Mechanism of Colchicine–Dimer Addition to Microtubule Ends: Implications for the Microtubule Polymerization Mechanism†

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ABSTRACT: We have studied the mechanism of colchicine inhibition of bovine brain microtubule assembly and have found, under steady-state conditions in vitro, that the normal equilibrium gain and loss of tubulin at the primary assembly end is retarded by the drug. We have quantitated the stoichiometry of colchicine binding to the ends of these microtubules and found that 50% inhibition of assembly occurs when the stoichiometry of colchicine–dimer complex bound per microtubule assembly end is ~0.5. This suggests that assembly inhibition occurs by the binding of one colchicine–dimer complex to a microtubule primary assembly end. Fivefold dilution of fully blocked microtubules results in complete retention of tubulin at the primary assembly end, as determined by using a pulse of [^{3}H]GTP to label the tubulin at this end. By contrast, dilution of untreated microtubules results in a biphasic loss of tubulin from the assembly end. These results indicate that under steady-state conditions at high colchicine concentrations, the drug acts by producing a tight assembly end "cap" in that the colchicine-bound dimers at the assembly end dissociate significantly more slowly than the normal dissociation of drug-free dimers. Additional sites on the microtubule are available for colchicine–dimer addition, to a saturation of 8.8 (±1.4) colchicine–dimer complexes/microtubule. This stoichiometry is independent of microtubule length. Since the binding values we measure reflect assembly addition of dimers at the primary assembly end, it is possible that this stoichiometry of colchicine binding may be reflecting the number of tubulin assembly sites at the assembly end. Thus, the data may be interpreted to indicate that assembly addition of tubulin is occurring in a helical manner on each of eight independent helical starts at the microtubule assembly end.

The assembly and disassembly of bovine brain microtubules, at steady state in vitro, consist of a net assembly reaction at one end of the polymer, which we have designated the primary assembly site, and a net disassembly reaction at the other end, which we have designated the primary disassembly site (Margolis & Wilson, 1978). Thus a unidirectional flow of tubulin through the microtubules ("treadmilling") occurs under steady-state conditions in vitro. However, the individual reactions at each end of the polymer, while not fully characterized, are reversible equilibria. For example, dilution of steady-state microtubules in vitro, thus producing a non-steady-state condition, results in loss of tubulin from the primary assembly site (Karr & Purich, 1979).

Microtubule assembly in vitro is poisoned in a substoichiometric manner by colchicine (Olmstead & Borisy, 1973; Margolis & Wilson, 1977). We have shown that colchicine acts by binding to free tubulin dimers in solution, and then the colchicine–dimer complexes add to the microtubule ends in an assembly reaction and inhibit further dimer addition (Margolis & Wilson, 1977). The colchicine–dimer complex addition to microtubule ends must be very tight under conditions of assembly blockage, since the free dimer concentration is 50-fold higher than the concentration of colchicine–dimer complex at half-maximal inhibition of assembly (Margolis & Wilson, 1977). It is possible the addition of colchicine–dimer complex represents a native irreversible addition of dimers which may occur at the primary assembly end. It is, on the other hand, also reasonable that the colchicine–dimer complex can bind to the primary assembly end with a substantially higher affinity than free tubulin, so that its equilibrium loss from that end would be less probable than the loss of drug-free dimer. The drug would, then, "cap" the microtubule assembly end. We report here that colchicine adds to bovine brain microtubules under steady-state conditions in vitro at the assembly end by means of a dimer-mediated assembly reaction and that under these conditions the drug cannot be chased from the microtubules once bound. The failure to chase the bound drug indicates the establishment of a drug-induced assembly end cap.

We have quantitated the extent of specific binding of colchicine to microtubule ends at steady state. Further, by measuring [^{3}H]GTP uptake, we have determined the inhibition constant (K_i) of the drug for the assembly reaction at steady state and have determined that at 50% inhibition of assembly rate there are ~0.5 colchicine–dimer complexes bound per microtubule. Thus, the data suggest that a single colchicine–dimer complex is able to block substantially the steady-state assembly of a microtubule at low drug concentrations. We have also determined that a maximum 8.8 ± 1.4 colchicine–dimer complexes are able to add to each microtubule at saturating drug concentrations under the conditions employed. These results may be interpreted in a manner which suggests that colchicine–dimer complexes can add onto each of eight independent helical starts at the microtubule assembly end and that this addition is a reflection of the molecular mechanism of microtubule assembly.

Experimental Procedures

Tubulin Preparation. Beef brain microtubule protein was purified by three cycles of microtubule polymerization–depolymerization essentially according to the method of Borisy et al. (1975). Modifications (Asnes & Wilson, 1979) included use of 2.5 mM GTP in each cycle and assembly at 30 °C for 20 min in the first cycle, 10 min in the second cycle, and 30

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min in the third cycle. Purification was carried out in a buffer composed of 20 mM sodium phosphate, 100 mM sodium glutamate, 1.0 mM $MgSO_4$, 1.0 mM EGTA,[1] and 2.5 mM GTP (pH 6.75) (PG buffer). All chemicals were supplied by Sigma Chemical Co. unless stated otherwise.

