Interactions of Taxol, Microtubule-associated Proteins, and Guanine Nucleotides in Tubulin Polymerization*

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Microtubule-associated proteins (MAPs), guanine nucleotides, and reaction temperature have substantial effects on the interaction of taxol with purified tubulin in 6.1 mM glutamate. Without MAPs, taxol induced tubulin to polymerize only if GTP was present and the reaction mixture warmed. GTP was hydrolyzed in tandem with polymerization in stoichiometric amounts, while GDP inhibited both polymerization and hydrolysis. The polymerized material contained few microtubules, consisting mostly of sheets of protofilaments which could be depolymerized at 0 °C. The addition of heat-treated MAPs (with minimal GTPase, ATPase, and nucleoside diphosphate kinase activities) to the reaction had dramatic effects on taxol-induced polymerization. Polymerization occurred with either GTP or higher temperatures. In the latter case, a single wave of polymerization, little affected by GDP, occurred on warming the reaction mixture. With MAPs and taxol, GTP-dependent polymerization occurred at 0 °C. This was associated with tandem and stoichiometric GTP hydrolysis. Both 0 °C polymerization and hydrolysis were totally inhibited by GDP. Once polymerization had reached a plateau at 0 °C, a second wave of polymerization occurred when the reaction mixture was warmed. This second wave of polymerization was independent of GTP hydrolysis and was little affected by GDP. During this second wave of polymerization, however, there was an apparently unrelated GTPase reaction which could be inhibited by GDP. With MAPs the taxol-induced polymer was largely cold-stable, and the predominant structures formed were microtubules. Morphology was unaffected by the presence of nucleotide or reaction temperature. The requirements for nucleotide, MAPs, or increased reaction temperature could also be satisfied by increasing the glutamate concentration of the reaction.

* Taxol, an antineoplastic agent derived from the plant Taxus brevifolia (1), has been shown by Schiff et al (2, 3) to interact with tubulin in a unique manner. Rather than inhibiting the formation of microtubules, taxol enhances their polymerization and prevents their depolymerization both in vitro (2) and in vivo (3). Most recently, Schiff and Horwitz have found that taxol will induce the polymerization of microtubule protein in the absence of GTP as well as the polymerization of purified tubulin (4).

While studying the effects of microtubule inhibitors on tubulin-dependent GTP hydrolysis, we observed impressive stimulation of the reaction by taxol. This led us to a further exploration of the effects of the drug on tubulin polymerization and GTP hydrolysis. We found that these effects were significantly modulated by other reaction components, including MAPs, reaction temperature, and GDP.

EXPERIMENTAL PROCEDURES

Materials—Taxol was obtained from the Natural Products Branch, Division of Cancer Treatment, National Cancer Institute, and dissolved in dimethylsulfoxide. All reaction mixtures contained 1% (v/v) dimethylsulfoxide. Colchicine, Mes, GTP, and GDP were obtained from Sigma, and the nucleotides were repurified by ion exchange chromatography. Calf brains were obtained from H. W. Stapf, Baltimore; monosodium glutamate from Grand Island; polyethyleneimine-cellulose thin-layer chromatography sheets from Brinkmann; [α-32P]GTP from Amer sham, and DEAE-Sephadex, Sephadex G-25 (coarse), and Sephadex G-200 from Pharmacia. Stock solutions of monosodium glutamate were adjusted to pH 6.6 with HCl.

Preparation of Tubulin—Calf brain tubulin, purified as described elsewhere (5), was electrophoretically homogeneous, free of nucleoside diphosphate kinase and ATPase activities, freed of unbound nucleotides by chromatography on Sephadex G-25, and contained approximately 1 mol each of GDP and GTP/mol of tubulin. We have assumed that the GTP is bound in the nonexchangeable site and the GDP in the exchangeable site, since a polymerization step followed by homogenization of the polymer in a GTP-free solution was a late step in the purification, and since further polymerization steps require at least stoichiometric amounts of GTP (5). After Sephadex G-25 chromatography (5), the tubulin solution was put into dialysis tubing and concentrated by several treatments with dry Sephadex G-200. The solution was then dialyzed against 1.0 M monosodium glutamate and centrifuged at 50,000 rpm for 30 min at 2 °C in a Beckman Ti 50 rotor. The tubulin was stored frozen at 30.5 mg/ml in liquid nitrogen.

