTP	61 (50–66; 7)
0.1 mM ara-GTP	70 (57–79; 5)
0.1 mM ddGTP	86 (84–88; 4)
0.1 mM ddGTP – MAPs	42 (36–48; 3)
0.1 mM 2′OMeGTP	68 (64–71; 3)
0.5 mM GTP	49 (46–52; 3)
0.5 mM 3′OMeGTP	47 (44–50; 3)
0.5 mM acyclo-GTP	39 (37–41; 3)
0.5 mM ox-red-GTP	43 (41–45; 3)

[a] Each reaction mixture contained tubulin, Mes, $MgCl_2$, and MAPs, except where indicated, as described in the text and the indicated nucleotide. They were incubated in the spectrophotometer as described in the text. After 1 h at 37 °C, they were centrifuged for 30 min at 40 000 rpm in a Beckman Ti 50 rotor. The protein concentrations of the total reaction mixtures and the postcentrifugation supernatants were determined by the method of Lowry et al. (1951) to determine the percent protein polymerized. [b] The initial value represents the average of all experiments, while those in parentheses represent the range of values obtained, followed by the number of experiments.

1 and 2 demonstrate that these analogues are also superior to GTP in MAP-dependent polymerization. With all four compounds, polymerization began earlier than with GTP,[2] and reactions supported by them consistently reached higher turbidity plateaus, particularly with ddGTP. In addition, when the polymer was isolated by ultracentrifugation, we have found a larger percentage of protein in the pellet with these four analogues than with GTP (Table I). Figure 1 also demonstrates that the reactions supported by dGTP, 3′dGTP, and ara-GTP are similar to the GTP-supported reaction in their cold reversibility. Penningroth & Kirschner (1978) have previously reported similar results with dGTP.

[2] This is due, at least in part, to a lower temperature threshold for polymerization with these analogues than with GTP, perhaps indicating enhanced nucleation. At 20 °C in this system, GTP-supported polymerization is minimal, but substantial reactions occur with all four analogues (data not presented).

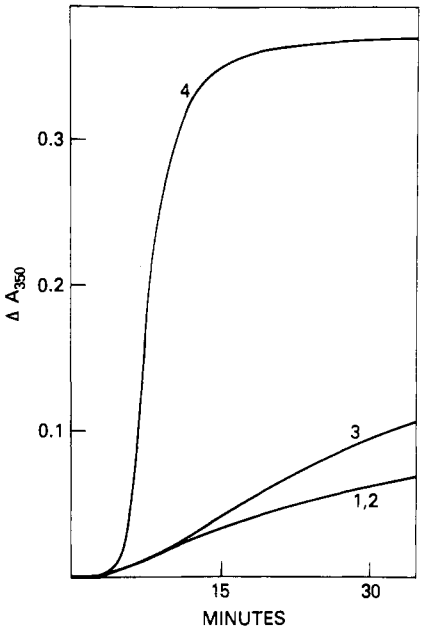

FIGURE 3: MAP-independent tubulin polymerization at higher nucleotide and tubulin concentrations. Each reaction mixture contained Mes and $MgCl_2$ as described in the text, 2.0 mg/mL tubulin, and the indicated nucleotide at 1.0 mM: curve 1, no nucleotide; curve 2, GTP; curve 3, $pp(CH_2)pG$; curve 4, ddGTP.

An unexpected finding with ddGTP, but not with any other analogue we have studied, was that polymerization occurred even without MAPs under the ionic conditions used in these experiments (Figure 2, curve 4). The reaction supported by ddGTP without MAPs was rapidly cold reversible, while with MAPs complete reversal in the cold was much slower (Figure 2, curve 3). Onset of polymerization was also delayed for about 10 min when MAPs were not included in the ddGTP-supported reaction. Although the turbidity plateau was generally higher with ddGTP – MAPs than with GTP + MAPs (Figure 2), a smaller amount of polymer was recovered by centrifugation (Table I).

Since these findings with ddGTP are reminiscent of those reported by Sandoval et al. (1977) with $pp(CH_2)pG$, we also examined the latter GTP analogue (Figure 2). We confirmed their observation that with MAPs intense turbidity developed with $pp(CH_2)pG$ and that the reaction was not cold reversible. We were, however, unable to obtain polymerization of purified tubulin without MAPs with $pp(CH_2)pG$, contrary to the report of Sandoval et al. (1977). Even at the higher nucleotide and tubulin concentrations used in the experiment shown in Figure 3, significant polymerization occurred only with ddGTP. The reason for this difference between our results and those of Sandoval et al. (1977) probably derives from significant differences in the tubulin preparations. They used charcoal-treated tubulin depleted of exchangeable nucleotide (Sandoval et al., 1977), while our tubulin has GDP initially bound in the exchangeable site (Hamel & Lin, 1981a). Tubulin freed of exchangeably bound nucleotide appears to have limited nucleotide specificity [Penningroth & Kirschner, 1978; Purich & MacNeal, 1978; cf. Lustbader & Hamel (1982) with Kirsch & Yarbrough (1981)] and may also have a reduced requirement for MAPs [cf. Kirsch & Yarbrough (1981)]. An additional difference is that our studies are with calf brain tubulin, while Sandoval et al. (1977) used rat brain tubulin.

