# Magnesium Requirements for Guanosine 5′-*O*-(3-Thiotriphosphate) Induced Assembly of Microtubule Protein and Tubulin†

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ABSTRACT: Two conflicting interpretations on the role of guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) in microtubule protein and tubulin assembly have been previously reported. One study finds that GTPγS promotes assembly while another study reports that GTPγS is a potent inhibitor of microtubule assembly. We have examined the potential role of $Mg^{2+}$ to learn if the conflicting interpretations are due to a metal effect. Turbidity, electron microscopy, and nucleotide binding and hydrolysis were used to analyze the effect of the $Mg^{2+}$ concentration on GTPγS-induced assembly of microtubule protein (tubulin + microtubule-associated proteins) in the presence of buffer ± 30% glycerol and in buffer with GTP added before or after GTPγS. GTPγS substantially lowers the $Mg^{2+}$ concentration required to induce cross-linked or clustered rings of tubulin. These cross-linked rings do not assemble well into microtubules, and GTP only partially restores microtubule assembly. However, taxol will promote GTPγS-induced cross-linked rings of microtubule protein to assemble into microtubules. The effect of GTPγS on microtubule protein assembly in the presence of $Zn^{2+}$ with and without added $Mg^{2+}$ suggests that GTPγS also effects the formation of $Zn^{2+}$-induced sheet aggregates. Purified tubulin was used in assembly experiments with $Mg^{2+}$, $Zn^{2+}$, and taxol to better understand GTPγS interactions with tubulin. The optimal $Mg^{2+}$ concentration for assembly of tubulin is lower with GTPγS than with GTP. However, GTPγS induces bundles of sheets and microtubules in the presence of 0.5–1 mM $Mg^{2+}$, and GTPγS is not hydrolyzed in the pelleted bundles. With higher $Mg^{2+}$, cross-linked rings are induced by GTPγS. These studies show that GTPγS promotes assembly of microtubule protein and tubulin into several polymeric forms. They further demonstrate the importance of $Mg^{2+}$ in microtubule assembly and that caution must be used when nucleotides or drugs are tested in the in vitro microtubule system. The nucleotide or drug may tie up needed $Mg^{2+}$, or it may interact with tubulin to substantially lower the $Mg^{2+}$ concentration needed to induce cross-linked rings or crystals of tubulin.

Tubulin, the major protein in microtubules, is a unique guanine nucleotide binding protein in that it contains one exchangeable binding site and one nonexchangeable binding site (Weisenberg et al., 1968). Models for tubulin assembly include a nucleated-condensation model and assembly from preformed rings and/or spirals containing tubulin and microtubule-associated proteins (MAPs),[1] protofilaments, and 6S tubulin (Gaskin et al., 1974; Dustin, 1984). Both models are oversimplifications. Both nucleotides and metal ions such as $Mg^{2+}$ and $Zn^{2+}$ have dramatic effects on microtubule assembly in vitro, most likely due to direct interaction with proteins as well as stabilization of certain aggregates. GTP at both sites is needed for optimal assembly in the growing microtubule, and the GTP at the exchangeable site is hydrolyzed after assembly (Dustin, 1984; Carlier & Pantaloni, 1981). Optimal assembly also requires $Mg^{2+}$ as well as GTP–Mg (Gaskin, 1981). Both nucleotides are essentially nonexchangeable in the microtubule. Nonhydrolyzable analogues of GTP will also promote assembly although at a slower rate. Guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) and chromium guanosine 5′-triphosphate (CrGTP) are two GTP analogues that also promote assembly, and they are only partially hydrolyzed (Kirsch & Yarbrough, 1981; MacNeal & Purich, 1978; Gaskin, 1981). Microtubules assembled with the nonhydrolyzable analogues, GTPγS or CrGTP, are more stable than GTP-induced microtubules. A more recent study showed that GTPγS can inhibit microtubule assembly (Hamel & Lin, 1984). The promoting and inhibiting effects of GTPγS on microtubule assembly might be due to important GTPγS–Mg and $Mg^{2+}$ interactions with the proteins in the assembly system. We used turbidity, electron microscopy, and nucleotide binding and hydrolysis to look at the effect of varying $Mg^{2+}$ and GTPγS on GTPγS-induced assembly of microtubule protein (tubulin + MAPs) in the presence of 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer ± 30% glycerol and in buffer with GTP added before or after GTPγS. We also studied the effect of GTPγS on microtubule protein assembly in the presence of $Zn^{2+}$ and taxol to learn if GTPγS could affect the formation of $Zn^{2+}$-induced sheet aggregates or taxol-induced microtubules. To better understand GTPγS interactions with tubulin, GTPγS effects on the assembly of tubulin purified by fast-performance liquid chromatography (FPLC) and free of MAPs and major enzymatic activities (Gaskin & Roychowdhury, 1986; Roychowdhury & Gaskin, 1986) were studied in the presence of varying concentrations of $Mg^{2+}$ and $Zn^{2+}$ and with taxol.

## Materials and Methods

*Preparation of Microtubule Protein.* Microtubule protein was prepared from porcine brain by 2 cycles of assembly–disassembly as described previously (Shelanski et al., 1973).

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[1] Abbreviations: $C_c$, critical concentration; CrGTP, chromium guanosine 5′-triphosphate; DTT, dithiothreitol; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FPLC, fast-performance liquid chromatography; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); MAPs, microtubule-associated proteins; MES, 2-(*N*-morpholino)ethanesulfonic acid; MTP, two-cycle microtubule protein (tubulin + microtubule-associated proteins); NDPK, nucleosidediphosphate kinase; PAGE, polyacrylamide gel electrophoresis; PEI, poly(ethylenimine); SDS, sodium dodecyl sulfate; TEAB, triethylammonium bicarbonate buffer.

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The protein (~10 mg/mL) was stored in small aliquots in 0.1 M MES, pH 6.6, in liquid nitrogen. On the day of the experiment, 1–2 mL of protein was dialyzed for 3 h against 3 × 500 mL of 0.1 M MES with or without 0.5 mM $MgCl_2$ and with 1 mM EGTA, pH 6.6 (buffer changed hourly), in a Crowe-Englander-type thin-film microdialyzer at 4 °C. The dialyzed sample was centrifuged at 100000*g* for 30 min at 4 °C and the supernatant diluted to the desired protein concentration in buffer containing 0.1 M MES, 1 mM EGTA, and 0.1 mM DTT. As indicated, $MgCl_2$ or 30% v/v glycerol was added. This protein did not assemble into microtubules at 37 °C unless appropriate cofactors such as GTP or taxol (supplied by Dr. M. Suffness, Natural Products Branch, NCI) were added. For experiments where trace amounts of GTP might promote nucleation, the protein was warmed to 37 °C for 30 min and cooled to 4 °C for 10 min before use. Protein concentration was determined by the Lowry method with BSA as a standard (Lowry et al., 1951).