Following the third cycle of polymerization, the preparation was cleaned by centrifugation of microtubules through 50% sucrose in PG buffer (200000*g*, 2.5 h, Beckman 50 Ti rotor, 30 °C) (Margolis & Wilson, 1978). Final pellets were stored in liquid nitrogen until use. The pellets used in these experiments contained 75% tubulin and 25% microtubule-associated proteins (MAPs), as determined below.

Methods. Each microtubule pellet was solubilized in PG buffer by dounce homogenization (ground glass on glass homogenizing mortar and pestle) and after 10 min on ice was centrifuged at 30000*g* for 10 min at 4 °C. GTP (Sigma Type IV) was added from a stock solution to the resulting supernatant, to a final concentration ranging from 0.1 to 2.5 mM, as determined by experimental requirements (see Results). The protein, at a final concentration ≥3.0 mg/mL, was then assembled by incubating at 30 °C for a period of time previously established as necessary to attain steady state, usually 30 min.

For assays of colchicine binding to microtubule ends, steady-state microtubules were sheared by rapid passage 3 times through a syringe (25-gauge needle) and, after 10 min, were added to test tubes in 0.5-mL aliquots. In one experiment (Table I, experiment 9) the microtubules were not sheared. Each tube contained colchicine at a known final concentration and 10 μCi of [^{3}H]colchicine (16.1 Ci/mmol, New England Nuclear). Following a 60-min incubation at 30 °C, the samples were layered over 50% sucrose in PG buffer and centrifuged (30 °C) for 2 h at 200000*g*. Pellets were analyzed as previously described (Margolis & Wilson, 1978). A colchicine-incubated microtubule sample was prepared for electron microscopy in order to quantitate polymer length distributions. Stoichiometries per microtubule were calculated on the basis of 8 nm for the longitudinal axis of a dimer in a microtubule (Amos & Klug, 1974) and 13 protofilaments/microtubule (Tilney et al., 1973). In other experiments, not shown, where the time of incubation in the drug was varied from 30 to 90 min, no change in stoichiometry and only a slight increase in K_A were observed.

Microtubule lengths were determined as previously described (Margolis & Wilson, 1978) by adding 10-μL aliquots of microtubules, after drug incubation, to 1.0 mL of warm (30 °C) 50% sucrose in PG. No change in microtubule lengths occurs in this buffer for up to 24 h. Samples were immediately prepared for negative-stain electron microscopy by the method of Olmsted et al. (1974) and photographed at a magnification of 3300× by using a Philips EM 300 operating at 80 kV. Mean microtubule lengths were obtained directly from photographic prints by measuring a minimum of 500 microtubules. We have previously established that microtubules sheared to widely different mean lengths adhere to the electron microscope grids with similar efficiency, since the rate of loss of [^{3}H]guanine nucleotide from steady-state microtubules under chase conditions is inversely proportional to the measured microtubule mean length (Margolis & Wilson, 1978).

Protein Quantitation by Lowry Assay and Disc Gel Electrophoresis. An accurate estimation of the concentration of tubulin in our preparations was essential for correct interpretation of binding stoichiometry. For this purpose, beef brain tubulin was purified by passage through a phosphocellulose (Whatman P-11) ion-exchange column (Weingarten et al., 1975), extensively dialyzed against distilled water, and lyophilized. An accurate dry weight was carefully determined, and weighed protein was both solubilized in 1 N NaOH for use as a protein standard (Lowry et al., 1951) and solubilized in 1% sodium dodecyl sulfate ($NaDodSO_4$) to generate a standard curve for tubulin concentration by $NaDodSO_4$–polyacrylamide gel electrophoresis.

Samples of microtubule protein used in experiments were analyzed by $NaDodSO_4$ gel electrophoresis performed according to the method of Weber & Osborn (1969). Areas under tubulin peaks, when compared with the tubulin standard curve, yielded a value of 75% in our microtubule protein preparations. A molecular weight of 110000 for tubulin was used in all calculations (Lee et al., 1973).

Results

The Colchicine–Dimer Complex as an Assembly Cap. Substoichiometric poisoning of microtubule assembly by colchicine (Olmsted & Borisy, 1973; Margolis & Wilson, 1977) requires that the microtubule assembly end retain bound colchicine–dimer complex almost indefinitely, so that the assembly site remains blocked to the addition of free dimers which are in great molar excess to colchicine–dimer complex [see Figure 5 and Margolis & Wilson (1977)]. We show below that colchicine binds very tightly to the assembly ends of bovine brain microtubules and that under steady-state conditions the bound colchicine–dimer complex acts as a cap, inhibiting dimer dissociation in addition to dimer association at the microtubule assembly end. This could be attributed either to a native irreversibility of dimer addition during assembly at the assembly end or to a uniquely high association constant for colchicine–dimer complex addition, so that the uncomplexed dimers dissociate from the assembly end with some rate constant but the colchicine–dimer complexes lock in and do not dissociate as readily.

