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Role of nucleotides in tubulin polymerization: Effect of guanylyl 5′-methylenediphosphonate

(microtubules/guanosine nucleotides/high molecular weight basic proteins/calcium/electron microscopy)

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ABSTRACT **Incubation of 48,000 × *g* rat brain supernatants for 30 min at 37° with 1–2 mM guanylyl 5′-methylenediphosphonate [Gmp(CH$_2$)pp] results in polymerization of 95–98% of the tubulin present. This is considerably more than the 50% polymerization that can be achieved with the natural nucleotide, GTP, under optimal conditions. Gmp(CH$_2$)pp is also much more effective than GTP in inducing polymerization of purified tubulin. Assembly of microtubules with Gmp(CH$_2$)pp occurs at tubulin concentrations one-third of those possible with GTP. Furthermore, with Gmp(CH$_2$)pp, microtubule assembly does not require the high molecular weight basic proteins needed with GTP. Polymerization of tubulin by Gmp(CH$_2$)pp is neither prevented nor reversed by concentrations of calcium (2 mM) that can either prevent microtubule assembly or disrupt already formed microtubules if the nucleotide used is GTP or guanylyl imidodiphosphate. When Ca^{2+} is added before or after microtubule assembly, electron microscopy of the Gmp(CH$_2$)pp preparations reveals normal microtubules turning into twisted ribbons. Low temperature (4°) can both prevent and disrupt the tubulin assembled with Gmp(CH$_2$)pp although disruption proceeds much more slowly than when GTP is used.**

At physiological concentrations (0.5–1 mM) of Mg, the polymerization of tubulin "*in vitro*" has an essential requirement for nucleoside triphosphates (1, 2) and for high molecular weight basic (HMWB) proteins (3). Recently, Arai and Kaziro (4) have assembled microtubules in the presence of guanylyl imidodiphosphate [Gmpp(NH)p] that are relatively resistant to disruption by millimolar concentrations of calcium, conditions that cause extensive disruption of the microtubules assembled in GTP. To explain this apparently greater resistance to calcium, Weisenberg *et al.* (5) has proposed that the conversion of GTP to GDP, which is well known to occur during microtubule assembly, may normally be required for microtubule disruption in the cell. Because of the existence of GDP in microtubule preparations, little attention has been given to the possible importance of hydrolysis or reactions involving the α-β bond of GTP. Nevertheless, Zeeberg *et al.* (6) recently have detected the existence of [β-^{32}P]GDP and [β-^{32}P]GTP in preparations of purified tubulin after incubation with $^{32}P_i$ at room temperature. This result suggests that hydrolysis of the α-β GTP (or GDP) bond might be needed in the assembly or disassembly of tubulin. The present studies examine the capacity of the GTP analog, guanylyl 5′-methylenediphosphonate [Gmp(CH$_2$)pp], which is resistant to α-β bond hydrolysis, to induce the polymerization of tubulin.

MATERIALS AND METHODS

To prepare 48,000 × *g* brain supernatants, adult rat brains were homogenized in 1 volume of buffer A [0.1 M 2-*N*-morpholinoethanesulfonic acid (Sigma)/1 mM 2-mercaptoethanol/1 mM $MgCl_2$, pH 6.75]. Tubulin was purified from rat brains by two cycles of polymerization-depolymerization, as described by Shelansky *et al.* (7) but omitting glycerol in the second polymerization cycle. Microtubule pellets, stored for no more than 1 week at −90°, were resuspended by gentle homogenization in buffer A and centrifuged at 100,000 × *g* (1 hr, 4°) to eliminate aggregates; the tubulin in the supernatant was adjusted to the desired concentration. Purified tubulin (5 mg) was freed of HMWB proteins (confirmed by 6.5% Acrylamide/1.0% sodium dodecyl sulfate gels) by chromatography on a 10-ml phosphocellulose column (3); the column, equilibrated with 10 mM 2-*N*-morpholinoethanesulfonic acid/1 mM 2-mercaptoethanol/1 mM $MgCl_2$, was washed with the same buffer, and the HMWB protein-free tubulin was collected in the breakthrough. Purified tubulin (60 mg; 8.5 mg/ml) was freed of 95% of the exchangeable GDP by two consecutive batch treatments (30 min, 4°) with 500 mg of Norit pretreated with 2% albumin. Tubulin was polymerized by incubating the 48,000 × *g* brain supernatants or the purified (2% glycerol) tubulin, prepared in buffer A solutions, at 37° for a minimum of 30 min.