*Preparation of FPLC Purified Tubulin.* Tubulin was purified from microtubule protein by Mono Q anion exchanger and Mono S cationic exchanger column chromatography using the Pharmacia fast-performance liquid chromatography system as previously described Gaskin & Roychowdhury, 1986; Roychowdhury & Gaskin, 1986). This tubulin is 99% pure on the basis of PAGE and contains little nucleosidediphosphate kinase (NDPK), adenylate kinase, and ATPase activities.

*Characterization of Assembly.* A Cary 118C recording spectrophotometer with a thermostable sample changer was used for turbidimetric measurements of $A_{350}$ as a function of time as described previously (Gaskin et al., 1974). Since $A_{350}$ is proportional to the weight concentration of microtubules, it can be used to quantitate microtubule assembly (Gaskin et al., 1974). However, it is not quantitative for sheets and other microtubule protein aggregates (Gaskin & Kress, 1977). Thus, assembled protein was also determined after centrifugation (1-mL sample) through a 1-mL of 40% glycerol in a buffer cushion in an ultracentrifuge at 100000*g* for 30 min or with a 200–400-μL sample at 100000*g* for 15 min in an airfuge at room temperature. Electron microscopy was done as previously described by negative staining and thin sectioning of pellets with a JEOL 1200 electron microscope (Gaskin & Kress, 1977). For each sample, four different areas of the pellets were embedded. One-micrometer sections were cut, and every tenth section (four to five sections per area) was examined.

*Purification of GTPγS, ATPγS, and [8-$^3$H]GTPγS.* To remove contaminating nucleotides, GTPγS and ATPγS (Boehringer Mannheim) were purified essentially as described by Kirsch and Yarbrough (1981). Fifteen to twenty milligrams of GTPγS or ATPγS in 5 mL of 0.05 M triethylammonium bicarbonate buffer (TEAB), pH 7.6, was applied to a column of DEAE-Sephadex A-25 (1.6 × 18 cm) equilibrated with 0.05 M TEAB, pH 7.6, and eluted with a linear gradient from 0.1 to 0.45 M TEAB (1000 mL each). The thionucleotides of interest eluted at about 0.4 M TEAB and were easily separated from GDP, GTP, ADP, and ATP. The fractions were pooled and dried under rotary evaporation, with repeated coevaporation with methanol. No impurities were detected by chromatography on PEI–cellulose thin-layer plates in 0.35 M $NH_4HCO_3$ as previously described (Kirsch & Yarbrough, 1981). [8-$^3$H]GTPγS was prepared by enzymatic transfer of the thiophosphate group from GTPγS to [8-$^3$H]-GDP (New England Nuclear) as described by Kirsch and Yarbrough (1981), modified from the procedure of Goody et al. (1972). The labeled GTPγS was purified as described for cold GTPγS and was 98% pure on the basis of PEI–cellulose TLC and radioactivity. About 1% of the counts were recovered in the GDP peak.

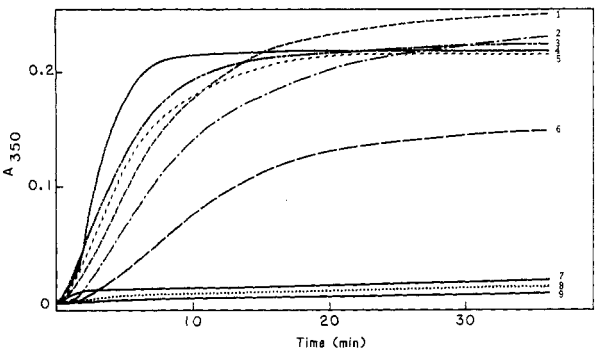


FIGURE 1: Comparison of kinetics of GTPγS- and GTP-induced assembly of MTP under standard assembly conditions with 0.5 mM $Mg^{2+}$ with and without 30% glycerol. MTP (1.5 mg/mL) in 0.1 M MES, 0.5 mM $MgCl_2$, 1 mM EGTA, 0.1 mM DTT, pH 6.6, and ±30% glycerol was incubated at 37 °C in the presence of GTP, GTPγS, or no added nucleotide. (1) 30% glycerol + 1 mM GTPγS; (2) 30% glycerol + 0.2 mM GTPγS; (3) 30% glycerol + 0.2 mM GTP; (4) 0.2 mM GTP; (5) 30% glycerol + 1 mM GTP; (6) 30% glycerol + 0.05 mM GTPγS; (7) 0.2 mM GTPγS; (8) 30% glycerol; (9) no additions. At 35 min, microtubules were found in samples 1–5. Microtubules and cross-linked rings were found in sample 6. Only rings and cross-linked rings were found in sample 7. Few aggregates were found in samples 8 and 9.

*Analysis of Radiolabeled Nucleotide Bound to Microtubules.* After the microtubules were pelleted as described under Characterization of Assembly, the pellets were resuspended in 5 mM MES, pH 6.6, containing 1 mM DTT. Aliquots were saved for protein and radioactivity determinations. The sample was treated with 1% SDS to release bound nucleotide and to determine the bound nucleotide according to the procedure described by Kirsch and Yarbrough (1981) in the preceding section. Tubulin is about 80% of the protein in the MTP fractions on the basis of PAGE and has a $M_r$ of 100000.

## RESULTS

*Comparison of GTPγS- and GTP-Induced Assembly of Microtubule Protein Using Standard Assembly Conditions with and without 30% Glycerol.* The kinetics of MTP assembly were followed with 0.2 mM GTP or GTPγS or no added nucleotide with our standard assembly conditions of 0.1 M MES, 0.5 mM $MgCl_2$, 1 mM EGTA, and 0.1 mM DTT, pH 6.6, 37 °C. As reported by Hamel and Lin (1984), we found that 0.2 mM GTPγS did not promote assembly on the basis of turbidity (see Figure 1). Higher GTPγS concentrations (0.5, 1, and 2 mM) also did not induce assembly into microtubules (data not shown). However, electron microscopic studies showed that GTPγS induced cross-linked or clusters of rings in the absence of glycerol. If 30% glycerol was included in the buffer, GTPγS promoted assembly (Figure 1). Although the lag time was longer with 0.2 mM GTPγS compared to 0.2 mM GTP (1.2 min vs. 0.2 min), the final extent of assembly was similar to that induced by GTP. Electron microscopy studies showed that 0.2 mM GTPγS promoted MTP assembly into microtubules in the presence of 30% glycerol. With MTP in buffer containing glycerol and 0.05 mM GTPγS, there was a longer lag and less assembly than with 0.2 mM GTPγS, whereas assembly kinetics with 1 mM GTPγS showed less lag and more assembly than those with 0.2 mM GTPγS. Microtubules, rings, and cross-linked rings were found at 0.05 mM GTPγS while microtubules were found with 1 mM GTPγS. Table I summarizes the different polymeric forms with 0.5 mM $Mg^{2+}$ and varying GTPγS.

