Proposed Mechanism for Colchicine Poisoning of Microtubules Reassembled in Vitro from *Strongylocentrotus purpuratus* Sperm Tail Outer Doublet Tubulin†

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ABSTRACT: We have examined the mechanism by which colchicine–tubulin dimer (CD) complexes inhibit tubulin dimer addition to microtubules reassembled to steady state from sperm tail outer doublet tubulin. The data support a mechanism in which a single CD complex binds to the microtubule assembly end and temporarily decreases the rate of free tubulin dimer addition. The block is not absolute, however. In time, some free tubulin dimer addition occurs over the CD block, eventually resulting in recovery from the effect of the block. The extent of the recovery is determined by the molar frequency of tubulin addition relative to that of CD addition. At high tubulin/CD ratios (>250) recovery is essentially complete and tubulin dimers add at the prepoisoned rate. The most important factor in determining the extent of inhibition of dimer addition to a microtubule is, therefore, the relative molar frequency of tubulin dimer and CD complex addition to the microtubule. The repeated cycles of transient blockage followed by repair result in the formation of a copolymer at the microtubule assembly end. Copolymer formation occurs at a reduced rate, compared with the polymerization of tubulin alone, and occurs without a reduction in the number of assembly-competent ends. Furthermore, the poisoning mechanism appears to change at very low tubulin/CD molar ratios (<14), where the frequency of tubulin dimer addition to CD complex addition falls below a critical ratio. Under these conditions, the poisoning effects of CD complexes become cooperative, with the result that inhibition of assembly becomes complete, in that the number of assembly-competent ends is reduced to zero.

Colchicine disrupts a variety of cell functions: for example, mitosis (Brinkley et al., 1967; Oppenheim et al., 1973), secretion (Lacy et al., 1968; Malaisse et al., 1975), cell elongation (Daniels, 1972; Piatigorsky, 1975), and cell morphology (Brown & Bouck, 1973). The high affinity of colchicine for tubulin, the subunit protein of microtubules [e.g., Olmsted & Borisy (1973a) and Wilson & Bryan (1974)], indicated that colchicine exerted its inhibitory effects by disrupting microtubule integrity.

Formation of a colchicine–tubulin dimer (CD) complex is a prerequisite for in vitro microtubule poisoning, since free colchicine does not detectably inhibit microtubule assembly (Margolis & Wilson, 1977). In vitro microtubule assembly is inhibited by substoichiometric concentrations of colchicine; half-maximal inhibition occurs when only 2% of the unpolymerized tubulin subunits is complexed with drug (Olmsted & Borisy, 1973b; Margolis & Wilson, 1977). A similar mechanism also appears to be applicable to in vivo poisoning; in the case of mitosis, colchicine exerts its influence when only a small fraction of the cellular pool of tubulin subunits is complexed with drug (Taylor, 1965).

Microtubule assembly in vitro is a biased polar phenomenon (Allen & Borisy, 1974; Dentler et al., 1974; Olmsted et al., 1974; Snell et al., 1974; Binder et al., 1975). Under steady-state conditions, net tubulin dimer addition and loss occur at opposite ends of the microtubules, resulting in a unidirectional flux of subunits through the microtubules (Margolis & Wilson, 1978; Farrell et al., 1979). CD complexes can therefore poison microtubule assembly by adding to the assembling ends of microtubules and obstructing dimer addition (Margolis & Wilson, 1977; Wilson & Margolis, 1978).

There are two good candidates for the mechanism by which CD complexes prevent further dimer addition. CD binding to a microtubule could render a microtubule end totally incapable of supporting further assembly. Microtubule assembly would therefore be poisoned owing to a reduction in the number of microtubules to which dimers could add.

Alternatively, Sternlicht & Ringel (1979) have proposed that CD complexes might decrease the affinity of a microtubule end for tubulin dimer addition. In this case, poisoning of assembly would result from a decrease in the apparent rate of tubulin addition to microtubules. In contrast to the previous mechanism, the number of assembly-competent ends would not decrease. Data supporting the latter mechanism have been obtained for in vitro reassembled vertebrate brain microtubules (Sternlicht & Ringel, 1979).

In the present study, we have examined the poisoning, by CD complexes, of microtubules reassembled to steady state from outer doublet tubulin. The data support a poisoning mechanism by which tubulin dimers and CD complexes copolymerize at the microtubule assembling ends. Copolymer formation occurs without a reduction in the number of assembly-competent ends until the molar ratio of Tu to CD complex addition falls below a critical value. At this point the poisoning effects of CDs appear to become cooperative and render the microtubule (MT) ends incompetent for assembly.

Materials and Methods

Tubulin Preparation. Sperm from the sea urchin *Strongylocentrotus purpuratus* was isolated from whole gonads by the method described by Binder & Rosenbaum (1979). Sperm was stored at −80 °C if not used immediately for preparation of outer doublets.

Procedures for isolating outer doublet microtubules and preparation of 200000*g* supernatants from them, containing 75–85% tubulin, have been described previously (Farrell & Wilson, 1978). These preparations, in 150 mM KCl, 1 mM

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$MgSO_4$, 1 mM EGTA,[1] and 5 mM Mes, pH 6.7, were further purified by one cycle of assembly–disassembly to yield preparations greater than 95% tubulin. During purification the microtubule pellets were resuspended in 100 mM Mes, 1 mM EGTA, and 1 mM $MgSO_4$, pH 6.7 (MEM reassembly buffer) for optimal buffering capacity.

