The Co-polymerization of Tubulin and Tubulin-Colchicine Complex in the Absence and Presence of Associated Proteins

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Tabulin-colchicine complex (TC) is a potent inhibitor of microtubule assembly whereas microtubule-associated proteins (MAPs) facilitate assembly. Previous studies done under MAP-depleted conditions (Sternlicht, H., and Ringel, I. (1979) Biol. Chem. 254, 10540) suggested that tubulin binds to the ends of the microtubule with an apparent dissociation constant (affinity$^{-1}$) which increases as the TC/tubulin ratio in the microtubule increases. Affinity$^{-1}$ was identified with the critical, i.e. minimum tubulin concentrations required for assembly. In this study, we examined the TC-tubulin co-polymerization reaction as a function of MAP availability. Critical tubulin concentrations, which increases in the presence of TC and decreases in the presence of MAP, could be approximated as a sum of contributions from MAPs and TC. An expression was derived which successfully predicted the per cent inhibition observed for a variety of TC, tubulin, and MAP concentrations. In all cases, increases in critical tubulin concentrations correlated with increases in the TC/tubulin ratio in the microtubules.

Our data suggest that substoichiometric inhibition by TC is not a consequence of impaired MAP function, nor is it a consequence of a marked increase in the apparent free energy of assembly in the presence of TC. Rather, the large per cent inhibition values observed at low TC concentrations ( TC $>$ 5 mm) appear to be a consequence of the small concentrations of tubulin typically used in assembly studies (2$>$ 4 mg/ml active tubulin) and the constraint that assembly in the presence of TC requires a minimum concentration of tubulin equal to affinity$^{-1}$. The molecular processes by means of which TC and MAPs affect affinity$^{-1}$ remain to be established.

Microtubules, a major component of the spindle fibers and cytoskeletal structure of eukaryotic cells, participate in a variety of cellular functions. They assemble primarily from tubulin, but contain significant amounts of other proteins, collectively referred to as MAPs$^{*}$(1-4). Colchicine has been used extensively to probe microtubule-dependent processes. TC, a 1:1 complex of tubulin with drug, directly inhibits microtubule assembly at substoichiometric concentrations of TC relative to tubulin in vitro (5, 6) and in vivo (7). Other potent antimicrotubule drugs, both competitive and noncompetitive inhibitors of colchicine binding, also inhibit assembly substoichiometrically (6, 8, 9). These substoichiometric inhibition processes are not fully understood. An elucidation of the molecular basis for this inhibition may be relevant to an understanding of microtubule assembly control mechanisms within the cell (7, 10).

MAP proteins are ubiquitous in eukaryotic cells, and at least several of these proteins appear to be conserved (11-13). Their biological functions remain to be established. MAPs co-assemble with tubulin and may constitute as much as 10 to 20% of the microtubule protein mass when tubulin is isolated by repetitive cycles of assembly-disassembly (4). Considerable efforts have been made to characterize the major MAP proteins and rank them with respect to their ability to facilitate microtubule assembly (2-4). MAPs affect the nucleation and elongation phases of the assembly reaction, and reduce the critical, i.e. minimum tubulin concentrations required for assembly (14-18). Recent in vitro studies have demonstrated that MAPs affect colchicine inhibition. Colchicine added to preformed microtubules, for example, will depolymerize microtubules more extensively if the microtubules are deficient in MAPs (19, 20). The molecular basis for this effect remains to be determined. In this study, we examine the effects that MAPs have on the TC inhibition of microtubule assembly.

Sternlicht and Ringel showed that TC co-polymerizes with tubulin to form microtubule co-polymers, and proposed a co-polymerization mechanism for the inhibition of microtubule assembly (21). The co-polymers had assembly-competent ends with reduced affinity for tubulin. Furthermore, tubulin dissociation constants, affinity$^{-1}$, correlated with co-polymer composition, and increased as the TC/tubulin ratio in the microtubule increased. At steady state, incorporated TC represented less than 1% of the microtubule mass and was presumed to be distributed randomly in the microtubule lattice. This model differed from the capping model of inhibition which hypothesized that TC poisons microtubule assembly by binding quasi irreversibly to the microtubule ends, thus preventing further tubulin addition (8, 22). The capping model was derived, in large measure, from studies involving TC addition to microtubules at or near steady state under conditions where TC concentrations were relatively high. The co-mercaptotethanol, 0.5 mM MgCl$_2$, and 25 mM glycerol; PB-4 M, a 4 M glycerol buffer similar to PB-2.5; 1x-, 2x-, and 3x-MTP, microtubule protein prepared by one, two, and three cycles of assembly-disassembly; Pipes, 1-piperazineethanesulfonate; Me$_2$SO, dimethyl sulfoxide; PC-tubulin, phosphocellulose chromatographed tubulin protein; D$_{nax}$, microtubule protein concentration; Y, the TC mole fraction in the microtubule; SDS, sodium dodecyl sulfate.
polymerization model, on the other hand, was derived from a study of tubulin assembly in the presence of TC under conditions where TC concentrations were relatively low (21). Recently, Farrell and Wilson (23) confirmed that TC inhibits assembly by a co-polymerization mechanism and suggested a plausible way to reconcile co-polymerization with the above-mentioned steady state inhibition studies (8).

The co-polymerization model was deduced initially from an assembly study done under MAP-depleted conditions. In that study, heparin, a MAP-complexing agent, was used as a convenient means of controlling MAP availability (21). Since TC was a potent inhibitor of assembly under MAP-depleted conditions, it was reasonable to assume that TC inhibits by a mechanism which does not directly involve MAPs. They generalized their findings and suggested that TC inhibition might be explained in terms of perturbations arising from tubulin-TC interactions rather than in terms of perturbations arising from TC-MAP interactions or altered tubulin-MAP interactions. We have carried out further studies in which tubulin and TC co-assembled in the presence of MAPs (~10 to 15% by weight) and in the absence of MAPs. Microtubule yield and composition was determined at steady state under different MAP conditions for a variety of TC and tubulin concentrations. We compare our results with the earlier MAP-depleted study using heparin (21), and confirm that MAPs have a significant effect on percent inhibition (19, 20). Our data suggest that TC does not inhibit assembly by interfering with MAP function. Rather, we find that assembly in the presence of TC requires a critical concentration of tubulin which is dependent on TC concentration and MAP availability. MAP effects on TC inhibition may be complex. However, we show that its effect can be modeled to a good first approximation by neglecting direct linkages between MAP and TC mechanisms of action and by assuming that MAPs and TC contribute independently to the tubulin critical concentrations.