Table I: Domains of Existence of Different Polymeric Forms When Microtubule Protein Was Assembled with Variable Concentrations of Magnesium and GTPγS or GTP in Buffer Containing 30% Glycerol

| Mg (mM)[a] | +0.05–2 mM GTP | +0.02 mM GTPγS | +0.05 mM GTPγS | +0.1–0.5 mM GTPγS | +1 mM GTPγS |
|---|---|---|---|---|---|
| 0 | MTs[b] | MTs | MTs | MTs | MTs, some spirals and open rings |
| 0.5 | MTs | MTs, few rings and C-L rings | MTs, few rings and C-L rings | MTs | MTs |
| 1 | MTs | MTs, rings and C-L rings | MTs, rings and C-L rings | MTs, rings and C-L rings | MTs, rings and C-L rings, some spirals and open rings |
| 2 | MTs | rings and C-L rings, few MTs | rings and C-L rings, few MTs | C-L rings, some rings, few MTs | rings and C-L rings, few MTs, some spirals and open rings |
| 3 | MTs | rings and C-L rings, infrequent MTs | rings and C-L rings, infrequent MTs | C-L rings, few rings, infrequent MTs | rings and C-L rings, few MTs, spirals and open rings |

[a] Added $MgCl_2$. [b] MTs, microtubules; C-L rings, cross-linked rings. No MTs or C-L rings were formed in absence of added nucleotides. Microtubule protein = 1.5 mg/mL. Assembly was at 37 °C, 30 min.

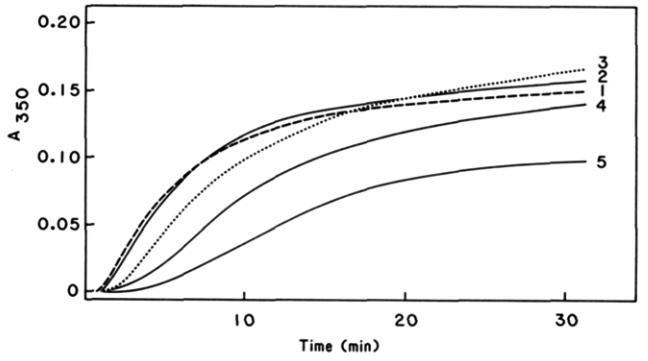


FIGURE 2: Effect of $Mg^{2+}$ on the kinetics of GTPγS-induced assembly of MTP in buffer plus 30% glycerol at 37 °C; 0.2 mM GTPγS was incubated with MTP (1.5 mg/mL). (1) No added $Mg^{2+}$; (2) 0.1 mM $Mg^{2+}$; (3) 0.5 mM $Mg^{2+}$; (4) 1 mM $Mg^{2+}$; (5) 2 mM $Mg^{2+}$. See Figure 3 for electron micrographs of structures found in curve 3 and curve 5.

Previous published studies did not show GTPγS to be a good promoter of MTP assembly even in the presence of glycerol (Kirsch & Yarbrough, 1981). In fact, it had been shown to inhibit GTP-induced assembly in buffer (Hamel & Lin, 1984). Since we had good assembly of MTP with GTPγS and glycerol, we reexamined our assembly conditions and found that our $Mg^{2+}$ was 0.5 mM instead of 1 mM as previously used by others. Thus, we designed several experiments to examine the role of $Mg^{2+}$ and GTPγS in MTP and purified tubulin aggregation and assembly.

*Effect of $Mg^{2+}$ on GTPγS-Induced Assembly of MTP in Buffer plus Glycerol.* Microtubule protein assembly was examined at a fixed GTPγS concentration with varying amounts of added $Mg^{2+}$ in buffer containing 30% glycerol. Figure 2 shows the kinetics of assembly of MTP with 0.2 mM GTPγS and 0, 0.1, 0.5, 1, and 2 mM $Mg^{2+}$. Optimal assembly is with no added $Mg^{2+}$, or 0.1 mM $Mg^{2+}$, on the basis of lag time and final turbidity. However, the final extent of assembly is similar with no added $Mg^{2+}$, 0.1 mM $Mg^{2+}$, and 0.5 mM $Mg^{2+}$. Comparison of 0.5, 1, and 2 mM $MgCl_2$ shows that the higher the $Mg^{2+}$ the longer the lag and the less the assembly. Negative staining electron microscopy shows primarily microtubules up to 0.5 mM $Mg^{2+}$ (see Figure 3A). However, a considerable number of cross-linked rings or clusters of rings as well as microtubules are found with 1 mM $Mg^{2+}$, and primarily cross-linked rings are found with 2 mM $Mg^{2+}$ (see Figure 3B). Table I summarizes these studies. Thin sections on pellets of structures induced by 0.2 mM GTPγS and 0.5 mM $Mg^{2+}$ showed only microtubules. (data not shown). At 2 mM $Mg^{2+}$ and 0.2 mM GTPγS, thin sections of pellets showed primarily short filaments and few rings with a suggestion of cross-linking. However, it has been shown that MAP-stabilized rings dissociate in the ultracentrifuge (Erickson, 1974; Marcum & Borisy, 1978). These GTPγS-induced cross-linked rings also are sensitive to ultracentrifugation.

