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Role of nucleotides in tubulin polymerization: Effect of guanosine 5′-methylene diphosphonate

(microtubules/guanosine nucleotides/calcium/electron microscopy)

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ABSTRACT **Incubation of purified rat brain tubulin with guanosine 5′-methylene diphosphonate [GMP(CH$_2$)P] (1 mM), a GDP analog resistant to hydrolysis, results in the polymerization of 20–30% of the total tubulin present. Analogous incubations with GDP (1 mM) do not result in tubulin polymerization. Polymerization with GMP(CH$_2$)P occurs in the presence of alkaline phosphatase (EC 3.1.3.1) under conditions that completely hydrolyze the likely phosphate donors (GTP, GDP, and GMP) as well as the potential product [GMP(CH$_2$)PP] of the transphosphorylase activity present in purified tubulin preparations. Tubulin polymerization *in vitro* thus can occur in the absence of γ-phosphate and phosphate bond hydrolysis at the exchangeable nucleotide-binding site of tubulin. Polymerization of tubulin by GMP(CH$_2$)P is neither prevented nor reversed by concentrations of calcium (2 mM) that prevent microtubule assembly and disrupt already formed microtubules induced by GTP. However, tubulin polymerized with GMP(CH$_2$)P is readily depolymerized by cold (4°, 30 min). The possible involvement of GTP α-β bond hydrolysis must be considered seriously as playing a role in the process of microtubule depolymerization.**

Current evidence supports an obligatory nucleoside triphosphate requirement for tubulin polymerization (1–5). Microtubule assembly induced by GTP occurs concomitantly with hydrolysis of GTP to GDP (3–5). However, Arai and Kaziro (6) and Penningroth *et al.* (7) have recently reported that microtubule assembly can be induced by GTP analogs that are resistant to γ-phosphate hydrolysis [i.e., GMPP(NH)P and GMPP(CH$_2$)P]. Weisenberg *et al.* (8, 9) have reported similar results, and because microtubules assembled in the presence of GMPP(NH)P were resistant to calcium-induced depolymerization (6, 9), they proposed that the conversion of GTP to GDP may be a prerequisite to microtubule disassembly.

We recently described some unique properties of microtubule assembly induced by guanylyl 5′-methylene diphosphonate [GMP(CH$_2$)PP], the analog of GTP that is resistant to hydrolysis at the α-β bond (10). When compared with GTP or GMPP(NH)P, GMP(CH$_2$)PP is a more favorable inducer of tubulin polymerization as shown by (*a*) a decrease in the minimal tubulin concentration required, (*b*) the absence of the lag period normally seen in turbidity measurements, (*c*) an increase in the absolute amount of tubulin polymerized, and (*d*) the lack of dependence on the high molecular weight basic proteins. In addition, microtubules assembled in the presence of GMP(CH$_2$)PP are fully stable to exposure to calcium (2 mM) but only partially to cold (4°). These differences were ascribed to the resistant nature of the α-β methylene bond, and it was suggested that production of the stable diphosphonate analog, guanosine 5′-methylene diphosphonate [GMP(CH$_2$)P], might be responsible for at least certain of these unusual properties (10).

This proposal has been examined directly by studying the capacity of GMP(CH$_2$)P to promote tubulin polymerization. Here we report that GMP(CH$_2$)P can indeed promote tubulin polymerization, and that this occurs under conditions in which other nucleotides capable of binding to the tubulin-exchangeable site are eliminated. Furthermore, these microtubules display the same resistance to calcium as those assembled with GMP(CH$_2$)PP.

MATERIALS AND METHODS

Rat brain tubulin was purified and stored as described (10). Purified tubulin was freed from high molecular weight basic proteins by placing 30 mg of purified tubulin on a 50-ml phosphocellulose column that had been equilibrated with 10 mM 4-morpholineethanesulfonic acid (Mes)/1 mM 2-mercaptoethanol/1 mM $MgCl_2$ and eluting with the same buffer (11).