**Materials and Methods**

**Tubulin Preparation.** Microtubule protein (MTP) was isolated by repetitive cycles of assembly-disassembly from bovine brains devoid of brain stem, following a procedure modified from Gaskin et al. (24). 1x-, 2x-, and 3x-MTP stocks (10 to 12 mg/ml), were stored at -20°C in a MTP-stabilizing buffer (PB-5 m). The pH was 6.3 at room temperature. Stock aliquots were diluted 1:1 with PB-0 (glycerol-free buffer) and clarified by centrifugation at 15,000 x g for 30 min at 4°C prior to use. Dilutions were made with PB-2.5 m. Studies done in the presence of MAPs were all done with 3x-MTP, except for the experiments comparing 1x-, 2x- and 3x-MTP (Fig. 1D). 3x-MTP contained ~5 to 90% tubulin and ~10% to 15% non-tubulin protein as estimated by densitometry of polyacrylamide gels (25).

MAP-free tubulin was prepared from microtubule protein chromatographed on precycled Whatman P11 phosphocellulose (Bethesda Research Laboratories). 2x-MTP was polymerized at 37°C, resuspended and homogenized in a minimum amount of cold Mss buffer at pH 6.7 (25 mm Mes, 2 mm EGTA, 1 mm mercaptoethanol) or cold Pipes buffer at pH 7.3 (20 mm Pipes, 1 mm EGTA, and 1 mm mercaptoethanol) to make ~25 to 30 mg/ml of solution. The solution was layered on top of a phosphocellulose column (~3 mg of tubulin/ml of phosphocellulose bed in Mes or Pipes) and eluted at a rate of ~1 bed volume/hour to give PC-tubulin (26). PC-tubulin in Mes buffer was combined with a buffer rich in Mes, Mg²⁺, and glycerc to obtain concentrated protein (~5 mg/ml) in a final buffer equivalent to PB-4 m, and stored at -20°C. Protein remained stable at -20°C for at least several days. PC-tubulin in Pipes buffer was brought to 0.1 M Pipes and 0.5 mM MgCl₂, and supplemented with Mes/SO₂ to a final Mes/SO₂ concentration of 10%. PC-tubulin in Pipes/MeSO₂ buffer was used within an hour of preparation. Protein concentrations were determined by the Lowry method with bovine serum albumin as standard (27).

**Preparation of TC.** TC was prepared in accord with a procedure described previously (21). About 30 μg 3x-MTP in PB-2.5 m buffer, or ~30 μl PC-tubulin in Pipes or Mes buffer were incubated for approximately 50 to 90 min at 37°C with [125I]Colchicine (~50 μM, ~50 Ci/mmol) from New England Nuclear. Free colchicine was removed by a rapid gel filtration procedure (28). Dilute TC solutions were concentrated by centrifuging through dry Sephadex G-25, or by centrifuging through Amicon filters (Centricon CF-50A). TC prepared from 3x-MTP was brought to a final buffer state equivalent to PB-2.5 m and stored at -20°C. TC prepared from PC-tubulin (Mes buffer) was brought to a final buffer state equivalent to PB-4 m and stored at -30°C for subsequent use, whereas TC prepared from PC-tubulin (Pipes buffer) was brought to a final buffer state equivalent to Pipes/MeSO₂ and used within 24 h of preparation. The stocks contained ~25 to 25 μM total protein and ~15 to 22 μM TC. Binding estimates were obtained by gel filtration of stock aliquots through Bio-Rad P-10 columns. At least 90 to 95% of the radioactive colchicine in the stocks was present as TC. TC is a relatively stable complex with little dissociation occurring over several hours at 37°C (29), and several days at -20°C.

**TC Mole Fraction in the Microtubule Phase.** Microtubule co-polymers were analyzed in accord with a procedure described elsewhere (21). MTP solutions, with and without 0.13 mM heparin, or PC-tubulin were assembled at 37°C in the presence of various concentrations of TC. The microtubules were collected at steady state either by pelleting 1 ml of microtubule suspension through 7 ml of 50% sucrose (Beckman 50, 198,000 x g for 90 min at approximately 30°C), or were collected as pellets by centrifuging the suspensions in 1.5 ml tubes (Sorvall SA-600) at 17,000 x g, h factor ~150, for ~45 min at approximately 30°C. The pellets obtained were washed in buffer, repelleted, and then resuspended in PB-0 m at 0°C. The TC mole fractions in the pellets, Y, were estimated from the radioactivity and protein mass corrected for non-tubulin protein. Y was set equal to (TC/TC + T₄), the TC mole fraction in the microtubule phase. The two methods used to recover microtubule pellets gave similar Y results.

**Absorbance Measurements.** Microtubule assembly at 37°C was monitored spectrophotometrically (14) by use of a Gilford 2400-2, a multichannel spectrophotometer capable of measuring the absorbances of four samples simultaneously. Changes in the absorbance at 350 nm with time, A(t), were recorded and analyzed, as described below. Previous studies of the light-scattering properties of microtubule solutions have shown that absorbance changes are proportionally related to the mass concentration of microtubule present.

**Activity Determinations.** The activity of tubulin available for assembly was determined using TC-free solutions for convenience. α is estimated from a mass balance as described previously (21), noting that active tubulin in solution is in equilibrium with tubulin in the microtubule phase at steady state, and had values of ~0.75 to 0.85 in the MTP study (heparin-free) and ~0.60 in the PC-tubulin study. Flourometric Analysis. We used a "C-methylmethylene method to increase the ability to detect non-tubulin protein. Microtubule pellets prepared under various conditions were described under "Results" were suspended in PB-0 m buffer, and were methylated reductively (31) by incubating the suspensions for approximately 15 min at 37°C with approximately 10 mM NaCNBH₃ and approximately 1 to 3 mm [14C]formaldehyde (New England Nuclear, 5 to 10 Ci/mmol). Alternating-colchicine inhibition of microtubule assembly
tively, heat-stable MAPs were obtained enriched in the supernatants from boiled microtubule suspensions (approximately 5 min at 100°C in 0.7 M NaCl buffer with 2 mM dithiothreitol (32), and were methylated reductively. The methylated materials were analyzed on 9% polyacrylamide gels using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (33). The gels were prepared for fluorography by a method modified from Bonner and Laskey (33). In our procedure gels were soaked in a scintillation-type fluid (EN'HANCE from New England Nuclear), for 1 ½ h, soaked in water for 1 to 2 h, dried, and then exposed to x-ray film (X-Omat XR-5) at -70°C for various lengths of time.

RESULTS

Microtubules were assembled at 37°C from reaction mixtures containing various concentrations of tubulin, TC, and MAPs. The increase in turbidity during assembly was monitored spectrophotometrically by measuring the absorbance change, ΔA(t) at 350 nm. Previous studies have shown that ΔA(t) is proportional to polymer mass present (14, 30). Electron microscopy examinations of negatively stained aliquots obtained during assembly confirmed that microtubules were the predominant polymeric structures. The bulk of the studies were done on bovine brain MTP solutions (~10 to 15% MAPs by weight) prepared by three cycles of assembly-disassembly in PB-2.5 M. These studies were supplemented with assembly studies done in the presence of heparin (the MAP-depleted case) and also with studies using PC-tubulin (MAP-free case).