Labeling of Microtubules. Microtubule formation was initiated by warming the tubulin supernatants to 37 °C in the presence of 0.05–0.10 mM GTP, 20 mM acetyl phosphate, and 0.05 IU of acetate kinase (EC 2.7.2.1) (MacNeal et al., 1977). Microtubule assembly was monitored by light scattering at 350 nm with a Gilford Model 2400 spectrophotometer and 2.0-mL cuvettes with 1.0-cm path lengths. Microtubules were deemed to be at steady state when the light absorption remained constant with time.

The methods employed for labeling microtubules with radioactive GTP have been described previously (Margolis & Wilson, 1978; Farrell et al., 1979). Briefly, to label microtubules uniformly, tubulin supernatants were reassembled to steady state in the presence of [^{3}H]GTP (ICN, 16.7 Ci/mmol; 10–20 μL per mL of MT suspension) or [^{14}C]GTP (Amersham, 472 Ci/mol; 5–25 μL per mL of MT suspension). Steady-state microtubules were pulsed labeled with [^{3}H]- or [^{14}C]GTP for 30–60 min to label the assembly (A) ends only. The pulse was terminated by adding a 40–50-fold excess of unlabeled GTP.

Assembly was terminated after timed intervals by centrifuging the microtubules through 50% sucrose (see Analysis of Microtubules). Alternatively, microtubule suspensions were added to warm (37 °C) 70% sucrose–MEM buffer to give a final sucrose concentration of 45%. In this condition, microtubules can be stored for at least 3 h with negligible change in microtubule-associated label [<0.01% for A ends and <0.3% for the net disassembly (D) ends].

Preparation of CD Complexes. Aliquots of outer doublet tubulin, purified by one cycle of assembly–disassembly and at a concentration of 5–7 mg/mL, were incubated with [^{3}H]colchicine (1×10^{-4} M, 10.1 Ci/mmol) for 2 h at 37 °C. [^{3}H]CD complexes were separated from uncomplexed [^{3}H]-colchicine over a Sephadex G-25 column equilibrated with MEM buffer.

CD complexes were prepared immediately prior to use, and their concentration was determined both spectrophotometrically and by Lowry assay. Since CDs dissociate slowly ($k = 4.2 \times 10^{-6}$ s^{-1}; Garland, 1978), initial CD concentrations decreased by less than 5% during the longest (3 h) experimental incubations.

Analysis of Microtubules. Labeled microtubules were separated from unincorporated label and free tubulin dimers by centrifuging 0.5-mL aliquots in either MEM buffer or 45% sucrose–MEM buffer through 4.5-mL 50% sucrose–MEM buffer (50 000 rpm, Beckman Ti50 rotor, 2 h, 30 °C). The microtubule pellets were depolymerized in 1.0 mL of ice-cold distilled water and aliquots removed for counting and protein assay.

By use of the double label ([^{3}H]CD and [^{14}C]GTP–tubulin) we were able to determine the molar ratios of CDs and drug-free dimers incorporated into microtubule A ends [$(Tu/CD)_{MT}$]. It should be emphasized that the tubulin in this ratio represents only the dimers incorporated into microtubules after addition of [^{14}C]GTP and [^{3}H]CDs and does not include dimers present in microtubules prior to label addition. Since, in a steady-state microtubule population, the microtubule number concentration will be the same for both CD and tubulin dimer addition, $(Tu/CD)_{MT}$ represents the relative frequency of tubulin dimer and CD complex addition to microtubule A ends.

The evidence for net tubulin dimer addition occurring at the microtubule A ends under our experimental conditions has been discussed previously (Margolis & Wilson, 1978; Farrell et al., 1979). Evidence that CD complexes also add to the A ends, rather than the D ends, will be presented in detail elsewhere (R. L. Margolis, C. Rauch, and L. Wilson, unpublished results). Briefly, however, CD complexes inhibit tubulin dimer addition to the microtubule A ends (e.g., Figure 2a). Secondly, [^{3}H]CD complexes remain bound to microtubules during a "chase" period with a molar excess of unlabeled colchicine, even though protein loss from the microtubules continues (from the D ends). Finally, the apparent K_D for net CD addition to microtubule D ends is $\sim 1.58 \times 10^{-5}$ M CD (K. W. Farrell and L. Wilson, unpublished data). Under our experimental conditions the CD concentration did not exceed 1.5×10^{-6} M.

In some experiments we examined the relationship between $(Tu/CD)_{MT}$ and the apparent rate constants for CD and tubulin addition. Experimental variation in $(Tu/CD)_{MT}$ values was obtained by altering the molar ratios of tubulin and CDs in solution. For ratios up to a value of 40, preformed [^{3}H]CD complexes were used. To obtain higher ratios, we used instead free [^{3}H]colchicine in varying concentrations for greater accuracy. Since the rate of CD complex formation is slower than the rate of CD addition to microtubules (data not shown), lower frequencies of CD addition to microtubules [i.e., greater $(Tu/CD)_{MT}$ ratios] could be obtained than with an identical concentration of preformed CDs.