Tubulin polymerization and transphosphorylase and phosphatase reactions were studied (37°) with purified tubulin solutions in the presence of 0.1 M Mes/1 mM $MgCl_2$/1 mM 2-mercaptoethanol, pH 6.75 (buffer A). GMP(CH$_2$)P, GMP(CH$_2$)PP, [8-^{3}H]GMP(CH$_2$) PP were obtained from ICN (HPLC Pure Biochemicals). No impurities were detected on polyethyleneimine (PEI) fluorescent cellulose thin-layer chromatography or paper electrophoresis. In all cases the nucleotides studied were equilibrated with the tubulin (30 min, 4°) before the reactions at 37° were started (30–60 min). When the effect of GMP(CH$_2$)P on tubulin polymerization was studied, the nucleotide was first incubated (60 min, 37°) with calf intestine alkaline phosphatase [1 unit/6 μmol of GMP(CH$_2$)P] (EC 3.1.3.1; Boehringer). Both the transphosphorylase and the phosphatase reactions (0.5-ml assay volume) were stopped with 125 μl of 5% sodium dodecyl sulfate (NaDodSO$_4$). The NaDodSO$_4$ was precipitated by cold (30 min, 4°) and removed by centrifugation (13,000 × *g*, 10 min, 4°) before quantitation of the enzymatic reactions by chromatography on PEI fluorescent cellulose (0.2 or 0.5 M NH_4CO_3, room temperature).

The content of phosphate donors (i.e., GTP products) was determined in the tubulin purified by two cycles of polymerization, which was used in the polymerization and phospho-

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Abbreviations: GMP(CH$_2$)P, guanosine 5′-methylene diphosphonate; GMP(CH$_2$)PP, guanylyl 5′-methylene diphosphonate; Mes, 4-morpholineethanesulfonic acid; NaDodSO$_4$, sodium dodecyl sulfate; PEI, polyethyleneimine.

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transferase studies, by resuspending microtubule pellets obtained by one cycle of polymerization in half of the initial volume of buffer A (0.5 mM GTP), depolymerizing (60 min, 4°), centrifuging at 100,000 × *g* for 30 min (4°), and again polymerizing the resultant supernatant with 1 mM [^{3}H]GTP (20 Ci/mol). The microtubules formed in the second polymerization cycle were collected by centrifugation (48,000 × *g*, 30 min, 37°) and resuspended in 1 ml of buffer A with 1% $NaDodSO_4$ to analyze the content of GTP, GDP, and GMP by chromatography on PEI fluorescence cellulose (0.2 M NH_4CO_3H, 2 hr, room temperature). In all cases the tested samples were chromatographed in parallel with GTP, $GMP(CH_2)PP$, GDP, $GMP(CH_2)P$, GMP, cyclic GMP, and guanosine standards, prepared in buffer A with 1% $NaDodSO_4$, and cooled (30 min, 4°) to eliminate the $NaDodSO_4$ before chromatography.

Tubulin polymerization (30 min, 37°) was quantitated in moles by subtracting the colchicine-binding activity remaining in the microtubule-free supernatants (48,000 × *g*, 30 min, 37°) from the colchicine-binding measured in the noncentrifuged, microtubule-containing controls (10). The incubation of the controls containing microtubules with the various nucleotides (buffer A plus 1% glycerol, 60 min, 37°) demonstrated constant colchicine-binding values equivalent to 16 nmol of tubulin per ml (Table 1). Only in the absence of nucleotide was this value decreased, probably as a result of tubulin denaturation (Table 1). The colchicine bound to tubulin (buffer A, 1 mM GTP) was quantitated by the DEAE-filter method (12, 13) by using protein concentrations (70–140 μg) that gave a linear dependence of colchicine binding when incubated (90 min, 37°) with 25 μM colchicine (7×10^2 Ci/mol). Electron microscopy was done on preparations of tubulin stained with uranyl acetate (1%) after incubation of the tubulin at 37° under different polymerization conditions (10).

GTP, GMP, and Mes were from Sigma; GDP and cyclic GMP were from Boehringer.

RESULTS

Polymerization of Purified Tubulin with $GMP(CH_2)P$. When tubulin (18 μM) was incubated (37°) with 1 mM $GMP(CH_2)P$, about 20–30% of the total tubulin polymerized after 30–60 min (Table 1). Microtubules, which appeared after the first 5 min of incubation, were generally short (Fig. 1) and, although they increased in length with time, did not cause apparent changes in turbidity (350 nm, 0.2 absorbance units scale, 30 min; and ref. 10). Thus, tubulin polymerization with $GMP(CH_2)P$ was not readily detected by spectrophotometric analysis. Replacement of $GMP(CH_2)P$ by $GMP(CH_2)PP$ resulted in much more efficient tubulin (Table 1) polymerization. In this case numerous and long microtubules were detectable within the first 5 min of incubation (Fig. 1), and large changes in turbidity were readily apparent immediately after addition of the nucleotide (10).

