Assembly of Microtubules from Tubulin Bearing the Nonhydrolyzable Guanosine Triphosphate Analogue GMPPCP [Guanylyl 5′-(β,γ-Methylenediphosphonate)]: Variability of Growth Rates and the Hydrolysis of GTP†

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ABSTRACT: The growth and shortening of microtubules in guanosine triphosphate- (GTP-) mediated dynamic instability has previously been observed to occur at rates which are remarkably variable (Gildersleeve et al., 1992, Chrétien et al., 1995). Neighboring microtubules observed simultaneously can grow or shorten at different rates, and a particular microtubule can undergo changes in rate with the passage of time. This paper addresses the question whether this variability has its origin in processes that involve GTP hydrolysis or whether it results from variations in the structure of microtubules that are independent of GTP hydrolysis. Tubulin was prepared with the nonhydrolyzable GTP analogue GMPPCP [guanylyl 5′-(β,γ-methylenediphosphonate)] bound to its exchangeable nucleotide-binding site and with GTP at its nonexchangeable site. Extensive measurements of length changes were obtained by DIC microscopy. Microtubules formed from the GMPPCP tubulin exhibited only growth. No shortening events were observed. Growth occurred at highly variable rates, indistinguishable from those exhibited by GTP tubulin. Subsequent analysis of nucleotides by high-pressure liquid chromatography (HPLC) revealed that some of the GTP that was initially present at the N-site underwent hydrolysis to produce microtubule-bound guanosine diphosphate (GDP). Despite this unexpected finding, one can conclude that variability of growth rate certainly occurs independently of dynamic instability and probably does not involve hydrolysis of GTP at the E-site.

Microtubules, in the presence of their tubulin subunits, spontaneously undergo large excursions of growth and shortening (polymerization and depolymerization) known as dynamic instability [Horio & Hotani, 1986; Mitchison & Kirschner, 1984; Walker et al., 1988; recent reviews: Erickson and O'Brien, (1992) and Bayley et al. (1994)]. Gildersleeve et al. (1992) observed that the growth and shortening occurs at highly variable rates. This variability has subsequently been observed in several other studies, both in vitro (Drechsel et al., 1992; Chrétien et al., 1995) and in vivo (Shelden & Wadsworth, 1993; Dhamodharan & Wadsworth, 1995). Earlier studies in which broad distributions of rates were measured have been reviewed by Gildersleeve et al. (1992). Observable variability of rates within cells may result from modulating factors present there. Indeed, the fundamental variables of dynamic instability (the rates of growth and shortening and the frequencies of transition between the growing and shortening states) are strongly modulated by the presence of MAPs[1] (Drubin & Kirschner, 1986; Bré & Karsenti, 1990; Pryer et al., 1992; Drechsel et al., 1992; Kowalski & Williams, 1993a; Panda et al., 1995) and affected by the ratio of GTP/GDP in the surrounding solution (Vandecandelaere et al., 1995). Nevertheless, in the absence of MAPs and in the presence of large excesses of GTP over GDP, the fundamental variability remains.

The large (severalfold) variability of rates that is observed would not be expected on the basis of a straightforward understanding of the processes of polymerization and depolymerization, such as that described by Walker et al. (1988). In that description, one imagines that a uniform population of tubulin dimers associate with, and dissociate from, a uniform population of microtubule ends [either (+)- or (−)-ends]. Association is taken to be a second-order process and dissociation a first-order one, and both processes occur during growth as well as during shortening. Because the occurrence of an observable change in length requires a large number of single association or dissociation events, variations in the rates of addition and removal of subunits would not be observable at the level of resolution of the light microscope (roughly 0.3 μm, or 500 subunits). Rather, they would be smoothed out, in effect, by the statistics of large numbers. Contrary to these expectations, a severalfold variation in rates is actually observed. This fact implies that some aspect of the straightforward view of the assembly/disassembly process is too simple.

Potential causes of variability can be divided into two classes: those which involve nucleotide hydrolysis as an essential part of the cause and those which rest upon variations of some kind in the contacts between tubulin

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[1] Abbreviations: DMSO, dimethyl sulfoxide; DTE, 1, 4-dithioerythritol; kDa, kilodalton; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; E-site, exchangeable nucleotide binding site of tubulin; GMPCPP, guanylyl 5′-(α,β-methylenediphosphonate); GMPPCP, guanylyl 5′-(β,γ-methylenediphosphonate); HPLC, high-pressure liquid chromatography; MAP, microtubule-associated protein; Mops, 3-[*N*-morpholino]propanesulfonic acid; N-site, nonexchangeable nucleotide-binding site of tubulin; Pipes, 1,4-piperazinediethanesulfonic acid; TBA, tetrabutylammonium phosphate. Buffer notation: P (0.1 M Pipes/NaOH, pH 6.9); E (EGTA); M ($MgSO_4$); D (DTE); subscripts (no subscript = 1 mM, subscript 2 = 2 mM, etc.). Therefore, PE_2MD_2 is 0.1 M Pipes/NaOH, pH 6.9, 2 mM EGTA, 1 mM $MgSO_4$, and 2 mM DTE.

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subunits. (Examples of such geometric variations might be changes in number of protofilaments with distance along the microtubule, incorporation of discontinuities into the tubulin lattice, or variability in shape of the microtubule's end during the course of growth and shortening.) A demonstration that nucleotide hydrolysis *can* be involved in the production of variable rates has been provided by the recent study of Vandecandelaere et al. (1995), which showed that the presence of large fractions of GDP (>0.25 of the total nucleotide) in tubulin solutions leads to variable observed rates of growth and shortening, chiefly through reduction of the lengths of growth and shortening events, an effect which lends the length-vs-time plot a meandering character. The phenomenon was modeled qualitatively by the lateral cap model (Martin et al., 1993) and could be understood as resulting from cooperativity of association behavior at the microtubule's end. This demonstration must be regarded, though, as explaining only part of the phenomenon. In the cellular context, and in experiments conducted with pure tubulin *in vitro*, the fraction of GDP is small, and yet variability is observed.

The class of proposed mechanisms that rely on variable geometry includes the explanation put forward by Chrétien et al. (1995). That work exploits the long-standing (Erickson, 1974; Kirschner et al., 1975; Detrich et al., 1985) but recently reinforced (Simon & Salmon, 1990; Mandelkow et al., 1991) understanding that microtubules grow by forming a sheetlike extension at the end which subsequently rolls up into a tubule. Addition of subunits to the microtubule's end is taken to be a uniform process, but the closure of the "seam" is supposed to take place at a non-uniform rate.

To investigate further the question of whether it is nucleotide hydrolysis or hydrolysis-independent geometry of the ends that is more influential in producing variability in dynamic instability, we have observed the assembly of individual microtubules from tubulin in which the GTP usually present at the exchangeable site (E-site) has been replaced by the nonhydrolyzable GTP analogue GMPPCP. Although it forms microtubules, this protein is not expected to support dynamic instability because of its inability to hydrolyze the E-site nucleotide (Mejillano et al., 1990a,b; Seckler et al., 1990, Shearwin & Timasheff, 1992). As expected, we observed only microtubule growth and no shortening events. The growth occurs at highly variable rates. This result implies that the shortening events characteristic of dynamic instability are not necessary to produce variability of microtubule assembly rates. We also made the unexpected observation that some of the GTP initially present at the nonexchangeable site (N-site) becomes hydrolyzed during assembly. This additional result lends complexity to the interpretation and suggests models for nucleotide exchange between the subunits of tubulin dimers.