For experiments using preformed CD complexes $(Tu/CD)_{MT}$ values were determined 30 min after CD addition to microtubules. Tubulin dimer loss from microtubule D ends did not significantly increase the native free tubulin concentration in this time (<10%), and the solution concentration of CD complexes was not significantly depleted (<5%). CD addition to microtubules therefore approximated a constant frequency during the experimental time course. This assumption is not valid for the case with free colchicine. A comparative analysis of poisoning with CD complexes and free colchicine will be presented elsewhere (unpublished experiments). However, the data obtained with colchicine may be compared qualitatively with those obtained by using preformed CD complexes.

Apparent rate constants for addition of CD complexes ($k_{app,CD}$) and tubulin dimers ($k_{app,Tu}$) to microtubules were estimated from the initial rates of [^{3}H]CD and [^{14}C]GTP–tubulin dimer incorporation during the first 20 min after CD addition. Uptake of both molecular species is linear over this time course (e.g., Figure 2). Solution concentrations of tubulin dimers and CD complexes were taken as the critical steady-state values (0.55 mg/mL; Farrell et al., 1979) and the added concentrations, respectively. No significant changes in the concentration of either molecular species occurred during the first 20 min of incorporation. The rate constant for tubulin addition to the microtubules is related to the observed rate of [^{14}C]GTP–tubulin addition ($d[Tu]_{MT}/dt$) by the equation

$$d[Tu]_{MT}/dt = k_{+1}[N][Tu]_I - k_{-1}[N]$$

where $[Tu]_I$ is the initial molar concentration of tubulin, [N] is the microtubule number concentration, and k_{+1} and k_{-1} are the rate constants for dimer addition and loss, respectively.

For a given microtubule population (i.e., fixed [N]) the

[1] Abbreviations used: Mes, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

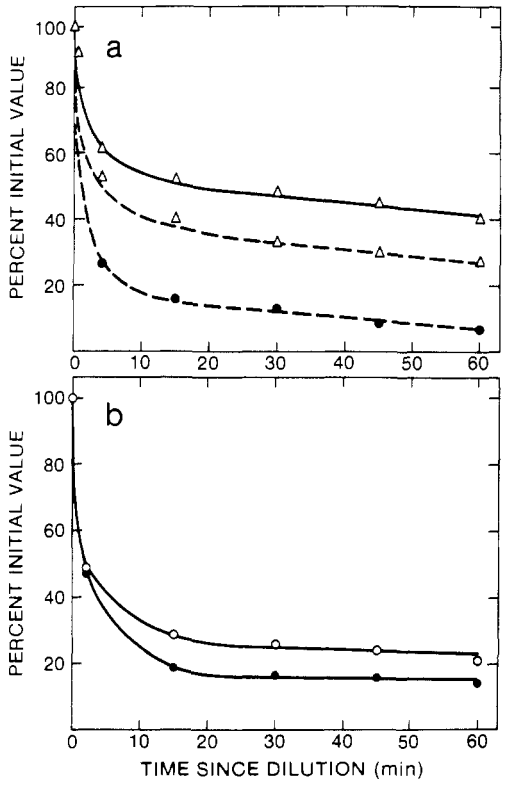

FIGURE 1: Loss of tubulin dimers and CD complexes from microtubule A ends. (a) Microtubules were reassembled to steady state from outer doublet tubulin. Microtubules in one aliquot were labeled at the A ends by incubating with [^{14}C]GTP for 30 min (control microtubules). A second microtubule aliquot was incubated both with [^{14}C]GTP (30 min) and subsequently with 1.3×10^{-5} M [^{3}H]colchicine for 1 h. Both microtubule preparations were centrifuged to a pellet and resuspended in warm (37 °C) MEM buffer containing a 40-fold excess of unlabeled GTP and, in the case of the colchicine-treated sample, a 1000-fold excess of unlabeled colchicine. ^{14}C-Labeled dimer loss from control microtubules (Δ—Δ) and colchicine-poisoned microtubules (Δ---Δ). [^{3}H]CD loss from colchicine-poisoned microtubules (●—●). (b) Microtubules reassembled to steady state from bovine brain tubulin were labeled at the A ends by incubating with [^{3}H]GTP for 30 min. Half of the microtubules were also incubated with colchicine (1.2×10^{-5} M) for 1 h at 30 °C. Both microtubule preparations were centrifuged to a pellet and resuspended in warm (30 °C) MEM buffer with a 40-fold excess of unlabeled GTP. Labeled dimer loss from control microtubules (●) and microtubules poisoned with colchicine (○).