The MAP-dependent reaction supported by GTP is almost unaffected by not adding Mg^{2+} to the reaction mixture (Figure 4, inset.[3] Similarly, with ddGTP if MAPs are present, added

Table II: Characteristics of Tubulin Polymerization Reactions Supported by GTP Analogues

nucleotide	min concn for polymerization[a] (μM)	onset of polymerization rel to GTP at 50 μM[b]	amount of tubulin polymerized rel to GTP[c]	polymer morphology	GTP-supported polymerization inhibited?[d]
GTP	10		1.0	microtubules	
ddGTP	5	earlier	1.7	microtubules	
ddGTP – MAPs	70	delayed	0.8	microtubules + sheets	
dGTP	5	earlier	1.4	microtubules	
3'dGTP	5	earlier	1.2	microtubules	
ara-GTP	5	earlier	1.4	microtubules	
2'OMeGTP	10	similar	1.3	80-nm tubules	
acyclo-GTP	30	delayed	0.8	microtubules	no
3'OMeGTP	40	delayed	1.0	microtubules + sheets	no
ox-red-GTP	50	delayed	0.9	80-nm tubules	no
2'PGTP[e]					no
3'PGTP[e]					no

[a] The minimum concentration at which a significant rise in turbidity relative to the base line without nucleotide was observed. No experiments were performed with less than 5 μM nucleotide or at concentrations between 5 and 10 μM. [b] Derived from Figures 1, 2, 7, and 8. [c] Derived from Table I. [d] The reaction mixtures contained tubulin, heat-treated MAPs, Mes, and $MgCl_2$ as described in the text and nucleotides as follows: in the experiments with acyclo-GTP, 3'OMeGTP, and ox-red-GTP, the GTP concentration was 10 μM and the concentration of the potential inhibitors was 50 μM; in the experiments with 2'PGTP and 3'PGTP, the GTP concentration was 50 μM and the concentration of the potential inhibitors was 250 μM. There was no significant difference in the time of onset, the rate of polymerization, or the turbidity plateau in any experiment between reaction mixtures containing GTP alone or GTP + a GTP analogue. [e] No significant polymerization observed.

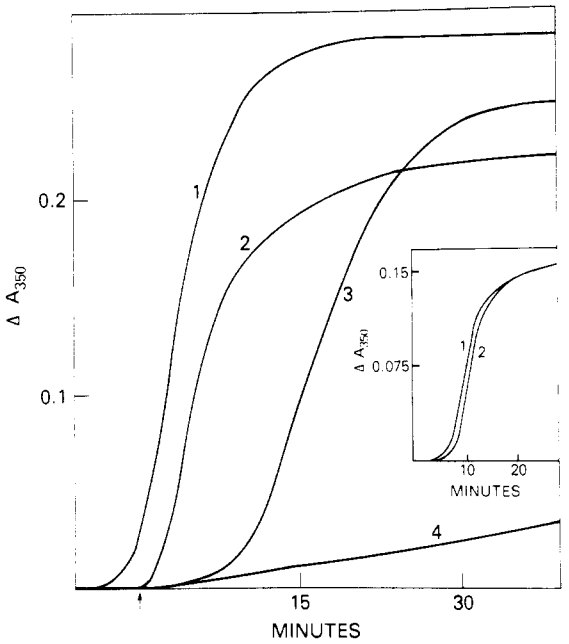

FIGURE 4: Mg^{2+} requirement for MAP-independent tubulin polymerization with ddGTP. Each reaction mixture contained tubulin and Mes as described in the text, 0.1 mM ddGTP, and if indicated 0.2 mg/mL heat-treated MAPs and 0.5 mM $MgCl_2$: curve 1, MAPs and $MgCl_2$; curve 2, MAPs only; curve 3, $MgCl_2$ only; curve 4, no addition. The arrow on the abscissa indicates the point at which the circulating water bath reached 37 °C (see text for further details). Inset: Each reaction mixture contained tubulin, Mes, and heat-treated MAPs as described in the text, 50 μM GTP, and either 0.5 mM $MgCl_2$ (curve 1) or no $MgCl_2$ (curve 2).

Mg^{2+} did not greatly affect the polymerization reaction (Figure 4, curves 1 and 2). Without MAPs, however, polymerization with ddGTP was totally dependent on added Mg^{2+} (Figure 4, curves 3 and 4).

[3] The solutions of Mes and glutamate [in which the tubulin and MAPs were stored (Hamel & Lin, 1981a; Hamel et al., 1981)] used here have been analyzed by atomic absorption spectroscopy and would contribute residual Mg^{2+} to the reaction mixtures of about 0.03 mM. In addition, heat-treated MAPs and tubulin were subjected to gel filtration chromatography in Mg^{2+}-depleted 0.1 M Mes and similarly analyzed. No significant Mg^{2+} was found in the MAPs, but up to 2 molar equiv of the cation remained associated with the tubulin.

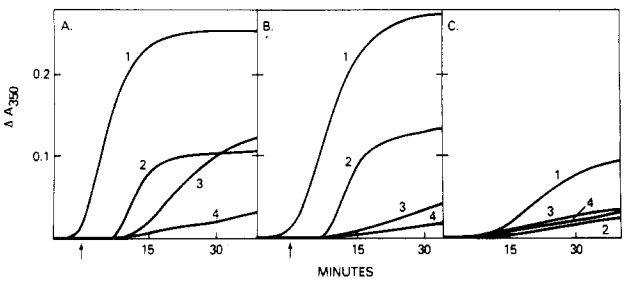

FIGURE 5: Effect of MAPs on the minimum concentration of ddGTP required for tubulin polymerization. Each reaction mixture contained tubulin, Mes, and $MgCl_2$ as described in the text, 0.2 mg/mL heat-treated MAPs if indicated, and the indicated nucleotide at 70 (A), 50 (B), or 5 μM (C): curves 1, ddGTP and MAPs; curves 2, GTP and MAPs; curves 3, ddGTP only; curves 4, MAPs only.

It should also be noted that a higher nucleotide concentration was required for ddGTP-supported polymerization without MAPs than with MAPs (Figure 5). As the ddGTP concentration was lowered, the MAP-independent reaction became progressively more sluggish. At 70 μM ddGTP, a significant reaction still occurred (Figure 5A), but this disappeared by 50 μM ddGTP (Figure 5B). With MAPs, brisk and complete polymerization was observed at 10 μM ddGTP (data not presented), and a delayed, partial reaction even occurred at the substoichiometric concentration of 5 μM ddGTP (Figure 5C). Polymerization also occurred with MAPs at 10 μM GTP, but there was no significant reaction at 5 μM GTP (Figure 5C). [Partial reactions were also observed with ara-GTP, dGTP, and 3'dGTP at 5 μM nucleotide (see Table II).]