Preparation of MAPs—Microtubule protein was prepared by two cycles of polymerization and depolymerization (6) as described by Weigarten et al. (7). In preliminary studies, MAPs and tubulin were initially separated from each other on DEAE-Sephadex (8). We found that the MAPs fraction contained substantial nucleoside diphosphate kinase, ATPase and GTPase activities (cf. Refs. 9–11) with which it would interfere with tubulin-dependent GTPase assays. We confirmed the result (11) that heat treatment destroyed most of the GTPase activity of the MAPs without affecting their stimulation of polymerization (11, 12); and we extended this observation to the nucleoside diphosphate kinase and ATPase activities originally present in the MAPs obtained from DEAE-Sepahex (8). We did find, however, residual heat-resistant components, representing about 2 to 3% of the original activity, of all three enzymes which were unaffected by an additional 15 min in a boiling water bath (data not presented).

The major preparative procedures were performed with 1.0 to 1.5 g of microtubule protein diluted to 10 mg/ml with Buffer A (0.1 M Mes pH 6.4 and 0.5 mM dithiothreitol). The solution was boiled for 20 min, chilled on ice, and centrifuged for 40 min at 2 °C in a Beckman 35 rotor at 35,000 rpm. The supernatant was applied to a column (2.5
x 20 cm) of DEAE-Sepharose equilibrated with Buffer A, and the column was developed with a 400-m NaCl gradient from 0 to 1.0 M containing Buffer A. A single symmetrical, but broad, protein peak was obtained centered at 0.1 M NaCl. The protein-containing fractions were pooled, put into dialysis tubing, concentrated with dry Sephadex G-200 and dialyzed against Buffer A. As a significant amount of GDP remained in the MAPs preparation, the solution was passed through 0.5 mM dithiothreitol, and frozen in liquid nitrogen. About 20 mg of MAPs were obtained/μg of microtubule protein.

The polyacrylamide gels displayed in Fig. 1 demonstrate that the order of heat treatment and DEAE-Sepharose chromatography had little effect on band patterns. The major components in the final preparation were MAP-2 and the τ proteins (8, 12), the MAP-1 band (8) disappearing with heat treatment.

Assays—Thin layer chromatography on polyethyleneimine-cellulose and autoradiography were used to assay GTP hydrolysis by measuring the formation of [α-32P]GDP from [γ-32P]GTP (5). All data are expressed as nmol/ml of reaction.

Turbidimetry was used to follow tubulin polymerization (13). Assays were performed in cuvettes with a 1-cm light path using a Gilford model 250 recording spectrophotometer equipped with a Lauda circulating water bath. The water bath was initially at 0 °C. After baseline was established, the thermostat of the water bath was set at 37 °C. Reaction times as recorded in the figures begin at the point when 37 °C was initially reached in the water bath, except when a reaction at 0 °C was studied.

When GTP hydrolysis and turbidity were followed simultaneously (5), the extent of polymerization in the reaction was determined. The protein concentration of the supernatant following centrifugation at 50,000 rpm for 30 min; 20 μg of MAPs prepared by boiling microtubule protein for 20 min followed by DEAE-Sepharose chromatography, as described in the text; and 5 μg of tubulin.

**Fig. 1.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of MAPs. From left to right, the gels contained the following: 20 μg of MAPs obtained by chromatography of microtubule protein on DEAE-Sepharose; 20 μg of the previous preparation after heating in a boiling water bath for 5 min and centrifugation at 50,000 rpm for 30 min; 20 μg of MAPs prepared by boiling microtubule protein for 20 min followed by DEAE-Sepharose chromatography, as described in the text; and 5 μg of tubulin.

**RESULTS**

Initially, while studying the effects of a variety of antimitotic agents on GTP hydrolysis by purified tubulin, we observed potent stimulation of the reaction by taxol, surpassing the similar stimulatory activity of colchicine (11, data not presented). Since taxol, unlike colchicine, also induces microtubule protein to assemble (2, 3), it seemed possible that this GTP hydrolysis was a consequence of tubulin polymerization. A study in which polymerization and GTP hydrolysis were measured simultaneously (Fig. 2A) confirmed this hypothesis. Purified tubulin, in the absence of taxol, developed little turbidity and hydrolyzed little GTP. With taxol there was an initial lag period followed by the simultaneous onset of polymerization and GTP hydrolysis in an apparent 1:1 stoichiometry. As turbidity development approached its plateau, however, GTP hydrolysis continued, although at a slower rate (about 0.25 nmol/min/ml).