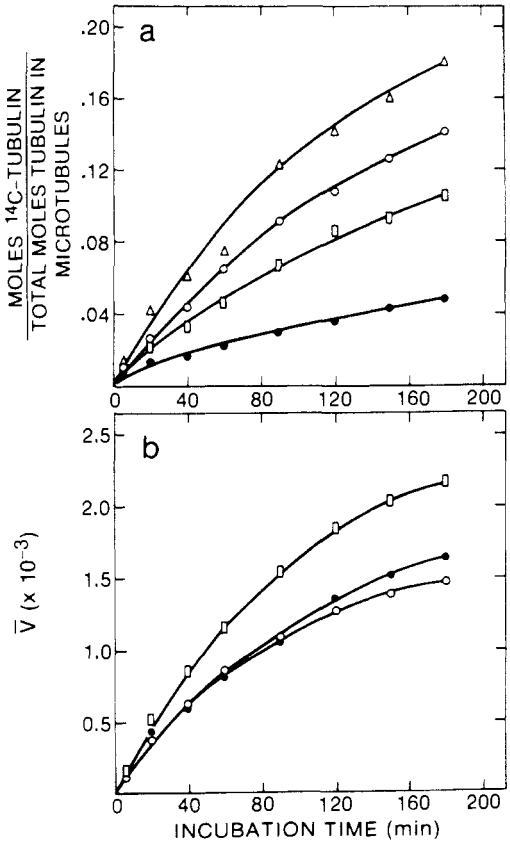

FIGURE 2: Effect of CD complexes on tubulin dimer incorporation. Microtubules were reassembled to steady state and then incubated with a steady-state concentration of [^{14}C]GTP–tubulin and varying concentrations of [^{3}H]CD complexes. (a) Uptake of [^{14}C]GTP-labeled tubulin dimers was measured in the absence (Δ) or presence of 6.77×10^{-8} (○), 1.35×10^{-7} (□), and 3.33×10^{-7} M (●) [^{3}H]CD complexes. (b) Incorporation of [^{3}H]CD complexes measured simultaneously and expressed as moles of [^{3}H]CD complex in experimental microtubule pellets per total number of moles of tubulin in control microtubule pellets ($\bar{V}$). Symbols indicate the same CD concentrations as in (a).

apparent rate constant for tubulin addition can be calculated at each $(Tu/CD)_{MT}$ ratio by using the simplified relationship $k_{app} = d[Tu]_{MT}/d[Tu]_f$. The $k_{app,CD}$ can be calculated in the same way from the [^{3}H]CD incorporation data. The values obtained for the rate constants are expressed in arbitrary units and reflect the relative magnitudes of $k_{app,Tu}$ and $k_{app,CD}$ within a given microtubule population.

Microtubule Length Determination. The procedure for estimating the mean length of microtubule populations has been described previously (Farrell et al., 1979).

Protein Estimation. Protein determination was carried out by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Results

CD Complexes Bound to Microtubule A Ends Dissociate upon Microtubule Dilution. The ability of substoichiometric concentrations of CD complexes to poison microtubule assembly at steady state indicated that CD complexes must remain bound to the microtubule assembly ends essentially irreversibly under these conditions [see Wilson & Margolis (1978) for discussion]. We examined whether CD binding to microtubules was irreversible per se or whether other factors rendered CD binding irreversible.

Steady-state microtubules were pulsed labeled for 30 min with [^{14}C]GTP to label the microtubule A ends uniquely. These microtubules were subsequently incubated for 1 h with [^{3}H]colchicine (1.3×10^{-5} M). This concentration of colchicine completely inhibited dimer addition to the microtubules and resulted in an average of seven CDs binding to each microtubule (data not shown). Control microtubules were incubated only with [^{14}C]GTP for 30 min. Both preparations were centrifuged to a pellet through sucrose and resuspended in warm (37 °C) MEM buffer containing a 40-fold excess of unlabeled GTP (control microtubules) or unlabeled GTP plus unlabeled colchicine in a 1000-fold excess (1×10^{-7} M) over microtubule-bound CDs. Aliquots were removed after timed intervals, centrifuged through sucrose, and prepared for analysis.

The results show that 93% of the [^{3}H]CDs were lost from colchicine-treated microtubules. In addition, 58% of the ^{14}C label was lost from control microtubules and 72% from drug-treated microtubules (Figure 1a). More ^{14}C-labeled dimers were lost from colchicine-treated microtubules since the unlabeled colchicine chase in the resuspension buffer decreased the apparent rate of tubulin readdition (e.g., Figure 2a).

These data demonstrated that CDs are not bound irreversibly to microtubule A ends under non-steady-state conditions of dilution. In addition, it is evident that microtubule A ends are in equilibrium with tubulin dimers in solution (Figure 1a, control) and that under steady-state conditions the equilibrium is displaced toward net dimer addition.

The loss of A-end CDs was curious since this was not observed upon a 1:3 dilution of in vitro reassembled bovine brain microtubules (R. L. Margolis, C. Rauch, and L. Wilson, unpublished results). Since our experimental conditions amounted to an infinite dilution of the initial free tubulin and CD concentrations, we tested whether the extent of CD loss was related to the degree of microtubule dilution.

Bovine brain microtubules were reassembled to steady state and pulse labeled for 30 min with [^{3}H]GTP to label the A ends. Half the microtubules were also incubated for 1 h with 1.2×10^{-5} M colchicine. The microtubules were pelleted by centrifugation and the pellets resuspended in warm (30 °C) MEM buffer with an unlabeled GTP chase. This amounts to an infinite dilution of the free tubulin and CDs since the initial concentrations are zero. Microtubule aliquots were removed after timed intervals, centrifuged through sucrose, and prepared for analysis.

Eighty to eighty-five percent of the A-end label was lost from both control and colchicine-treated microtubules (Figure 1b). Other experiments have also shown that CD complexes are lost from the A ends of microtubules reassembled from outer doublet tubulin and diluted only 1:5. However, the rate and extent of CD loss under these conditions were less than those under the extreme dilution conditions (data not shown).

Interestingly, the extent of CD loss from microtubules reassembled from outer doublet tubulin and diluted 1:5 was greater than that from similarly diluted bovine brain microtubules. This indicated that the apparent rate constant for CD binding to brain microtubules may be greater than that for echinoderm microtubules.

These data demonstrate that CD binding to microtubule A ends is in itself readily reversible and that the extent of CD loss is related to the amount of microtubule dilution. Therefore, under substoichiometric poisoning conditions other factors must conspire to render CD binding irreversible.