Figure 6 demonstrates the morphology of the polymer formed with the deoxy-GTP analogues (including ara-GTP). With MAPs, microtubules were the predominant polymerization product with all four analogues (Figure 6A–D). The polymer formed with ddGTP without MAPs was a mixture of microtubules, open structures (sheets), and tubules with attached sheets (Figure 6E). If no nucleotide was added to the reaction mixture, a small pellet with an amorphous appearance was obtained (Figure 6F).

Ribose-Substituted and Open Ribose Ring GTP Analogues. In the glutamate-dependent polymerization reaction, GTP analogues with an open ribose ring or bearing a methyl or

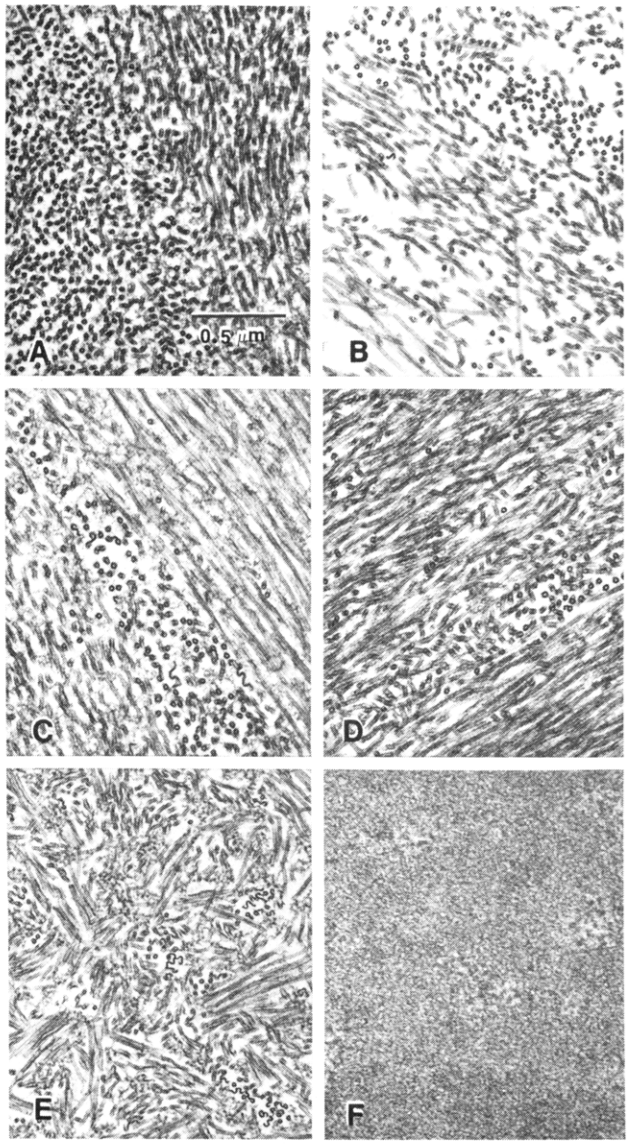

FIGURE 6: Morphology of polymers formed with dGTP, 3′dGTP, ara-GTP, and ddGTP. Reaction mixtures contained in a 0.3-mL volume Mes, $MgCl_2$, tubulin, and heat-treated MAPs as described in the text, unless indicated otherwise, and nucleotides as indicated. After 1 h at 37 °C, the reaction mixtures were centrifuged, the pellets fixed with glutaraldehyde, and thin sections prepared and examined in the electron microscope as described previously (Hamel et al., 1981). All magnifications are 26000×. (A) Thin section of polymer formed with dGTP and MAPs. The reaction mixture contained 0.2 mM dGTP. (B) Thin section of polymer formed with 3′dGTP and MAPs. The reaction mixture contained 0.2 mM 3′dGTP. (C) Thin section of polymer formed with ara-GTP and MAPs. The reaction mixture contained 0.2 mM ara-GTP. (D) Thin section of polymer formed with ddGTP and MAPs. The reaction mixture contained 0.2 mM ddGTP. (E) Thin section of polymer formed with ddGTP without MAPs. The reaction mixture contained 0.2 mM ddGTP, but the MAPs were omitted. (F) Thin section of protein pellet obtained with no nucleotide. The reaction mixture contained 2 mg/mL tubulin and 0.4 mg/mL MAPs but no nucleotide.

phosphate group at the 2′- or 3′-position were all deficient in supporting tubulin polymerization relative to GTP (Hamel & Lin, 1981b; Lustbader & Hamel, 1982). Particularly at lower nucleotide concentrations, reactions supported by these analogues began later than the GTP-supported reaction, and turbidity development was generally slower. If a reaction was initiated, however, similar turbidity plateaus were reached with all nucleotides (Hamel & Lin, 1981a; Lustbader & Hamel, 1982). These experiments had demonstrated the following order of activity: GTP > acyclo-GTP > 2′OMeGTP > 3′OMeGTP > ox–red-GTP > 3′PGTP > 2′PGTP.

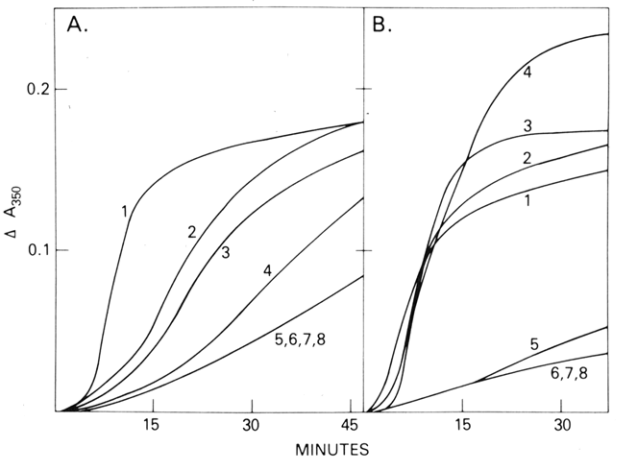

FIGURE 7: MAP-dependent polymerization supported by acyclo-GTP, 3′OMeGTP, and ox–red-GTP. Each reaction mixture contained tubulin, heat-treated MAPs, Mes, and $MgCl_2$ as described in the text and the indicated nucleotide at either 50 μM (A) or 0.5 mM (B): curves 1, GTP; curves 2, acyclo-GTP; curves 3, 3′OMeGTP; curves 4, ox–red-GTP; curves 5, 3′PGTP; curves 6, 2′PGTP; curves 7, ATP; curves 8, no nucleotide.