Since heat treatment largely abolished the tubulin-independent GTPase activity of MAPs without altering their
effect on tubulin polymerization (11, see “Experimental Procedures”), we extended our examination of the effects of taxol to the MAP-dependent reaction (Fig. 2B). In the absence of taxol there was now a brisk reaction occurring after a lag phase. Onset of polymerization and GTP hydrolysis were again simultaneous with an initial 1:1 stoichiometry. Again, as polymerization approached its plateau, hydrolysis continued at a reduced rate (about 0.15 nmol/min/ml). This relationship of polymerization and hydrolysis in the MAP-dependent reaction is similar to that reported by David-Pfeuty et al. (17), as well as similar to what we have observed in a glutamate-dependent system (5).

With taxol a very different pattern was observed. In this experiment, the base-line was established in the cold prior to the addition of taxol. As taxol was added, the thermostat of the water bath was set at 37 °C so that the temperature immediately began to rise, with zero time defined as the point at which the water bath reached 37 °C. Under these conditions there was an almost instantaneous onset of polymerization, following a lag of about 5 s. This was accompanied by a burst of GTP hydrolysis, apparently in excess of the tubulin polymerized. After the initial bursts of hydrolysis and polymerization, GTP hydrolysis continued at a steady linear rate of about 0.3 nmol/min/ml, while the rate of turbidity development progressively decreased as polymerization approached its plateau.

To better characterize these effects of taxol, we explored further the requirements for the polymerization reactions. Fig. 3 demonstrates that with purified tubulin, and without MAPs, no polymerization occurred in the cold unless both taxol and GTP were added. In addition, a 10-fold excess of GDP over GTP completely inhibited the polymerization reaction (Fig. 3), as well as associated GTP hydrolysis (Table I). Finally, in contrast to the results reported by Schiff et al. (2) with microtubule protein, taxol- and GTP-dependent polymerization of purified tubulin was substantially, although slowly, cold-reversible.

A much more complicated pattern was observed in the presence of MAPs (Fig. 4). At 0 °C, a reaction beginning within 5 s of the addition of taxol was observed only if both the drug and GTP were present (curve 1). This 0 °C polymerization reaction was completely inhibited by a 10-fold excess of GDP (curve 1a). Warming the cuvettes resulted in a second wave of turbidity development which was much less affected by GDP, although its onset was delayed and its plateau somewhat lower. Moreover, in the presence of MAPs, GTP was no longer required for polymerization at 37 °C (curve 2), as a brisk reaction occurred in the absence of added nucleotide. Curiously, GDP still exerted a mild inhibitory effect on this GTP-independent polymerization reaction (curve 2a). Without taxol, but with GTP, a typical cold-reversible reaction occurred at 37 °C (curve 3), and it was inhibited by GDP in 10-fold excess (curve 3a) in agreement with the results of others using microtubule protein (18-20). There was sluggish turbidity development at 37 °C without GTP (curve 4) in the presence of MAPs (but not in their absence, cf. Fig. 3). This may represent aggregation or ring formation (7, 8) as it was not cold-reversible.

In agreement with the findings of Schiff et al. (2) with microtubule protein, the polymerization reaction occurring with tubulin, MAPs, and taxol is largely cold-irreversible (Fig. 4, curves 1, 1a, 2, and 2a), no matter whether or not GTP or GDP was present. This contrasts to the rapid cold reversibility seen with MAPs and GTP (curve 3) or the sluggish cold reversibility with taxol and GTP (Fig. 3, curve 1).

Since tubulin polymerization in the presence of taxol, MAPs, and GTP has at least two distinct temperature phases, we re-examined GTP hydrolysis with an initial 0 °C incubation (Fig. 5). Under this condition, hydrolysis and polymerization reaction began immediately. Under these conditions, GTP hydrolysis continued at a steady linear rate of about 0.15 nmol/min/ml. This relationship of polymerization and hydrolysis in the MAP-dependent reaction is similar to that reported by David-Pfeuty et al. (17), as well as similar to what we have observed in a glutamate-dependent system (5).