MATERIALS AND METHODS

Reagents. $MgSO_4$ and EGTA were from Fisher (Stone Mountain, GA), glycerol was from Mallinckrodt (St. Louis, MO), and Affi-Gel 10 was from Bio-Rad (Hercules, CA). Pipes, GTP, GDP, and GMPPCP were from Boehringer Mannheim (Indianapolis, IN). Very pure stocks of GTP, used for calibrations and in certain controls where contamination with GDP was undesirable (see below), were obtained from U.S. Biochemical Corp. (Cleveland, OH). Ultrol brand of Pipes, taxol, and GMPPCP were purchased from Calbiochem (La Jolla, CA). GMPCPP was the gift of Dr. J. J. Correia. Sea urchin (*Lytechinus pictus*) sperm were a gift of Dr. Leslie Wilson.

Preparation of Tubulin and Axonemes. Bovine brain tubulin was prepared by three cycles of assembly and disassembly followed by phosphocellulose chromatography [Williams and Lee (1985) as modified by Correia et al. (1987)] and stored at −70 °C after dropwise freezing in liquid nitrogen. In some instances, the protein was concentrated to 25−30 mg/mL in a Centriprep 30 centrifugal concentrator (Amicon, Beverly, MA) before freezing. It was prepared for use by rapid thawing, centrifugation, and immediate exchange into the buffer of choice (PE_2MD_2 or PM_2) by the rapid gel-filtration method of Penefsky (1977). All handling of proteins was done either on ice or at 4 °C. Preparation of axonemes is described in detail in Williams (1992).

Immobilized Alkaline Phosphatase. Calf intestine alkaline phosphatase (immunoassay grade, Boehringer Mannheim) was coupled to Affi-Gel 10 beaded agarose at pH 7.5 in 0.1 M Mops buffer, according to the manufacturer's instructions. An amount of 900 units of enzyme/mL of gel was added to the coupling reaction.

Removal of E-Site Nucleotide. Complete removal of E-site nucleotide was accomplished according to Seckler et al. (1990) by treatment of GDP/GTP tubulin (the notation implies an inferred content of 1 GTP and 1 GDP/tubulin dimer) with immobilized alkaline phosphatase in the presence of GMPPCP, followed by immediate buffer exchange by means of a centrifuged gel-filtration column (Penefski, 1977). To do this, tubulin containing 0.1 mM GTP was thawed, centrifuged briefly to remove small amounts of denatured protein, made 5 or 10 mM in GDP, kept on ice for 10 −15 min, and then exchanged into $PE_2M_2D_2$. GMPPCP was immediately added to a final concentration of 2 mM. One milliliter of the resulting tubulin solution was then applied to a 3-mL column of Affi-Gel 10/alkaline phosphatase, which had previously been equilibrated in incubation buffer containing 2 mM GMPPCP and drained. The tubulin was allowed to remain in the column at 4 °C for 2−3 h and was then eluted with 5 mL of incubation buffer and collected in 1-mL fractions. Tubulin-containing fractions were pooled and frozen dropwise in liquid nitrogen. Tubulin thus prepared, when rapidly thawed and immediately exchanged twice into nucleotide-free PM_2 buffer by means of a centrifuged gel-filtration column of Sephadex G-50 fine and analyzed by HPLC, was shown to contain essentially 1 mol of GTP/mol of tubulin dimer (see Results). This material was frozen dropwise in liquid N_2 and stored at −70 °C until use. It is referred to as −/GTP tubulin, to indicate the absence of nucleotide from one site, presumably the E-site.

Microtubule Assembly in Taxol. Where noted, taxol was employed to stimulate microtubule assembly. An amount was added from a 10 mM stock in dimethyl sulfoxide sufficient to produce a solution of 100 μM. The solubility of taxol in water is near 30 μM but has recently been measured as 76 μM in 0.1 M Pipes, pH 6.9, at 20 °C (Melki et al., 1996). After allowance for binding of some 10 μM to tubulin, this amount probably produces a barely saturated solution. It is referred to as 100 μM taxol below, even though some of the taxol may not be in solution.

Nucleotide Extraction and Analysis. Nucleotides were extracted from tubulin solutions by the method of Seckler et al. (1990) with minor modifications. After addition of ice-cold $HClO_4$ to a final concentration of 0.5 M to precipitate

the protein, the solution was incubated on ice for 10 min and then centrifuged for 10 min in an Eppendorf microcentrifuge at 4 °C. The supernatant was removed and $^1/_6$ volume of 1 M K_2HPO_4 was added, followed by an additional $^1/_6$ volume of 3 M KOH. The solution was incubated on ice for 10 min and centrifuged as before. The supernatant was filtered through a Millipore filter (Type HV 0.45 μm) and diluted 1:5 with mobile-phase buffer (see below) before injection into HPLC. The method was modified as follows for small samples or those with low concentration. (1) Instead of removing precipitated protein by direct centrifugation, it was removed by filtration in centrifugal ultrafiltration units (Millipore Ultrafree-MC, 10 kDa nominal molecular weight limit), and (2) the final dilution with mobile phase was omitted. Injection of undiluted extracts increased the void volume disturbance but had no effect on the separation or quantitation of controls. To confirm that the process of extraction did not hydrolyze GTP to GDP, controls were conducted with the use of GTP uncontaminated by GDP (Calbiochem). Nucleotide analysis was by isocratic reversed-phase ion-pair HPLC (Perrone & Brown, 1984) on octadecylsilica (Vydac 5 μm C18, 250 $\times$ 4.6 mm), with detection of A_{253}. Peaks were identified by comparison of their retention times and order of elution to standards run daily. The mobile phase was 150 mM KH_2PO_4/KOH, pH 5.9, plus 0.5 mM tetrabutylammonium phosphate (TBA). Injection volume was 20 μL and the flow rate was 1.0 mL/min. These conditions were found to be optimal for separation. The system was quantitatively calibrated before each day of operation by the use of GMPPCP whose concentration was determined by absorption, using $\epsilon_{253} = 13\,700$ M^{-1} cm^{-1}, (Dawson et al., 1986). Quantities eluted were determined by comparison of their areas to the area produced by the injection of GMPPCP of known concentration. Blanks of the buffer in which the protein was dissolved were run at regular intervals to assure there were no interfering contaminants. (Batches of Pipes from Boehringer Mannheim were received containing a contaminant which, due to absorption at 253 nm, interfered with quantitation. Ultrol brand of Pipes from Calbiochem was used thereafter.) DTE and EGTA absorb light at 253 nm and interfered with quantitation of nucleotides; therefore, solutions were cleared of these constituents by buffer exchange prior to HPLC. Small fluctuations in the retention times (cf. Figure 1) were noted. These were especially noticeable in mixtures of nucleotides. Concentration of protein prior to extraction was determined by absorbance at 280 nm. The absorbance was first adjusted for small amounts of scattering by subtracting $[(320)^4/(280)^4]A_{320}$. An extinction coefficient of 1.20 mL mg^{-1} cm^{-1} (Detrich and Williams, 1978) was applied to the corrected absorbance of native tubulin and an adjusted value of 1.14 mL mg^{-1} cm^{-1} (calculated by subtracting the A_{280} due to 1 GTP) to the absorbance of tubulin containing no E-site nucleotide. A molecular weight of 100 kDa was assumed for the tubulin dimer.