Production of $GMP(CH_2)PP$ from $GMP(CH_2)P$ by Purified Tubulin. Because of the unusual efficacy of $GMP(CH_2)PP$ in inducing microtubule assembly (ref. 10; and Table 1), it was essential to demonstrate that the effect of $GMP(CH_2)P$ on inducing polymerization did not result from enzymatic production of $GMP(CH_2)PP$ from $GMP(CH_2)P$ and some phosphate donor (i.e., GTP, GDP, or GMP) present in the preparations of tubulin purified with GTP.

The search for phosphate donors in preparations of purified tubulin revealed that when tubulin purified by a cycle of polymerization was depolymerized and then polymerized again with 1 mM [^{3}H]GTP (20 Ci/mmol), the nucleotide content per mg of tubulin polymerized was 23 nmol of GTP, 24 nmol of GDP, and 6.8 nmol of GMP (data corresponding to three separate experiments; no effort was made to wash out the contaminating nucleotide not bound directly to tubulin). Furthermore, incubation (30 min, 37°, 0.5-ml assay volume) of purified tubulin (14 μM) with 1 mM $GMP(CH_2)P$ and [γ-^{32}P]GTP (20 Ci/mmol, 10 μCi) revealed that the GTP contained in the tubulin preparation was enough to produce 3.4×10^{-11} mol of $GMP(CH_2)PP$ (68 nM) after the 30-min incubation (37°) period normally used in the studies of tubulin polymerization (Fig. 2).

Table 1. Effect of $GMP(CH_2)P$, GDP, GTP, and $GMP(CH_2)PP$ on tubulin polymerization and the effect of calcium

Additions				Tubulin		
	$CaCl_2$					
	0	30		Total,	Polymerized	
Nucleotide	min	min	AP	nmol/ml	nmol/ml	%
Exp. 1						
None	−	−	−	13.5	0.5	3.5
GDP	−	−	−	16	0.5	3
	+	−	−	16	0.8	5
$GMP(CH_2)P$	−	−	+	16	5	31
	+	−	+	16	4	25
	−	+	+	16	4.5	28
$GMP(CH_2)PP$	−	−	−	16	15.8	99
	+	−	−	16	15	95
	−	+	−	16	15	95
Exp. 2						
GTP	−	−	−	18	10.8	60
	+	−	−	18	0.2	1

Purified tubulin (18 μM in 0.5 ml of buffer A plus 1% glycerol) was preincubated (60 min, 37°) with (+) or without (−) alkaline phosphatase (AP) (75 milliunits/mg of tubulin) prior to centrifugation (100,000 × *g*, 1 hr, 4°) and use in the polymerization experiments. Before the incubation with tubulin, and in order to eliminate any possible trace contamination of $GMP(CH_2)PP$, the $GMP(CH_2)P$ was always preincubated (1 hr, 37°) with calf intestine alkaline phosphatase [1 unit/6 μmol of $Gp(CH_2)P$] under conditions in which an added trace amount (4.6 μM, 3.49 Ci/mmol) of [^{3}H]$GMP(CH_2)PP$ was completely converted into $GMP(CH_2)P$. Tubulin polymerization was carried out for 60 min at 37°. When required, $CaCl_2$ (2 mM) was added at the beginning of the polymerization (0 min) or after the microtubules had been assembled (30 min). All the nucleotides were tested at 1 mM. Tubulin assembly was quantitated by colchicine binding (see *Materials and Methods* and ref. 10). The polymerization experiments with $GMP(CH_2)P$ and GDP have been repeated five times. Polymerization values with $GMP(CH_2)P$ ranged from 20 to 30%. In one experiment GDP gave an apparent tubulin polymerization of 10%, but no microtubules were observed by electron microscopy. Results are presented as nmol of tubulin polymerized and not polymerized and as percent of the total tubulin polymerized.