The nucleotide concentrations and polymerization periods used were those found to be optimal for GTP.* The rates of tubulin polymerization were measured by the turbidity developed at 350 nm (8), and polymerization was quantitated by subtracting the colchicine binding activity remaining in the microtubule-free 48,000 × *g* (30 min, 37°) supernatants from the total colchicine binding measured in the noncentrifuged controls containing the microtubules.* The colchicine binding activity was measured in parallel in both microtubule-free and corresponding control aliquots within a range of protein concentrations that gave linear colchicine binding when incubated (90 min, 37°) with 25 μM colchicine (970 Ci/mol) in buffer A containing 1 mM GTP. The colchine bound to tubulin was quantitated by the DEAE-filter method (9, 10).

Electron microscopy was done on preparations stained with 1% uranyl acetate.

RESULTS

Tubulin Polymerization in Crude Brain Preparations. Only 40–50% of the tubulin contained in adult rat brain supernatants (48,000 × *g*) polymerized when incubated (37°, ½–2 hr) with 1 mM GTP or Gmpp(NH)p (Table 1). In contrast, replacement of GTP by Gmp(CH$_2$)pp (1 mM) resulted in a 2-fold increase in the polymerization of tubulin, and less than 5% of the total tubulin could be detected in the microtubule-free supernatants. Attempts to polymerize (with 1 mM GTP) the tubulin (50%) that remained in the supernatant after the first GTP poly-

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Abbreviations: HMWB proteins, high molecular weight basic proteins; Gmpp(NH)p, guanylyl imidodiphosphate; Gmp(CH$_2$)pp, guanylyl 5′-methylenediphosphonate.

* I. V. Sandoval and P. Cuatrecasas, unpublished data.

Table 1. Effect of calcium and cold (4°) on induced polymerization of tubulin

$CaCl_2$ added		Time, min		Tubulin polymerized					
				Supernatant		Purified tubulin		1st polymer supernatant	
At 0 min	At 30 min	At 37°	At 4°	nmol/ml	%	nmol/ml	%	nmol/ml	%
				GTP-induced					
		60		45	50	10.5	67	4	9
		30	30	9	10	0	0		
+		60		0	0	1	6.4		
	+	60		3	3	1.5	9.5		
				Gmp(CH_2)pp-induced					
		60		86	95	15.5	99	37	83
		30	30	54	60	10.5	67		
+		60		88	97	15	95		
	+	60		89	98	15.5	99		
				Gmpp(NH)p-induced					
		60		37	41	8.5	54		
		30	30	—	—	0	0		
+		60		3	3	1.5	9.5		
	+	60		3	3	1	6.4		

Polymerization was studied on 48,000 × *g* brain supernatants (9×10^{-8} mol of tubulin/ml), first polymerization supernatants (4.5×10^{-8} mol/ml), or purified tubulin (15.7×10^{-8} mol/ml). $CaCl_2$ (2 mM) was added at the beginning of the incubation (60 min, 37°) or for 30 min (37°) after 30 min of preincubation (37°) to study the capacity to prevent or to disrupt, respectively, the tubulin polymers. The ability of cold to disrupt the tubulin polymers was studied by cooling, for 30 min (4°), tubulin samples preincubated for 30 min at 37°. With the exception of the cooled samples which were centrifuged at 4°, the tubulin polymers were pelleted by 48,000 × *g* centrifugation (30 min) at 37°. The nucleotides were tested at 1 mM; identical results were obtained in separate experiments using 2 mM concentrations. Tubulin polymerization was quantitated by colchicine binding.*

merization consistently failed or gave poor yields. However, replacement of GTP with Gmp(CH_2)pp resulted in high polymerization of this GTP-insensitive tubulin.