Microscopy, Measurement, and Analysis. Techniques were essentially those described by Williams (1992) and by Gildersleeve et al. (1992). Pieces of sea urchin sperm axonemes, which serve as nuclei for microtubule growth, were applied to a glass coverslip and allowed to adhere. Purified tubulin in PM_2 buffer, augmented with either 1 mM GTP or 2 mM GMPPCP as appropriate, was then added, and the mixture was sealed between slide and coverslip. Microtubule growth was started by placing the sample on the stage of the microscope, maintained at 37°. Microtubules were observed by the use of video enhanced differential interference contrast microscopy and recorded on videotape. Measurement of microtubule lengths as a function of time was carried out at intervals of approximately 5 s from the videotaped images by means of a computer program similar to that described by Kowalski and Williams (1993a). As explained in Results, because it was not possible to distinguish microtubule plus-ends from minus-ends in the case of GMPPCP-mediated growth, the distinction between plus and minus ends was neglected in measurement of control microtubules as well.

The resulting data of length *vs* time were numerically differentiated to yield rates of growth and shortening, as follows. The rate corresponding to a particular time-point was measured by selecting a number of adjacent points, usually 5 on each side, fitting a quadratic equation to the local curve of length-*vs*-time which they describe, and obtaining the slope at the middle point (i.e., the rate of growth or shortening), as well as the standard deviation of the slope (i.e., its statistical uncertainty). The next point was then selected as the middle point for fitting and the process was repeated, until all time-points of a particular microtubule had been treated. These successive measurements of slopes and their uncertainties were used to find the occurrences of catastrophes and rescues in a statistically well-defined way, as follows. First, each slope was categorized as being certainly positive, certainly negative, or indeterminate, according to whether its absolute value differed from zero by more than 1.65 times its standard deviation (95% confidence). Then this list of categories was used to identify transitions between growth and shortening. A catastrophe was deemed to occur when a period of growth (i.e., one or more windows of certainly positive slope) was followed by a period of shortening (one or more windows of certainly negative slope) or by one or more windows of indeterminate slope followed by shortening. A rescue was similarly detected, although with opposite signs of the successive slopes. These techniques provide a robust and statistically objective measure of the quantities involved.

The virtue of the kind of numerical differentiation algorithm employed here is that it does not require a descriptive "model" of the process being observed. Because the current study aims to examine the applicability of a model, an algorithm which assumed, for instance, growth and shortening at constant rates (e.g., Walker et al., 1988) would be inappropriate. The inevitable drawback of the algorithm chosen is that the apparent variation in rates that it detects depends on the width of the "window" of points (*i.e.*, the number of points on each side of the central point) employed in the fit. A narrow window provides relatively variable apparent derivatives, while a broader window smoothes out the variations. This issue has been examined by Gildersleeve et al. (1992), who showed that the sensitivity of the results to the width of the window is not great in the context of similar measurements. In addition, when the same data set was examined by this algorithm and by similar algorithms previously used (Gildersleeve et al., 1992; Kowalski & Williams, 1993a), the frequencies of transitions and the distributions of growth and shortening rates were found to agree well.

For electron microscopy, spontaneously nucleated microtubules were prepared from tubulin in PM_2 buffer in the presence of 1 mM GTP or 2 mM GMPPCP by addition of

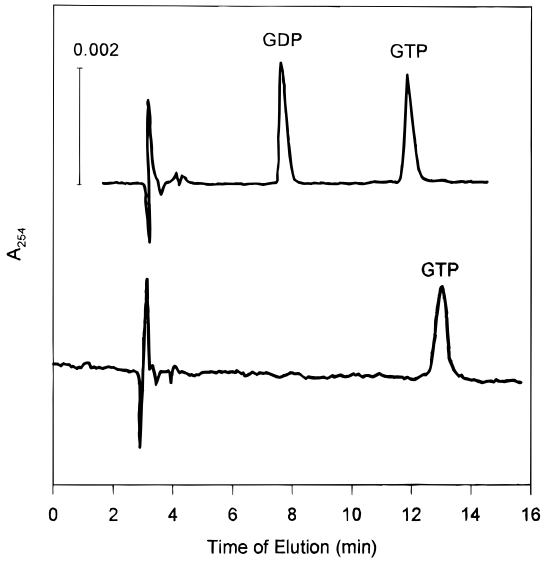

FIGURE 1: Removal of exchangeable-site nucleotide from tubulin. Reverse-phase ion-pair HPLC separation of tubulin-bound nucleotides. *Upper trace:* GDP/GTP-tubulin prior to treatment with immobilized alkaline phosphatase for removal of E-site nucleotide. *Lower trace:* The same tubulin after treatment with alkaline phosphatase and passage through a gel-filtration column to remove unbound nucleotides. This material is referred to as −/GTP tubulin. In each case, bound nucleotides were extracted in perchloric acid, neutralized, and subjected to HPLC on a C-18 column in 150 mM potassium phosphate and 0.5 mM tetrabutylammonium phosphate, pH 5.9. Peaks were identified by time and sequence of elution. The bipolar peak which appears at 3−4 min is caused by passage of the solvent front. Small variations in retention times were noted.

taxol. After 15 min of assembly, 5 μL of solution was rapidly diluted about 10-fold in buffer, applied directly to a freshly glow-discharged, carbon-coated, Formvar-covered grid, allowed to adhere for 30 s, rinsed with 0.01 M ammonium acetate, pH 6.5, and negatively stained by application of 10 μL of 0.5% uranyl acetate (Williams, 1981).

RESULTS

Preparation and Analysis of GMPPCP/GTP Tubulin. As noted in Materials and Methods, preparation of −/GTP tubulin involves the initial replacement of the E-site nucleotide by GDP, followed by hydrolysis of the GDP by alkaline phosphatase. The upper trace in Figure 1 shows a typical HPLC separation of nucleotides extracted from tubulin equilibrated with GDP prior to its application to the alkaline phosphatase column. One can see that the nucleotides are well separated and can easily be quantitated from the chromatogram. Roughly equal amounts of GDP and GTP are bound to the tubulin. The lower trace in Figure 1 shows the content of bound nucleotide after the GDP/GTP tubulin was exposed to immobilized alkaline phosphatase. No GDP was detected. Table 1 shows the ratios of nucleotide to tubulin obtained from chromatographic analyses of several samples of GTP/GTP (unmodified) tubulin, GDP/GTP tubulin, and −/GTP tubulin. Unmodified (GTP/GTP) tubulin contained, on average, somewhat more than 2 molecules of bound GTP/dimer, and no appreciable GDP, consistent with the idea that both the E-site and N-site contained bound GTP. The origin of the slight excess of GTP over the theoretical value of 2.0 per unmodified tubulin dimer is not completely understood; similar deviations appear in most such analyses. It may represent residual unbound nucleotide released by minor denaturation prior to the exchange into nucleotide-free buffer. GDP/GTP tubulin contained essentially equal amounts of GDP and GTP, consistent with the idea that the E-site GTP had been fully exchanged for GDP. The −/GTP tubulin had very little remaining GDP but 1 molecule of GTP, consistent with the idea that the N-site had retained all of its GTP while the E-site nucleotide was entirely removed during exposure to the alkaline phosphatase column.

Table 1: Nucleotide Content of Empty E-Site Tubulin and Controls

	mol/mol of tubulin dimer	
	GTP	GDP
unmodified tubulin[a]	2.28 (SD 0.35; range 2.62−1.68)	<0.01
GDP/GTP tubulin[b]	1.17 (SD 0.07; range 1.11−1.28)	0.90 (SD 0.15; range 0.74−1.09)
−/GTP tubulin[c]	0.99 (SD 0.17; range 0.72−1.22)	<0.01 (SD <0.01; range 0.0−0.06)

[a] Native tubulin, equilibrated with GTP-containing buffer, was gel-filtered into nucleotide-free buffer and then extracted and analyzed in the same way as −/GTP tubulin. Mean nucleotide content of seven analyses of six separate preparations is reported. [b] Nucleotide contents of GDP/GTP tubulin prepared by addition of excess GDP to GTP/GTP tubulin, followed by gel filtration. Mean of six analyses of three separate preparations is reported. [c] Nucleotide contents of −/GTP tubulin. Mean of 15 analyses of nine separate preparations is reported.