$GMP(CH_2)PP$ Hydrolysis by Purified Tubulin and by Alkaline Phosphatase. When tubulin (14 μM) was incubated for 30 min at 37° (0.5-ml assay volume) with 3.7 nmol of [^{3}H]-$GMP(CH_2)PP$, nearly 1 nmol of $GMP(CH_2)P$ was produced by hydrolysis of the $GMP(CH_2)PP$ (Fig. 3). Similar incubations containing calf intestine alkaline phosphatase (0.35 unit/ml) result in complete hydrolysis of the $GMP(CH_2)PP$ added (Fig. 3), even at concentrations of the nucleotide as high as 10 mM (Fig. 3), an amount three orders of magnitude higher than the maximal amount that could be produced from contaminating GTP (40 μM) and 1 mM $GMP(CH_2)P$ (Fig. 2). No $GMP(CH_2)PP$ was detected when purified tubulin (14 μM) was incubated with 1 mM $GMP(CH_2)P$ and [γ-^{32}P]GTP (20 Ci/mmol, 10 μCi) in the presence of 0.35 unit of calf intestine alkaline phosphatase per ml (Fig. 2).

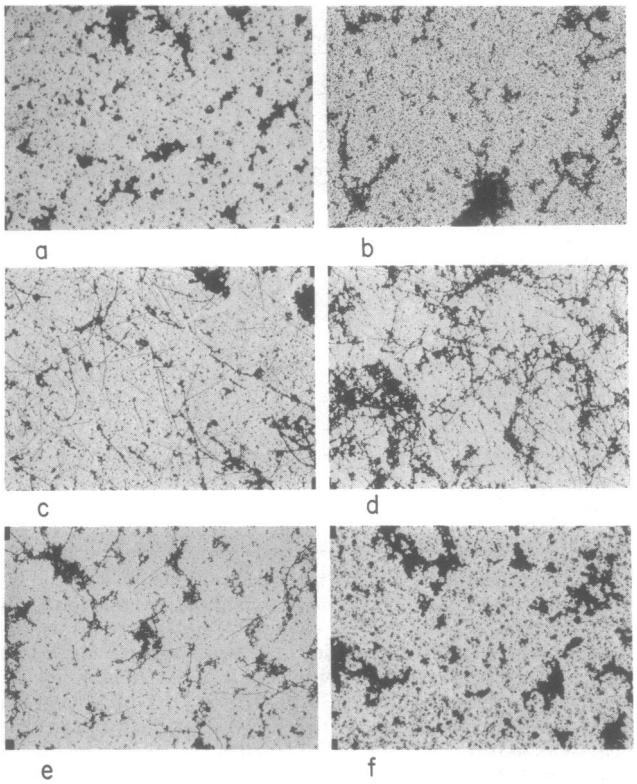

FIG. 1. Morphological correlates of the capacity of $GMP(CH_2)P$ to promote microtubule assembly and of the ability of calcium to prevent or to disrupt the assembled microtubules. Purified tubulin (18 μM) was incubated at 37° with no nucleotide for 15 min (*a*). At 16 min, 1 mM $GMP(CH_2)P$ was added and the incubation was continued for an additional 5 (*b*) or 30 min (*c*). $CaCl_2$ (2 mM) was added at the termination (*d*) or initiation (*e*) of the 30-min incubation with 1 mM $GMP(CH_2)P$, and the incubation mixture was examined for microtubules 35 min after initiation of the incubation. Tubulin was polymerized with 1 mM $GMP(CH_2)P$ for 30 min, cooled to 4° for an additional 60 min, and examined for microtubules (*f*). All the incubations done with $GMP(CH_2)P$ contained 1 unit of alkaline phosphatase per 6 μmol of $GMP(CH_2)P$. (×4000.) The same fields examined at ×53,000 showed the double-wall morphology characteristic of microtubules. Some preparations of tubulin showed a higher or lower rate of assembly than the more typical experiment shown in *a–c*. Microtubule length showed some variation from preparation to preparation. Ribbons were not a consistent finding under the conditions described for *e*.