To determine whether the colchicine–dimer complex inhibits dimer dissociation at the assembly end, we assembled bovine brain microtubules to steady state and then pulsed them for 1 h with [^{3}H]GTP. Under these conditions, ~10% of the microtubule is labeled uniquely at the assembly end (Margolis & Wilson, 1978). Following the pulse, a half aliquot of the microtubule protein was briefly incubated with 0.1 mM colchicine. Finally, small aliquots of the microtubule protein, with or without colchicine, were diluted 1:5 into warm assembly buffer. After various periods of time in dilute state, microtubules were isolated by centrifugation through sucrose cushions and analyzed for protein content and radioactivity.

Under dilution conditions in the absence of colchicine (Figure 1), there was a distinctly biphasic loss of label, with an initial rapid depletion of label that became apparently first order before the first dilution time point was taken (5 min). Thus a reversible equilibrium reaction must occur at the primary assembly ends of these microtubules, since tubulin can readily dissociate from the assembly ends under non-steady-state conditions.

By contrast, there was no tubulin lost from the assembly ends of microtubules that were incubated at steady state with colchicine prior to dilution (Figure 1). Since the loss of tubulin at the primary assembly end is prevented by the drug, these colchicine-blocked microtubules must be capped at their assembly ends.[2]

[1] Abbreviations used: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; MAPs, high molecular weight microtubule associated proteins; PG buffer, 20 mM sodium phosphate, 100 mM sodium glutamate, 1.0 mM $MgSO_4$, 1.0 mM EGTA, and 2.5 mM GTP, pH 6.75.

Table I: Binding of [^{3}H] Colchicine to Bovine Brain Microtubules at Steady State[a]

expt no.	binding constant K_D ($\times 10^{-6}$ M)	mean microtubule length (μm)	max molecules of colchicine bound/microtubule	molecules of colchicine bound/microtubule at half-maximal inhibition of assembly[b]
1	2.2	1.42	6.6	0.32
2	3.2	2.35	9.1	0.36
3	2.7	1.42	8.7	0.36
4	1.2	1.43	8.8	0.81
5	1.4	1.40	12.0	0.68
6	2.6	0.94	10.0	0.47
7	2.4	1.21	8.0	0.38
8	2.0	1.22	11.0	0.61
9	3.4	6.37	7.8	0.32
10	0.9	0.62	5.5	0.49
mean values	2.2		8.8 ($\pm$1.4)[c]	0.48 ($\pm$0.12)[c]

[a] All experiments were carried out at 3.8–4.2 mg/mL total microtubule protein and were similar to the experiment shown in Figure 2. [b] Calculated from the linear regression line of the binding data by using a value of 1.15×10^{-7} M for the concentration of added colchicine which inhibits steady-state microtubule assembly by 50%. This value is the average determined for the experiment of Figure 5 and a second identical experiment. [c] Variance at the 95% confidence level. Student's t test.

Binding of Colchicine to Microtubule Assembly Ends. The forgoing results suggest that colchicine–dimer complexes can add very tightly to the primary assembly ends of steady-state bovine brain microtubules. We have investigated the binding of tritium-labeled colchicine to these steady-state microtubules to determine the stoichiometry of colchicine–dimer addition under conditions of half-maximal assembly inhibition and also the maximum stoichiometry of colchicine–dimer addition at these ends.

The measurement of colchicine binding to microtubules requires sensitive techniques since one must accurately quantitate binding when only a few molecules of colchicine are bound per several thousand dimers in microtubules. Binding measurements were performed by incubating microtubules at steady state with [^{3}H]colchicine and then centrifuging the product through sucrose cushions to separate microtubules from free subunits and unbound drug. Microtubule pellets prepared in this manner were assayed for radioactivity and for protein content. The results were then recalculated on the basis of molecules of colchicine bound per microtubule by measuring the mean microtubule length independently for each experiment.

The result of a typical binding stoichiometry experiment is shown in Figure 2 in the form of a double-reciprocal plot, in which the reciprocal of the binding stoichiometry is plotted against the reciprocal of the total added colchicine concentration. This experiment was performed by assembling microtubules to steady state, shearing them by repeated passage through a syringe (final polymer length = 0.62 μm), and then incubating the microtubules with various concentrations of unlabeled colchicine in the presence of [^{3}H]colchicine. Microtubules were then isolated by centrifugation through sucrose cushions and assayed for protein content and radioactivity. A colchicine-incubated sample of microtubules taken just prior to centrifugation was prepared for electron microscopy, and microtubule lengths were measured from electron micrographs. By use of these values, the drug binding data were recalculated on the basis of molecules of colchicine bound per microtubule. The data (e.g., Figure 2) indicated the presence of a single affinity class of saturable binding sites, with a very small number of sites on each microtubule and a binding constant very similar to that of the binding of colchicine to 6S tubulin [0.9×10^{-6} M (see Table I)].