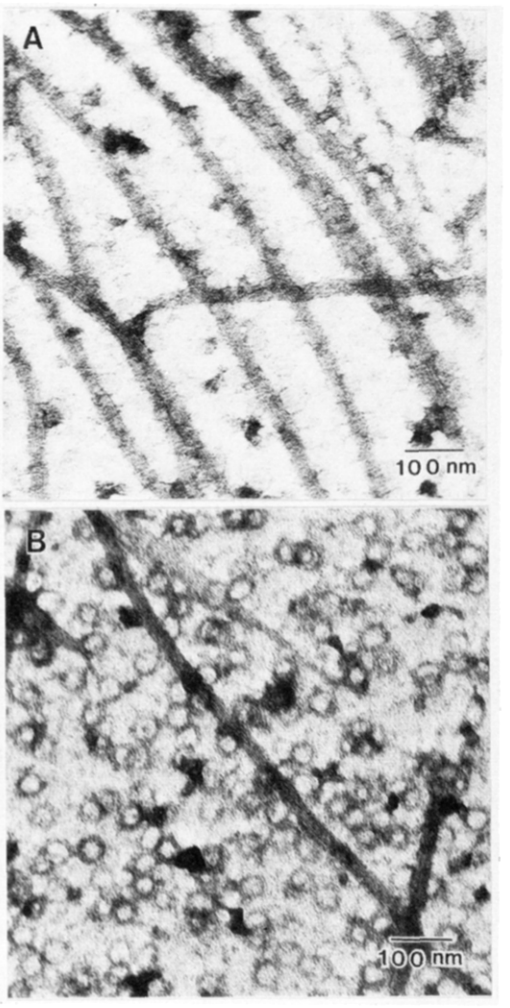


FIGURE 3: Electron micrographs of GTPγS-induced microtubules and cross-linked rings on samples from Figure 2: (A) microtubules and few rings are found with 0.5 mM $Mg^{2+}$ (curve 3), and (B) cross-linked rings and few microtubules are found with 2 mM $Mg^{2+}$ (curve 5). Magnification 80000×.

The effect of $Mg^{2+}$ on the binding of [$^3$H]GTPγS to aggregated MTP was studied to learn if $Mg^{2+}$ affected the nucleotide binding to tubulin. Table II demonstrates that approximately 0.8 mol of labeled nucleotide is bound per tubulin dimer in the pelletable protein. Pelletable protein is constant up to 1 mM $Mg^{2+}$ and decreased slightly at 2 mM and by 50% at 3 mM $Mg^{2+}$. Analysis by TLC showed that 89% of the radioactivity was GTPγS and 11% was GDP. This agrees with the published value of 90% GTPγS and 10% GDP (Kirsch

Table II: Effect of $Mg^{2+}$ on Binding of [$^3$H]GTPγS to Aggregated MTP in Buffer Containing 30% Glycerol[a]

| added $Mg^{2+}$ (mM) | pelletable protein (mg/mL) | mol of [$^3$H]nucleotide/tubulin dimer | [$^3$H]GTPγS/[$^3$H]GDP | structures by negative-staining EM | structures by thin-sectioning EM |
|---|---|---|---|---|---|
| 0 | 1.35 | 0.83 | not done | MTs[b] | MTs |
| 0.5 | 1.47 | 0.80 | 8.1 | MTs, few rings | MTs |
| 1 | 1.40 | 0.80 | 8.1 | C-L rings and MTs | MTs, few short protofilaments |
| 2 | 1.26 | 0.79 | not done | C-L rings, few MTs | not done |
| 3 | 0.71 | 0.90 | not done | C-L rings | short protofilaments, few C-L rings and rings |

[a] MTP (1.98 mg/mL) in 0.1 M MES, 1 mM EGTA, 0.1 mM DTT, 30% glycerol, pH 6.6, and 0.25 mM [$^3$H]GTPγS was incubated at 37 °C with various concentrations of added $Mg^{2+}$ for 45 min. The polymers were centrifuged through a 40% glycerol cushion at 34000 rpm for 40 min at 25 °C. Pellets were resuspended in buffer and analyzed for both protein content and radioactivity. TLC analysis showed that 89% of the labeled nucleotide was GTPγS and 11% was GDP. [b] MTs, microtubules; C-L rings, cross-linked rings.

Table III: Effect of Variable Concentrations of GTPγS and Magnesium on Domains of the Existence of Different Polymeric Forms with Microtubule Protein in Buffer Containing 0.1 mM GTP

| Mg (mM)[a] | no added GTPγS | +0.05 mM GTPγS | +0.1 mM GTPγS | +0.25 mM GTPγS | +0.5 mM GTPγS | +0.7 mM GTPγS |
|---|---|---|---|---|---|---|
| 0 | MTs[b] | MTs, some spirals and open rings | spirals and open rings | spirals and open rings | spirals and open rings | spirals and open rings |
| 0.5 | MTs | MTs | MTs | MTs | mixture of MTs, rings and C-L rings | rings and C-L rings, few MTs |
| 1 | MTs | MTs | MTs | MTs and C-L rings | MTs, rings and C-L rings | rings and C-L rings, few MTs |
| 2 | MTs | MTs | rings and C-L rings | C-L rings and some rings | C-L rings and some rings | C-L rings and rings |

[a] Added $MgCl_2$. [b] MTs, microtubules; C-L rings, cross-linked rings. Microtubule protein = 1.5 mg/mL. Assembly was at 37 °C, 30 min.

& Yarbrough, 1981). Again, electron microscopy studies suggest that cross-linked rings are sensitive to ultracentrifugation. However, they also suggest that microtubules are the main pelletable structures with 0.5 mM $MgCl_2$ and 0.25 mM GTPγS and contain primarily unhydrolyzed GTPγS.

*Effect of $Mg^{2+}$ on GTPγS-Induced Ring Aggregation of Microtubule Protein in Buffer.* $Mg^{2+}$ was varied from none added to 0.06, 0.08, 0.1, 0.15, 0.2, 0.5, 1, 2, 3, and 4 mM with MTP (1.7 mg/mL) in buffer and 0.35 mM GTPγS. After a 30-min incubation at 37 °C, micrtubules were not found by electron microscopy. With no added $Mg^{2+}$, nonspecific aggregates and few rings were found. From 0.06 to 0.5 mM $Mg^{2+}$, GTPγS induced increasing numbers of rings and cross-linked rings. From 1 to 4 mM $Mg^{2+}$, increasing numbers of cross-linked rings were found with GTPγS.