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zation again initially coincided with about a 1:1 stoichiometry. The rate of GTP hydrolysis slowed to a linear rate of about 0.12 nmol/min/ml as polymerization reached its 0 °C plateau. As the cuvette was warmed, the temperature-dependent phase of polymerization began. About 5 min later, when this second phase of turbidity development was about 50% complete, GTP hydrolysis suddenly accelerated about 2.5-fold to about 0.05 nmol/min/ml. Fig. 5 also demonstrates that a 10-fold excess of GDP completely inhibits 0 °C GTP hydrolysis by tubulin in the presence of MAPs and taxol, as well as reduces the rate of temperature-dependent hydrolysis by 6-fold to about 0.05 nmol/min/ml. In addition, in the presence of GDP in 10-fold excess over GTP, polymerization occurring at 37 °C substantially exceeded the amount of GTP hydrolyzed.

The effect of taxol on GTP hydrolysis by tubulin, MAPs, and the combination of tubulin plus MAPs is presented in Fig. 6. In this experiment, samples were rapidly warmed to 37 °C, and the incubation begun as soon as taxol was added. Taxol had no effect on the minimal GTPase activity of the MAPs themselves in the absence of tubulin. It is also clear that without the 0 °C “head start” under the conditions described in Fig. 2, the complete system of tubulin, MAPs, and taxol has no advantage over taxol and tubulin and little advantage over tubulin and MAPs.

It should also be emphasized that the tubulin-taxol interaction is significantly influenced by solvent conditions. In the preceding studies, 0.1 mM glutamate was always used, a level well below that required for the polymerization of purified tubulin (5). When the glutamate concentration was raised to 0.33 mM glutamate, a level which induces GTP-dependent tubulin polymerization (5), the temperature requirement for taxol-induced polymerization disappeared (Fig. 7A). When the glutamate concentration was raised to 1.0 mM glutamate, each 0.5-ml reaction mixture contained the indicated concentration of monosodium glutamate and 1.0 mg/ml of tubulin as well as the following, as indicated: 50 μM GTP and 10 μM taxol, Curve 1, no addition; curve 2, GTP only; curve 3, taxol only; curve 4, taxol and GTP. Temperature changes in these experiments were performed as described in Fig. 5 with the arrows having the same significance.

0.2 mM, the absolute requirement for GTP disappeared in taxol-induced polymerization of purified tubulin (Fig. 7A). When the glutamate concentration was raised to 1.0 mM glutamate, a level which induces GTP-dependent tubulin polymerization (5), the temperature requirement for taxol-induced polymerization disappeared (Fig. 7B). The pattern observed with 1.0 mM glutamate was similar to that with MAPs at 0.1 mM glutamate: a 0 °C reaction dependent on GTP, a reaction occurring at higher temperatures independent of nucleotide.

Finally, we examined the electron microscopic appearance of tubulin polymerized in the presence of taxol (Figs. 8 to 10). In the complete system at 0.1 mM glutamate (Fig. 8)
Taxol Effects on Tubulin Polymerization and GTP Hydrolysis

FIG. 8. Electron micrographs of tubulin polymerized with GTP, MAPs, and taxol. All reaction mixtures contained 0.1 M monosodium glutamate, 1.0 mg/ml of tubulin, 0.33 mg/ml of MAPs, 10 μM taxol, and 50 μM GTP. A, thin section of material polymerized at 0 °C for 1 h (× 128,900); B, negatively stained preparation polymerized at 0 °C for 1 h (× 128,900); C, thin section of material polymerized at 37 °C for 1 h (× 45,700); D, thin section of material polymerized at 37 °C for 1 h and then maintained at 0 °C for 1 h prior to centrifugation (× 45,700).