Since our goal was to obtain a binding stoichiometry of colchicine at the microtubule assembly ends, it was first necessary to determine that the bound [^{3}H]colchicine we were detecting actually added to microtubules in a specific manner at the assembly ends. To determine this, we incubated mi-

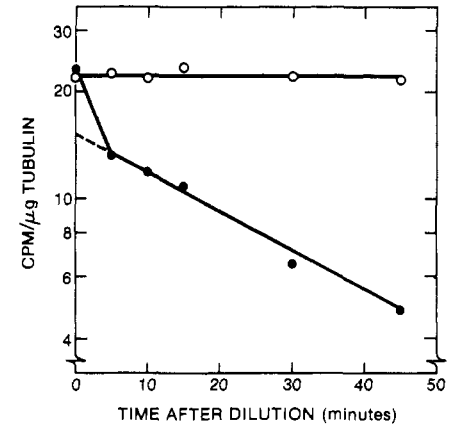

FIGURE 1: Capping of microtubule assembly ends by colchicine. Microtubules were assembled to steady state at 30 °C with 80 μM unlabeled GTP with a GTP regenerating system. After reaching steady state, the microtubule assembly ends were pulse-labeled by incubation with 10 μCi of [^{3}H]GTP for 1 h. The microtubule suspension (2.45 mg/mL total protein) was then split into two equal aliquots, and incubation of one aliquot was continued for an additional 15 min with 1×10^{-4} M colchicine in the presence of a 2.5 mM unlabeled GTP chase. Drug-treated microtubules (0.4-mL aliquots) were diluted to 2.0 mL in buffer containing 10^{-4} M colchicine and 2.5 mM unlabeled GTP and incubated for the times indicated (open circles). Drug-free microtubules were similarly diluted, but into buffer containing the GTP chase only (closed circles). Microtubules were collected by centrifugation and analyzed for incorporation of label and protein as described under Experimental Procedures. Background counts, subtracted from all samples (10% of maximal incorporation) were determined by incubating microtubules under chase conditions prior to addition of the labeled GTP.

[2] The initial rapid loss of label in the absence of colchicine (Figure 1) represented ~25% of the total label incorporated. Since in a 1-h pulse, 10% of each microtubule was labeled at its assembly end, 2.5% of the microtubule's length, or ~200 nm of length in a typical 8-μm microtubule, was rapidly lost upon dilution. The remaining assembly end label was lost slowly in an apparent first-order manner ($t_{1/2}$ = 30 min). Before dilution the protein concentration was 2.5 mg/mL. With an assembly critical concentration of 0.27 mg/mL (Asnes & Wilson, 1979), ~50% of the microtubule population must disassemble before the critical concentration, and therefore a new steady state is reestablished following dilution. At the end point of the experiment (45 min), only 15% net disassembly had occurred [calculation includes loss from assembly and disassembly end (data not shown)], and so the microtubule population was still far from reaching a new steady state. The cause of the biphasic loss of tubulin from microtubule assembly end which occurs upon dilution of steady-state microtubules is currently under investigation.

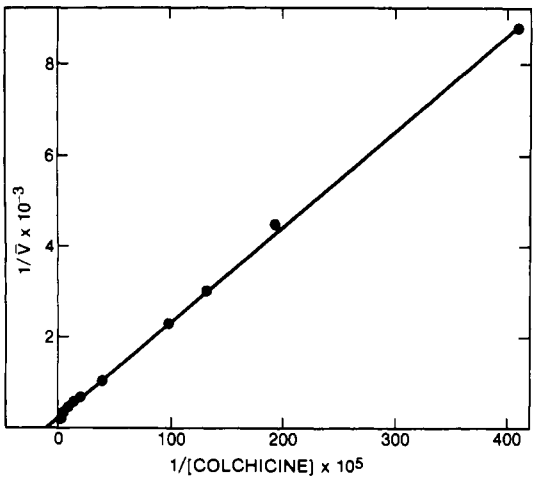

FIGURE 2: Double-reciprocal plot showing binding of [^{3}H]colchicine to steady-state microtubules. Aliquots (0.5 mL) of a sheared microtubule suspension at steady state (3.8 mg/mL total microtubule protein, mean microtubule length 0.62 μm) were incubated with increasing concentrations of [^{3}H]colchicine (10 μCi of colchicine/0.5-mL aliquot, 2.5×10^{-8}–1.0×10^{-5} M colchicine) for 1 h at 30 °C. [^{3}H]Colchicine and protein were determined in microtubule pellets after centrifugation through 50% sucrose cushions as described under Experimental Procedures. The K_D for colchicine was 0.9×10^{-6} M. $\bar{V}$ = moles of colchicine bound per mole of tubulin in microtubules. $\bar{V}_{max}$ in this experiment was 5.3 mol of colchicine bound/1000 mol of tubulin in microtubules. Since the mean microtubule length was 0.62 μm (1035 dimers/microtubule) there were 5.5 molecules of colchicine bound/microtubule at infinite colchicine concentration.