The results of HPLC extractions of unmodified tubulin controls (Table 1), either with GTP at both binding sites or with GDP at the exchangeable site (through exchange with free nucleotide or by hydrolysis of GTP to GDP during assembly), demonstrate slight excesses of GTP (2.28 and 1.17 mol/mol). Nevertheless they are in generally good agreement with theoretical values and the previous literature. In approximately 10% of cases, the gel filtration columns when used singly did leave some small traces of free nucleotides as revealed by a small peak of guanosine when it was present in the solution. It should be noted that two such columns were always used when analyzing −/GTP tubulin in the course of experiments but were often used singly when preparing unmodified tubulin for analysis. It might also be noted that preparations of −/GTP tubulin which showed excess nucleotide were not used in experiments and thus not included in the data in Table 1, whereas no such elimination occurred with samples of unmodified tubulin done over the course of the work as controls.

The nonhydrolyzable nucleotide analog GMPPCP was added to this −/GTP tubulin in PE_2MD_2 buffer at a concentration of 2 mM to make GMPPCP/GTP tubulin. Because of its weak affinity for the E-site ($K_a \cong 5 \times 10^4$ M^{-1}; Mejillano et al., 1990b), GMPPCP dissociates from tubulin during the removal of free nucleotide by gel filtration. The nucleotide content of this tubulin therefore could not be directly measured, but from knowledge of the binding constant, the degree of saturation of the E-site was inferred to be 0.99. When this GMPPCP/GTP tubulin was warmed to 37 °C, polymerization ensued upon addition of 4 M glycerol or 100 μM taxol, or in the presence of axonemal pieces employed as seeds. By electron microscopy, the structures formed appeared as microtubules (Figure 2). There was no apparent difference between microtubules formed in the presence of 2 mM GMPPCP and those formed from unmodified GTP/GTP tubulin. By light microscopy, the GMPPCP/GTP microtubules were found to be cold-labile: they disassembled completely when the sample was placed on ice for 20 min and reassembled when it was returned to the 37 °C stage of the microscope. (Figure 2

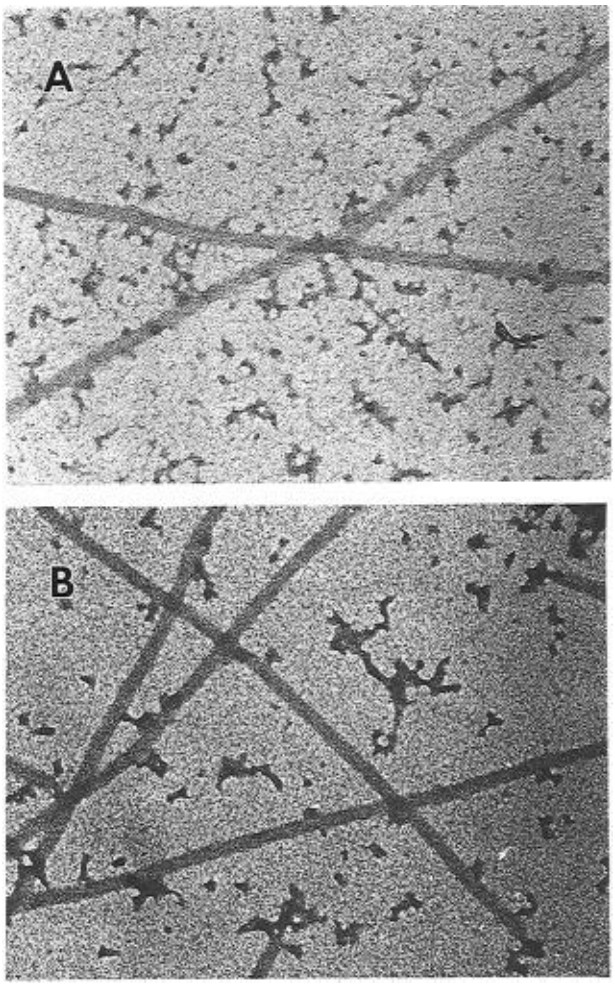

FIGURE 2: Electron micrographs of microtubules formed by assembly of −/GTP tubulin and GMPPCP/GTP-tubulin. *(A)* −/GTP tubulin; *(B)* GMPPCP/GTP tubulin. Assembly was performed in 0.1 M Pipes/NaOH, pH 6.9, 2 mM $MgSO_4$, 2 mM GMPPCP, 100 μM taxol, and 1% dimethyl sulfoxide (DMSO) at 37 °C for 20 min. Tubulin concentration was 13.4 μM.

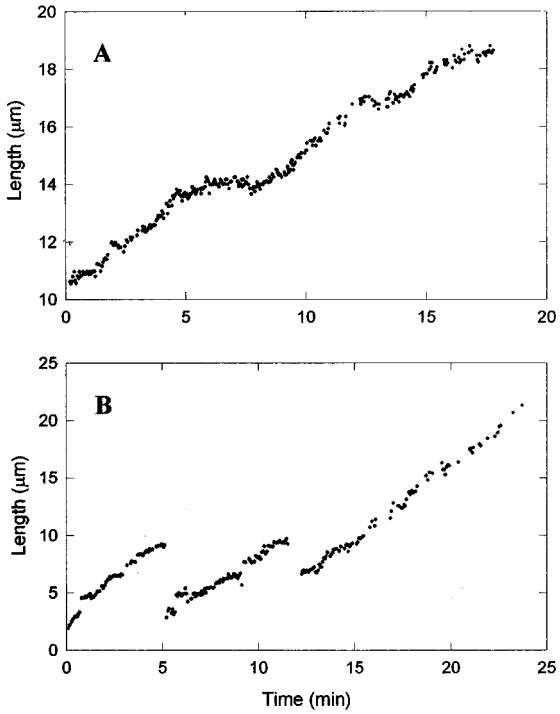

FIGURE 3: Growth (and shortening) of representative microtubules as observed by DIC microscopy. *(A)* Microtubule assembled from GMPPCP/GTP tubulin in the presence of 2 mM GMPPCP and 50−60 μM tubulin. *(B)* Control microtubule assembled from GTP/GTP tubulin in the presence of 2 mM GTP and 18 μM tubulin. A solution of tubulin with an initially vacant E-site (*i.e.*, −/GTP tubulin) and without free nucleotide was divided into two aliquots. The appropriate nucleotide was then added and assembly was observed.

also shows that −/GTP tubulin forms microtubules in the presence of taxol.)

Measurement of Dynamics of GMPPCP/GTP and GMPCPP/GTP Tubulin. Figure 3 shows the time course of assembly of a single typical microtubule from GMPPCP/GTP tubulin, together with the assembly/disassembly of a typical GTP/GTP tubulin microtubule. One can easily see that assembly of the GMPPCP/GTP microtubule does not occur at a constant rate. Rather, numerous rate changes, including apparent periods of no growth, characterize the length-vs-time plot. Observation of a large number of such microtubules revealed only a single apparent shortening event of approximately 1 μm in length in over 20 microtubule-hours of growth.[2] This single event may not have been meaningful.