Tubulin Polymerization in Preparations of Purified Tubulin. Polymerization of tubulin into microtubules (Fig. 1) was much more efficient with Gmp(CH_2)pp than with GTP at all tubulin concentrations tested between 1.6 and 32 nmol/ml (Fig. 2). Furthermore, although microtubule assembly with both GTP and Gmpp(NH)p required a minimum tubulin concentration of 1.6 nmol/ml, this threshold concentration fell to about 0.6 nmol/ml when GTP was replaced by an equal concentration of Gmp(CH_2)pp. When the rates of tubulin (1.5 mg/ml) polymerization were measured by turbidimetry (8), the short time lag ordinarily found with GTP was absent with Gmp(CH_2)pp (Fig. 3). With only 5% of the total exchangeable nucleotide binding sites of the tubulin filled with GDP, half of the maximal assembly occurred at 0.1 mM Gmp(CH_2)pp (Fig. 4). Tubulin treatment with Norit caused a 50% decrease in the capacity of tubulin to polymerize with GTP but affected the polymerization with Gmp(CH_2)pp only slightly. Determination of the GTP concentration required to cause 50% of the maximum polymerization was hampered by both the degradation of GTP in the absence of regenerating system and its catalytic effect on polymerization in the presence of GDP (5%) and regenerating system (10) (Fig. 5). At 1 mM, Gmp(CH_2)p failed to promote polymerization as measured by turbidimetry (Fig. 3).

HMWB Proteins Are Not Required for Microtubule Assembly with Gmp(CH_2)pp. As described by Weingarten *et al.* (3), removal of HMWB proteins from purified tubulin destroys its ability to assemble into microtubules with 1 mM MgGTP (Fig. 5). Nevertheless, tubulin free of HMWB proteins did assemble effectively into normal microtubules upon incubation with MgGmp(CH_2)pp (Fig. 5).

Effect of Calcium on Tubulin Polymerization in Crude Brain Preparations. When 48,000 × *g* brain supernatants were incubated with 2 mM $CaCl_2$, 1 mM GTP or Gmpp(NH)p failed to induce polymerization of tubulin (Table 1). Notably, tubulin readily polymerized (97%) in 2 mM $CaCl_2$ if Gmp(CH_2)pp was present. Although 2 mM $CaCl_2$ could completely disrupt the microtubules formed by incubating the brain supernatants at 37° for 30 min with GTP or Gmpp(NH)p, it had no effect on the tubulin assembled with Gmp(CH_2)pp.

Effect of Calcium on the Polymerization of Purified Tubulin. At 2 mM, $CaCl_2$ prevented tubulin polymerization and disrupted (into amorphous aggregates) microtubules preassembled with either 1 mM GTP or Gmpp(NH)p (Fig. 2; Table 1). The same concentration of calcium did not prevent or reverse the polymerization of tubulin if 1 mM Gmp(CH_2)pp was used (Figs. 2 and 3; Table 1). When the rates of tubulin polymerization with Gmp(CH_2)pp were measured with and without 2 mM $CaCl_2$, faster rates were observed in the absence of the cation (Fig. 3). Electron microscopy of the tubulin polymerized with 1 mM Gmp(CH_2)pp and 2 mM $CaCl_2$ revealed no microtubules but a large amount of twisted ribbons (Fig. 1*g*). Treatment (30 min) of microtubules preassembled in Gmp(CH_2)pp with 2 mM $CaCl_2$ resulted in normal microtubules in a field crowded with a large number of twisted ribbons (Fig. 1*d–f*); the proportion of ribbons increased as the concentration of tubulin decreased. The calcium-resistant microtubules and ribbons assembled in mM Gmp(CH_2)pp became sensitive to calcium after addition of 2 mM GDP (Fig. 6).