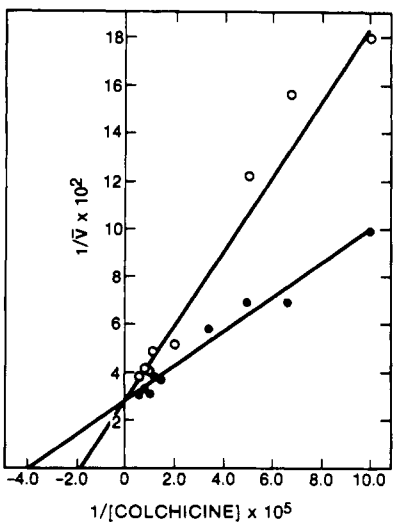

FIGURE 3: Competitive inhibition of colchicine binding to steady-state microtubules by podophyllotoxin. Aliquots (0.5 mL) of a microtubule suspension at steady state (4.1 mg/mL total microtubule protein), sheared to a mean length of 1.33 μm, were incubated with increasing [^{3}H]colchicine concentrations (6.25×10^{-7}–1.0×10^{-5} M) in the absence (closed circles) or presence (open circles) of 4×10^{-6} M podophyllotoxin for 1 h at 30 °C and analyzed for colchicine binding to microtubules as described under Experimental Procedures. $\bar{V}$ = moles of colchicine bound per mole of tubulin in microtubules. The K_D for colchicine was 2.51×10^{-6} M. The K_i for podophyllotoxin was 3.3×10^{-6} M.

crotubules at steady state with increasing concentrations of labeled colchicine, in the presence or absence of another drug, podophyllotoxin. Microtubules were then collected by centrifugation through a sucrose cushion and assayed for radioactivity and protein content. Podophyllotoxin competes with colchicine for a common binding site on tubulin (Wilson & Bryan, 1974) and, like colchicine, blocks microtubule assembly in vitro in a substoichiometric manner (Wilson & Margolis, 1978). Figure 3 shows result of such a competition experiment. Podophyllotoxin interfered with [^{3}H]colchicine binding to microtubules in a competitive manner with a inhibition constant of 3.3×10^{-6} M.

Thus, in the presence of absence of podophyllotoxin, colchicine binding to microtubules ($K_D = 2.2 \times 10^{-6}$ M) appeared specific and saturable at low stoichiometry, and we can conclude that the [^{3}H]colchicine binding that we detect represents a specific binding to tubulin and, therefore, to the microtubules at a limited number of available sites at steady state.

The maximum stoichiometry of colchicine binding per microtubule in the experiment depicted in Figure 2 was 5.5 molecules of colchicine/microtubule. Very similar stoichiometries per microtubule were obtained in nine similar experiments, with mean microtubule lengths varying from 0.62 to 6.37 μm (Table I). These results indicate that the binding is not occurring along the surface of the microtubules and, therefore, must be at one or both of the microtubule ends.

Previously, we reported that colchicine blocks microtubule assembly in vitro by adding to the assembling polymer only when bound to a tubulin dimer (Margolis & Wilson, 1977). The [^{3}H]colchicine addition to microtubules that we measure, therefore, may represent colchicine–dimer assembly addition at assembly sites. Since assembly occurs uniquely at microtubule ends (Johnson & Borisy, 1975; Bryan, 1976; Margolis & Wilson, 1978) and both ends of the microtubule are capable of supporting net assembly under non-steady-state conditions (Olmsted et al., 1974; Dentler et al., 1974), it was necessary to establish whether the [^{3}H]colchicine binding that we measure was occurring at one or both ends of the microtubule.

To establish where colchicine was adding, we assembled microtubules to steady state and then incubated them with [^{3}H]colchicine for 1 h at a concentration adequate to more than half-saturate the available microtubule binding sites. We then chased by addition of a 100-fold excess of unlabeled colchicine for varying lengths of time, simultaneously with a fivefold dilution of the microtubules. Microtubules were then collected by centrifugation through 50% sucrose, and pellets were assayed for protein content and radioactivity.

If the colchicine were binding tightly to microtubule assembly ends, there should be significant retention of the bound labeled colchicine under conditions of dilution and chase with excess unlabeled colchicine, since colchicine produces a tight assembly end cap under these conditions (Figure 1). Further, if the colchicine were binding uniquely at the microtubule assembly ends and not at the disassembly ends, there would be protein loss from the microtubules due to dissociation of tubulin from the disassembly ends but no loss of bound labeled colchicine.