*Effect of $Mg^{2+}$ on GTPγS Inhibition of GTP-Induced Assembly of MTP in Buffer.* It has been reported by Hamel and Lin (1984) that increasing the GTPγS concentration progressively reduced the MAP-dependent GTP-supported tubulin polymerization. With MTP and 1 mM $Mg^{2+}$ in buffer + 0.1 mM GTP, we also find that increasing GTPγS from none to 0.1, 0.2, 0.5, and 0.7 mM progressively resulted in less assembly from 0.2 to 0.7 mM GTPγS (Figure 4). The effect of $Mg^{2+}$ was examined with 0, 0.5, 1, and 2 mM $Mg^{2+}$ with 0.1 mM GTP and 0.25 mM GTPγS (see Figure 5). No assembly into microtubules was found with 0.1 mM GTP and 0.25 mM GTPγS and no added $Mg^{2+}$ or 2 mM $Mg^{2+}$. Spirals and open rings were found with no added $Mg^{2+}$, and cross-linked rings were found with 2 mM $Mg^{2+}$. At 1 mM $Mg^{2+}$ and 0.1 mM GTP, 0.25 mM GTPγS resulted in both microtubules and cross-linked rings. At 0.5 mM $Mg^{2+}$ and 0.1 mM GTP, 0.25 mM GTPγS resulted in a slower rate of assembly into microtubules than MTP containing 0.1 mM GTP. However, the final level of assembly was similar. Table III demonstrates the effects of variable concentrations of GTPγS and $Mg^{2+}$ on the domains of the existence of different polymeric forms of MTP in buffer containing 0.1 mM GTP at 37 °C. Evidence that GTP-induced assembly of MTP preincubated with GTPγS in buffer depends on the $Mg^{2+}$ concentration is shown in Figure 6. MTP was incubated at 37 °C for 20 min with 0.25 mM GTPγS and 0 (sample 1), 0.5

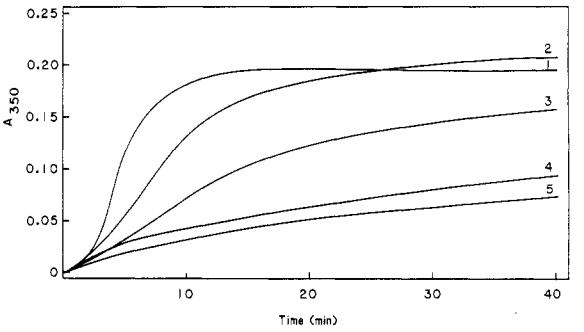


FIGURE 4: GTPγS inhibition of GTP-supported assembly of MTP. MTP (1.55 mg/mL) in buffer containing 1 mM $Mg^{2+}$ plus 0.1 mM GTP was incubated at 37 °C in the presence of the following GTPγS concentrations: (1) 0, (2) 0.1, (3) 0.2, (4) 0.5, and (5) 0.7 mM. With increasing GTPγS, increasing numbers of cross-linked rings are found.

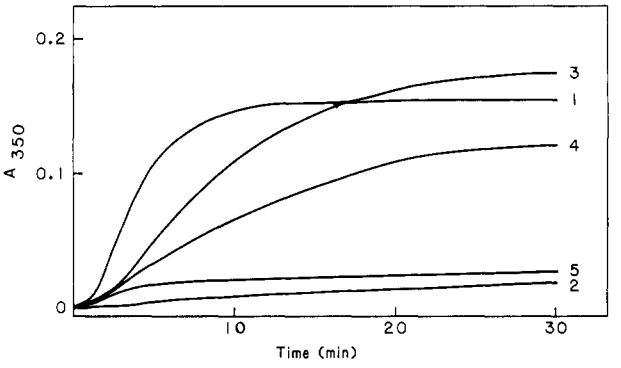


FIGURE 5: Effect of $Mg^{2+}$ on GTPγS inhibition of GTP-induced assembly of MTP. MTP (1.4 mg/mL) in buffer plus 0.1 mM GTP was incubated at 37 °C with various GTPγS and $Mg^{2+}$ concentrations: (1) no added GTPγS and $Mg^{2+}$; (2) 0.25 mM GTPγS; (3) 0.25 mM GTPγS + 0.5 mM $Mg^{2+}$; (4) 0.25 mM GTPγS + 1 mM $Mg^{2+}$; (5) 0.25 mM GTPγS + 2 mM $Mg^{2+}$. Electron microscopy studies show (curve 1) microtubules, (curve 2) spirals and open rings, (curve 3) microtubules, (curve 4) microtubules, rings, and cross-linked rings, and (curve 5) rings and cross-linked rings.

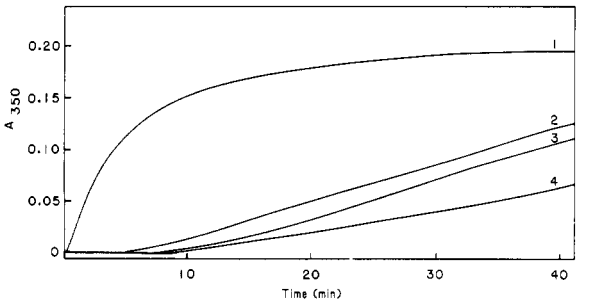


FIGURE 6: GTP-induced assembly of MTP preincubated with GTPγS in buffer without glycerol dependes on the $Mg^{2+}$ concentration. MTP (1.6 mg/mL) in buffer and 0.25 mM GTPγS was preincubated at 37 °C for 20 min in the presence of (1) 0, (2) 0.5, (3) 1, and (4) 2 mM $Mg^{2+}$. Kinetics of assembly were followed after adding 0.5 mM GTP. In the sample with no $Mg^{2+}$ in the buffer (curve 1), 0.5 mM $Mg^{2+}$ was also added with GTP.

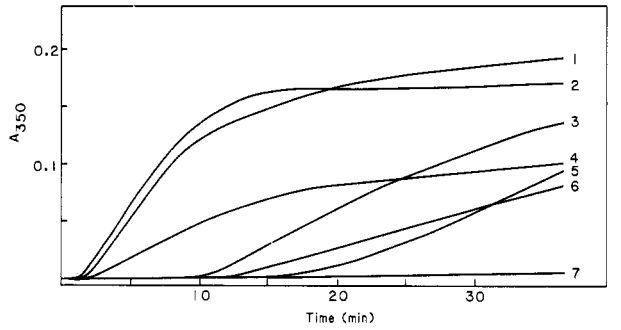


FIGURE 7: Kinetics of assembly of MTP with GTP, GTPγS, ATPγS, and GDP. MTP (1.4 mg/mL) in buffer containing 0.5 mM $Mg^{2+}$ and 30% glycerol was incubated at 37 °C in the presence of (1) 0.2 mM GTPγS, (2) 0.2 mM GTP, (3) 1.0 mM GDP, (4) 1.0 mM ATPγS, (5) 0.2 mM ATPγS, (6) 0.2 mM GDP, and (7) no added nucleotide. At 35 min, microtubules were found in samples 1–6. No significant aggregation was found in sample 7.

(sample 2), 1, and 2 mM $Mg^{2+}$. Both 0.5 mM GTP and 0.5 mM $Mg^{2+}$ were added to sample 1 while 0.5 mM GTP was added to sample 2. Much better asembly is found in the sample to which $Mg^{2+}$ was added with GTP. Also, the higher the initial $Mg^{2+}$, the less the assembly into microtubules. These results suggest that GTPγS requires $Mg^{2+}$ to cross-link rings and that, with no added $Mg^{2+}$, GTPγS may bind available $Mg^{2+}$ so that GTP cannot promote assembly.