Figure 4 and Table 2 summarize data from a large number of microtubules such as those shown in Figure 3. Figure 4A reinforces the qualitative impression that microtubules formed from GMPPCP/GTP tubulin grow at variable rates despite the fact that they do not display dynamic instability. The variability does not, of course, extend to shortening rates, since essentially no shortening was observed. Because neither shortening events nor visible asymmetry of the nucleating axonemes is present to provide a means of distinguishing growing (+)-ends of these microtubules from growing (−)-ends, the data from both types of ends are necessarily mixed together. In order to provide a reasonably suitable control, data from tubulin with GTP at its E-site were obtained without attempt to distinguish the ends. The breadth of the distribution of growth rates of GMPPCP/GTP tubulin (Figure 4A) is the same as, or marginally greater than, that of GTP tubulin (Figure 4B), and it is closely comparable to that of a second control, namely, −/GTP tubulin assembled in the presence of GTP (Figure 4C). Comparison of panels A and C in Figure 4 shows that dynamic instability not only is fully suppressed by the presence of GMPPCP but also is restored by the readdition of GTP to the same −/GTP tubulin from which the GMPPCP/GTP tubulin was prepared. As a byproduct of the systematic analysis of the data to yield statistically significant rates of growth and shortening, some series of points did not produce slopes which were statistically distinguishable from zero with 95% confidence. These are shown in the right-hand panels of Figure 4. Both true "pauses" and apparent periods of no length change are expected to appear in this category. It is evident that GMPPCP/GTP tubulin differs little from GTP/GTP tubulin in its tendency to display periods of no significant change. Table 2 records the quantitative measures of these distributions of rates. One can see that the normalized width of the distributions of growth rates (the coefficient of variation), as measured by the ratio of the standard deviation to the mean, are essentially the same for GMPPCP/GTP tubulin and GTP/GTP tubulin. The slight difference that may be noted in the shapes of the frequency distributions of growth rates between panels A and C of Figure 4 is of unknown origin.

[2] A microtubule-hour corresponds, e.g., to observation of a single microtubule for one hour, two microtubules for a half-hour each, etc.

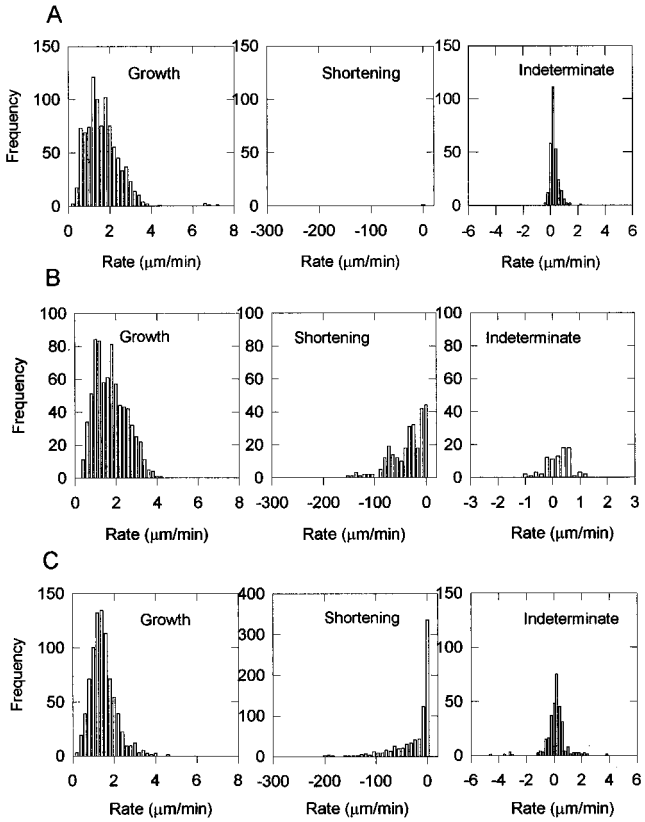

FIGURE 4: Distributions of rates of growth and shortening. The histograms represent rates of assembly/disassembly obtained by numerical differentiation of extensive data similar to those in Figure 3. In each section, the *left-hand panel* represents rates of growth; *center panel*, rates of shortening; *right-hand panel*, rates not meeting either growth or shortening criteria (see Materials and Methods). *(A)* Tubulin with an initially vacant E-site assembled in the presence of 2 mM GMPPCP (*i.e.*, GMPPCP/GTP tubulin). Tubulin concentration was 50−60 μM. *(B)* Untreated GTP/GTP-tubulin assembled in the presence of 2 mM GTP. Tubulin concentration was 15 μM. *(C)* Tubulin with an initially vacant E-site assembled in the presence of 2 mM GTP (*i.e.*, GTP/GTP tubulin). The concentrations of tubulin in *(B)* and *(C)* (20 μM) were chosen to produce mean growth rates comparable to those observed in *(A)*. Each of the rates contributing to the frequency histograms represents the slope obtained by a fit of a quadratic equation to 11 points measured at intervals of approximately 5 s (see Materials and Methods). (+)-ends were not distinguished from (−)-ends in these data. Measurements and analysis were conducted by equivalent techniques to provide comparability of the final data. Assemblies carried out at 37 °C, nucleated by sea urchin axonemes, and in 0.1 M Pipes/NaOH, pH 6.9, and 2 mM each EGTA, $MgSO_4$, and DTE.

Results of experiments carried out at lower concentrations of GMPPCP tubulin are also shown in Table 2, as are those of an experiment with GMPCPP/GTP tubulin. Both the mean rate and the breadth of the distributions are smaller, as would be expected at the lower concentration. However, the ratio of standard deviation to mean (the coefficient of variation, chosen as an appropriate way to normalize the data for comparison) is not greatly altered by dilution. It therefore appears that a broad distribution of growth rates is obtained, regardless of concentration, whether or not dynamic instability is suppressed by the presence of nonhydrolyzable GTP analogues.

Changes in Nucleotide Content. To ascertain the nucleotide content of the microtubules formed from GMPPCP/GTP tubulin, the protein was polymerized in the presence of 100 μM taxol, which was added to reduce the critical concentration and thus to raise the fraction of tubulin which assembled. The resulting microtubules were pelleted by centrifugation. The pellet was rinsed with nucleotide-free buffer and extracted with $HClO_4$ without prior disassembly of the microtubules. Figure 5 shows a typical chromatogram resulting from analysis of the nucleotides in a pellet of microtubules. It was surprising to find that, in addition to GMPPCP and GTP, some GDP was also present in the assembled microtubules. Table 3 reveals the results. Slightly more than 1 mol of GMPPCP/mol of tubulin dimer was recovered in the pellets of GMPPCP/GTP microtubules. The remaining mole of nucleotide, which had been GTP at the initiation of assembly, was recovered as somewhat more than half GTP and somewhat less than half GDP. This observation of unexpected hydrolysis upon assembly did not depend

Table 2: Growth Rates of Microtubules with Different Nucleotides at the E-Sites[a]

tubulin	mean growth rate (μm/min)	standard deviation (μm/min)	SD/mean rate (coefficient of variation)
GMPPCP/GTP (55 μM)	1.6	0.91	0.57
GTP/GTP (native)[b]	1.6	0.75	0.47
GTP/GTP (added back)[c]	1.4	0.62	0.44
GMPPCP/GTP (50 μM)[d]	1.0	0.51	0.51
GMPPCP/GTP (46 μM)[d]	0.4	0.20	0.5
GMPCPP/GTP (3.0 μM)[e]	0.5	0.3	0.6

[a] As noted in the text, (+)-ends could not be differentiated from (−)-ends in the samples containing no hydrolyzable nucleotide. Data from both (+)- and (−)-ends are therefore combined in all reported data for purposes of comparability. [b] Tubulin not subjected to prior removal of nucleotide from its E-site. [c] −/GTP tubulin to which GTP (2 mM) was added. [d] A sample of GMPPCP tubulin was diluted with GMPPCP-containing buffer to produce slower rates of growth. [e] −/GTP tubulin to which GMPCPP (2 mM) was added. The protein concentration of this sample (3.0 μM) was made considerably smaller than that of the others to avoid the spontaneous nucleation that is observed. The data set collected was quite small but is thought to be reliable.