The result of the experiment described above is shown in Figure 4. Under dilution and chase conditions, there was a 20–25% loss of protein 20 min after dilution of the microtubules. However, more than 95% of the [^{3}H]colchicine remained bound to the microtubules under dilution conditions in the presence of the unlabeled colchicine chase. Similar results were obtained under nondilution conditions, with the tubulin being lost at the disassembly end at a rate of 10% per hour as previously reported (Margolis & Wilson, 1978). We therefore conclude, since the microtubules disassembled appreciably and there was little loss of bound colchicine, that the colchicine is adding to microtubule assembly ends and is not binding to the disassembly ends.

Stoichiometry of Colchicine Binding to Microtubules. Having determined that the colchicine binding to the microtubule that is measurable is physiologically significant and that it represents binding via dimer addition at the microtubule assembly end, we could then interpret the stoichiometry of

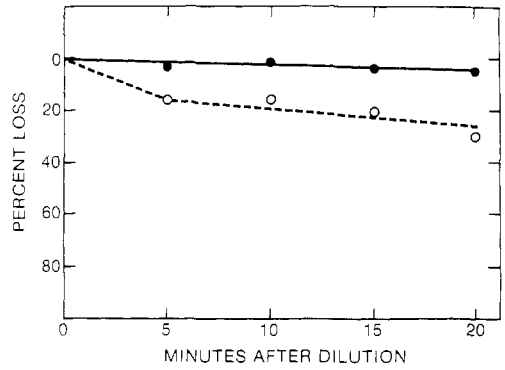

FIGURE 4: Stability of [^{3}H]colchicine binding to steady-state microtubules. Aliquots (0.5 mL) of a suspension of unsheared microtubules at steady state (2.95 mg/mL total microtubule protein, mean microtubule length 8 μm) were incubated with 1.0×10^{-6} M [^{3}H]-colchicine for 1 h at 30 °C. A 100-fold excess of unlabeled colchicine (final concentration, 1×10^{-4} M) was then added to each aliquot, and incubation at 30 °C was continued for the times indicated. [^{3}H]Colchicine (closed circles) and protein (open circles) were determined in microtubule pellets after centrifugation through 50% sucrose cushions as described under Experimental Procedures. The zero-time sample (data point not shown) was not diluted and did not receive any unlabeled colchicine chase. Background radioactivity was determined by incubating a 0.5-mL aliquot of steady-state microtubules with 1.0×10^{-6} M [^{3}H]colchicine simultaneously with the 100-fold excess colchicine chase. The stoichiometry of [^{3}H]colchicine binding to the steady-state microtubules at zero time (prior to addition of the colchicine chase) was 1 mol of colchicine/1870 mol of tubulin in microtubules or ~7 molecules of colchicine/microtubule (>75% of maximal binding).

colchicine addition as obtained in the binding analyses (e.g., Figure 2). There are two values of interest: (1) the number of molecules of colchicine bound per microtubule assembly end under conditions of half-maximal inhibition of assembly and (2) the maximum number of colchicine molecules bound per microtubule at infinite colchicine concentration.

From a group of ten binding experiments identical with that shown in Figure 2, we determined the stoichiometry of binding at saturation to be 8.8 (±1.4) colchicine dimer complexes per microtubule (Table I).

In order to determine the number of molecules of colchicine bound per microtubule assembly end under conditions of half-maximal inhibition of assembly, it was first necessary to determine accurately the concentration of added colchicine that would produce a 50% decrease in assembly rate, utilizing experimental conditions very similar to those employed in the determination of colchicine binding to the microtubules. Microtubule protein was assembled to steady state in a buffer that contained acetyl phosphate and acetate kinase in order to maintain a constant GTP concentration (MacNeal et al., 1977; Margolis & Wilson, 1978), and the microtubules were sheared to 1.5-μm mean length. At steady state, colchicine was added to aliquots of the microtubule protein to obtain a distribution of final colchicine concentrations, and incubation was carried out for 1 h. These aliquots were then each incubated with [^{3}H]GTP for 30 min, and finally the incubation was terminated by centrifuging the microtubules through a sucrose cushion. [^{3}H]GTP uptake at each colchicine concentration was then determined (Figure 5). Since [^{3}H]GTP uptake measures the steady-state asembly rate of microtubules (Margolis & Wilson, 1978), we can use these data to calculate an apparent inhibition constant (K_i) for colchicine inhibition of assembly at steady-state. The calculated value, $K_i = 0.13$ μM, is close to the value previously determined under assembly-initiating conditions, $K_i = 0.18$ μM (Margolis & Wilson, 1977).