*Possible Role of Nucleosidediphosphate Kinase in GTPγS-Induced Assembly of MTP.* Porcine MTP prepared by 2 cycles of assembly–disassembly contains nucleosidediphosphate kinase activity (NDPK) on the order of 60 munits $mg^{-1}$ $min^{-1}$ (Roychowdhury & Gaskin, 1986). Previous studies suggested GTPγS was not promoting assembly through NDPK since ATPγS did not promote assembly of MTP in buffer containing 1 mM $Mg^{2+}$ and 30% glycerol (Kirsch & Yarbrough, 1981). However, we have optimized the assembly conditions for GTPγS, and so we reexamined assembly of MTP using GTP, GTPγS, ATPγS, and GDP in buffer containing 0.5 mM $Mg^{2+}$ and 30% glycerol. Figure 7 shows that ATPγS and GDP do promote assembly, presumably through NDPK, but there is a longer lag and less assembly than with GTPγS at 0.2 mM nucleotide, and GTPγS results in 3 times as much assembly as 0.2 mM ATPγS in 30 min. ATPγS at 1 mM results in about 50% of the assembly of that found with 0.2 mM GTPγS. The better rate of assembly with GTPγS than with ATPγS or GDP can be explained by (1) NDPK is more active with GTPγS than with ATPγS or GDP, (2) tubulin–GTPγS may be incorporated in a growing microtubule but does not promote nucleation. Since pelleted microtubules contain approximately 0.7 mol of labeled GTPγS and 0.08

Table IV: Relative Affinity of Binding of GTPγS and GTP to Microtubule Protein That Assembles into Microtubules

| sample | +GTPγS (mM) | +GTP (mM) | mol of GTPγS/ mol of tubulin[a] | mol of GTP/mol of tubulin | relative affinity, GTP/ GTPγS |
|---|---|---|---|---|---|
| 1 | 0.25 | | 0.80 | | |
| 2 | 0.25 | 0.05 | 0.43 | 0.20 | 2.3 |
| 3 | 0.25 | 0.1 | 0.40 | 0.31 | 1.9 |
| 4 | 0.25 | 0.25 | 0.23 | 0.46 | 2.0 |
| 5 | 0.25 | 0.5 | 0.16 | 0.54 | 1.7 |
| 6 | | 0.25 | | 0.65 | |

[a] MTP (1.35 mg/mL) in buffer containing 30% glycerol and 0.5 mM $Mg^{2+}$ was incubated at 4 °C for 15 min with either 0.25 mM [$^3$H]-GTPγS and various concentrations of GTP or 0.25 mM GTPγS and various concentrations of [$^3$H]GTP. After assembly at 37 °C, 30 min, microtubules were found in all the samples. The protein was pelleted and assayed as described in Table II. Numbers are nucleotide binding, and hydrolysis was not determined.

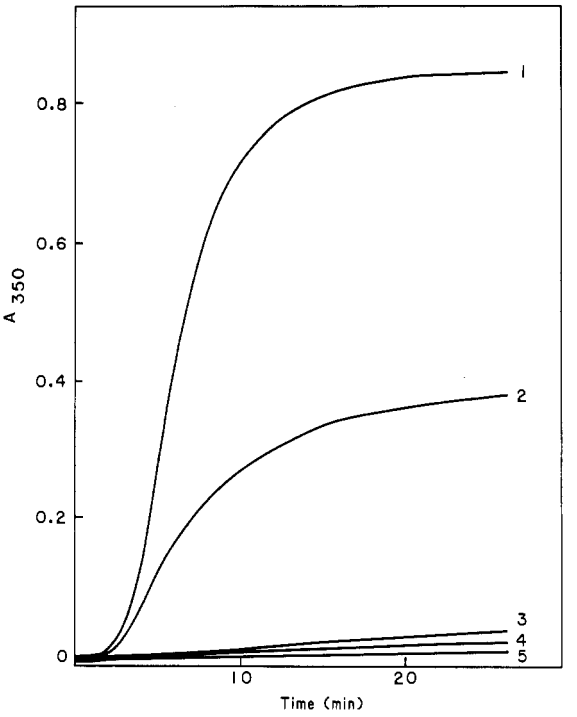


FIGURE 8: Kinetics of assembly of tubulin with GTP, GTPγS, ATPγS, and GDP. Mono Q–Mono S purified tubulin (1.45 mg/mL) in buffer containing 1 mM $Mg^{2+}$, 25% glycerol, and 10% $Me_2SO$ was incubated at 37 °C in the presence of 0.6 mM nucleotide: (1) GTPγS; (2) GTP; (3) ATPγS; (4) GDP; (5) none.

mol of labeled GDP when [$^3$H]GTPγS is used to promote assembly of MTP in buffer + glycerol, it is clear that tubulin–GTPγS is incorporated in the growing microtubule (see Table II).

*Relative Affinities of GTP and GTPγS for Microtubule Protein.* To learn about the relative affinity of microtubule protein for GTPγS and GTP, the binding to assembled microtubules was studied at 0.25 mM GTPγS and varying GTP concentrations in buffer containing glycerol and 0.5 mM $Mg^{2+}$ (see Table IV). For example, at equal concentrations of GTP and GTPγS, GTP has a relative affinity twice that of GTPγS.

*GTPγS-Induced Assembly of Purified Tubulin.* Tubulin purified by Mono Q–Mono S anion–cation exchanger column chromatography is low in NDPK activity, <1 munit $mg^{-1}$ $min^{-1}$ (Roychowdhury & Gaskin, 1986). GTP (0.6 mM) promotes assembly of tubulin in buffer containing 1 mM $Mg^{2+}$, 25% glycerol, and 10% $Me_2SO$ (Figure 8), and electron microscopy (EM) showed microtubules and sheets. Under the same conditions, GTPγS (0.6 mM) promotes bundles of sheets

Table V: Comparison of Effect of Magnesium on GTPγS and GTP-Induced Assembly of Tubulin

| Mg (mM)[a] | +GTPγS | +GTP |
|---|---|---|
| 0.5 | bundles of sheets and MTs, $A_{350}$ = 0.80[b] | MTs and thin sheets |
| 1 | bundles of sheets and MTs, $A_{350}$ = 0.92 | MTs and thin sheets, $A_{350}$ = 0.39 |
| 3 | rings and C-L rings, $A_{350}$ = 0.62 | MTs, few thin sheets |
| 5 | C-L rings and crystalline-like aggregates of rings, $A_{350}$ = 0.59 | MTs, few thin sheets, $A_{350}$ = 0.69 |

[a] Added $MgCl_2$. [b] MTs, microtubules; C-L rings, cross-linked rings. Mono Q–Mono S purified tubulin (1.5 mg/mL) was incubated with 0.6 mM GTPγS or GTP with varying concentrations of $MgCl_2$ in buffer containing 25% glycerol and 10% $Me_2SO$ for 30 min at 37 °C. $A_{350}$ is the turbidity at 30 min.

and microtubules, and the high turbidity is probably due to the bundles (Figure 8). Figure 9 demonstrates the bundles by negative-staining EM and thin-sectioning EM. ATPγS and GDP were not effective in promoting assembly of tubulin.