CD Complexes Copolymerize with Tubulin Dimers to Poison Microtubule Assembly. Aliquots of steady-state microtubules were added to steady-state concentrations of free [^{14}C]GTP–tubulin and increasing concentrations of preformed [^{3}H]CD complexes. Samples were removed at timed intervals and prepared for analysis.

Use of the double label allowed CD incorporation and assembly inhibition to be monitored simultaneously on the same preparation of microtubules. This was preferable to measuring these parameters on separate microtubule preparations, since it obviated the necessity for correcting apparent rate data for variation in microtubule number concentration.

CD incorporation into microtubules continued for at least 3 h without reaching a plateau (Figure 2b). Drug-free dimers were also incorporated into CD-treated microtubules, although neither the rates nor the extents of incorporation approached those of untreated, control microtubules (Figure 2a).

Dimer incorporation into both control and drug-treated microtubules was not linear with time. This probably resulted from tubulin denaturation ($t_{1/2}$ = 4 h; K. W. Farrell and A. S. Barloon, unpublished data), rather than GTP exhaustion, since the added GTP was always in a molar excess over tubulin and increasing the concentration of GTP regenerating system components (Materials and Methods) failed to prevent the decay of the tubulin uptake rates.

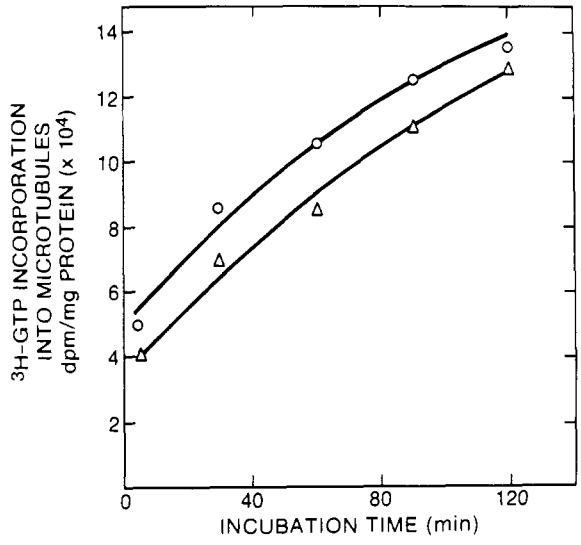

FIGURE 3: Assembly of tubulin dimers onto colchicine-blocked microtubules. Steady-state microtubules were incubated with (△) or without (○) 1.8×10^{-5} M colchicine for 45 min at 37 °C. From a simultaneous experiment with the same microtubule preparation, but using [^{3}H]colchicine, we calculated that this resulted in microtubules each incorporating an average of six CD complexes (data not shown). The microtubules were then pelleted and resuspended in 0.86 mg/mL drug-free tubulin labeled with [^{3}H]GTP.

Essentially identical data have also been obtained for CD poisoning of in vitro reassembled bovine brain microtubules. Furthermore, the data are not peculiar to preformed CD complexes, since qualitatively similar data were obtained by using [^{3}H]colchicine, both with microtubules reassembled in vitro from outer doublet and bovine brain tubulins (data not shown).

Tubulin Dimers Can Assemble over a CD Block. Microtubules reassembled to steady state from outer doublet tubulin were incubated for 45 min with 1.8×10^{-5} M colchicine. This colchicine concentration is sufficient to completely block microtubule assembly (data not shown). The colchicine-blocked microtubules were centrifuged to a pellet through sucrose and resuspended in 0.86 mg/mL drug-free tubulin labeled with [^{3}H]GTP. Microtubule aliquots were removed at timed intervals for analysis and compared with control microtubules not treated with colchicine.

The data show that tubulin dimers assemble onto colchicine-poisoned microtubules at the same rate as unblocked, control microtubules (Figure 3). A second experiment, using [^{3}H]colchicine and unlabeled GTP, was run simultaneously using the same preparation of microtubules. From these data we were able to determine that CDs remained bound to microtubules during the experimental procedures and estimated that on the average each microtubule contained six CDs (data not shown).

We corrected for dimer addition to unblocked self-nucleated microtubules in the drug-free tubulin solution by subtracting the assembly occurring in simultaneously processed tubulin solutions (0.86 mg/mL) with no added microtubules. The data in Figure 3 therefore represent assembly onto colchicine-blocked microtubules.

Furthermore, dimer addition also occurred onto colchicine-blocked microtubules when a steady-state concentration of drug-free tubulin was employed. Self-nucleation of microtubules under these conditions should not have occurred to any significant extent, again indicating that tubulin dimers assemble over a CD block (data not shown).