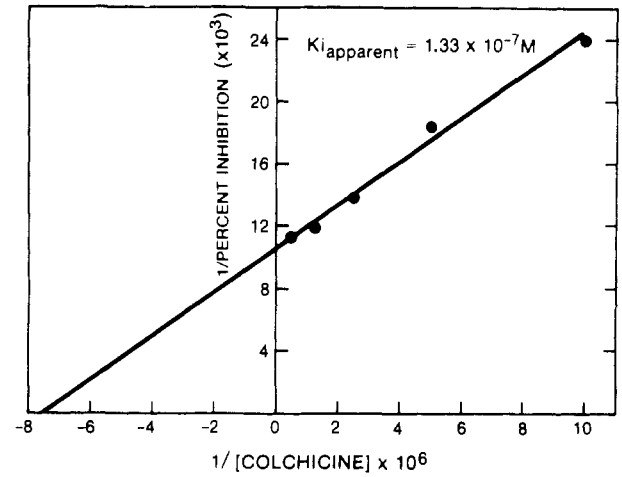

FIGURE 5: Inhibition of microtubule assembly at steady state by colchicine. Microtubules were assembled to steady state at 30 °C with unlabeled GTP with a GTP regenerating system [see Margolis & Wilson (1978)] and sheared to a mean length of 1.5 μm. Aliquots (0.5 mL, 4 mg/mL total protein) were then incubated with increasing concentrations of colchicine (1×10^{-7}–2×10^{-6} M) for 1 h at 30 °C and then pulsed with 10 μCi of [^{3}H]GTP for 30 min to measure the rate of dimer addition to microtubule assembly ends. Microtubules were collected by centrifugation through 50% sucrose and analyzed for incorporation of the label as described under Experimental Procedures. The apparent inhibition constant (K_i) for colchicine in this experiment was 1.33×10^{-7} M. At 50% inhibition of assembly (i.e., 1.33×10^{-7} M colchicine), 2.1% of the soluble tubulin was drug bound.

The inhibition constant represents the colchicine concentration added to steady-state microtubules which results in half-maximal inhibition of the microtubule assembly rate. Using data from the 10 colchicine binding experiments described previously, similar to that shown in Figure 2, we could directly determine the number of colchicine–dimer complexes bound per microtubule at the added drug concentration that yields 50% inhibition of the assembly rate at steady state. A mean value of 0.48 ± 0.12 was obtained for all experiments carried out (Table I). Thus, there are ~0.5 colchicine–dimer complexes bound per average microtubule when the assembly rate is inhibited by 50%. The data support a poisoning mechanism in which a single colchicine–dimer complex binds to the microtubule assembly end and decreases the apparent rate of free tubulin dimer addition to zero or near zero.

Discussion

Mechanism of Substoichiometric Inhibition of Assembly by Colchicine. We have found that a single colchicine–dimer complex bound at the assembly end of bovine brain microtubules can reduce the tubulin addition rate to zero or near zero, under conditions where the free tubulin concentration is at or near the steady-state critical concentration. Further, we find that colchicine–dimer complex binding to the assembly end can also stabilize or cap the end, so that the dissociation rate of tubulin from this end, as revealed by dilution of the microtubules, is reduced as compared with the normal dimer dissociation rate.

The binding we have detected to the microtubule assembly ends occurs to an apparent single affinity class of sites (mean affinity, 4.5×10^5 L/mol at 30 °C), with a maximum stoichiometry of 8.8 ± 1.4 colchicine dimer complexes per microtubule. It is of considerable interest that the affinity of colchicine for purified 6S bovine brain tubulin at 30 °C is essentially identical, at 5.9×10^5 L/mol (data not shown). Further, the binding of colchicine that we detect at the microtubule assembly ends is prevented competitively by podophyllotoxin, with an apparent inhibition constant very similar

to that obtained for the ability of podophyllotoxin to inhibit colchicine binding to 6S bovine brain tubulin. Thus, the binding of colchicine that we are detecting at the microtubule assembly ends is indistinguishable from the binding of colchicine to soluble tubulin.

Previous evidence indicates that colchicine must bind to soluble tubulin first, before it can effectively block microtubule assembly (Margolis & Wilson, 1977). Since the binding of colchicine to microtubule assembly ends actually reflects the sequential binding of colchicine to soluble tubulin and then the binding of the colchicine–dimer complex to microtubule assembly ends and since the characteristics of colchicine binding to the microtubules resemble the binding reaction between soluble tubulin and colchicine, we can infer that the rate-limiting reaction in the two-reaction sequence is the binding reaction between colchicine and soluble tubulin.