The effect of $Mg^{2+}$ on GTPγS-induced assembly was examined with 0.6 mM GTPγS and 0.5, 1, 3, and 5 mM $MgCl_2$ in buffer containing 25% glycerol and 10% $Me_2SO$ (Table V). Bundles of sheets and microtubules were found at 0.5 and 1.0 mM $Mg^{2+}$. Although the turbidity was high with 3.0 mM $Mg^{2+}$, only rings and cross-linked rings were found. At 5.0 mM $Mg^{2+}$, GTPγS promoted assembly of tubulin into cross-linked rings and crystalline-like ring structures. With 0.6 mM GTP and 0.5–5 mM $Mg^{2+}$, only microtubules and thin sheets were found. No assembly was found in the absence of added nucleotide.

Pelleted bundles of sheets and microtubules assembled from tubulin and 0.5 mM [$^3$H]GTPγS in buffer containing 1 mM $Mg^{2+}$, 25% glycerol, and 10% $Me_2SO$ contained 0.41 mol of $^3$H-labeled nucleotide/tubulin dimer. Thin-layer chromatography of bound nucleotide showed 99% of the label in GTPγS and 1% in GDP. Thus, GTPγS is not hydrolyzed during tubulin assembly under these conditions.

*Effect of GTPγS on Zinc-Induced Assembly of MTP and Tubulin.* Zinc acts directly on tubulin to promote sheet structures of protofilaments (Gaskin, 1981). With MTP, wide sheets containing 20 or more protofilaments are found with $Zn^{2+}$ (0.01–1 mM) and 1 mM GTP, whereas wrapped sheets (~200–250 nm) are found with purified tubulin and $Zn^{2+}$ (0.1–0.5 mM) and 1 mM GTP (Larson et al., 1976; Gaskin & Kress, 1977). We looked at zinc-induced assembly of MTP and tubulin with GTPγS to see if GTPγS could affect $Zn^{2+}$-induced abnormal structures. With no added $Mg^{2+}$ or EGTA in buffer, 0.2 mM $Zn^{2+}$ and 0.32 mM GTPγS induced MTP (37 °C, 30 min) to assemble into sheets, which were conjugated to microtubules. As expected, in the presence of 0.32 mM GTP and 0.2 mM $Zn^{2+}$ (37 °C, 20 min), only flat sheets were found. In the absence of added nucleotide there was no assembly. $Zn^{2+}$ (at $Zn^{2+}$/EGTA ratios of 1, 1.2, 1.25, and 1.5) could not induce assembly of MTP in the presence of 0.3 mM GTPγS in buffer containing 0.5 mM $Mg^{2+}$. Zinc at $Zn^{2+}$/EGTA = 1.2–1.5 induced pure tubulin (1.5 mg/mL) to assemble into wrapped sheets with 1 mM GTPγS in buffer, 37 °C, 30 min.

*Effect of GTPγS on Taxol-Induced Assembly of MTP and Tubulin in Buffer.* Taxol does not require added GTP or MAPs for assembly of tubulin (Schiff et al., 1981). We studied taxol induced assembly of GTPγS-treated MTP and tubulin in buffer. On the basis of turbidity and electron microscopy studies, taxol (0.01 mM) promoted microtubule assembly of cross-linked rings of MTP (1.4 mg/mL), which has been pretreated with 0.2 mM GTPγS at 37 °C for 30 min in buffer containing 2 mM $Mg^{2+}$. Tubulin pretreated with GTPγS and 0.5, 1, or 2 mM $Mg^{2+}$ in buffer at 37 °C for 30 min and then with taxol assembled into a mixture of microtubules and thin sheets. Binding studies showed that [$^3$H]-GTPγS was incorporated in the taxol-induced microtubules, i.e., 0.8 mol of GTPγS/tubulin dimer with MTP and 0.5 mol of GTPγS/tubulin dimer with purified tubulin.

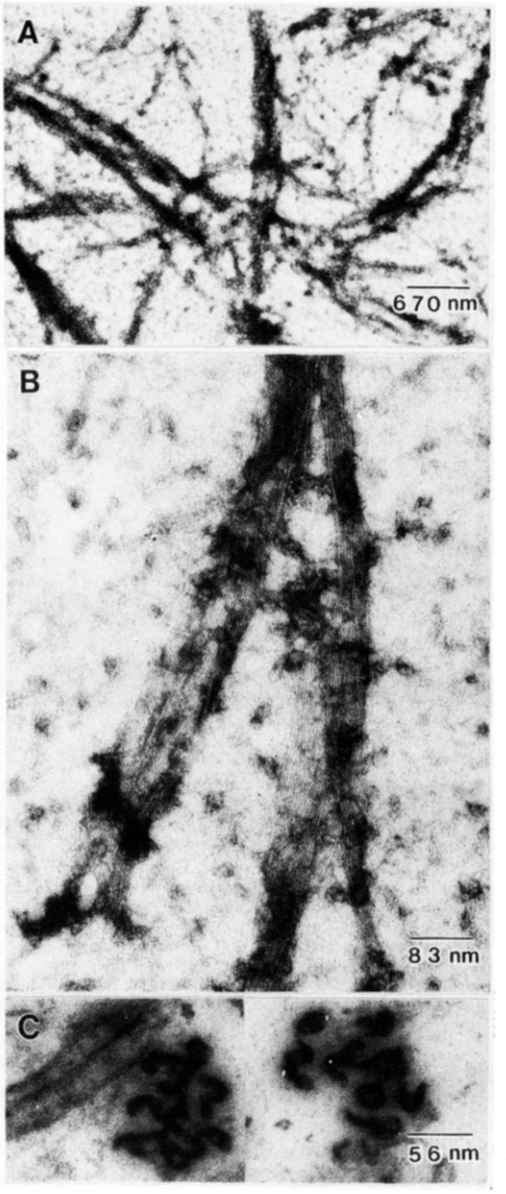


FIGURE 9: Electron micrographs of GTPγS-induced bundles of sheets and microtubules from purified tubulin (1.5 mg/mL) in buffer containing 30% glycerol, 10% $Me_2SO$, 0.5 mM $Mg^{2+}$, and 0.6 mM GTPγS, 37 °C, 30 min. (A) Typical bundles demonstrated by negative staining. Magnification 12000×. (B) Higher magnification showing the end of a bundle, which reveals sheets and possibly microtubules. Magnification 96000×. (C) Thin section of pelleted bundles showing two groups of bundles in cross-section demonstrating structures suggestive of sheets and microtubules. Longitudinal sections compatible with sheets are also seen in (C). Magnification 140000×.