Depolymerization by Cold of Tubulin Assembled with Gmp(CH_2)pp. Microtubule assembly with Gmp(CH_2)pp did not occur at 4° (Fig. 1*i*), but the microtubules were partially resistant to cold. Incubations at 4° for as long as 60 min failed to achieve complete disruption (Table 1; Fig. 1*h*) but very quickly and completely disrupted those microtubules assembled with GTP.

Gmp(CH_2)pp Is Hydrolyzed into Gmp(CH_2)p by Preparations of Purified Tubulin. Incubation (30 min, 37°, final volume 0.5 ml) of 1 mM [^{3}H]Gmp(CH_2)pp with 18 μM tubulin

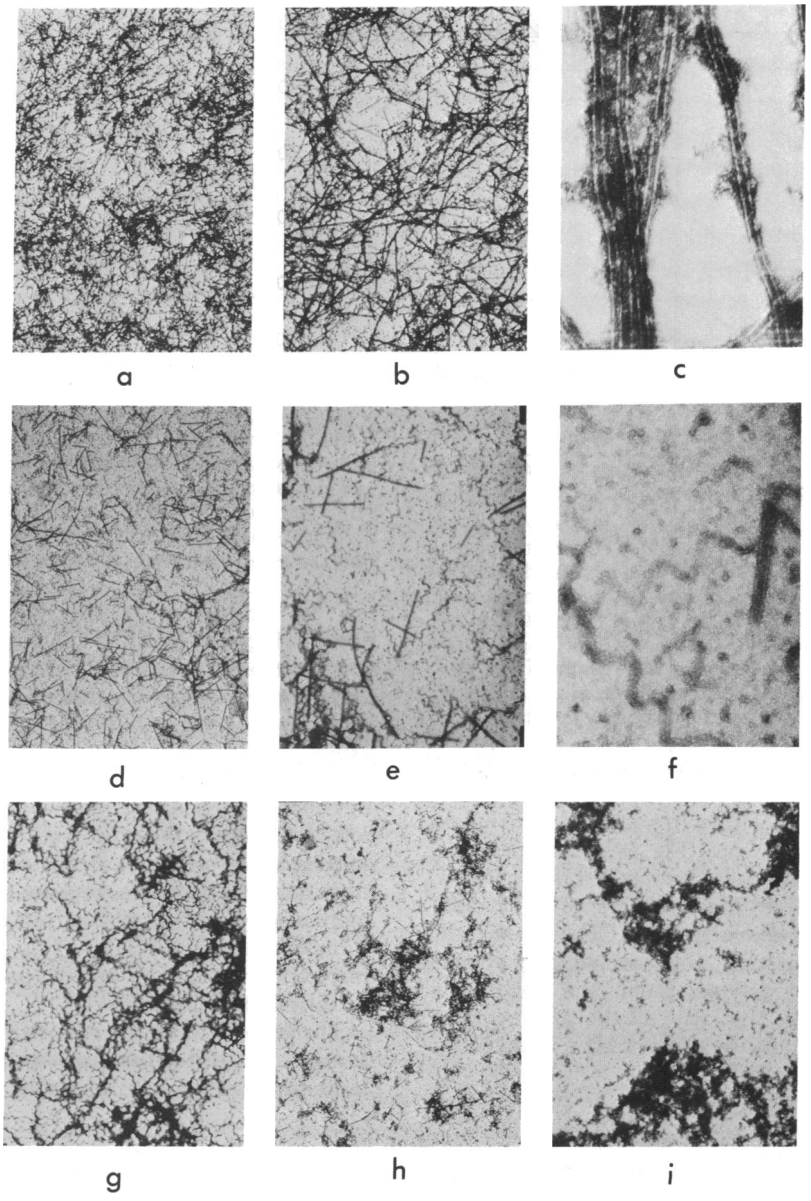

FIG. 1. Morphological study of the ability of calcium and cold to prevent and to disrupt microtubules assembled with Gmp(CH_2)pp. (*a*, *b*, and *c*) Purified tubulin (0.5 mg/ml) was incubated with 1 mM Gmp(CH_2)pp at 37° for 30 min. (*a*, ×2600; *b*, ×6500; *c*, ×65,000.) (*d*, *e*, and *f*) Purified tubulin (0.5 mg/ml) was incubated with 1 mM Gmp(CH_2)pp at 37° for 30 min and then for an additional 30 min at 37° with 2 mM $CaCl_2$. (*d*, ×2600; *e*, ×6500; *f*, ×65,000.) (*g*) Purified tubulin (1.5 mg/ml) was incubated with 1 mM Gmp(CH_2)pp and 2 mM $CaCl_2$ at 37° for 30 min. (×6500.) (*h*) Purified tubulin (0.5 mg/ml) was incubated with Gmp(CH_2)pp at 37° for 30 min and then for an additional 60 min at 4° (×2600.) (*i*) Purified tubulin (0.5 mg/ml) was incubated with 1 mM Gmp(CH_2)pp at 4° for 30 min. (×2600.) Similar incubations of tubulin at 1.5 mg/ml with GTP and Gmp(CH_2)pp revealed microtubules under incubation conditions identical to those described in *a*,*b*, and *c* but only amorphous aggregates under the conditions described in *d*–*i*.

resulted in the production of 50 nmol of Gmp(CH_2)p, in excess of the 9 nmol of tubulin in the assay; however, no GMP was detected (Fig. 7). Analogous incubations of tubulin with GTP resulted in both GDP (95 nmol) and GMP (9.5 nmol) production.