Figure 7 demonstrates that with five of these analogues essentially comparable results were obtained with MAPs. At 50 μM nucleotide (Figure 7A), progressively later and more sluggish reactions occurred with acyclo-GTP, 3′OMeGTP, and ox–red-GTP, while 3′PGTP and 2′PGTP were inactive. At 0.5 mM nucleotide, there was an equivocal reaction with 3′PGTP, while the more active analogues all supported brisk polymerization reactions. At concentrations over 0.1 mM, we have consistently observed higher turbidity plateaus with ox–red-GTP and 3′OMeGTP than with GTP, while the turbidity plateaus with acyclo-GTP have been similar to those with GTP. When the extent of polymerization with these analogues was assessed by centrifugation, however, in comparison to GTP, less protein was in the pellet with ox–red-GTP and acyclo-GTP and a comparable amount with 3′OMeGTP (Table I).

Figure 7 also demonstrates that ATP is without activity in this system, a result consistent with the lack of nucleoside-diphosphate kinase activity in both the purified tubulin (Hamel & Lin, 1981a) and the heat-treated MAPs (Hamel et al., 1981). We also could obtain no polymerization with 2′,3′-dideoxyadenosine 5′-triphosphate (data not presented).

One ribose-substituted GTP analogue, 2′OMeGTP, had strikingly different activity with MAPs as compared to glutamate (Hamel & Lin, 1981b; Lustbader & Hamel, 1982). In Figure 8, 2′OMeGTP is compared to GTP, as well as dGTP and ddGTP, at 50 μM nucleotide. The onset of polymerization was simultaneous with 2′OMeGTP and GTP, but the turbidity plateau was much higher with the analogue, exceeding even that obtained with ddGTP. By centrifugation, even though more protein was polymerized with 2′OMeGTP than with GTP, the proportion of protein polymerized was more comparable to the results obtained with dGTP than with ddGTP (Table I). Polymerization with 2′OMeGTP was comparable in its onset to polymerization with GTP at concentrations as low as 10 μM, while neither nucleotide was active at 5 μM (see Table II).

The polymerization reactions supported by acyclo-GTP, 2′OMeGTP, 3′OMeGTP, and ox–red-GTP were all cold reversible and required MAPs in 0.1 M Mes–0.5 mM $MgCl_2$. Not adding $MgCl_2$ did not abolish the polymerization reactions supported by these analogues but, in preliminary experiments,

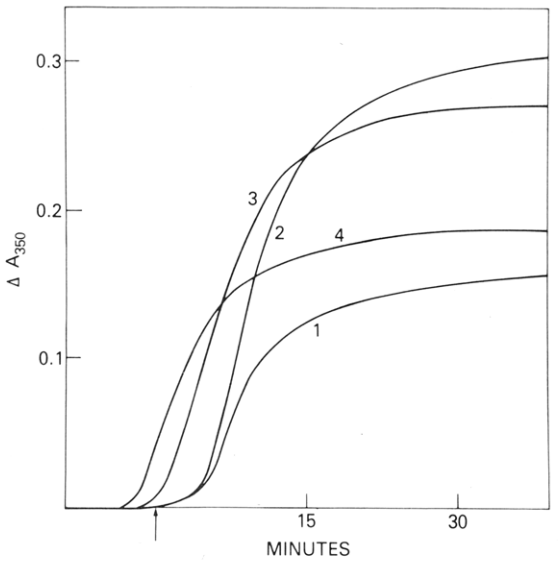

FIGURE 8: MAP-dependent polymerization supported by 2′OMeGTP. Each reaction mixture contained tubulin, heat-treated MAPs, Mes, and $MgCl_2$ as described in the text and the indicated nucleotide at 50 μM: curve 1, GTP; curve 2, 2′OMeGTP; curve 3, ddGTP; curve 4, dGTP. The arrow on the abscissa indicates the point at which the circulating water bath reached 37 °C (see text for further details).

appeared to result in slower reactions with lower turbidity plateaus (data not presented).

When polymer morphology was examined (Figure 9), we found that only the polymer formed with acyclo-GTP had the appearance of microtubules (Figure 9A). With 3′OMeGTP, a mixture of microtubules and open sheets (Figure 9B), similar to the polymer formed with ddGTP without MAPs, was formed. With both 2′OMeGTP (Figure 9C) and ox–red-GTP (Figure 9D), unusual and relatively uniform, large tubular structures were formed, along with what appears to be many fragments of these structures. The diameter of these large tubules averages 82.5 nm with 2′OMeGTP and 81.9 nm with ox–red-GTP, as compared to a diameter of 28.9 nm for microtubules formed with GTP (measurements were made on micrographs of polymers fixed with glutaraldehyde–tannic acid; see below).

Our findings with ox–red-GTP differ significantly from those reported by Kirsch & Yarbrough (1981) using microtubule protein. These workers found ox–red-GTP to be as effective as GTP in supporting formation of microtubules, while we have observed reduced activity and formation of aberrant structures. Microtubule protein has often been reported to contain nucleosidediphosphate kinase activity (Gaskin et al., 1974; Kobayashi, 1974; Weisenberg et al., 1976; Penningroth & Kirschner, 1977; Jacobs & Huitorel, 1979; Terry & Purich, 1979), and we found that adding exogenous nucleosidediphosphate kinase to our reaction mixtures resulted both in substantial apparent enhancement of the ox–red-GTP-supported polymerization reaction [data not presented; cf. Lustbader & Hamel (1982)] and in formation of microtubules with the analogue (Figure 9E). Although a few large tubules were still observed, 80–90% of the polymer had the appearance of typical microtubules.