FIG. 9. Electron micrographs of tubulin polymerized with taxol and either MAPs or GTP. All reaction mixtures contained 1.0 mg/ml of tubulin, 0.1 M monosodium glutamate, 10 μM taxol, and, if indicated, 0.33 mg/ml of MAPs or 50 μM GTP. A, thin section of material polymerized at 37 °C with MAPs for 1 h (× 45,600). B, thin section of material polymerized with MAPs at 37 °C for 1 h and then maintained at 0 °C for 1 h prior to centrifugation (× 45,600). C, thin section of material polymerized with MAPs at 37 °C for 1 h and then maintained at 0 °C for 1 h prior to centrifugation (× 45,600). D, Negatively stained preparation polymerized with GTP at 37 °C for 1 h (× 52,600). E, thin section of material polymerized with GTP at 37 °C for 1 h and then maintained at 0 °C for 1 h prior to centrifugation (× 26,300).

containing taxol, MAPs and GTP, most of the structures observed had the appearance of microtubules. There was no morphologic difference in the structures formed at 0 °C (Fig. 8A) or 37 °C (Fig. 8C) or in those persisting when a preparation was shifted from 37-0 °C (Fig. 8D). Fig. 8, A and B, demonstrates that the structures formed at 0 °C have the characteristic appearance of microtubules whether examined in thin section or when negatively stained.
When GTP was eliminated, the structures formed with taxol and MAPs at 37 °C (Fig. 9A) or persisting after a shift from 37-0 °C (Fig. 9B) were also predominantly microtubules. Without MAPs, however, the structures formed with taxol and GTP in 0.1 M glutamate at 37 °C contained only occasional microtubules (Fig. 9, C and D). The predominant structures formed appeared to be sheets of protofilaments, most obvious in negatively stained material (Fig. 9D), including structures in which an open sheet appears to be attached to a closed tubule. These sheet structures probably account for the significantly greater turbidity developed in the absence of MAPs as compared to their presence (cf. Fig. 2A to Fig. 2B and Fig. 3 to Fig. 4). We have observed a similar effect of morphology on degree of turbidity development in sulfonate-induced tubulin polymerization (26). Fig. 9E demonstrates the cold reversibility of the structures formed at 37 °C in the presence of taxol and GTP. Although an occasional tubule-like structure remained, the bulk of the pellet obtained after the shift from 37-0 °C appears to be an amorphous aggregate.

In 1.0 M glutamate, because of an intense chemical reaction between glutaraldehyde and glutamate, we were able to examine only unembedded, negatively stained material. Without taxol and with GTP at 37 °C, sheets of varying width with parallel protofilaments are formed. Similar structures are formed with taxol at both 0 °C and 37 °C if GTP is present and at 37 °C without GTP (Fig. 10).

DISCUSSION

Schiff et al. (2) have demonstrated with microtubule protein that taxol lowers the critical concentration of tubulin required for polymerization, abolishes the usual lag period at 37 °C prior to polymerization, and increases the number, but decreases the length of microtubules formed. Therefore, they concluded that one of the effects of taxol is to enhance the nucleation phase of microtubule assembly.

Our studies clearly demonstrate that the interaction of taxol and tubulin is profoundly influenced by other components of the reaction system. Excluding solvent conditions and tubulin itself, the system we have examined in detail here has four components: taxol, GTP, MAPs, and heat (0 °C being considered negative, 37 °C positive for heat). Rapid tubulin polymerization occurred if any three of these components were present. Microtubules were always formed, although, without MAPs, sheets of protofilaments predominated. Of particular note is the almost instantaneous polymerization of tubulin which occurs at 0 °C in the presence of GTP, MAPs, and taxol. Under this condition, nucleation must be virtually instantaneous.

These observations suggest that the critical feature of the nucleation phase of microtubule assembly is a conformational change in the tubulin molecule rather than the generation of intermediate polymeric forms. The final “polymerizable” conformation of tubulin may be reached through several intermediates, with each of the four components we have examined (taxol, GTP, MAPs, and heat) having different but additive

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effects on the shift from the "nonpolymerizable" to the "polymerizable" conformation. In this model, the critical concentration under specific reaction conditions would reflect the overall equilibrium between the polymerizable and nonpolymerizable conformations. The biphasic reaction, as a function of temperature, observed in the complete system probably reflects a shift in this equilibrium in favor of the polymerizable conformation when reaction temperature is raised.

The studies presented here and those of Schiff and Horwitz (4) demonstrate that, under appropriate conditions, taxol eliminates the requirement for exogenous GTP or a nonhydrolyzable GTP analog (9, 21–23) for microtubule nucleation, and confirms the conclusion that exogenous GTP is not required for microtubule elongation (18, 24). We have also observed that the mole of bound GTP present in our tubulin preparation is not hydrolyzed in taxol-induced polymerization occurring without exogenous GTP\(^2\) (data not presented), supporting our assumption that this GTP is bound at the nonexchangeable site. Taxol thus can induce the efficient polymerization of tubulin with GDP in the exchangeable site. This can be achieved with either MAPs or altered solvent conditions and requires warmer temperatures.