DISCUSSION

The analog of GTP, Gmp(CH_2)pp, may be useful for probing the mechanism of tubulin polymerization and the role of nucleotides in this process. Although, like GTP, it permits the polymerization of tubulin into normal microtubules (Fig. 1*c*), Gmp(CH_2)pp displays some outstanding properties that differentiate its effects from those of GTP.

First, although acting at the GTP exchangeable binding site (Fig. 6), Gmp(CH_2)pp appears to be much more effective than GTP in facilitating polymerization because it permits the process to occur at much (i.e., 3 times) lower concentrations of tubulin in both crude brain supernatants and preparations of purified tubulin (Table 1; Fig. 2). In addition, the polymerization process proceeds much more extensively and efficiently because nearly all of the detectable tubulin can be made to polymerize with Gmp(CH_2)pp in both crude and purified preparations of tubulin whereas with GTP it is difficult to effect polymerization of more than 50% of the total tubulin present in crude preparations, more than 9% of the first polymerization supernatants, and more than 70% of the purified tubulin (Table 1).

Tubulin polymerization in the presence of Gmp(CH_2)pp

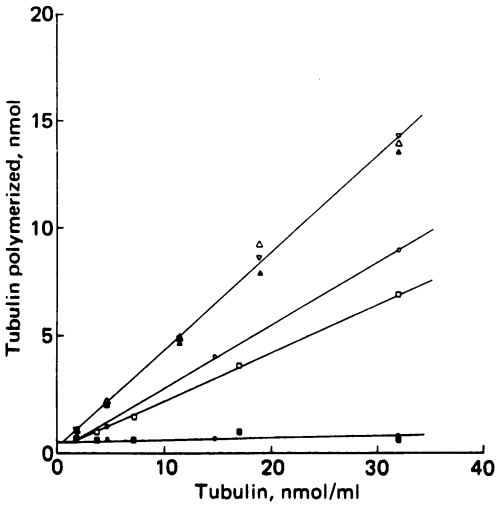

FIG. 2. Effect of nucleotides on the relationship between concentration of tubulin and microtubule assembly. Different concentrations (2–32 nmol/ml) of purified tubulin were incubated with 2% glycerol for 60 min at 37°, and the polymerization of tubulin was quantitated by colchicine binding.* ○, GTP; ●, GTP plus $CaCl_2$; △, $Gmp(CH_2)pp$; ▲, $Gmp(CH_2)pp$ plus $CaCl_2$; ▽, $Gmp(CH_2)pp$ plus $CaCl_2$ after 30 min of polymerization; □, Gmpp(NH)p; ■, Gmpp(NH)p plus $CaCl_2$. All nucleotides were assayed at 1 mM, and 2 mM $CaCl_2$ was added at the start of the incubation except in ▽.