Assembly in the Presence of TC—The absorbance versus time plots shown in Fig. 1, A and B, were obtained at 37°C from solutions containing microtubule protein and TC at the indicated total concentrations (Dtotal = 2.5 and 1.5 mg/ml, respectively; TCtotal = 3.5 μM). Significant concentrations of TC were incorporated into the microtubules indicating a copolymerization reaction. This is discussed in more detail below (e.g., Table I). Absorbance measurements confirmed that TC inhibits microtubule assembly (22). Aα, the absorbance change observed at steady state, decreased with increasing TC (Fig. 1A), whereas the assembly lag time (Fig. 1B), increased with increasing TC. In Fig. 1C, we plot Aα against TCtotal for three concentrations of microtubule protein: Dtotal = 2.5, 1.5, and 1.0 mg/ml. Although Aα values were dependent on Dtotal, the rate at which Aα decreased with increasing TCtotal, as measured by initial slopes and also curvature (Fig. 1C), appeared to be independent of Dtotal. This finding was reproduced consistently. Initial slope values from independent brain preparations typically varied no more than approximately 25% from that in Fig. 1C.

The dependence of Aα on TCtotal was investigated further. The slopes (Fig. 1C) correspond to the rate of change of Aα with respect to TCtotal. The observation that the slopes decreased in magnitude as TCtotal increased indicated that the assembly process was less sensitive to changes in TC at high TC concentrations than at low concentrations. Wallin and Larsson recently proposed from studies of MTP crudes that the microtubule sensitivity to disruption by colchicine correlates decreased in magnitude as TCtotal increased indicated that the observation that the slopes de-

![Fig. 1. Assembly as a function of TC concentration. MTP concentrations in the reaction mixtures were held constant, while TC concentrations varied. GTP concentration was 1 mM. Absorbances, ΔA(t), were recorded during the assembly reaction, and the steady state absorbances, Aα, were determined. Assembly occurred spontaneously in those preparations lacking heparin, and were initiated by raising temperature from 0 to 37°C. Assembly in the presence of 0.13 mM heparin (Panel D) did not occur spontaneously at 37°C, but required the addition of microtubule fragments as initiator seeds (21). A, absorbance versus time for reaction mixtures containing MTP (2.5 mg/ml) and TC (0 to 3.5 μM); B, absorbances during the initial phase of the assembly reaction. MTP concentration was 1.5 mg/ml and TC concentrations varied from 0 to 3.5 μM. Lag periods characterized by no assembly were evident at the start of the assembly reaction and increased with increasing TC. Lag periods are presumably related to the time required to form nucleation centers spontaneously; C, Aα plotted against TC for 25 (●), 1.5 (○), and 0.95 (△) mg/ml of MTP; D, Aα plotted against TC for 2.5 mg/ml of 1×(●), 2×(○)), and 3×(△)-MTP without heparin, and 1×(●) and 3×(△)-MTP with 0.13 mM heparin. Initial slopes were ~0.083 ± 0.007 and ~0.083 ± 0.003 A/μM TC in the absence and presence of heparin, respectively. These slope values are similar to those from (C).](image-url)
The above analysis suggests that $A_\infty$ plots (Figs. 1, C and D) show a common dependence on $T_{C_{total}}$ which is insensitive to such factors as $D_{total}$, the number of cycles of purification, and MAP availability, because the critical tubulin concentrations corrected for MAPs, TC, and inactive tubulin. $[T]_{w}$ values are averages of several measurements with standard error of mean values indicated by the horizontal bars. $D_0$ depends on $T_{C_{total}}$ (Panel A) and were calculated from Equation 3. A linear relationship was observed between $(D_0 - D_{0w})$ and $T_{C_{total}}$. $D_0$ had an estimated value of approximately 1.6 $\mu$M. The constants in Equation 3 were derived from the slope and intercept values of this double reciprocal plot. $D_0$ values, estimated either from the abscissa intercepts in Panel A (C) or from Fig. 1C (D), coincide well with the calculated values $(- -)$ from Equation 3. Fig. 1C was analyzed with the aid of Equation 1B. $E$, $A_\infty$ plotted against $aT_{total}$, for assembly reactions in the presence of 0.13 $\mu$M heparin.

Equation 3 was derived from the absorbance difference, $A_\text{yield}$ when assembly occurs in the presence of TC, is proportional to the absorbance difference, $A_\text{with TC} - A_\text{with no TC}$. If one ignores small contributions from incorporated TC, $A_{\text{yield}}$ can be taken as equal to $-(D_0 - D_{0w})$ (cf. Equation 1B). Below we examine how microtubule yield varies with changes in $T_{C_{total}}$, $Y$, and $F_T$, the free energy associated with the co-assembly reaction; and we also examine to what extent these changes are related to MAP concentration and availability (e.g. Table IV.) The per cent to which the assembly reaction is inhibited by TC follows from Equation 1B,

\[
\text{% Inhibition} = 100\% \times \frac{[A_{\infty, w TC} - A_{\infty, w no TC}]}{A_{\infty, w TC}}
\]

and is dependent on total tubulin and MAP concentrations, and also on $D_0 - D_{0w}$. $D_0 - D_{0w}$ in Equation 2 denotes the change in critical tubulin concentration induced by TC under conditions of constant MAP concentration.
vary with TC_{total} in a manner which is insensitive to D_{total}, the number of cycles of purification, and MAP availability. Direct determinations of the critical tubulin concentrations from assembly studies done in the absence and presence of heparin demonstrated that D_{i} - D_{0} values increased in a similar manner with increasing TC_{total} (Fig. 2, A and B). In the MTP study (no heparin) where it was practical to use high TC concentrations, changes in critical tubulin concentrations showed a nonlinear dependence on TC_{total} which could be approximately represented by the expression

\[ D_{i} - D_{0} = \frac{4.3\text{TC}_{total}}{1 + 0.5\text{TC}_{total}} \]  

(3)

where TC_{total} \approx 5 \mu M and D_{0} \approx 1.6 \mu M (Fig. 2D). In the MAP-depleted study (0.13 mM heparin), changes in the critical tubulin concentrations could be fitted to an expression of the form

\[ D_{i} - D_{0} = 3.8\text{TC}_{total} \]  

(4)

where D_{0} \approx 6.5 \mu M, and TC_{total} was of the order of 1 \mu M or less (21). In the TC range \leq 1 \mu M, Equation 4 is a good approximation for Equation 3. We also examined the effects of intermediate concentrations of heparin (<0.13 mM) on the assembly reaction and could resolve D_{i} into two terms, a TC-dependent term similar to that reported in this study, and a MAP-dependent term (D_{0}) which decreased with decreasing heparin. These findings suggest that D_{i} can be approximated by a sum of independent contributions from MAPs and TC. If one substitutes Equation 3 for D_{i} - D_{0} in Equation 1, one obtains an equation for A_{i} in terms of TC_{total} which gives absorbance values in good agreement with the observed values. As noted above, \Delta A_{yield} can be set equal to \(- (D_{i} - D_{0})\). We therefore estimate from Equations 3 and 4 that microtubule yield decreases initially by \(-4.2 \pm 0.3 \mu M/\mu M \text{TC}_{total}\) present, a rate of decrease which, to a good approximation, is independent of heparin and the number of cycles of assembly-disassembly used for purification.