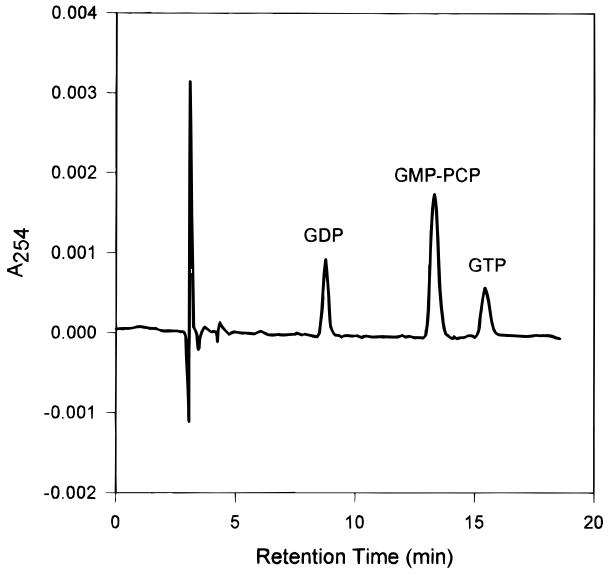

FIGURE 5: Nucleotide content of microtubules assembled from GMPPCP/GTP tubulin. GMPPCP (2 mM) was added to tubulin with an initially vacant E-site (*i.e.*, −/GTP tubulin), and assembly was initiated by addition of taxol. Microtubules were pelleted and extracted without prior disassembly, and their nucleotides were subjected to HPLC as described in the text.

Table 3: Nucleotide Content of Microtubules Assembled from Tubulin Bearing Different Nucleotides

	GTP	GDP	GMPPCP
GMPPCP/GTP tubulin	0.54 (SD 0.07; range 0.48−0.64)	0.34 (SD 0.09; range 0.25−0.46)	1.12 (SD 0.15; range 0.98−1.30)
unmodified tubulin after assembly[b]	1.17 (SD 0.14; range 0.95−1.39)	0.99 (SD 0.22; range 0.74−1.28)	na

[a] GMPPCP/GTP tubulin was polymerized in the presence of 2 mM GMPPCP and 100 μM taxol. The results of four analyses of three separate preparations are shown. [b] Native tubulin was polymerized in nucleotide-free buffer. The resulting microtubules were pelleted, resuspended in nucleotide-free buffer, extracted and analyzed. The results of eight analyses of seven separate preparations are shown.

Table 4: Analysis of Merged Data for Growth of Identified (+)-Ends and (−)-Ends[a]

(+)-ends		(−)-ends		merged (+)- and (−)-ends		
mean ± SD (μm/min)	coeff of var	mean ± SD (μm/min)	coeff of var	mean ± SD (μm/min)	coeff of var	reference
2.15 ± 0.89	0.41	1.09 ± 0.6	0.6	1.61 ± 0.92	0.57	Kowalski and Williams (1993b)
2.28 ± 0.67	0.29	1.18 ± 0.50	0.42	1.73 ± 0.80	0.46	Gamblin and Williams (1995)[b]

[a] Data for dynamic instability of well-identified (+)- and (−)-ends were combined and analyzed. The merged frequency distributions of rates (i.e., the sum of the distributions for (+)- and (−)-ends) were unimodal and yielded the descriptive statistics shown. [b] These values have been corrected to compensate for a computational error which appears in Table I of Gamblin and Williams (1995).

on the presence of GMPPCP or on the use of taxol. In 4 M glycerol, for instance, 90% of the tubulin assembled, and the pellet contained 1.19 GMPPCP, 0.49 GTP, and 0.25 GDP/tubulin dimer. The nucleotide content of microtubules assembled from GTP/GTP tubulin is also reported in Table 3. This control is consistent with the extensive literature and with the long-standing conclusion that E-site GTP is hydrolyzed upon assembly and N-site GTP is not.

DISCUSSION

Complete Replacement of E-Site Nucleotide. As reported above and as previously observed by Seckler et al. (1990) and by Shearwin and Timasheff (1992), treatment with immobilized alkaline phosphatase yields tubulin which has nearly 1 bound GTP/tubulin dimer. This tubulin, which before treatment had 1 GTP and 1 GDP/dimer, after treatment had, on average, 1 GTP/dimer but no detectable GDP. The remaining GTP evidently resisted hydrolysis by alkaline phosphatase. It is generally accepted that α-tubulin binds one GTP in an irreversible fashion (Weisenberg et al., 1968; Berry & Shelanski, 1972; Weisenberg et al., 1976), at the N-site on the α-chain (Nath et al., 1985), and can protect it from hydrolysis by alkaline phosphatase. It is therefore reasonable to infer that the GTP is present at the N-site of this −/GTP tubulin. Because of the free exchange at the E-site (Brylawski & Caplow, 1985), when GMPPCP, GTP, or other guanosine nucleotides bind to this tubulin, it must be the E-site, on the β-chain, to which they are bound.

Nonhydrolyzable Analogues Yield Normal Morphology but No Dynamic Instability. When a nonhydrolyzable nucleotide is added to this −/GTP tubulin, cold-labile microtubules of normal structure are readily assembled from it, in agreement with earlier reports (Mejillano et al., 1990a; Seckler et al., 1990). Also in accord with previous inferences (Mejillano et al., 1990b; Hyman et al., 1992), in the presence of the nonhydrolyzable GMPPCP or GMPCPP, microtubules grow but do not exhibit dynamic instability. Dynamic instability is restored when GTP is added, demonstrating that −/GTP tubulin has not been permanently altered in its assembly properties. It must therefore be the absence of a hydrolyzable nucleotide at the E-site which causes the loss of dynamics. This result would, of course, be predicted from the general understanding that the large length-excursions of dynamic instability result from processes that depend on the hydrolysis of GTP.

Variability of Growth Rates Not Linked to Dynamic Instability. Microtubules lacking hydrolyzable nucleotide, like the polymers of sickle-cell hemoglobin (Eaton & Hofrichter, 1990) or of tobacco mosaic virus coat protein (Lauffer, 1971), must be subject to the kinetic rules which apply to ordinary reversible association reactions. The length fluctuations that do occur in those cases must be too short to measure (see below). Despite the lack of dynamic instability, the growth of GMPPCP/GTP microtubules occurs at highly variable rates, as exemplified in Figure 3A and observed in the data at large. Therefore, one may conclude qualitatively that variability of growth rates is not directly a result of the dynamic instability process. This conclusion argues strongly against such hypothetical sources of variability as "microcatastrophes"—shortening events too small to be seen in the light microscope, but frequent enough to slow the *observed* growth rate in a fluctuating way.