## Discussion

Our studies suggest that the concentration of $Mg^{2+}$ is critical for GTPγS interaction with tubulin. Although high concentrations of $Mg^{2+}$ (10–20 mM) and GTP have been shown to induce crystals of tubulin rings (Erickson, 1978), the presence of GTPγS results in cross-linked rings of tubulin at consid-

erably lower $Mg^{2+}$ concentrations, i.e., 1 mM $Mg^{2+}$ with MAPs in buffer and 2–3 mM $Mg^{2+}$ with or without MAPs in buffer containing 30% glycerol. The $Mg^{2+}$ concentration (0–3 mM) does not have a significant effect on the amount of GTPγS bound to pelletable microtubules and cross-linked rings. Relative binding affinities of GTP and GTPγS to pelleted microtubules suggest that GTP has a binding affinity twice that of GTPγS. The apparent GTPγS inhibition of GTP-induced MTP assembly in buffer may be due to GTPγS with $Mg^{2+}$ inducing cross-linked rings of tubulin (see Figures 4 and 5) or at low $Mg^{2+}$ concentrations (none added) tying up free $Mg^{2+}$ so GTP does not bind. Also, if MTP is incubated at 37 °C with GTPγS ± 0.5 mM $Mg^{2+}$, GTP induces more assembly if it is added with fresh $Mg^{2+}$ then if it is added to the sample preincubated with $Mg^{2+}$ (Figure 6). These studies can be explained by trapping tubulin in rings and cross-linked rings in the preincubation with GTPγS and $Mg^{2+}$. These studies also suggest that lateral interactions are not strong with GTPγS. In fact, lateral associations of short protofilaments are important intermediates in microtubule assembly (Erickson & Voter, 1986).

Although GTPγS and $Zn^{2+}$ induced sheets of MTP in buffer at 37 °C, inclusion of 0.5 mM $Mg^{2+}$ in buffer inhibited $Zn^{2+}$-induced GTPγS MTP assembly. That GTPγS can promote tubulin assembly is further supported by our assembly studies with purified tubulin and the binding of GTPγS to bundles of sheets and microtubule pellets. It is also of interest that GTPγS is not hydrolyzed in bundles of sheets and microtubules assembled from tubulin and GTPγS and 0.5 mM $Mg^{2+}$ in buffer containing 25% glycerol and 10% $Me_2SO$ or with taxol in buffer. As previously reported (Kirsch & Yarbrough, 1981), it is only partially hydrolyzed (11%) with microtubules assembled from MTP in buffer containing 30% glycerol. The studies with taxol added to Mg–GTPγS-induced cross-linked rings of MTP or tubulin show that these aggregates can be induced to form microtubules and that GTPγS is still bound in the taxol-induced microtubule. They suggest that taxol may shift a cross-linked rings ⇌ ring ⇌ spiral equilibrium and support lateral association.

Our studies with GTPγS demonstrate the importance of $Mg^{2+}$ in microtubule assembly and that caution must be used when nucleotides or drugs are tested in the in vitro microtubule assembly system. Although the nucleotide or drug may tie up needed $Mg^{2+}$, it may also substantially lower the $Mg^{2+}$ concentration needed to induce cross-linked rings or crystals of tubulin. A drug such as taxol, which binds to microtubules and possibly to cross-linked rings of tubulin, may reverse the apparent inhibiton.

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**Registry No.** GTPγS, 37589-80-3; 5′-GTP, 86-01-1; ATPγS, 35094-46-3; 5′-GDP, 146-91-8; Mg, 7439-95-4; Zn, 7440-66-6; taxol, 33069-62-4; nucleosidediphosphate kinase, 9026-51-1.

REFERENCES

Carlier, M. F., & Pantaloni, D. (1981) *Biochemistry 20*, 1918–1924.

Dustin, P. (1984) in *Microtubules*, 2nd ed., Springer-Verlag, New York.

Erickson, H. P. (1974) *J. Supramol. Struct. 2*, 393–411.

Erickson, H. P. (1978) in *Electron Microscopy*, Vol. III, pp 483–494, Microscopical Society of Canada, University of Toronto, Toronto.

Erickson, H. P., & Voter, W. A. (1986) *Ann. N.Y. Acad. Sci 466*, 554–565.

Gaskin, F. (1981) *Biochemistry 20*, 1318–1322.

Gaskin, F., & Kress, Y. (1977) *J. Biol. Chem. 252*, 6918–6924.

Gaskin, F., & Roychowdhury, S. (1986) *Ann. N.Y. Acad. Sci. 466*, 622–625.

Gaskin, F., Cantor, C. R., & Shelanski, M. L. (1974) *J. Mol. Biol. 89*, 737–755.

Goody, R. S., Eckstein, F., & Schirmer, R. H. (1972) *Biochim. Biophys. Acta 276*, 155–161.

Hamel, E., & Lin, C. M. (1984) *J. Biol. Chem. 259*, 11060–11069.

Kirsch, M., & Yarbrough, L. R. (1981) *J. Biol. Chem. 256*, 106–111.

Larson, H., Wallin, M., & Edström, A. (1976) *Exp. Cell Res. 100*, 104–110.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

MacNeal, R. K., & Purich, D. L. (1978) *Arch. Biochem. Biophys. 191*, 233–243.

Marcum, J. M., & Borisy, G. G. (1978) *J. Biol. Chem. 253*, 2852–2857.

Roychowdhury, S., & Gaskin, F. (1986) *J. Neurochem. 46*, 1399–1405.

Schiff, P. B., & Horwitz, S. B. (1981) *Biochemistry 20*, 3247–3252.

Shelanski, M. L., Gaskin, F., & Cantor, C. R. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 765–768.

Weisenberg, R. C., Borisy, G. G., & Taylor, E. W. (1968) *Biochemistry 7*, 4466–4679.