exhibits, in addition, some notable properties that are qualitatively different from those observed with GTP. Despite the fact that the microtubules formed with both nucleotides are indistinguishable by electron microscopy (Fig. 1*c*), subsequent addition of 2 mM calcium completely disrupts and thus reverses the microtubules formed in the presence of GTP and Gmpp(NH)p but those formed with $Gmp(CH_2)pp$ are only partially affected and are partially transformed to an apparently intermediate state of polymerization, the ribbon-like structures (11) (Fig. 1 *d–f*). Similarly, all the tubulin polymerization induced by GTP can be prevented by 2 mM calcium, but the ribbon-like state still forms if $Gmp(CH_2)pp$ is present (Figs. 1*g*, 2, and 3; Table 1). Thus, this nucleotide appears to stabilize a stage of tubulin polymerization (i.e., ribbons) that ordinarily is only transitory when tubulin is incubated for short periods of time with GTP (11). By virtue of this stabilization, the formation of a subsequent, more advanced and favored stage, that of microtubule formation, may be promoted by $Gmp(CH_2)pp$. These observations also point to the possible role of calcium in the process of disaggregation, because they suggest an involvement at the stage of microtubule–ribbon transition. Furthermore, because cold prevents and reverses more readily (without ribbon formation) the tubulin polymers formed with $Gmp(CH_2)pp$ (Fig. 1), the effects on depolymerization by calcium and low temperature might be exerted by different mechanisms.

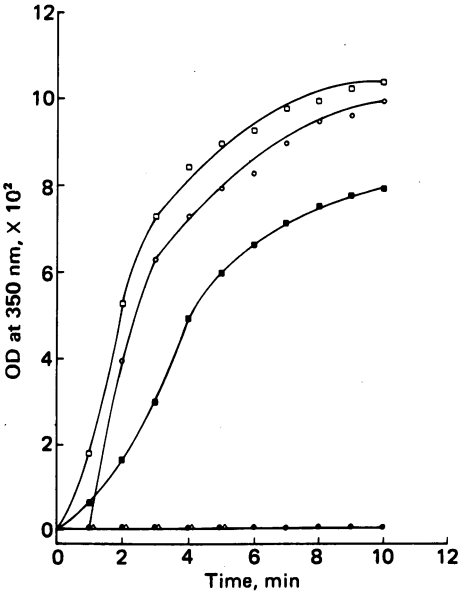

FIG. 3. Influence of calcium on the rates of tubulin polymerization with GTP or $Gmp(CH_2)pp$, and inability of $Gmp(CH_2)p$ to induce tubulin polymerization. Tubulin (1.5 mg/ml) was polymerized at 37° with (filled figures) or without (open figures) 2 mM $CaCl_2$ plus 1 mM GTP (○, ●), 1 mM $Gmp(CH_2)pp$ (□,■), or 1 mM $Gmp(CH_2)p$ (△), and the turbidity at 350 nm was measured.

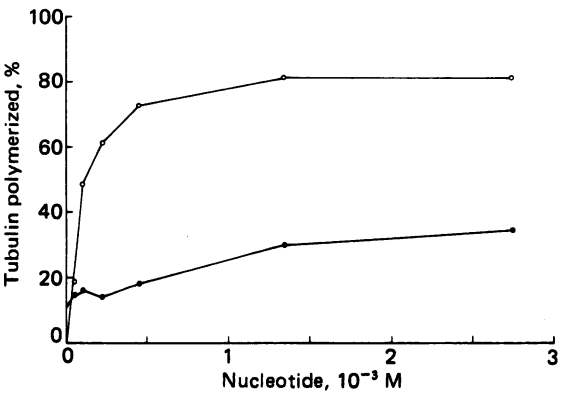

FIG. 4. Relationship between the concentrations of GTP and $Gmp(CH_2)pp$ and tubulin polymerization. Purified tubulin was freed (95%) of exchangeable GDP by Norit treatment, adjusted to 2 mg/ml, and incubated at 37° for 30 min with 1 mM GTP (●) or $Gmp(CH_2)pp$ (○) in the presence of a GTP-regenerating system consisting of creatine phosphate (1.6 mg/ml) and creatine kinase (0.28 mg/ml). Tubulin polymerization was quantitated by colchicine binding.