**Microtubule-MAP Association in the Presence of TC**—Both TC and heparin cause increases in D_{i}, and consequently, cause microtubule yields to decrease. Heparin increases D_{i} by complexing with MAPs and reducing MAP-microtubule associations. However, TC increases D_{i} by a different mechanism. This conclusion was reached in part from the following experiments: Microtubules were assembled under heparin-free conditions from MTP in the presence of varying concentrations of TC (0 to 3.5 \mu M), and were analyzed with respect to MAP content using SDS-polyacrylamide gel electrophoresis (Fig. 3A). In these samples, D_{i} varied from approximately 1.6 to 5 \mu M, and the TC/tubulin ratio in the microtubule co-polymers varied from 0 to about 0.05 (Table I). A control polymerization with no TC present was also done in the presence of 0.13 mM heparin, and the microtubules from this control were analyzed (Fig. 3A, Lane 1). The D_{i} value of the heparin control was -6.5 \mu M and was similar to that of the heparin-free sample assembled in the presence of 3.5 \mu M TC, but the MAP patterns of these two samples were distinctly different (c.f. Fig. 3A, Lanes 1 and 4). Microtubules assembled in the presence of heparin were observed to be depleted of Band I, the high molecular weight MAPs, a group of proteins known to facilitate microtubule assembly (2). However, microtubules assembled without heparin do have high molecular weight MAPs (Fig. 3A, Lanes 2 to 4). Furthermore, the 3.5 \mu M TC sample (Fig. 3A, Lane 4) gave a pattern similar to the patterns from microtubules spontaneously assembled with no TC present (Fig. 3A, Lane 2) and with 1 \mu M TC present (Fig. 3, Lane 3). Similar high molecular weight MAP patterns, irrespective of TC content in the polymerizing samples (Fig. 3, Lanes 2 to 4), argue against MAP-TC complexation as a cause of TC inhibition. In a further effort to identify possible TC-induced MAP differences, we isolated heat-stable MAPs from microtubule pellets derived from reaction mixtures containing MTP and 0 or 3 \mu M TC, and analyzed these MAPs using SDS-polyacrylamide gel electrophoresis (Fig. 3B). Previous studies have shown that heat-stable MAPs from MTP are rich in high molecular weight-MAP 2 and contain Tau (37), a group of non-tubulin proteins (M, 55,000 to 70,000) capable of facilitating microtubule assembly (3, 37). A com-

**Fig. 3.** SDS-polyacrylamide gel electrophoresis analysis of microtubule co-polymers. A, microtubules were assembled to steady state from reaction mixtures containing 2.5 mg/ml of MTP and varying concentrations of TC_{total}, and were collected as pellets by centrifugation. Eighty-microgram aliquots from the resuspended pellets were electrophoresed on approximately 6% polyacrylamide gels, and stained by Coomassie Blue R250. TC_{total} concentrations were 0.0, 1.0, and 3.5 \mu M (Lanes 2, 3, and 4, respectively). The control in Lane 1 was derived from microtubules assembled in a 0.13 mM heparin buffer free of TC. The control is observed to be nearly devoid of Band I. Band I is a multiplet consisting of high molecular weight MAPs 1 and 2 (terminology in accord with Sloboda et al. (2)) whose molecular weights are greater than 3 \times 10^6. Band V is a doublet consisting of \( \alpha \) and \( \beta \) subunits of tubulin. Bands III to IV come from proteins whose molecular weights were, respectively, 225, 175, and 70 \times 10^3. These three bands appear to be similar to the triplet of proteins ascribed to neurofilament contamination (4, 36). B, fluorograms of heat-stable MAPs. Microtubules were assembled from MTP (2.5 mg/ml) solutions in the presence of 0 or 3 \mu M TC. Heat-stable MAPs were obtained from pelleted microtubules, and were[^{[\text{14C}]methy}lated and analyzed as described under "Materials and Methods" (fluorogram analysis). The fluorograms shown were obtained after 6% and 15 h of exposure. Lanes 2 and 5, TC_{total} = 0.0 \mu M; Lanes 3 and 6, TC_{total} = 3 \mu M. A heparin control derived from microtubules assembled in a 0.13 mM heparin buffer without TC, is displayed in Lanes 4 and 7. Lane 1 was obtained from a[^{[\text{14C}]methy}lated microtubule control (TC = 0.0 \mu M, no heparin) prepared as described in A. C, a comparison of MTP and PC-tubulin. Microtubules were assembled from MTP in PB-2.5 M and PC-tubulin in PB-4 M, pelleted and then analyzed on 9% gels. \( \alpha \)- and \( \beta \)-tubulin polypeptides are the predominant components by far in the PC-tubulin samples (Lane 2).
Colchicine Inhibition of Microtubule Assembly

Comparative analysis of aliquots from the 0 and 3.5 μM TC studies indicated no significant differences in high molecular weight MAPs, Tau proteins, or in any of the residual heat-stable associated proteins (Fig. 3B, compare Lane 2 with Lane 3, and Lane 5 with Lane 6). Our inability to detect any significant TC-induced changes (Fig. 3, A and B) suggest to us that MAP functions are unaltered in the presence of TC. This is consistent with our earlier observations above, which suggested that, to a good approximation, MAPs and TC make independent contributions to D.

PC-Tubulin Studies—We regard the observation that heparin causes a significant reduction in the amount of high molecular weight MAPs found in association with microtubules (Fig. 3) yet does not affect the degree to which TC increases critical tubulin concentrations (Fig. 2 and Equations 3 and 4) as conclusive evidence that TC inhibits by a mechanism which can function independently of high molecular weight MAPs. Evidence that TC inhibits assembly by a mechanism which can function independently of Tau and also high molecular weight MAPs was obtained from assembly studies using PC-tubulin (Fig. 4). SDS-polyacrylamide gel electrophoresis analysis indicated that PC-tubulin was >99% pure with no significant concentrations of either Tau or high molecular weight MAPs found in association with microtubule samples assembled from PC-tubulin in PB-4 M (Fig. 4, C and D). Our inability to detect any significant increases in the scattering factor used to relate turbidity to polymer mass concentration (38, 39). We chose a Pipes/Me2SO buffer at pH 7.2 to 7.4 to minimize the number of altered forms (38, 40) while the PB-4 M buffer was chosen to maintain conditions as close as possible to our standard PB-2.5 M buffer. Electron microscopy examinations of negatively stained aliquots of polymerized material from the Pipes/Me2SO assembly study indicated that complete microtubules were the predominant forms, although there were also twinned and branched microtubules present reminiscent of the incomplete forms observed by Burton and Himes using a similar Pipes/Me2SO buffer system (40). Despite the fact that altered forms were observed in the PC-tubulin study, TC caused comparable decreases in polymer yield, and incorporated to a similar extent into polymeric materials assembled.