We also attempted to determine the fine structure of the polymers formed with 2′OMeGTP and ox–red-GTP. In particular, we wished to determine whether the polymers had protofilaments, but we were unsuccessful in obtaining adequate negatively stained specimens. We therefore turned to glutaraldehyde–tannic acid fixation to enhance resolution in thin sections (Figure 10). The polymers formed with both

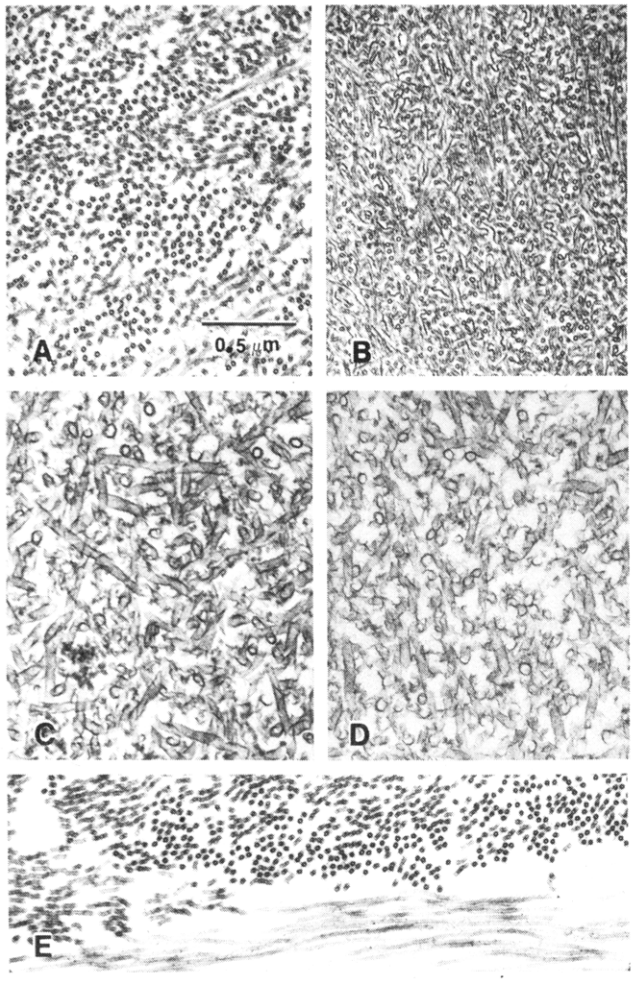

FIGURE 9: Morphology of polymers formed with acyclo-GTP, 3′OMeGTP, 2′OMeGTP, and ox–red-GTP. Reaction mixtures contained in a 0.3-mL volume Mes, $MgCl_2$, tubulin, and heat-treated MAPs as described in the text and GTP analogues and nucleosidediphosphate kinase as indicated. After 1 h at 37 °C, the reaction mixtures were centrifuged, the pellets fixed with glutaraldehyde, and thin sections prepared and examined in the electron microscope as described previously (Hamel et al., 1981). All magnifications are 26000×. (A) Thin section of polymer formed with acyclo-GTP. The reaction mixture contained 0.2 mM acyclo-GTP. (B) Thin section of polymer formed with 3′OMeGTP. The reaction mixture contained 0.2 mM 3′OMeGTP. (C) Thin section of polymer formed with 2′OMeGTP. The reaction mixture contained 0.2 mM 2′OMeGTP. (D) Thin section of polymer formed with ox–red-GTP. The reaction mixture contained 0.2 mM ox–red-GTP. (E) Thin section of polymer formed with ox–red-GTP and nucleosidediphosphate kinase. The reaction mixture contained 0.2 mM ox–red-GTP and 5 units of yeast nucleosidediphosphate kinase.

2′OMeGTP and ox–red-GTP were more labile when fixed with glutaraldehyde–tannic acid than with glutaraldehyde alone, but many of these structures appeared to be well preserved. No definite fine structural detail was visualized in the polymers formed with either 2′OMeGTP (Figure 10A) or ox–red-GTP (Figure 10B). As reported by others (Tilney et al., 1973; Kim et al., 1979), microtubules formed with GTP displayed beadlike protofilaments in cross section (Figure 10C). The walls of the bizarre, large tubules did appear to be thicker than the walls of microtubules. Average wall thickness was 8.1 nm for microtubules, 9.7 nm for the structures formed with 2′OMeGTP, and 10.8 nm for the structures formed with ox–red-GTP. Even if protofilaments are present in these polymers, the significant increase in wall thickness implies that they would be staggered rather than side by side as in microtubules.

We have also found, however, that neither 2′OMeGTP nor

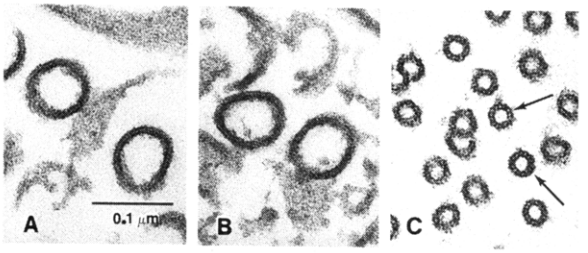

FIGURE 10: High magnification views of microtubules and the polymers formed with 2′OMeGTP, ox–red-GTP, and GTP. Reaction mixtures contained in a 0.3-mL reaction volume Mes, $MgCl_2$, tubulin, and heat-treated MAPs as described in the text and GTP or GTP analogues as indicated. After 1 h at 37 °C, the reaction mixtures were centrifuged as described previously (Hamel et al., 1981). The pellets were fixed with tannic acid and glutaraldehyde and further processed as described by Kim et al. (1979), except that the pellets were embedded in Quetol 651 (Kushida, 1974). All magnifications are 108000×. (A) Thin section of polymer formed with 2′OMeGTP. The reaction mixture contained 0.2 mM 2′OMeGTP. (B) Thin section of polymer formed with ox–red-GTP. The reaction mixture contained 0.2 mM ox–red-GTP. (C) Thin section of polymer formed with GTP. The reaction mixture contained 0.2 mM GTP.