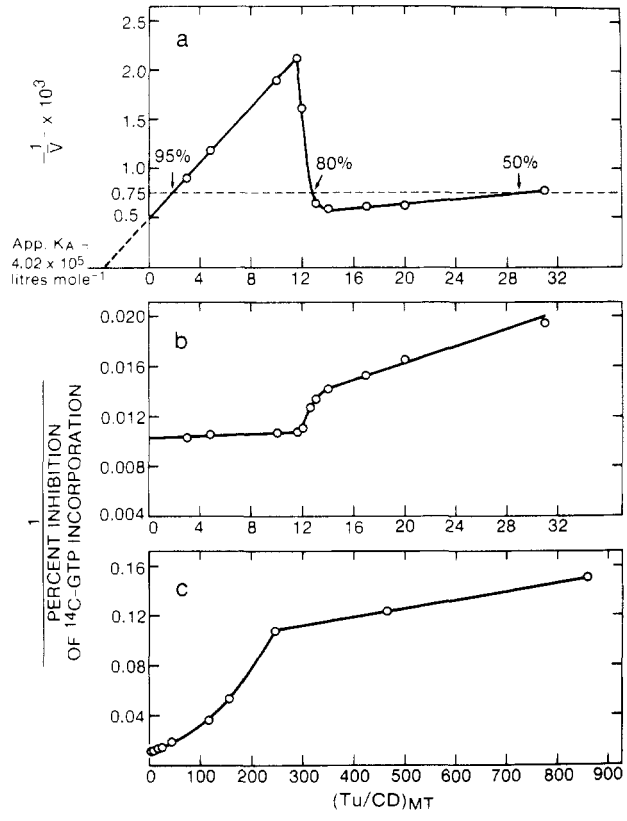

FIGURE 4: Tubulin dimer and CD complex incorporation into microtubules as a function of the relative molar frequency of tubulin and CD addition. Aliquots of steady-state microtubules were incubated for 30 min with steady-state concentrations of [^{14}C]GTP-labeled tubulin (control microtubules) or tubulin plus increasing concentrations of preformed [^{3}H]CD complexes (5.7×10^{-8}–3.33×10^{-6} M). (a) Moles of [^{3}H]CD incorporated into microtubule pellets per total number of moles of tubulin in the control pellet ($\bar{V}$) as a reciprocal function of $(Tu/CD)_{MT}$. The dashed line parallel to the abscissa and at a distance of $1/\bar{V} = 0.75 \times 10^3$ intersects the graph at three points, corresponding to three percentages of inhibition of dimer incorporation. (b) CD inhibition of ^{14}C-labeled dimer incorporation into microtubule pellets, relative to control microtubules not treated with CD, plotted as a reciprocal function of $(Tu/CD)_{MT}$. (c) Same as (b), except the microtubules were incubated with [^{3}H]colchicine (1.56×10^{-8}–5.02×10^{-6} M) to achieve high $(Tu/CD)_{MT}$ ratios.

Tubulin Dimer and CD Incorporation into Microtubules as a Function of the $(Tu/CD)_{MT}$ Ratio. A peculiarity of the data in Figure 2b is that at certain CD concentrations the stoichiometry of CD binding decreased with increasing CD concentration. We investigated this phenomenon by incubating steady-state microtubules for 30 min with steady-state concentrations of [^{14}C]GTP-labeled tubulin and increasing [^{3}H]-CD concentrations. The microtubules were separated from unbound label by centrifugation through sucrose and prepared for analysis.

The stoichiometry of [^{3}H]CD incorporation as a function of $(Tu/CD)_{MT}$ is shown in Figure 4a. CD incorporation increased with decreasing $(Tu/CD)_{MT}$ ratios for values greater than 14. However, decreasing the $(Tu/CD)_{MT}$ ratio below 14 initially caused a decrease in stoichiometry, followed by the stoichiometry again increasing linearly with the $(Tu/CD)_{MT}$ ratio.

At $(Tu/CD)_{MT}$ values in excess of 250, CD poisoning of assembly is relatively ineffective; decreasing the $(Tu/CD)_{MT}$ ratio from 850 to 250 resulted in only a 2 to 3% increase in inhibition of dimer incorporation (Figure 4c).

Dimer addition to microtubules was increasingly prevented by decreasing $(Tu/CD)_{MT}$ ratios. Inhibition of assembly was not linear but approximated an exponential function (Figure 4c).

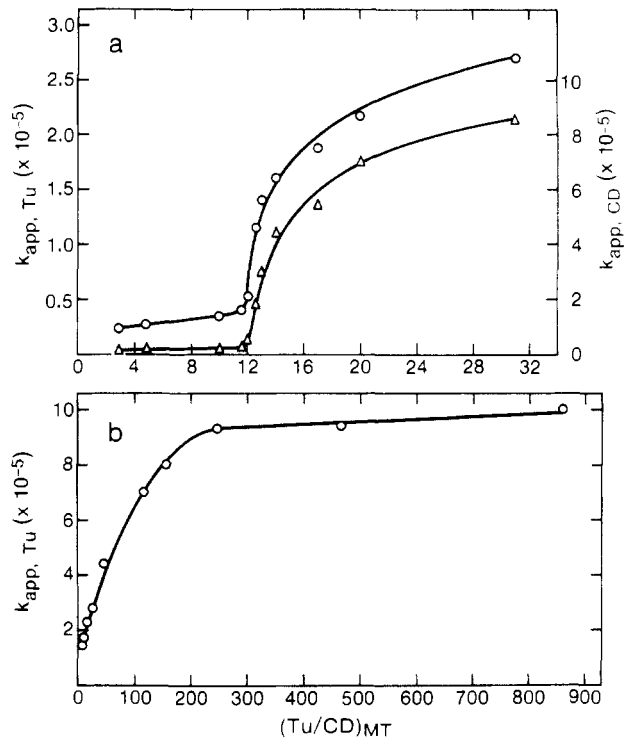

FIGURE 5: Variation in the apparent rate constants for tubulin dimer ($k_{app,Tu}$) and CD complexes ($k_{app,CD}$) addition to microtubules as a function of $(Tu/CD)_{MT}$. (a) Data used in parts a and b of Figure 4 recalculated to yield $k_{app,Tu}$ (○) and $k_{app,CD}$ (△), expressed as arbitrary units. (b) Data used in Figure 4c recalculated to yield $k_{app,Tu}$, expressed as arbitrary units.