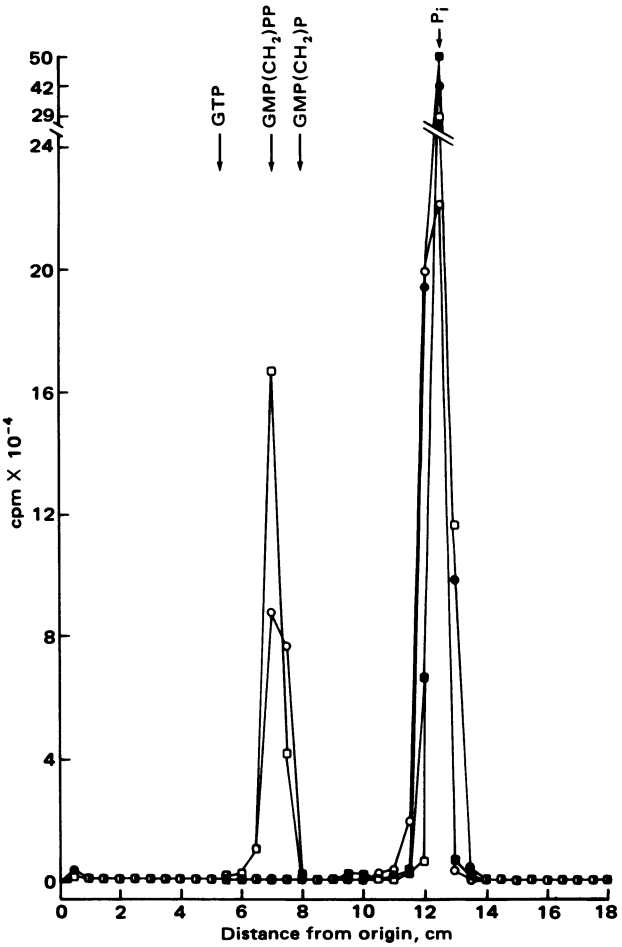

FIG. 2. Production of $GMP(CH_2)PP$ by transphosphorylation of $GMP(CH_2)P$ by the GTP present in preparations of purified tubulin, and efficiency of hydrolysis of $GMP(CH_2)PP$ to $GMP(CH_2)P$ by alkaline phosphatase. Tubulin (14 μM in 0.5 ml of buffer A) was preequilibrated (30 min, 4°) either with 5.6 μM [γ-^{32}P]GTP (3.49 Ci/mmol) (○,●) or with 15 μM [γ-^{32}P]GTP (1.1 Ci/mmol) (□,■) in the presence of 1 mM $GMP(CH_2)P$ prior to incubation (30 min, 37°) with (●, ■) or without (○,□) 0.35 unit of calf intestine alkaline phosphatase. The reactions were stopped by the addition of 125 μl of 5% $NaDodSO_4$. The $NaDodSO_4$ was removed by cold precipitation (30 min, 4°) and centrifugation (3,000 × *g*, 10 min, 4°), and 20-μl aliquots of each sample were chromatographed on PEI fluorescent cellulose (2 hr, room temperature) with 0.5 M NH_4CO_3H as the mobile phase. The developed chromatograms were cut in 0.5-cm strips and ^{32}P was measured in 10 ml of Bray's solution. R_F values for the standards are indicated by arrows.

Tubulin Polymerization with $GMP(CH_2)P$ Occurs without Production of $GMP(CH_2)PP$. Pretreatment of tubulin (30 min, 37°) with calf intestine alkaline phosphatase destroyed all intrinsic phosphate donors (Fig. 4). When such a tubulin preparation was incubated (30 min, 37°) with 1 mM $GMP(CH_2)P$ that had been freed from any contaminating trace of $GMP(CH_2)PP$ by incubation with alkaline phosphatase (legend to Fig. 1), 30% of the tubulin polymerized into a form sedimentable at 48,000 × *g* (Table 1). Concomitantly, characteristic short microtubules appeared by electron microscopy (Fig. 1). Thus, $GMP(CH_2)P$ can induce polymerization under conditions where $GMP(CH_2)PP$ cannot exist or be formed.

Effect of Calcium on Tubulin Polymerization by $GMP(CH_2)P$. When GTP was the nucleotide used, 2 mM $CaCl_2$ could prevent tubulin polymerization as well as disrupt the preassembled microtubules (10). Nevertheless, this same concentration of calcium did not prevent or reverse the polymerization achieved with 1 mM $GMP(CH_2)P$ (Table 1). Electron microscopy of the tubulin assembled with 1 mM $GMP(CH_2)P$ and 2 mM $CaCl_2$ revealed a considerable number of short microtubules and, in some experiments, ribbons (Fig. 1*e*). Addition of the calcium after microtubule assembly resulted in no microtubule depolymerization (Fig. 1*d*).