ox–red-GTP prevents formation of either protofilaments or microtubules when purified tubulin was polymerized without MAPs. With high concentrations of glycerol and Mg^{2+} (Lee & Timasheff, 1975), typical microtubules with parallel protofilaments were the predominant polymer formed with 2′OMeGTP (Figure 11A) and ox–red-GTP (Figure 11B), as well as with GTP (Figure 11C).

Minimum Analogue Concentrations Required for Polymerization and Failure of Weakly Active Analogues To Inhibit GTP-Supported Polymerization. Table II presents a summary of our findings with this series of ribose-modified GTP analogues, including the observations described in detail above on polymer morphology, centrifugal analysis of the proportion of protein polymerized, and relative time of onset of polymerization. In addition, Table II summarizes our experience in establishing threshold nucleotide concentrations required for polymerization and our inability to demonstrate inhibition of GTP-supported polymerization with nucleotides less active than GTP.

Without exception in the MAP-dependent reactions, there is a clear correlation between the onset of polymerization and the threshold concentration required for the reaction. The four nucleotides (ddGTP, dGTP, 3′dGTP, and ara-GTP) which initiate polymerization before GTP have partial activity at the substoichiometric concentration of 5 μM. The reactions supported by GTP and 2′OMeGTP, which have a similar time of onset, require at least 10 μM nucleotide. Finally, the analogues which support progressively later reactions (acyclo-GTP, 3′OMeGTP, and ox–red-GTP) are required in progressively higher concentrations. Moreover, the reactions with all analogues at least as active as GTP were more extensive than the GTP-supported reaction, while the reactions with the weaker analogues were either comparable or less extensive than the reaction with GTP.

It seems likely from these findings that the more active analogues would bind at least as readily as GTP in the exchangeable site. The less active analogues might bind in the site but be deficient at a subsequent step in the polymerization reaction. If this were the case, they should inhibit GTP-dependent polymerization. This interesting possibility was excluded, however, because we were unable to demonstrate any inhibition of this reaction with a 5-fold molar excess of acyclo-GTP, 3′OMeGTP, ox–red-GTP, 3′PGTP, or 2′PGTP (Table II), confirming similar observations with glutamate-induced polymerization (Hamel & Lin, 1981b). With a 5-fold molar excess of GDP, polymerization was almost completely inhibited [data not presented; cf. Weisenberg et al. (1976) and MacNeal & Purich (1978)].

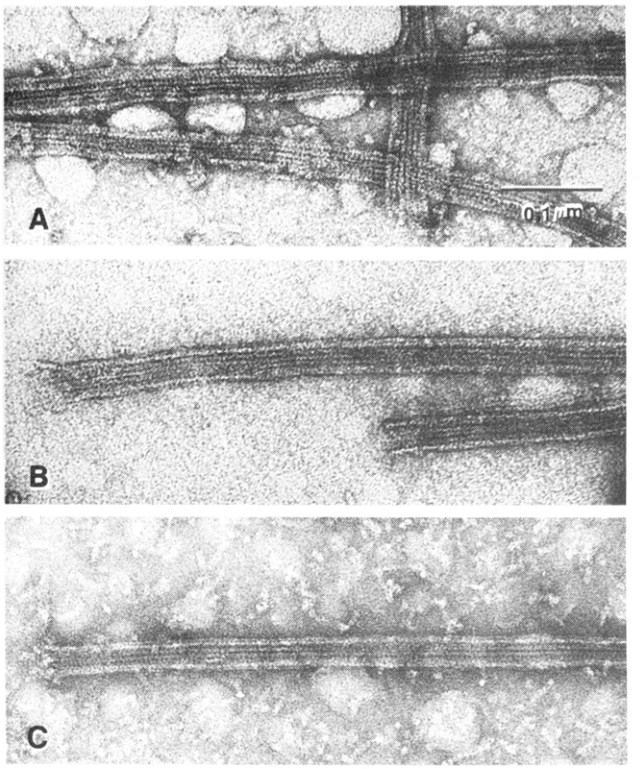

FIGURE 11: Morphology of polymers formed from purified tubulin with 2′OMeGTP, ox–red-GTP, and GTP in glycerol–$MgCl_2$. Negatively stained specimens were prepared as described previously (Hamel et al., 1981) after 1 h at 37 °C. All magnifications are 132600×. Each 50-μL reaction mixture contained 4.0 mg/mL tubulin, 3.4 M glycerol, 6 mM $MgCl_2$, 50 mM Mes, 0.5 mM EGTA, and 1.0 mM 2′OMeGTP (A), ox–red-GTP (B), or GTP (C).

Discussion

With the exception of only 2′OMeGTP, the order of relative nucleotide activity in MAP-dependent tubulin polymerization is the same as that reported earlier for the glutamate-induced reaction (Hamel & Lin, 1981b; Lustbader & Hamel, 1982). The deoxy-GTP analogues dGTP, 3′dGTP, and ddGTP, as well as ara-GTP, are superior to GTP, while the ribose-substituted and open ribose ring analogues acyclo-GTP, 3′OMeGTP, ox–red-GTP, 3′PGTP, and 2′PGTP are progressively less active than GTP. With MAPs, 2′OMeGTP appears to have activity comparable to GTP, whereas with glutamate, it was substantially less active than both GTP and acyclo-GTP (Lustbader & Hamel, 1982).