At $(Tu/CD)_{MT}$ ratios below 14, CD poisoning became synergistic and assembly was completely poisoned by ratios below 12 (Figure 4b).

The data in Figure 4 have been replotted to show the apparent rate constants for tubulin and CD addition as a function of $(Tu/CD)_{MT}$ (Figure 5). The $k_{app,Tu}$ was essentially constant at $(Tu/CD)_{MT}$ ratios greater than 250 but decreased sharply at ratios below 250 (Figure 5b). At ratios above 14, the graph approximated a hyperbola. Below this point both rate constants decreased more rapidly and attained minimum values by a $(Tu/CD)_{MT}$ ratio of 12 (Figure 5a).

These data also demonstrated that the apparent rate constant for CD addition to microtubules is 2 to 3 times greater than that for tubulin dimer addition.

Discussion

Addition of increasing concentrations of CD to steady-state microtubules increasingly inhibited both the initial rates and final extents of tubulin dimer addition to the microtubules (Figure 2a). Also, the number of CDs associated with the microtubules did not plateau, even after 3 h of incubation (Figure 2b). At an added CD concentration of 1.35×10^{-7} M (Figure 2b) we calculate that each microtubule had incorporated an averge of 30 CD complexes after 3 h. Furthermore, on the average, 10% of each microtubule had become labeled with [^{14}C]GTP–tubulin dimers (Figure 2a).

These data argue strongly that inhibition of assembly involves CD complexes coassembling into the microtubules with tubulin dimers and decreasing the affinity of the microtubule ends for free tubulin.

A CD complex does not act as a permanent and irreversible "cap" which completely prevents further dimer addition or loss.

This mechanism is untenable since CD binding to microtubule A ends is not irreversible per se (Figure 1). CD complexes at microtubule ends would be free to dissociate, thereby permitting further tubulin dimer addition. Clearly, this is inconsistent with colchicine poisoning microtubule assembly substoichiometrically (Olmsted & Borisy, 1973b; Margolis & Wilson, 1977) since the large molar excess of tubulin dimers under these conditions would mean that assembly could continue with little inhibition.

Furthermore, a permanent and irreversible end-capping mechanism requires that the poisoned microtubule ends be totally incapable of supporting dimer addition. In contrast, copolymerization of CD complexes and tubulin dimers yields an end-conserving mechanism (Sternlicht & Ringel, 1979) in which the microtubule ends are still competent for assembly. Experimentally, we observed that CD-poisoned microtubules are capable of supporting dimer addition (Figure 3).

The ability of CD complexes to poison microtubule assembly at concentrations substoichiometric to the unpolymerized tubulin concentration argues that CD binding to microtubules must be essentially irreversible under these conditions (Wilson & Margolis, 1978). This is apparently contradictory to our observation that CD binding to microtubules is rapidly reversible (Figure 1).

However, CD binding to microtubules under substoichiometric conditions would appear irreversible if the large molar excess of tubulin under these conditions assembled over CD complexes bound at microtubule ends and embedded them within the microtubule lattice.

Our data are consistent with this hypothesis. Tubulin dimers can assemble over microtubule-bound CD complexes (Figure 3). Also, the apparent affinity of microtubules for CD complexes decreases as the molar ratio of tubulin to CD complex decreases (Figure 4a); at the higher ratios there is a sufficient molar excess of tubulin dimers to embed CD complexes within the microtubule lattice. In contrast, at lower ratios the frequency of tubulin dimer addition is insufficient to retain the CDs in the microtubule and they are free to dissociate, resulting in a decreased apparent association constant.

Finally, under non-steady-state conditions of microtubule dilution, CD complexes within the microtubule lattice would become exposed at the microtubule ends through tubulin dimer loss. These CDs would be free to dissociate from the microtubules (Figure 1). In contrast, under steady-state conditions of substoichiometric poisoning, net tubulin dimer addition would retain the CDs within the microtubules.

A Proposed Mechanism for CD Poisoning of Microtubule Assembly. We would like to propose the following mechanism for CD poisoning of microtubule assembly. The mechanism derives from data obtained with preformed steady-state microtubules. However, available data for CD poisoning of microtubule formation de novo from tubulin solutions (Sternlicht & Ringel, 1979) indicate that the mechanism is generally applicable.

CD poisoning of microtubule assembly may arbitrarily be considered in two phases. At low drug concentrations a "poisoning" phase occurs when a CD complex binds to a microtubule end and slows the apparent rate of subsequent tubulin dimer addition (and also CD complex addition) (Figures 2, 4, and 5).

This is followed by a "recovery" phase, during which tubulin dimers assemble onto the microtubule over the CD complex at a rate which increasingly approaches the rate of tubulin dimer addition prior to CD complex poisoning. Figure 5b shows the variation in the apparent rate constant for tubulin dimer addition to a microtubule population as a function of the frequency of CD addition relative to that of tubulin dimer addition. Most probably this also represents the increase, with time in the recovery phase, in the rate of tubulin dimer addition to *individual* microtubules poisoned with a CD complex; decreasing the relative frequency of CD complex addition to microtubule A ends [i.e., increasing the $(Tu/CD)_{MT}$ ratio] is equivalent to increasing the mean length of time individual microtubules spend in the recovery phase before a subsequent CD complex addition initiates a new poisoning phase.