Under conditions in which nucleotide is not required for taxol-dependent polymerization, if GTP is present the polymerization and hydrolytic reactions appear to occur independently. Specifically, when the reaction mixture containing taxol, MAPs, and GTP reached the turbidity plateau at 0 °C hydrolysis continued at a reduced rate. When the reaction mixture was warmed, polymerization resumed without a change in the rate of GTP hydrolysis. About halfway through the second phase of polymerization, GTP hydrolysis abruptly accelerated. While the second phase of polymerization was little affected by GDP, the GTPase reaction was significantly inhibited.

The linear rate of GTP hydrolysis occurring at the turbidity plateau with taxol, tubulin, and MAPs (the reaction which bears little relationship to tubulin polymerization) differs little from the analogous rates with taxol and tubulin or with tubulin and MAPs (conditions in which the initial phase of hydrolysis is closely coupled to polymerization, Fig. 2, A and B). This was most obvious when the three reactions were examined together without a prior 0 °C incubation (Fig. 6). These comparable rates suggest similar mechanisms.

David-Pfeuty et al. (17, 25) have postulated that the continuing GTP hydrolysis at the turbidity plateau with MAPs and tubulin represents turnover of tubulin dimers at the ends of microtubules. They found that this reaction could be enhanced by fragmenting the tubules or abolished by removing the tubules by centrifugation. Yet an almost identical GTPase reaction occurs with MAPs, tubulin, and taxol, which is associated with microtubules so stable that they cannot be depolymerized by dilution, Ca\(^{2+}\), or cold (2, 4, Fig. 5). It seems unlikely that such stable structures would have the high turnover rate implied by the GTPase activity at the polymerization plateau. Moreover, in preliminary studies we have found that the linear rate of GTP hydrolysis at the polymerization plateau varies directly with GTP concentration.\(^3\) As the amount of tubulin-bound GTP was not greatly altered, the increased rate of GTP hydrolysis is apparently a function of the free GTP concentration. This preliminary finding is also difficult to explain on the basis of tubulin turnover. We, therefore, conclude that the GTPase activity demonstrated by David-Pfeuty et al. (17, 25) to be a function of microtubule ends is an intrinsic property of these ends rather than a function of the turnover rate. This obviously implies that a few tubulin molecules in the polymer have a high, repetitive GTPase activity while most are inert. This interpretation is also consistent with the finding that the bulk of the nucleotide in polymerized tubulin is not exchangeable (17, 22). The function of this postulated high rate of GTP hydrolysis at microtubule ends is unknown, but the activity may be an artifact of the in vitro system or may reflect the loss of controlling factors during purification.

Under some reaction conditions, taxol-dependent polymerization is totally dependent on added GTP. When this occurs, GTP hydrolysis is initially closely coupled to polymerization and also requires taxol. Both reactions are almost totally inhibited by GDP.

The taxol-induced polymerized forms of tubulin that we have observed differ morphologically from those described by Schiff et al. (2, 4) in two cases. While they found microtubules (4), we observed sheets of protofilaments as the predominant structure with purified tubulin. With microtubule protein and GTP at 0 °C, Schiff et al. observed ribbons with a few protofilaments (2). With a reconstituted system of tubulin and heated MAPs together with GTP at 0 °C, we found that microtubules are formed. These differences probably arise from the different solvent conditions used. Without taxol, we have observed significant differences in polymer formed in glutamate as compared to that formed in Mes, the buffer anion used by Schiff et al. (2, 4).

Acknowledgments—We would like to thank Dr. D. G. Johns for his encouragement and a critical reading of the manuscript; Dr. S. B. Horwitz for communicating her results to us prior to their publication; and Ms. E. Olausen for her help in preparing the manuscript.

REFERENCES


\(^{2}\) Protein and tubulin-bound nucleotide were separated by gel filtration chromatography on Sephadex G-100 in 8 m urea. The nucleotide peak was then analyzed by ion exchange chromatography on DEAE-Sephadex A-25.

\(^{3}\) Unpublished observations, E. Hamel.
Taxol Effects on Tubulin Polymerization and GTP Hydrolysis