Effect of $GMP(CH_2)P$ on Polymerization of Tubulin Purified by Phosphocellulose. When measured by the colchicine-binding method (10), purified tubulin treated with phosphocellulose, which effectively removes the tubulin-associated high molecular weight basic proteins (11), was still capable of being polymerized (30 min, 37°) by 1 mM $GMP(CH_2)P$ at high protein concentrations (18 μM tubulin) (data not shown). The extent of polymerization (10–17%), however, was less marked, in a manner that parallels the decreased efficiency (about 50–70%) of $GMP(CH_2)PP$-induced polymerization of phosphocellulose-purified tubulin (10). Also, in analogy with the observations with $GMP(CH_2)PP$ (10), the polymerization achieved with $GMP(CH_2)P$ was not reversed by 2 mM $CaCl_2$ (30 min, 37°) but could be reversed by cold (4°,

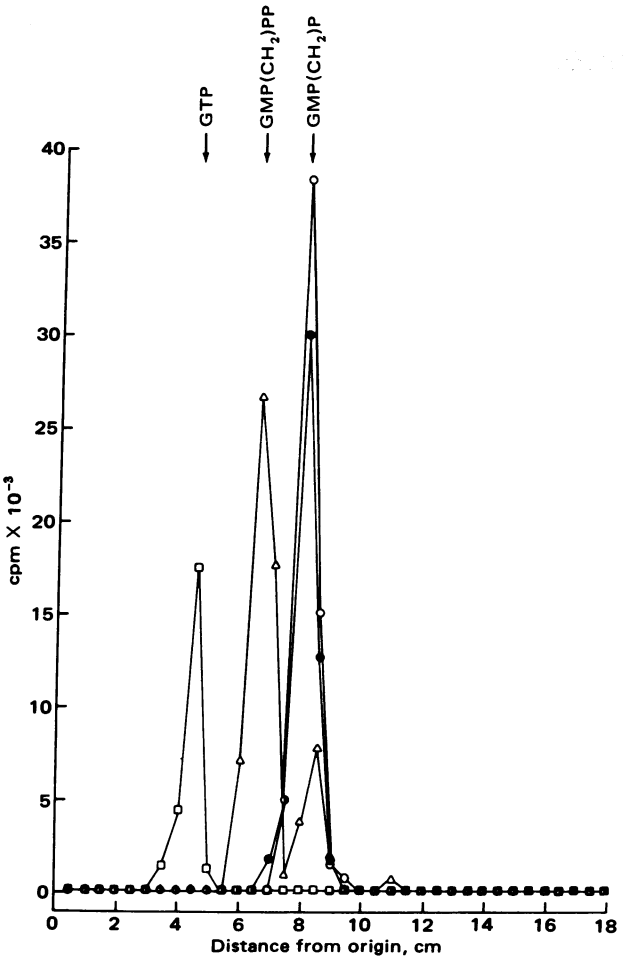

FIG. 3. Hydrolysis of $GMP(CH_2)PP$ by purified tubulin and by alkaline phosphatase. Tubulin (14 μM in 0.5 ml of buffer A) was preequilibrated (30 min, 4°) with [^{3}H]$GMP(CH_2)PP$ at 7.4 μM (3.49 Ci/mmol) (△, ○) or at 10 mM (1.3 Ci/mmol) (●) before being incubated (37°) for an additional 30 min with (○,●) or without (△) 0.35 unit of calf intestine alkaline phosphatase. [^{3}H]GTP standard (□). A 10-μl aliquot of each sample was processed as described in the legend of Fig. 2. The developed chromatograms were cut in 0.5-cm strips and ^{3}H was measured in 10 ml of Bray's solution (30% efficiency).