The magnitude of the variability, as measured by the ratio of the standard deviation of the rate to the mean rate (i.e., the coefficient of variation), is quite close to that observed when GTP occupies the E-site (Table 2). The strength of the quantitative conclusion that the variability is identical is reduced, however, by the fact that (+)-ends were not distinguishable from (−)-ends in the cases where microtubules did not shorten. Because of this, both the experimental and control distributions of rates contain merged data gathered from both kinds of ends, and in consequence, some of the measured breadth of the distribution of rates must arise from their potentially different mean rates of growth. The ratio of (+)-ends to (−)-ends in each data set is not known, although many axonemes in each case had microtubules growing from both ends, showing that *some* data from each type of end was included in each data set. The extent to which this problem is likely to interfere with the conclusions drawn here can be assessed by examining data in the literature (Kowalski & Williams, 1993b; Gamblin & Williams, 1995) in which dynamic instability of microtubules of definitely known end-types was measured. Plus-ends and (−)-ends show overlapping distributions of rates with differing mean rates. When (+)- and (−)-ends are arithmetically recombined in these data, as shown in Table 4, one finds unimodal distributions with coefficients of variation in the vicinity of 0.5. This fact reflects the fact that the "spread" in the behavior of individual ends is large in comparison to the difference in mean behavior between (+)-

and (−)-ends, at least in normal (GTP/GTP) microtubules. Although one cannot be absolutely certain that the behavior of GMPPCP/GTP microtubules is not different, the unimodal nature of the observed distributions argues that the mean rates of (+)- and (−)-ends are not much more disparate than in normal microtubules. It thus seems unlikely that the qualitative conclusions drawn here are much affected by the unknown differences between ends.

Variation Not Due to Fluctuations in a Simple Structure. So what *is* the cause of the observed variability in rates− Three possibilities can be considered. The first is that it results from simple fluctuations of growth rate of the sort that would occur at the end of a growing polymer of simple structure (*e.g.*, the hemoglobin S fibers or tobacco mosaic virus coat protein polymers mentioned above). These, however, are expected to be quite small, as may be seen from the following discussion. Consider the growth of a hypothetical linear polymer (essentially a microtubule composed of one protofilament), which occurs by addition of subunits to its end, without nucleotide hydrolysis or other complicating factors. Imagine an ensemble of these polymers, each long enough to avoid exposure of its nucleus by a fluctuation. Such an ensemble will add subunits at a rate given by k_+c_1, where k_+ is the second-order rate constant for association and c_1 is the subunit (tubulin dimer) concentration, and it will lose them at a rate equal to k_-, the first-order rate constant for dissociation. The variance, $\langle \delta i^2 \rangle$, of the number of subunits per polymer in such an ensemble at equilibrium is given by

$$\langle \delta i^2 \rangle = \langle i \rangle \tag{1}$$

where $\langle i \rangle$ is the mean number of subunits in the polymers. When the system is far from equilibrium, as is the case for our growing polymers, the variance is somewhat larger. As shown by Oosawa (1970), the variance (i.e., the mean-square deviation) of the number of subunits added in a particular interval of time, t, is given by

$$\langle \delta i^2 \rangle \cong \langle i \rangle + 2k_- t \tag{2}$$

where $\langle i \rangle$ is the mean number of subunits added during the time-interval.

$$\langle i \rangle = (k_+c_1 - k_-)t \tag{3}$$

The data of Walker et al. (1988), obtained under conditions similar to those employed here, give $k_+ = 8.9\ \mu M^{-1}\ s^{-1}$ and $k_- = 44\ s^{-1}$ during the growing phase. At 12 μM tubulin, an 8 s period of growth yields a mean change in length $\langle i \rangle = 500$ subunits, near the minimum resolvable. Substituting these numbers into eq 2, one finds that $\langle \delta i^2 \rangle \cong 500 + 704 = 1204$ subunits. The fractional root-mean-square deviation, for comparison with the observed coefficients of variation in Table 2, is $[\langle \delta i^2 \rangle]^{1/2}/500 = (\pm 35\ \text{subunits})/(500\ \text{subunits})$, or 7% of the number of subunits added in the time period. [Identical results are obtained with a more frankly statistical Markov-chain analysis, following methods described, i.e., in Goel and Richter-Dyn (1976).] Because the fractional deviation drops as the length change increases, and because 500 subunits is the smallest change that can be observed with the light microscope, 7% is the largest fractional deviation that would be expected. This figure is nearly an order of magnitude smaller than the values of approximately 50% actually seen (Table 2). Hence, ordinary fluctuations are not expected to cause the observed variability of rates.

Denatured Subunits or Contaminating GDP Unlikely To Cause Observed Variation. A second possibility is that the binding of a small fraction of subunits that are in some way altered acts to "poison" the microtubule end by associating with it but inhibiting addition of further monomers. Such an explanation seems unlikely in view of the consistency of the results, but it cannot be ruled out entirely. Candidates for such molecules would be partially denatured tubulin (Maccioni, 1983; Croom et al., 1985) or tubulin with GDP bound at its E-site. The latter species could result from a process in which unfolding of a small number of α-chains during the experiment would release GTP into the solution. This GTP would be expected to displace some of the GMPPCP from the E-sites on the β-chains of other dimers, producing traces of GTP/GTP tubulin. This tubulin, if it entered into microtubules, might undergo hydrolysis of its E-site GTP to produce GDP/GTP tubulin.

This scenario is in qualitative accord with the finding that GDP (which must have originated, at the start of the assembly reaction, as GTP irreversibly bound to N-sites) is found incorporated in the subunits of pelleted microtubules. It seems unlikely, however, that the incorporation of these dimers would greatly affect the rate of growth. First, the dimers entering the microtubule would be GTP-containing. Second, because the microtubules essentially grow without shortening, the GDP-containing dimers, and their nucleotide, would be trapped within the microtubules (Caplow et al., 1984), preventing the release of GDP dimers into solution. Since the fraction of GDP dimers required to have a measurable effect on assembly rates is quite large and the effect fairly small (Vandecandelaere et al., 1995), it is unlikely that enough of them would be present to produce the several fold fluctuations in rates observed.

Variable Structure of Microtubule End? Likely causes of the observed variability appear to be those which involve variable geometry of intersubunit contacts. It has long been noted (Weisenberg, 1980; Erickson & Pantaloni, 1981; Erickson & Voter, 1986; Martin et al., 1993) that the kinetics of addition and removal of a subunit must depend on the microscopic configuration of its binding site. Because of the multiprotofilament nature of microtubules, there can be several distinct kinds of sites. In addition, a microtubule which has a long, sheetlike extension can be expected to have a different complement of binding sites (for instance, more of those which are present on the side of a protofilament) than one with a blunt end would have. Gildersleeve et al. (1992), in their investigation of the underlying cause of variability, concluded that the structural differences responsible were unlikely to remain as a permanent part of the microtubule lattice. Rather, they appear to be transient in nature, most probably associated with evanescent features of the microtubule end. The model of Chrétien et al. (1995) extends this view. It supposes that the growing tip of the microtubule is sheetlike and grows at a constant rate and that the tubular form results from rolling up of the sheet and closure of the resulting seam. The extensions are both too short and of too low contrast to be visible in the light microscope. Closure and GTP hydrolysis are imagined to be coupled. The rate of closure is supposed to be variable, for reasons not fully elaborated.

We speculate that the variability could as easily originate in a series of changes in the shape of the end, which would

be expected to occur on a scale of time (seconds) and length (order of a micrometer) long in comparison to the scale of addition of a subunit but short in comparison to the length of a microtubule. The variation in rate observed at the light-microscope level would therefore be the result of changes in the shape of the end—for instance, longer sheets changing to shorter ones, and back again. Regardless of the validity of this speculation, though, the results reported here show that variability of growth rate is an intrinsic part of the microtubule's *structural* nature, independent of dynamic instability and probably independent of E-site GTP hydrolysis as well.