![Fig. 4. Assembly of PC-tubulin as a function of TC concentration.](image)

**Table 1** Microtubule yield and composition at steady state as a function of TC

<table>
<thead>
<tr>
<th>TC, μM</th>
<th>Microtubule yield</th>
<th>ΔYield</th>
<th>Observed</th>
<th>Predicted</th>
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<td>2.5</td>
<td>−5.2</td>
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</tr>
</tbody>
</table>

A. No heparin

B. 0.13 mm heparin (Sternlicht and Ringel (21))

Microtubulin, (T + TC)₄, were estimated from Aₙ values using the appropriate scattering factor L. L was corrected for non-tubulin protein content in the case of the microtubule protein study. L was taken as −0.020 and 0.057 A/μM of polymerized tubulin in the microtubulin protein and PC-tubulin studies, respectively.

Per cent inhibition is defined in Equation 2a. The predicted per cent inhibition was calculated from Equation 2b using the parameters listed in Table II and the appropriate equation for D = D₋ (Equations 3, 4, and 5).

α = 2.5 ± 0.2 mg/ml in PB-2.5 M (Figs. 1C and 2).

Based on L = 0.020 A/μM of polymerized tubulin.

α = 5.2 ± 0.3 mg/ml in PB-4 M (Fig. 4, C to E).
from PC-tubulin and MTP (Table I, Fig. 4). We estimated that polymer yields decreased by ~ 4 μM/μM TCtotal when TC and PC-tubulin co-polymerized in the presence of 10% Me2SO or in the presence of 4 M glycerol. In Fig. 4D, for example, we plot A50, the steady state absorbances, as a function of PC-tubulin and TC concentrations. This study done in PB-4 M buffer was analogous to the study done earlier with MTP (Fig. 2), and suggested that TC inhibits PC-tubulin assembly by raising the minimum tubulin concentrations required for assembly. We obtained

\[ D_5 - D_6 = 4(TC)_{total} \]  

where \( D_5 \sim 7 \text{ μM} \) and \( [TC]_{total} \approx 3 \text{ μM} \).

Summarizing, we observed similar but not identical values for \( D_5 - D_6 \) as a function of TC for assembly studies done in the absence or presence of MAPs. While \( D_5 - D_6 \) values were nearly identical at low TC concentrations (TC_{total} \approx 1 μM), differences were apparent at higher TC concentrations (Equations 3 and 5). These findings presumably are not a consequence of different buffer conditions used (2.5 M versus 4 M glycerol) since similar difference in \( D_5 - D_6 \) behavior were obtained when a Pipes/Me2SO buffer was substituted for PB-4 M glycerol in the PC-tubulin study. The results from the previous sections (e.g., Equations 3 and 4, Fig. 3) were consistent with independent modes of action for MAPs and TC, and could be understood in terms of a model in which MAPs affect \( D_5 \) while TC affects \( D_5 - D_6 \). However, the results of this section suggests that the relationship between MAPs and the TC inhibition process may be more complex.

TC Inhibition and Microtubule Composition—In a previous heparin study (21), we noted that \( D_5 \) correlates with Y, the TC mole fraction in the microtubule phase. We examined the implications of this correlation further. MTP with and without heparin, and PC-tubulin were assembled into polymer in the presence of varying concentrations of TC, and analyzed at steady state. Y, ΔYield, and per cent inhibition were evaluated as a function of TC_{total} (Table I). The observed values for per cent inhibition were compared with the predicted values calculated from Equation 2 using the parameters listed in Table I. We noted the following: Microtubules from MTP or from PC-tubulin have similar compositions as they assemble in the presence of similar TC_{total} concentrations; substoichiometric but significant concentrations of TC co-polymerized with tubulin and inhibited assembly. (In the MTP study, for example, Y values as large as approximately 0.05 were observed when TC_{total} \sim 3.5 μM); the observed and predicted per cent inhibition values were in excellent agreement, and inhibition correlated with Y. ΔYield was sensitive to TC_{total} and, to a good approximation, independent of MAPs (TC_{total} \approx 1 μM); per cent inhibition was sensitive to TC_{total} but was dependent on MAP availability. These differences in behavior are consistent with our earlier results (Fig. 1, C and D, Equations 1 to 5). The larger per cent inhibition values in the heparin study are attributed to the fact that α_{TC} and \( D_5 \) in the heparin study differ by a relatively small amount compared to the corresponding difference in the other two cases (Table II). The excellent agreement between predicted and observed per cent inhibition values, illustrated with preparations containing different tubulin, TC, and MAP concentrations (Table I), indicates that we have successfully begun to unravel the inter-relationships between tubulin, TC, and MAPs in the inhibition process. In fact, we have used Equation 2 and the general concepts developed above to successfully model many properties of the co-polymerization reaction (41, 42).

We observed that small but significant amounts of TC co-polymerize with tubulin. Appropriate controls demonstrated that Y values do not reflect adventitious binding of TC to microtubules, but arise from a TC incorporation process which occurs by addition of TC to the microtubule ends in a manner similar to tubulin (21). The Y values for the heparin-free study (Table I) were obtained from studies involving a wide

### Table II

**Derived from studies displayed in Figs. 2 and 4.**

| Microtubule protein  
|---|---|---|---|---|
| --- | αTubulin | D5 (μM) | DeltaTubulin (μM) | Y  
| Total protein (mg/ml) | 2.5 ± 0.2 | 2.5 ± 0.2 | 5.2 ± 0.3 |  
| Activity factor (α) | ~0.8 | ~0.6 | ~0.6 |  
| Total active tubulin (μM) | ~18 | ~12 | ~27 |  
| (αTubulin) |  
| D5 (μM) | ~1.6 | ~6.5 | ~7 |  
| DeltaTubulin (μM) | ~16 | ~5.5 | ~20 |  
| Y |  
| TC50 |  

---

α In PB-2.5 M. Non-tubulin protein pelleting with the microtubules constituted ~15% of the pellet mass in the absence of heparin (–) and approximately 5% in the presence of 0.13 mM heparin (+). Neurofilament protein contaminants and Tau proteins were the majority non-tubulin proteins in the heparin study (Fig. 3).