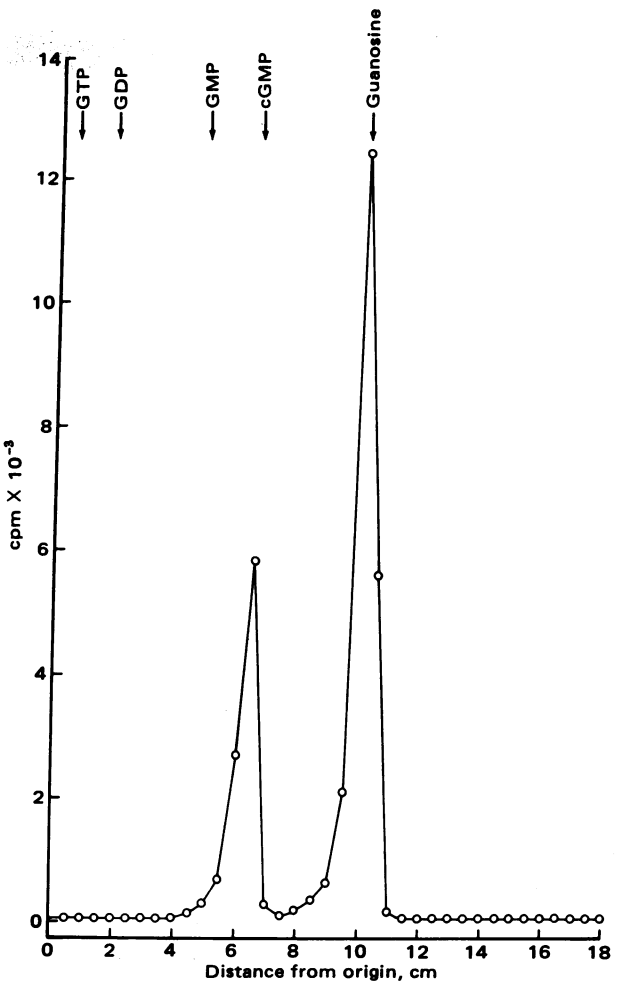

FIG. 4. Hydrolysis by alkaline phosphatase of the GTP, GDP, and GMP present in preparations of purified tubulin. The microtubules resulting from a second cycle of polymerization were resuspended in 0.5 ml of buffer A (18 μM tubulin) and preequilibrated (15 min, 4°) with 1 mM [^{3}H]GTP (86 Ci/mol) before being incubated (37°, 30 min) with 0.35 unit of calf intestine alkaline phosphatase. The phosphatase reaction was stopped as described in the legend to Fig. 2 and 20-μl aliquots of each sample were chromatographed on PEI fluorescent cellulose (2 hr, room temperature with 0.2 M NH_4CO_3 as the mobile phase). ^{3}H was measured in strips (0.5 cm) of the chromatograms. R_F values for standards are indicated by arrows.

30 min) (data not shown). Nevertheless, negative stain of these preparations failed to reveal the presence of microtubules.

DISCUSSION

The role of nucleotides in tubulin polymerization has been studied extensively (1–5, 12). Two molecules of GTP bind per molecule of tubulin dimer (3–5, 12). One of these (E) exchanges rapidly with GTP in the medium, while the second does not (N) (3–5, 12). During tubulin polymerization, the E-GTP is hydrolyzed to GDP (4, 12). Whether this hydrolysis is related to GTP production at the N binding site (3), and whether hydrolysis of the latter is required for tubulin polymerization, are controversial subjects (3–5, 7). Recently, Jacobs and Caplow (14) and MacNeal *et al.* (15) have suggested that complete filling of the E tubulin-binding site with GTP might not be a requisite for tubulin polymerization. Furthermore, the latter authors (16) have presented data which suggest that tubulin polymerization may be the result of hydrolysis of GTP to GDP at the N site, in the absence of nucleotide bound to the E binding site.

The present studies demonstrate that microtubule assembly is promoted by $GMP(CH_2)P$, the α-β methylene analogue of GDP (Fig. 1 and Table 1). The experimental conditions chosen, which include the presence of alkaline phosphatase in the polymerization incubation, exclude the possibility that the $GMP(CH_2)P$ effect could be the result of $GMP(CH_2)PP$ (10) formation by the transphosphorylase activity present in tubulin preparations (Fig. 2). Both the potential phosphate donors (GTP, GDP, and GMP) (Fig. 4) and the possible product [$GMP(CH_2)PP$] of this enzymatic reaction (Figs. 2 and 3) are completely destroyed by alkaline phosphatase under the polymerization conditions used here.

The discrepancy between the colchicine-binding assay and electron microscopy, both of which confirm microtubule formation, and the turbidity measurements, which do not, is not readily explained. The microtubules (Fig. 1*c*) are many fold longer than the wavelength of light (350 nm) and, if 20–30% of the tubulin was assembled into this form, we should expect an increase in turbidity. It is possible that only a portion of the turbulin that polymerized formed typical microtubules. The remainder may have polymerized into some intermediate form, large enough to sediment at 100,000 × *g* but not large enough