More striking than the relatively greater activity of 2′OMeGTP, however, was the morphology of the structures this analogue, as well as ox–red-GTP, induced in the MAP-dependent reaction. These were thick-walled, relatively short, large tubules in which no definite protofilaments could be distinguished. In MAP-independent polymerization induced by glycerol–Mg^{2+} (Lee & Timasheff, 1975), however, 2′OMeGTP and ox–red-GTP supported formation of microtubules with parallel protofilaments indistinguishable from those formed with GTP. Preliminary electron micrographic studies in 1.0 M glutamate have also demonstrated that the polymers formed with 2′OMeGTP and ox–red-GTP have parallel protofilaments (data not presented), as does the polymer formed with GTP (Hamel et al., 1982).

We are aware of only one other report in which use of a GTP analogue resulted in structures of altered morphology in the presence of MAPs: Sandoval & Weber (1980) reported that MAP-2 with pp(CH_2)pG induced the formation of short ribbons with an average of six protofilaments while MAP-2 with GTP induced the formation of microtubules.

The results reported here remain consistent with the hypothesis that the ribose-substituted and open ribose ring analogues have a reduced affinity for tubulin, resulting in relative exclusion from the exchangeable site, as a result of steric factors (Hamel & Lin, 1981b; Lustbader & Hamel, 1982). Either because of the MAPs or because of the ionic environment (0.1 M Mes–0.5 mM $MgCl_2$ as opposed to 1.0 M glutamate), this exclusion no longer extends to 2′OMeGTP.

Only one of the ribose-substituted and open ribose ring analogues, acyclo-GTP, supported formation of microtubules, while a second, 3′OMeGTP, supported formation of a mixture of microtubules and open sheets. The other compounds with significant activity, 2′OMeGTP and ox–red-GTP, supported the formation of highly abberant polymerization products rather than microtubules when bound in the exchangeable site in the MAP-dependent reaction examined here. Apparently, when the ribose ring is disrupted in a sterically unfavorable manner, polymer morphology as well as the nucleation and elongation phases of polymerization can be affected.

A second unexpected finding in our study of MAP-dependent polymerization with GTP analogues was that ddGTP was able to support tubulin polymerization without MAPs in 0.1 M Mes–0.5 mM $MgCl_2$. Only ddGTP had this property in our system. With MAPs, morphologically typical microtubules were the polymerization product formed with ddGTP while without MAPs a mixture of microtubules and open sheets was formed. The MAPs also had other significant effects on both the reaction and the polymer formed with ddGTP.

With MAPs, both the GTP- and ddGTP-supported reactions have at most a minimal Mg^{2+} requirement [cf. Buttlaire et al. (1980)], since added $MgCl_2$ had little effect on polymerization. Without MAPs, however, added Mg^{2+} was essential for polymerization. With MAPs, the onset of polymerization occurred much earlier, and both the extent of polymerization and the turbidity plateau were higher. With MAPs, there was also a significantly lower threshold for ddGTP. Without MAPs, at least 50–70 μM ddGTP was required for sluggish polymerization of tubulin at 1 mg/mL (9 μM), but with MAPs, partial polymerization occurred with 5 μM ddGTP.

The cold stability of the polymer formed with ddGTP was also affected by the presence of MAPs. If MAPs were not added with ddGTP, the polymer was as cold labile as microtubules formed with GTP and MAPs. But with MAPs and ddGTP, the microtubules formed were more cold stable than the polymer formed with any other nucleotide we have examined except pp(CH_2)pG [cf. Sandoval et al. (1977)], although ultimately they were completely disrupted.

These observations with ddGTP indicate that the MAPs enhance the affinity of nucleoside triphosphates for the exchangeable binding site of tubulin, reduce the requirement for Mg^{2+} in polymerization [Herzog & Weber (1977) have, in fact, demonstrated polymerization of purified tubulin at high Mg^{2+} concentrations in 0.1 M piperazine-*N*,*N*′-bis(2-ethanesulfonate)], and help stabilize the microtubule once it has formed [cf. Zackroff et al. (1980) and Carlier & Pantaloni (1982)].

Finally, we have found that in 0.1 M Mes–0.5 mM $MgCl_2$ both the turbidity plateau and the amount of polymer formed varied greatly, depending on which nucleotide was used to support polymerization. [Glutamate-induced polymerization had shown much less variability (Hamel & Lin, 1981b; Lustbader & Hamel, 1982; unpublished results).] With GTP, an average of half the protein was polymerized, although a range of 43–61% was observed.[4] In experiments in which direct comparisons were made, the higher turbidities observed with dGTP, ara-GTP, and ddGTP + MAPs were directly proportional to the higher amounts of polymer, in the form of microtubules, recovered with these nucleotides as compared to GTP. Even though microtubules are also formed with 3′dGTP and acyclo-GTP, the turbidity readings with these analogues were almost always disproportionately higher than the amount of polymer recovered by centrifugation. The reason for this is not known but could conceivably result from differences in protofilament number. With ddGTP – MAPs, 3′OMeGTP, 2′OMeGTP, and ox–red-GTP, the turbidity readings were also disproportionately higher than the amount of polymer recovered. In all four cases, aberrant structures were prominent in the polymer. Both the sheets formed with ddGTP – MAPs and 3′OMeGTP [cf. Himes et al. (1977) and Waxman et al. (1981)] and the 80-nm tubules formed with 2′OMeGTP and ox–red-GTP were considerably wider than microtubules and apparently scatter light to a greater degree.

In summary, modification of the ribose ring of GTP can have substantial effects on the polymerization of tubulin beyond the apparent relative affinity of the modified analogue for the exchangeable site. Once bound, analogues can affect polymer morphology, the proportion of tubulin polymerized (and hence probably the critical concentration), the requirement for MAPs, and polymer stability. In turn, MAPs may alter the relative affinity of some analogues, as exemplified here by the enhanced activity of 2′OMeGTP, they appear to affect polymer morphology, depending on the nucleotide in the exchangeable site, and they perhaps increase the affinity of all GTP analogues for tubulin if the 10-fold reduction by MAPs of the minimum effective concentration of ddGTP represents a general phenomenon. We are presently preparing radiolabeled analogues to study these tubulin–MAP–nucleotide interactions in further detail.