β In PB-4 M. Non-tubulin proteins were estimated to represent less than 1% of protein mass.

### Table III

**A comparison of solution phase and microtubule phase compositions at steady state**

<table>
<thead>
<tr>
<th>TC mole fraction ratios</th>
<th>TC50</th>
<th>Reaction mixture</th>
<th>Solution phase (X)</th>
<th>Microtubule phase (Y)</th>
<th>X/Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>[TC50]</td>
<td>μM</td>
<td>[TC50]</td>
<td>[TC50]</td>
<td>[TC50]</td>
<td>[TC50]</td>
</tr>
<tr>
<td>Microtubulin*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| A. No heparin | 0.25 | 1.4 | 5.1 | 0.6 | 8.4  
| 0.50 | 2.7 | 8.3 | 1.1 | 7.5  
| 1.0 | 5.3 | 13 | 1.9 | 7.0  
| 2.0 | 10 | 21 | 3.4 | 6.0  
| 3.5 | 16 | 27 | 5.4 | 5.0  
| Ave. 6.8 ± 1.3 |  
| B. 0.13 mM heparin (Sternlicht and Ringel (21)) | 0.17 | 1.3 | 1.8 | 0.4 | 4.4  
| 0.34 | 2.5 | 3.3 | 0.6 | 5.5  
| 0.68 | 5.0 | 6.0 | 0.85 | 7.1  
| 1.2 | 8.4 | 9.6 | 1.4 | 6.9  
| Ave. 6.0 ± 1.3 |  
| PC-tubulin* |  
| 0.82 | 3.0 | 5.9 | 1.0 | 5.9  
| 1.8 | 6.3 | 8.8 | 2.5 | 3.4  
| 2.9 | 9.7 | 12 | 3.8 | 3.1  
| Ave. 4.1 ± 1.5 |  

* Corrected for MAP content (cf. Table I).

**Reaction mixture ratio = [TC50]/([αTubulin] + [TC50])**, [αTubulin] values are tabulated in Table II.

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range of MTP and TC concentrations (also Ref. 42). In general, we have not observed Y values appreciably larger than 0.1. The significance of this finding is not clear, but it may indicate that TC/tubulin ratios in stable co-polymers are restricted to values less than a maximal or critical ratio of the order of 0.1 (23, 43).

Previously we proposed (42, 43) that 

$$\frac{\text{affinity}_{x} - \text{affinity}_{TC}}{\text{affinity}_{x}}$$

the ratio of the affinity of the microtubule end for tubulin to its affinity for TC, can be determined from an analysis of solution and microtubule phase compositions, and can be approximated by $X/Y$, the ratio of the TC mole fraction in the solution phase at steady state ($X$) to the TC mole fraction in the microtubule phase at steady state ($Y$). We consequently determined $X/Y$ (Table III) as a function of MAP availability in an effort to estimate the degree to which the affinity ratio, $\frac{\text{affinity}_{x}}{\text{affinity}_{TC}}$, depends on MAP concentration. $X/Y$ ratios were obtained from a composition analysis which made use of the data and parameters in Tables I and II, respectively. An average $X/Y$ value of approximately $5.6 \pm 1.4$ was obtained which we interpret as suggesting that $\frac{\text{affinity}_{x}}{\text{affinity}_{TC}}$ is only weakly dependent on MAP content. Although we did observe a correlation between $X/Y$ and MAP content, these values were generally averages from several independent measurements done under different conditions of MAP availability. $Y$ correlated linearly with $TC_{total}$, and were only weakly dependent on MAP concentrations. The slope from the MTP study (no heparin) was $-1.3 \pm 0.2$ and 1.5 times larger, respectively, than the slopes obtained from the 0.13 mM heparin and PC-tubulin studies. Differences in slope values obtained from heparin and PC-tubulin may be related to their different tubulin protein concentrations ($-2.5 \text{ versus } 5.3 \text{ mg/ml}$). The slopes obtained for the three different cases shown in Fig. 5 had an average value of $-1.6 \pm 0.3 \times 10^{-2}/\mu M\text{ TC}_{total}$, which indicated that TC mole per cent in the microtubule phase increases by $\sim 1.6\%$/each $\mu M$ increase in $TC_{total}$ concentration. Upon dividing this estimate for the rate of change of $Y$ with respect to $TC_{total}$ into the earlier estimate of $-4$ for the rate at which microtubule yields decrease with increasing $TC_{total}$ (cf. Equations 3 to 5), we obtained a quotient of approximately $-250 \mu M$, which indicated that microtubule yield decreases by approximately $2.5 \mu M$ per cent increase in the TC content in the microtubule (cf., Table IV). This finding of similar decreases in microtubule yield with increasing $Y$ suggests to us that if incorporated TC alters affnity by perturbing the microtubule lattice (21), the magnitude of the perturbations are not strongly linked to MAP concentration.

In an effort to better quantitate the perturbations induced by TC when assembly occurs under different MAP concentrations, we estimated the change in $\Delta F^T$ with $Y$ and with yield, where $\Delta F^T$ denotes the molar free energy change for the binding of tubulin to the microtubule ends during the elongation phase of the co-polymerization reaction. Estimates were made for MTP with and without heparin, and for PC-tubulin (Fig. 6, Table IV). The free energy change for the co-polymerization reaction was calculated from the observed variation of $Y$ with $TC_{total}$ (e.g. Table I, Fig. 5).
assembly reaction, is presumed to be a stoichiometric weighted average of free energy contributions from tubulin and TC binding with the major contribution, $\Delta F^T$, coming from tubulin since tubulin is the major component in the microtubule. $\Delta F^T$ was calculated from the apparent binding constants, $D_i$, at 37°C using the standard thermodynamic equation which relates free energy changes to equilibrium-binding constants: 

$$\Delta F^T = \frac{R}{1-k_B T} \ln D_i \text{ kcal/mol}$$

In Fig. 6A, we plotted $\Delta F^T$ against $Y$, and in Fig. 6B, we plotted $D_i$ against $Y$. The $D_i$ and $\Delta F^T$ increased as $Y$ increased. When TC was absent, $D_i = D_{ic}$ and $\Delta F^T$ had the apparent value of approximately $-7.4 \text{ kcal/mol}$ in the PC-tubulin study and the apparent values $-7.4$ and $-8.2 \text{ kcal/mol}$ in MTP studies with and without 0.13 mM heparin, respectively (Fig. 6A). These estimates for $\Delta F^T$ (no TC) are in good agreement with previous estimates (21, 45). However, since the MTP and PC-tubulin studies were done in different buffers, $\Delta F^T$ values are not directly comparable (40, 45). Nevertheless, we assume that changes in $\Delta F^T$ with TC or with $Y$ are less sensitive to glycerol content, and can be compared directly.