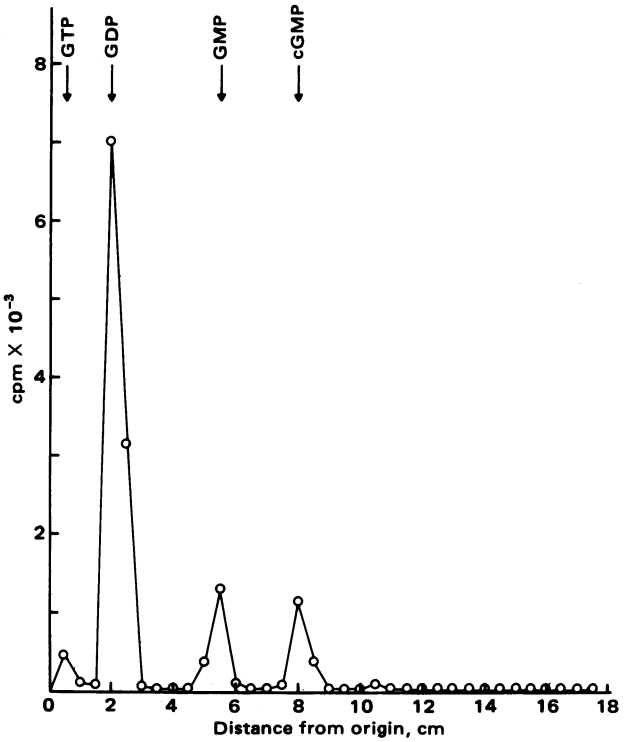

FIG. 5. Products of GTP obtained by tubulin polymerization with GTP. Tubulin (18 μM), purified by two cycles of polymerization-depolymerization, was incubated (37°, 30 min) with 1 mM [^{3}H]GTP (86 Ci/mol) and the GTP products resulting from the incubation were analyzed (20 μl) by PEI fluorescent cellulose chromatography (0.2 M NH_4CO_3, 2 hr, room temperature). R_F values of the standards are indicated by arrows.

to scatter light. This intermediate form may be short microtubules or perhaps an initiation site that has not undergone appreciable elongation.

The capacity of GMP(CH_2)P to induce tubulin polymerization (Table 1) excludes the possibility that binding of a nucleotide triphosphate to tubulin, followed by hydrolysis of the γ-phosphate bond, is an essential prerequisite for tubulin polymerization *in vitro*. Nevertheless, it is important to note that tubulin polymerization with GMP(CH_2)PP occurs with considerably greater efficiency than with GMP(CH_2)P (Fig. 1 and Table 1). The complete resistance of GMP(CH_2)P to hydrolytic cleavage (Fig. 3) also excludes any nucleotide hydrolysis or phosphate transfer at the E tubulin-binding site as a requisite for microtubule assembly. However, it is premature to conclude from these data that microtubule polymerization *in vitro* can occur as a process totally independent of metabolic or chemical energy. Although a requirement for chemical energy provided in the form of an externally added nucleotide can be excluded, energy could, in principle, be supplied if GTP hydrolysis were to occur at the N tubulin site, possibly as a consequence of a conformational change induced by the simple binding of a proper nucleotide to the E site. Alternatively, microtubule assembly *in vitro* may possibly occur in the total absence of phosphate bond hydrolysis, as the result of a favorable change in the conformation of the tubulin dimers (allosterism) induced by the binding of an appropriate diphospho- [GMP(CH_2)P] or triphospho-nucleoside [GTP or GMP(CH_2)PP] to the E site of tubulin.

The complete resistance to hydrolysis of the α-β methylene bond of GMP(CH_2)P may account for the capacity of this nucleotide, but not GDP (Table 1), to induce tubulin polymerization. Weisenberg *et al.* (8) have proposed that the calcium resistance of the microtubules assembled with GMPP(NH)P could result from the resistance of this nucleotide to hydrolysis. Our results with GMP(CH_2)PP (10), which show a more effective assembly of tubulin and a high degree of resistance to calcium, are consistent with an important role of GTP (especially α-β bond) hydrolysis in microtubule depolymerization. Furthermore, the demonstrated conversion of GMP(CH_2)PP into GMP(CH_2)P by preparations of purified tubulin (Fig. 3; and ref. 10) and the capacity of GMP(CH_2)P to promote the assembly of calcium-resistant microtubules, suggest that further conversion to GMP of the GDP produced by hydrolysis of GTP could be a requirement for microtubule disassembly in general and, in particular, for disassembly by calcium. Consistent with this hypothesis are our preliminary results showing the constant presence of GMP in microtubule pellets obtained after the incubation of purified tubulin with [^{3}H]GTP under polymerization conditions (Fig. 5). In addition to GMP, these same microtubule preparations also show the constant presence of a guanine nucleotide with the same R_F as cyclic GMP (Fig. 5).

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