Acknowledgments

We thank B. Cohen for her help in preparing the manuscript.

Registry No. GTP, 86-01-1; dGTP, 2564-35-4; 3′dGTP, 55968-37-1; ara-GTP, 72490-81-4; ddGTP, 68726-28-3; 2′OMeGTP, 61556-44-3; 3′OMeGTP, 61556-45-4; acyclo-GTP, 66341-18-2; ox–red-GTP, 59122-53-1.

References

Arai, T., & Kaziro, Y. (1977) *J. Biochem. (Tokyo) 82*, 1063–1071.

[4] Although this suggests a critical concentration of about 0.5 mg/mL, these experiments were performed with subsaturating amounts of heat-treated MAPs, due to their scarcity. [We usually obtain about 20 mg from each gram of microtubule protein (Hamel et al., 1981).] Turbidity as a function of MAP concentration was parabolic. The amount of MAPs used here gave 60–70% of the turbidity obtained with 3 times as much MAP, with the tubulin and GTP concentrations held constant. We have also determined critical concentrations by the dilutional method (Weisenberg & Deery, 1976; Karr et al., 1979), but only with GTP + MAPs and ddGTP + MAPs. These values were, respectively, 0.1 and 0.05 mg/mL. It is unclear why the unpolymerized protein obtained with the centrifugal assay is higher than the apparent critical concentrations in the dilutional studies, but it is probably related to our use of subsaturating amounts of MAPs.

Bryan, J. (1972) *Biochemistry 11*, 2611–2616.
Buttlaire, D. H., Czuba, B. A., Stevens, T. H., Lee, Y. C., & Himes, R. H. (1980) *J. Biol. Chem. 255*, 2164–2168.
Carlier, M.-F., & Pantaloni, D. (1982) *Biochemistry 21*, 1215–1224.
Cleveland, D. W., Hwo, S.-Y., & Kirschner, M. W. (1977) *J. Mol. Biol. 116*, 207–225.
David-Pfeuty, T., Erickson, H. P., & Pantaloni, D (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5372–5376.
David-Pfeuty, T., Laporte, J., & Pantaloni, D. (1978) *Nature (London) 272*, 282–284.
Fellous, A., Francon, J., Lennon, A. M., & Nunez, J. (1977) *Eur. J. Biochem. 78*, 167–174.
Gaskin, F., Cantor, C. R., & Shelanski, M. L. (1974) *J. Mol. Biol. 89*, 737–758.
Hamel, E., & Lin, C. M. (1981a) *Arch. Biochem. Biophys. 209*, 29–40.
Hamel, E., & Lin, C. M. (1981b) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3368–3372.
Hamel, E., del Campo, A. A., Lowe, M. C., & Lin, C. M. (1981) *J. Biol. Chem. 256*, 11887–11894.
Hamel, E., del Campo, A. A., Lowe, M. C., Waxman, P. G., & Lin, C. M. (1982) *Biochemistry 21*, 503–509.
Herzog, W., & Weber, K. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1860–1864.
Himes, R. H., Burton, P. R., & Gaito, J. M. (1977) *J. Biol. Chem. 252*, 6222–6228.
Jacobs, M., & Huitorel, P. (1979) *Eur. J. Biochem. 99*, 613–622.
Karr, T. L., Podarsky, A. E., & Purich, D. L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5475–5479.
Kim, H., Binder, L. I., & Rosenbaum, J. L. (1979) *J. Cell Biol. 80*, 266–276.
Kirsch, M., & Yarbrough, L. R. (1981) *J. Biol. Chem. 256*, 106–111.
Kobayashi, T. (1974) *J. Biochem. (Tokyo) 76*, 201–204.
Kobayashi, T. (1975) *J. Biochem. (Tokyo) 77*, 1193–1197.
Kushida, H. (1974) *J. Electron Microsc. 23*, 197.
Lee, J. C., & Timasheff, S. N. (1975) *Biochemistry 14*, 5183–5187.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Lustbadter, J., & Hamel, E. (1982) *Biochim. Biophys. Acta 719*, 215–222.
MacNeal, R. K., & Purich, D. L. (1978) *J. Biol. Chem. 253*, 4683–4687.
Penningroth, S. M., & Kirschner, M. W. (1977) *J. Mol. Biol. 115*, 643–673.
Penningroth, S. M., & Kirschner, M. W. (1978) *Biochemistry 17*, 734–740.
Purich, D. L., & MacNeal, R. K. (1978) *FEBS Lett. 96*, 83–86.
Sandoval, I. V., & Weber, K. (1980) *J. Biol. Chem. 255*, 8952–8954.
Sandoval, I. V., MacDonald, E., Jameson, J. L., & Cuatrecasas, P. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4881–4885.
Terry, B. J., & Purich, D. L. (1979) *J. Biol. Chem. 254*, 9469–9476.
Tilney, L. G., Bryan, J., Bush, D. J., Fujiwara, K., Mooseker, M., Murphy, D. G., & Snyder, D. H. (1973) *J. Cell Biol. 59*, 267–275.
Waxman, P. G., del Campo, A. A., Lowe, M. C., & Hamel, E. (1981) *Eur. J. Biochem. 120*, 129–136.
Weisenberg, R. C., & Deery, W. J. (1976) *Nature (London) 263*, 792–793.
Weisenberg, R. C., Borisy, G. G., & Taylor, E. W. (1968) *Biochemistry 7*, 4466–4479.
Weisenberg, R. C., Deery, W. J., & Dickinson, P. J. (1976) *Biochemistry 15*, 4248–4254.
Zackroff, R. V., Deery, W. J., & Weisenberg, R. C. (1980) *J. Mol. Biol. 139*, 641–659.