The present studies are consistent with and provide support for the scheme of sequential steps of polymerization from tubulin proposed by Kirschner *et al.* (12) and which involves a

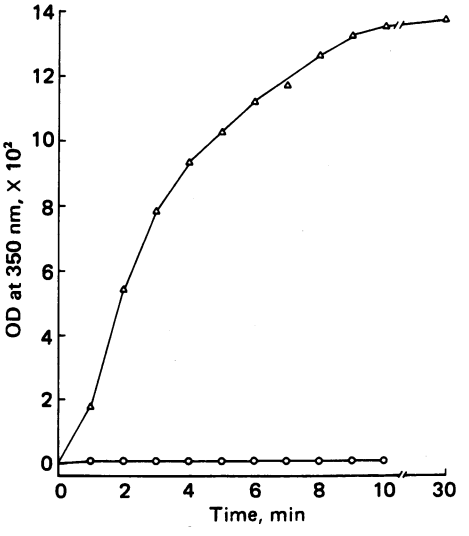

FIG. 5. Ability of $Gmp(CH_2)pp$ to induce the polymerization of tubulin freed of HMWB proteins by chromatography on a phosphocellulose column. Tubulin (2 mg/ml) polymerization was measured by turbidimetry. Both GTP (○) and $Gmp(CH_2)pp$ (△) were tested at 1 mM. Parallel quantitation of tubulin polymerization by colchicine binding showed 88% polymerization with $Gmp(CH_2)pp$ and 2% with GTP.

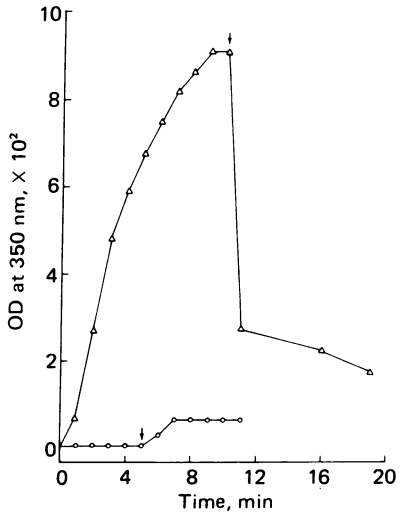

FIG. 6. Reversal by GDP of calcium-insensitive tubulin assembled in Gmp(CH_2)pp, and Gmp(CH_2)pp-induced polymerization of tubulin preincubated with GTP and calcium. Tubulin (1.5 mg/ml) was polymerized at 37° in 2 mM $CaCl_2$ with 1 mM GTP (○) or 0.1 mM Gmp(CH_2)pp (△). At the times indicated (arrows), 2 mM GDP (△) or 2 mM Gmp(CH_2)pp (○) was added and the incubation was continued at 37°.