We noted that small increases in $\Delta F^T$ of the order of 1 kcal/mol correlated with significant reductions in microtubule yield of the order of 10% (i.e., $\sim 1 \text{ mg/ml}$). This conclusion was reached from the following considerations: The rate of change of $\Delta F^T$ with respect to $Y$ was estimated from Fig. 6B. $\Delta F^T$ increased initially at a rate equal to $-0.37$ and $0.25 \text{ kcal/mol}$ each per cent increase in the TC content of the microtubule phase when assembly occurred with relatively high concentrations of MAPs (MTP, no heparin) or with reduced concentrations of MAPs (e.g., PC-tubulin, and MTP with heparin), respectively. Earlier we obtained values for the rate of change of yield with respect to $Y$. By combining those results with our estimates for the rate of change of $\Delta F^T$ with respect to $Y$, we calculate the rate of change of yield with respect to $\Delta F^T$ (Column 5 in Table IV). The calculated values varied with MAP availability and suggested that MAPs stabilize microtubules against TC perturbations. However, the differences were small and may have arisen from small systematic errors in the analysis. In summary, the finding that there were no large MAP-dependent differences in the change of $\Delta F^T$ with $Y$ (Fig. 6), nor in the various initial rates of change evaluated in this study (Table IV) suggests to us that TC perturbations are not strongly linked to MAP concentrations.

### DISCUSSION

Previous investigators have shown that colchicine added to microtubule suspensions at steady state will depolymerize microtubules to a much greater extent if the microtubules are deficient in MAPs (19, 20). Their findings raised questions concerning the inter-relationship between inhibition and MAP concentration, which this study sought to answer. Our investigation, however, was restricted to the copolymerization reaction where low concentrations of TC were used (TC$_{total}$ < 10 μM), and insignificant amounts of free colchicine was present in the reaction mixture. Our data indicate that TC co-polymerizes with tubulin and inhibits assembly in the absence and presence of MAPs, and confirm that MAPs influence the TC inhibition process. A comparison of tubulin critical concentrations (e.g., Equations 3 to 5), co-polymer compositions (Fig. 5), and the rates of change of various factors (Table IV) as a function of TC and MAP content indicated values which varied typically no more than a factor of about 2 over the range of MAP concentrations considered. On the basis of these observations we suggest that TC inhibition occurs by a process which is not strongly linked to MAP content. We find, in accord with this suggestion, that the MAP role in TC inhibition can be analyzed assuming that MAPs and TC independently affect tubulin critical concentration. That is, the MAP role can be understood qualitatively in terms of the effect that MAPs have on $D_i$, the critical tubulin concentration in the absence of TC, an important factor in the per cent inhibition expression, Equation 2. Although we have found this model capable of rationalizing the dependence of TC inhibition on MAP content (see below), the data are suggestive that MAPs may participate in a more complex manner in the TC inhibition process. That is, in addition to affecting $D_i$, MAPs may also directly affect the TC inhibition process in a variety of ways. MAPs may, for example, affect affinity/affinity$_{TC}$, the ratio of the affinity of the microtubule end for tubulin to its affinity for TC, although our composition data suggest that this effect is probably small (Table III). MAPs may also stabilize microtubules against TC perturbations as suggested by estimates for the rate of change of $\Delta Y$ with respect to $\Delta F^T$ (Table IV), and $D_i - D_{ic}$ as a function of TC$_{total}$ at large TC (Equations 3 and 5).

Heparin was used as a convenient method to control MAP availability whereas phosphocellulose chromatography was used to prepare MAP-free tubulin. Microtubules formed in the presence of polyanions, such as heparin, appear to be morphologically indistinguishable from MAP-depleted microtubules prepared by high-speed centrifugation and chromatographic techniques (15, 18, 21). High molecular weight MAPs

### TABLE IV

<table>
<thead>
<tr>
<th>Microtubule protein</th>
<th>$(\frac{d\text{Yield}}{dTC_{total}})$</th>
<th>$(\frac{d\text{Yield}}{dTC_{total}})$</th>
<th>$(\frac{d\text{Yield}}{dY})$</th>
<th>$(\frac{d\Delta F^T}{dY})$</th>
<th>$(\frac{d\text{Yield}}{d\Delta F^T})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>No heparin</td>
<td>$-5.0$</td>
<td>$0.019$</td>
<td>$-240$</td>
<td>$37 \pm 3$</td>
<td>$-6.5 \pm 0.8$</td>
</tr>
<tr>
<td>0.13 mM heparin</td>
<td>$-3.8$</td>
<td>$0.016$</td>
<td>$-250$</td>
<td>$25 \pm 2$</td>
<td>$-16 \pm 2$</td>
</tr>
<tr>
<td>PC tubulin</td>
<td>$-4.0$</td>
<td>$0.013$</td>
<td>$-300$</td>
<td>$-24 \pm 2$</td>
<td>$-12 \pm 2$</td>
</tr>
</tbody>
</table>

* The derivative notation is used to designate rates of change. Initial rates are tabulated. These values are approximately the observed rates when TC$_{total}$ ≈ 1 μM.

* From Equations 3 to 5. The small TC contribution to microtubule yield is neglected.

* From Fig. 6A.

* Column (1)/Column (2).

* From Fig. 6B.

* Column (3)/Column (4).

* In PB-2.5 M.

* In PB-4 M.
and Tau proteins are the two major classes of non-tubulin proteins in association with microtubules, and both have been shown to facilitate microtubule assembly (2, 3). SDS-polyacrylamide gel electrophoresis analysis (Fig. 3) indicated that heparin formed a complex with high molecular weight MAPs and reduced high molecular weight MAP availability, but that heparin did not significantly affect the availability of a group of intermediate molecular weight non-tubulin proteins (M, ~ 55,000 to 70,000) previously identified as Tau proteins (37). However, further studies using PC-tubulin, which was free of both high molecular weight MAPs and Tau, gave results consistent with the general conclusions reached with heparin.

Our findings suggest that TC inhibits co-polymer assembly by a mechanism which functions without MAPs. In all cases, irrespective of MAP content, the assembly reaction in the presence of TC conforms to the following: (I) A minimum tubulin concentration, , which increases with increasing TCtotal, is required for assembly. Tubulin concentrations in excess of , appear in the microtubule phase (Figs. 2 and 4); (II) D, values can be expressed as the sum of a MAP-dependent term, , and a TC-dependent term which, at sufficiently low TC concentrations (TCtotal = 1 ,um), could be taken as independent of MAP concentration (e.g. Equations 3 to 5). However, when TCtotal concentrations were greater than 1 ,um, D, - D,0 values varied with MAP content (cf Equations 3 and 5). The D, - D,0 term for microtubule protein free of heparin (Equation 3) deviated from a linear dependence on TCtotal and increased more slowly with increasing TCtotal than did the D, - D,0 term for PC-tubulin (Equation 5). This difference in behavior is suggestive of MAP-microtubule interactions which stabilize the microtubule against further TC perturbations. Although this is the simplest interpretation of the data, caution needs to be exercised. Portions of the study were carried out in different buffer systems. Furthermore, Y values of the microtubule protein samples were always larger than the corresponding values in the PC-tubulin samples (Fig. 5). If TC-TC neighbor effects are important (23), neighbor effects would be more evident at high TCtotal in the microtubule protein study than in the PC-tubulin study.