Figure 5b therefore indicates that the rate of tubulin dimer addition to individual CD-poisoned microtubules increases hyperbolically with time allowed for recovery [increasing $(Tu/CD)_{MT}$ ratios] up to a maximum value achieved at $(Tu/CD)_{MT}$ ratios greater than 250.

The maximum attainable rate of dimer addition to microtubules during the recovery phase appears to approximate the prepoisoned rate for the following reasons. First, microtubule assembly is little affected by CD additions at $(Tu/CD)_{MT}$ ratios in excess of 250. At the highest ratio observed, 850, assembly is inhibited by less than 6% and decreasing the $(Tu/CD)_{MT}$ ratio to 250 resulted in only a further 3% inhibition of assembly (Figure 4c).

Second, if complete recovery of the rate of dimer addition were not possible, dimer addition to CD-blocked microtubules should not have occurred at the same rate as to control microtubules (Figure 3).

If CD complexes exert their influence through a perturbation of the microtubule lattice, these data indicate that the upper limit over which this effect is transmitted is of the order of 0.15 μm, assuming a helical assembly mechanism.

Successive CD additions to a microtubule appear to decrease the rate of dimer addition to the same initial value as previous CD complexes. If successive CDs were less efficacious, a time-dependent attenuation of microtubule assembly inhibition by CDs should be observed. In fact, assembly is inhibited to a constant extent over a period of 3 h for each of three added CD concentrations (Figure 2a).

A similar line of reasoning argues against successive CDs decreasing the initial rate of tubulin dimer addition to successively greater extents.

As the frequency of CD additions to microtubules increases [decreasing $(Tu/CD)_{MT}$ ratios], the mean length of time of the recovery phase between successive CD additions will decrease. On average, lower maximum rates of tubulin dimer addition to microtubules will therefore be achieved during the recovery phase by increasing the frequency of CD addition. Moreover, the proportion of microtubules in a population exhibiting decreased apparent rates of dimer addition also becomes greater. These combined influences decrease both the initial rates (Figures 2a and 5) and final extents (Figure 2a) of microtubule assembly with decreasing $(Tu/CD)_{MT}$ ratios.

The most important factor in determining microtubule assembly inhibition is not therefore the absolute number of CDs in a microtubule but rather the relative frequency with which CDs become incorporated into the microtubule. This is apparent from Figure 4a; for a fixed stoichiometry of CD incorporation (e.g., $1/\bar{\nu} = 0.75 \times 10^3$) three degrees of assembly inhibition were seen. In contrast, each $(Tu/CD)_{MT}$ value yields a unique degree of assembly inhibition.

At $(Tu/CD)_{MT}$ ratios below 14, the poisoning effects of CD complexes become cooperative. The apparent rate constant for tubulin addition decreased more rapidly than at ratios greater than 14 (Figure 5), and microtubule assembly became

completely poisoned (Figure 4b).

Similarly, the apparent affinity of microtubules for CD complexes decreased more rapidly at $(Tu/CD)_{MT}$ ratios below 14 (Figure 5a), with the result that the stoichiometry of CD binding decreased with decreasing $(Tu/CD)_{MT}$ ratios down to 12 (Figure 4a). Below a $(Tu/CD)_{MT}$ ratio of 12 a further decrease in the apparent rate constant did not occur, and the stoichiometry of CD binding to microtubules again increased with decreasing $(Tu/CD)_{MT}$ ratios.

The mechanism of the cooperativity between CDs is unclear. Possibly, disruption of the microtubule lattice, produced when the relative frequency of CD addition exceeds a critical value [i.e., $(Tu/CD)_{MT}$ less than 14], is greater than the additive effects of CDs at lower frequencies [$(Tu/CD)_{MT}$ ratios greater than 14]. As a result, the apparent association constant for tubulin and CD binding would abruptly decrease once the critical frequency of CD addition was exceeded.

A further contributing factor to the abrupt decrease in microtubule affinity for CDs may be the frequency of tubulin dimer addition. At high $(Tu/CD)_{MT}$ ratios tubulin dimers could add with sufficient frequency to retain the CDs within the microtubule. In contrast, at lower $(Tu/CD)_{MT}$ ratios CD complexes may not be retained in the microtubules by tubulin dimers and would be free to dissociate. Consequently, the apparent association constant for CDs would decrease.

It should be stressed, however, that although an inability of tubulin dimers to retain CD complexes in the microtubules might contribute to the abrupt decrease in CD binding, it does not appear to be solely responsible. If this were the case, the reciprocal plot of the inhibition of dimer addition should be a linear function of the $(Tu/CD)_{MT}$ ratio. This is not observed (Figure 4b).

The fact that the binding of tubulin dimers also abruptly decreases at $(Tu/CD)_{MT}$ ratios below 14 indicates that a cooperative interaction between CD complexes occurs which not only inhibits dimer addition more effectively but probably also contributes to the abrupt decrease in CD binding.

In summary, complete inhibition of microtubule assembly is achieved only at $(Tu/CD)_{MT}$ ratios below 12. At higher ratios tubulin dimers and CD complexes copolymerize into microtubules at decreased rates determined by the relative tubulin and CD complex composition of the microtubule ends.

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