Apparent Hydrolysis of N-Site GTP. How more than a third of the single GTP initially bound (presumably irreversibly) to tubulin's N-site becomes hydrolyzed during assembly of microtubules from GMPPCP/GTP tubulin is not fully understood. It is imaginable that the presence of a nonhydrolyzable nucleotide on the β-chain somehow induces assembly-mediated hydrolysis of some GTP to GDP on the α-chain. It is also possible that nucleotides might somehow migrate from the N-site to the E-site in −/GTP tubulin. Both these possibilities seem unlikely. However, even if either of them were the case, the results show that dynamic instability is not induced in the microtubule. The consistent finding that 1 GMPPCP/dimer is present in pelleted microtubules is also a puzzle. Either all of this nucleotide is present at the E-site, in which case hydrolysis has occurred at the N-site, or some of it is present at the N-site, in which case there has been migration of the nucleotide. These questions about GTP hydrolysis are under investigation.

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REFERENCES

Bayley, P. M., Sharma, K. K., & Martin, S. R. (1994) in *Microtubules* (Hyams, J., & Lloyd, C., Eds.) pp 111−137, Wiley-Liss, New York.
Berry, R. W., & Shelanski, M. L. (1972) *J. Mol. Biol. 71*, 71−81.
Bré, M. H., & Karsenti, E. (1990) *Cell Motil. Cytoskel.* 15, 88−98.
Brylawski, B. P., & Caplow, M. (1983) *J. Biol. Chem. 258*, 760−763.
Caplow, M., Brylawski, B. P., & Reid, R. (1984) *Biochemistry 23*, 6745−6752.
Chrétien, D., Fuller, S. D., & Karsenti, E. (1995) *J. Cell Biol. 129*, 1311−1328.
Correia, J. J., Baty, L. T., & Williams, R. C., Jr. (1987) *J. Biol. Chem. 262*, 17278−17284.
Croom, H. B., Correia, J. J., Baty, L. T., & Williams, R. C., Jr. (1985) *Biochemistry 24*, 768−775.
Damodharan, R., & Wadsworth, P., (1995) *J. Cell Sci. 108*, 1679−1689.
Dawson, R. M. C., Elliott, D. C., Elliot, M. H., & Jones, K. M. (1986) *Data for Biochemical Research*, 3rd ed., pp 108−109, Oxford University Press, New York.
Detrich, H. W., III, & Williams, R. C, Jr. (1978) *Biochemistry 17*, 3900−3907.
Detrich, H. W., III, Jordan, M. A., Wilson, L., & Williams, R. C., Jr. (1985) *J. Biol. Chem. 260*, 9479−9490.
Drechsel, D. N., Hyman, A. A., Cobb, M. H., & Kirschner, M. W. (1992) *Mol. Biol. Cell 3*, 1141−1154.
Drubin, D., & Kirschner, M. W. (1986) *J. Cell Biol. 103*, 2739−2746.
Eaton, W. A., & Hofrichter, J. (1990) *Adv. Protein Chem. 40*, 63−279.
Erickson, H. P. (1974) *J. Cell Biol. 60*, 153−167.
Erickson, H. P., & Pantaloni, D. (1981) *Biophys. J. 34*, 293−309.
Erickson, H. P., & Voter, W. A. (1986) *Ann. N.Y. Acad. Sci. 466*, 552−565.
Erickson, H. P., & O'Brien, E. T. (1992) *Annu. Rev. Biophys. Biomol. Struct. 21*, 145−166.
Gamblin, T. C., & Williams, R. C., Jr. (1995) *Anal. Biochem. 232*, 43−46.
Gildersleeve, R. F., Cross, A. R., Cullen, K. E., Fagen, A. P., & Williams, R. C., Jr. (1992) *J. Biol. Chem. 267*, 7995−8006.
Goel, N. S., & Richter-Dyn, N. (1976) *Stochastic Models in Biology*, pp 10−11, Academic Press, New York.
Horio, T., & Hotani, H. (1986) *Nature 321*, 605−607.
Hyman, A. A., Salser, S., Drechsel, D. N., Unwin, N., & Mitchison, T. J. (1992) *Mol. Biol. Cell 3*, 1155−1167.
Kirschner, M. W., Honig, L. S., & Williams, R. C., Sr. (1975) *J. Mol. Biol. 99*, 263−276.
Kowalski, R. J., & Williams, R. C., Jr. (1993a) *J. Biol. Chem. 268*, 9847−9855.
Kowalski, R. J., & Williams, R. C., Jr. (1993b) *Cell Motil. Cytoskel. 26*, 282−290.
Lauffer, M. A. (1971) in *Subunits in Biological Systems, Part A* (Timasheff, S. N., & Fasman, G. D., Eds.) pp 149−199, Marcel Dekker, New York.
Maccioni, R. B. (1983) *Biochem. Biophys. Res. Commun. 110*, 463−469.
Mandelkow, E.-M., Mandelkow, E., & Milligan, R. A. (1991) *J. Cell Biol. 114*, 977−991.
Martin, S. R., Schilstra, M. J., & Bayley, P. M. (1993) *Biophys. J. 65*, 578−596.
Mejillano, M. R., Barton, J. S., & Himes, R. H. (1990a) *Biochem. Biophys. Res. Commun. 166*, 653−660.
Mejillano, M. R., Barton, J. S., Nath, J. P., & Himes, R. H. (1990b) *Biochemistry 29*, 1208−1216.
Melki, R., Fievez, S., & Carlier, M.-F. (1996) *Biochemistry 25* (in press).
Mitchison, T., & Kirschner, M. W. (1984) *Nature 312*, 237−242.
Nath, J. P., Eagle, G. R., & Himes, R. H. (1985) *Biochemistry 24*, 1555−1560.
Oosawa, F. (1970) *J. Theor. Biol. 27*, 69−86.
Panda, D., Goode, B. L., Feinstein, S. C., & Wilson, L. (1995) *Biochemistry 34*, 11117−11127.
Penefsky, H. (1977) *J. Biol. Chem. 252*, 2891−2899.
Perrone, P. A., & Brown, P. R. (1984) *J. Chromatogr. 317*, 301−310.
Pryer N. K., Walker, R. A., Skeen, V. P., Bourns, B. D., Soboeiro, M. F., & Salmon, E. D. (1992) *J. Cell Sci. 103*, 965−976.
Seckler R., Wu, G.-M., & Timasheff, S. N. (1990) *J. Biol. Chem. 265*, 7655−7661.
Shearwin, K. E., & Timasheff, S. N. (1992) *Biochemistry 31*, 8080−8089.
Shelden, E., & Wadsworth, P. (1993) *J. Cell Biol. 120*, 935−945.
Simon, J. R., & Salmon, E. D. (1990) *J. Cell Sci. 96*, 571−582.
Vandecandelaere, A., Martin, S. R., & Bayley, P. M. (1995) *Biochemistry 34*, 1332−1343.
Walker, R. A., O'Brien, E. T., Pryer, N. K., Soboeiro, M. F., Voter, W. A., Erickson, H. P., & Salmon, E. D. (1988) *J. Cell Biol. 107*, 1437−1448.
Weisenberg, R. C. (1980) *J. Mol. Biol. 139*, 660−677.
Weisenberg, R. C., Borisy, G. G., & Taylor, E. W. (1968) *Biochemistry 7*, 4466−4478.
Weisenberg, R. C., Deery, W. J., & Dickinson, P. J. (1976) *Biochemistry 15*, 4248−4254.
Williams, R. C., Jr., (1992) in *The Cytoskeleton: A Practical Approach* (Carraway, K. L., Ed.) pp 151−166, Oxford University Press, Oxford, England.
Williams, R. C., Jr., & Lee, J. C. (1985) *Methods Enzymol. 85B*, 376−385.
Williams, R. C., Sr. (1981) *J. Mol. Biol. 150*, 399−408.

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