The elongation phase of the assembly reaction involves the addition of tubulin and TC to the microtubule ends (21, 42). Our estimates for the free energy change during the elongation reaction, 2, were based on the apparent affinity constants, D, -1, and are approximations. A rigorous evaluation of 2 would require an extensive study of many factors (e.g. GTP hydrolysis, solvent effects, etc. (45, 46)). However, we believe that our estimation of 2, which does not consider these factors explicitly, is reasonable, and is consistent with the approach taken by others for assembly studies without TC. 2 versus Y were calculated from D, versus Y data (Fig. 6B) for microtubule protein and PC-tubulin assembling in PB-2.5 m and PB-4 m glycerol buffers, respectively, and rates of change of 2 with respect to Y and microtubule yield were estimated and compared (Table IV). Lee and Timasheff (39) and Timasheff (45) have shown that glycerol contributes thermodynamically to the assembly reaction, and consequently the validity of our free energy analysis needs to be discussed. In this free energy investigation we were more concerned with changes in 2 with TC than with 2 values. That is, although we recognized that 2 values in the absence of TC depend on glycerol content (39, 45), we assumed that if studies are carried out at constant glycerol concentrations (2.5 m or 4 m) and varying TCtotal, the changes in 2 with TCtotal or with Y would not depend strongly on glycerol content. This assumption is reasonable, and consistent with the data. We concluded, for example, that MAPs appear to stabilize microtubules against TC perturbations by comparing rates of change of 2 for microtubule protein assembly in PB-2.5 m glycerol buffer (no heparin) with the corresponding rates for PC-tubulin in PB-4 m glycerol buffer (Table IV, Column 5). Similar conclusions were also reached by comparing rates of change of 2 for microtubule protein assembly in PB-2.5 m glycerol buffer (no heparin) with the corresponding rates obtained with heparin (Table IV), and with PC-tubulin in Pipes/Me&SO buffer (data not shown). Similar conclusions, independent of buffer systems, lend credence to our assumption that changes of 2 with TCtotal or with Y are relatively insensitive to glycerol content for the narrow range of glycerol concentrations considered (2.5 m to 4 m).

We showed that substoichiometric inhibition by TC is not due to impaired MAP function, nor is it due to a marked TC-induced increase in 2 (Tables I and IV, Fig. 6). We noted, for example, in the 0.13 m heparin study that ~1 ,um TC in the reaction mixture inhibited assembly by approximately 60%, but 2 only underwent an apparent increase of approximately 4.5% (Y ~ 0.014). The large per cent inhibition values observed with substoichiometric concentrations of TC can be understood in terms of assembly properties I and II, discussed above, and the fact that 2TC was not much larger than D,0, the critical tubulin concentration in the absence of TC. Thus, as TCtotal increased while tubulin concentration was held constant, D, increased and became comparable to or larger than 2TC causing a significant or complete reduction in microtubule yield. The inter-relationship between MAPs and TC inhibition can be qualitatively understood in this model in terms of the effects MAPs have on D, and D, increases with MAP concentration decreases. Thus, if tubulin concentrations are held constant as MAP concentrations decrease, the per cent inhibition achieved with a fixed concentration of TCtotal increases since progressively smaller concentrations of TC are required to make D, ~ 2TC. While we find this simplified model useful, a quantitative understanding of the inter-relationship between MAPs and TC inhibition presumably will require a better understanding of the direct effects that MAPs appear to have on TC inhibition then we are presently able to provide.

Summarizing, we perceive microtubule assembly in the presence of TC to be a co-polymerization reaction involving tubulin, MAPs and TC as "monomer" components. We have examined this reaction under various combinations of concentrations involving tubulin, MAPs, and TC. We envisage the tubulin and MAP co-polymerization reaction as one that has a lower free energy than the corresponding assembly reaction involving tubulin by itself. A consequence of this, as is well known, is that a lower tubulin concentration is required for assembly in the presence of MAPs (15). However, when the co-polymerization reaction of tubulin and MAPs occurred in the presence of TC, we observed increases in the apparent free energy relative to the TC-free case which were not very sensitive to MAP concentration (Fig. 6). This observation suggests to us that it may be more fruitful to relate the apparent free energy increases in the presence of TC to TC-tubulin interactions or changes in tubulin-tubulin interactions rather than to MAP-tubulin or MAP-TC interactions. This suggestion is consistent with the earlier inhibition study done under MAP-depleted conditions (21) and the recent study by David-Pfety et al. (48) which suggests that TCs may inhibit lateral tubulin-tubulin associations. We observed that inhibition correlated with Y, the TC mole fraction in the microtubule (Table I). However, we do not know which of two possibilities prevails—whether Y directly determined D, or whether other factors are responsible for the changes in D, with increasing TCtotal. If other factors are responsible, then the Y values observed are incidental to the inhibition process.
and simply reflect the reaction mixture compositions and the affinity values for tubulin and TC. The former of the two possibilities appears most plausible to us if TC primarily causes structural perturbations, while the second of the two possibilities appears more plausible if TC primarily causes biochemical perturbations. We suspect that TC-induced structural perturbations contribute to the inhibition process. TC differs conformationally from tubulin (47), and presumably incorporates into the microtubule lattice in a somewhat altered manner from tubulin. As a result, incorporated TCs may perturb both short and long range organization. These structural perturbations would be expected to correlate with Y, and to be reflected in altered assembly kinetics and affinity constants. Thus, affinity and rate constants would be strongly linked to Y. The nature of these structural perturbations are not known. However, microtubules do have long range order. For example, microtubules show 96 nm axial periodicities which arise from specific interactions between tubulin sub-units (49), and furthermore these periodicities are observed in MAP-free microtubules (49, 50). Biochemical factors may also be contributing. For example, GTP is a co-factor in the assembly process (46). Wallin and Larsson observed extensive depolymerization of microtubules by colchicine in microtubule protein crudes obtained from certain species. Sensitivity to colchicine appeared to correlate with an exogenous GTPase activity (34). However, we found no evidence for an essential exogenous protein factor under the conditions we used to isolate and purify microtubule protein. Our data do not exclude the possibility that an endogenous GTPase activity, intrinsic to tubulin and linked to polymerization, plays a role in the TC inhibition process (48). Further studies are required to elucidate in detail the molecular processes involved in assembly inhibition by TC.

REFERENCES