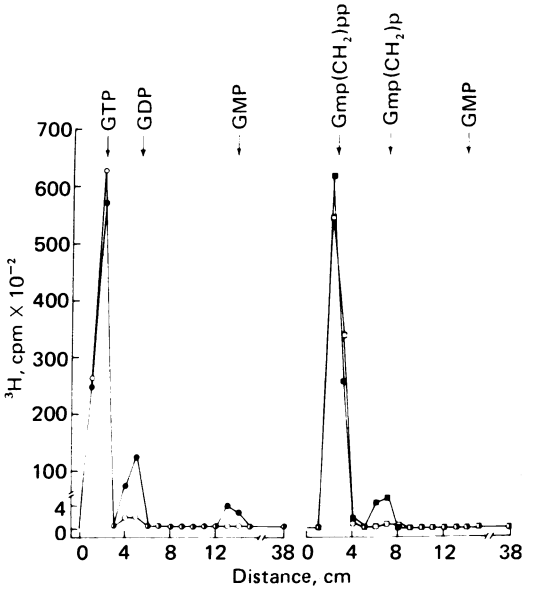

FIG. 7. Gmp(CH_2)pp conversion into Gmp(CH_2)p by purified tubulin preparations. Purified tubulin at 18 μM (○ and □, boiled; ● and ■, native) was incubated (37°, 0.5 ml of buffer A) with 1 mM [^{3}H]Gmp(CH_2)pp (16 Ci/mol) (○, ●) or 1 μM [^{3}H]GTP (16 Ci/mol) (□,■). The incubation at 37° was stopped after 30 min with 125 μl of 5% sodium dodecyl sulfate. The detergent was precipitated by cooling the samples at 0° (15 min) and removed by centrifugation. The samples then were freed of protein by filtration through a 0.22-μm pore size Millipore filter, and 10 μl of them and of the standards (GTP, Gmp(CH_2)pp, GDP, Gmp(CH_2)p, and GMP, all 1 mM) were run on PEI-F cellulose thin layers. The chromatography was done in duplicate with 0.2 NH_4HCO_3 (4 hr, room temperature) and the resulting chromatograms cut in 0.5-cm strips and assayed for ^{3}H.

proposed penultimate ribbon-like structure that rapidly equilibrates into a microtubule by closure or folding of the individual ribbons. Although formation of microtubules under physiological conditions (1 mM $MgCl_2$) appears to require HMWB proteins (3), it is notable that these proteins are not required when Gmp(CH_2)pp is used (Fig. 5). Clearly, although these proteins may normally assist or in some way facilitate formation of the microtubular state, they are not absolutely required for this process. The tubulin molecule must possess, within itself, all of the information necessary for the proper assembly into microtubules. These considerations suggest that perhaps the normal role of the HMWB proteins may be to stabilize (and thus perhaps to "trap") the final microtubular structures which, when formed in the presence of Gmp(CH_2)pp, are sufficiently stable not to require the assistance of other proteins. In addition, the possibility must also be considered that the effects of calcium on the processes discussed above may result in large part by antagonizing the microtubule-stabilizing effect of these HMWB proteins.

The exact reasons for the unique properties of Gmp(CH_2)pp, compared to those of GTP and other analogs, are not yet clear. It is known (13) that, during the normal process of polymerization, the exchangeable GTP bound to tubulin is converted to GDP, although it is not known whether the hydrolytic process is in itself absolutely required for this stage to occur. Weisenberg *et al.* (5) interpreted the higher resistance to calcium of the microtubules assembled with Gmpp(NH)p (compared to GTP) as evidence that GTP hydrolysis may not be essential for microtubule formation but rather may render the microtubules susceptible to subsequent disruption, such as occurs with calcium. The possibility that the unusual α-β methylene bond of Gmp(CH_2)pp may distort the geometry of the molecule (compared to GTP) with respect to the β-γ bond, such that GTPase hydrolysis would be impaired, has been discarded by demonstrating that as much as 50 nmol of Gmp(CH_2)p is generated when 18 μM tubulin is incubated (30 min, 37°) with 1 mM GMP(CH_2)pp and because the effects of Gmp(CH_2)pp and Gmpp(NH)p on tubulin polymerization are different quantitatively and qualitatively. It is more reasonable to predict that the highly unique character of Gmp(CH_2)pp is derived from its α-β bond, which is certainly not susceptible to hydrolysis. In this case, the product analogous to tubulin-GDP would be tubulin-Gmp(CH_2)p, a distinctly different derivative compared to GDP, Gmpp(NH_2)p, or Gmpp(NH_2). Thus, the possibility must be seriously considered that the normal process of GTP involvement in microtubule assembly-disassembly may involve, at some stage, not only hydrolysis of the terminal β-γ but also of the α-β phosphate bond.

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