It is of significant interest that under conditions of half-maximal inhibition of microtubule assembly rate at steady state, the stoichiometry of colchicine–dimer complex addition to microtubule ends is $\sim$0.5 mol of colchicine/microtubule. That is, 1 h after the addition of 1.3×10^{-7} M colchicine to steady-state microtubules, regardless of the length of the microtubules, the rate of assembly is reduced by 50% and approximately half of the microtubules contain one bound colchicine–dimer complex at the assembly end. The simplest interpretation of these results is that a single colchicine–dimer complex binds at the microtubule assembly end and reduces the assembly rate at that end to zero, as long as the colchicine–dimer complex remains bound at that end. However, this interpretation is inconsistent with the results of Sternlicht & Ringel (1979) who have recently reported that colchicine–dimer complex can coassemble onto microtubule ends along with free dimers to form microtubule copolymers. They find that in the presence of colchicine under their conditions of assembly there is a reduced tubulin association rate, a lower tubulin binding affinity for the microtubule ends, and an increase in the critical concentration for assembly accompanying a substantial incorporation of colchicine–tubulin dimer into the microtubule polymer. However, the results of Sternlicht and Ringel, though different than those reported here, may be compatible with the present results, as some interesting explanations may exist to account for the differences.

We have recently investigated the mechanism for colchicine poisoning of microtubules reassembled in vitro from *Strongylocentrotus purpuratus* sperm tail outer doublet tubulin (Farrell & Wilson, 1980). The data support a mechanism in which a single colchicine–dimer complex binds to the microtubule assembly end and temporarily decreases the rate of free tubulin dimer addition to below an experimentally detectable level. The block is not absolute, however. In time, some free tubulin dimer addition appears to occur over the colchicine–dimer complex block, eventually resulting in recovery from the effect of the colchicine–dimer block. Thus, colchicine–dimer complex poisoning of microtubule assembly at low colchicine concentrations may be considered in two phases, with the first phase occurring through establishment of a block produced by the permanent addition of a colchicine–dimer complex to the microtubule assembly end and second "recovery" phase during which dimers assemble onto the microtubule over the colchicine–dimer complex block.

We have not detected any evidence of colchicine–dimer copolymerization upon addition of colchicine to the steady-state bovine brain microtubules under the conditions employed in this study. Thus, it appears that the recovery phase is not occurring. It is important to emphasize that all of the experiments reported in this work have involved addition of colchicine to preformed microtubules under steady-state conditions and that the bovine brain microtubules we have investigated depolymerize very slowly under conditions of assembly-end blockage [$\sim$0.7 μm/h (Margolis & Wilson, 1978)]. Thus in a typical experiment in which microtubules were incubated with labeled colchicine for 1 h, the amount of tubulin released from disassembly ends would raise the soluble tubulin level by no more than 0.4 mg/mL. This increase may be insufficient to compensate for the decrease in tubulin affinity for microtubule assembly ends produced by the presence of colchicine–dimer complexes at those ends. Thus under these conditions and perhaps as well because of the properties of the specific type of microtubules being studied, there is little or no further tubulin addition. Copolymerization could perhaps occur when the free tubulin level became sufficiently high to overcome the assembly-end block. Our results indicate that under conditions in which the free tubulin concentration approximates the critical concentration for assembly of the bovine brain microtubules used in this study, a single colchicine–dimer complex bound at the assembly end is sufficient to produce a long-lasting assembly-end block, with no copolymer formation.

The peculiar characteristics of colchicine binding to 6S tubulin have been well documented [for a review, see Wilson & Bryan (1974)], and recent evidence indicates that the unusually slow kinetics of colchicine binding to tubulin is due to a colchicine-induced conformational change in tubulin (Garland, 1978). It is conceivable that colchicine-induced capping of bovine brain microtubules at steady state, which results in a significantly increased affinity of the colchicine–tubulin complex for the microtubule assembly end as compared with free tubulin, may be due to the conformational change induced in the subunit by the binding of colchicine. Thus, colchicine may be acting by binding to free tubulin in solution, thereby causing a conformational change in the protein that results in a significantly greater affinity of the tubulin for microtubule assembly ends. Thus, once bound under steady-state conditions, colchicine–dimer complexes remain bound, essentially as a cap, preventing both normal dimer loss and gain at the microtubule assembly end.

A single colchicine–dimer complex can block the assembly of a microtubule, and yet a further seven colchicine–dimer complexes can be added to the microtubule at infinite concentration of drug. Given these parameters, we picture blockage of assembly occurring by a colchicine–dimer complex adding to one helical start and blocking assembly on that helix. Assembly on seven other helices (Amos & Klug, 1974) occurs independently and continues either until a colchicine–dimer complex addition blocks their assembly or until the incoming dimer cannot form a stabilizing longitudinal bond due to colchicine–dimer complex blockage of the helix below. Following a single colchicine–dimer complex addition, all unblocked helices will thus continue to assemble until they collect on the edge of a "precipice" caused by the absence of dimers on the blocked helix below. One colchicine–dimer complex addition can thus cause a propagated assembly failure. If a sufficient concentration of colchicine–dimer complexes is present, each of the other seven helices can bind a colchicine–dimer complex before it terminates on the end of the blocked helix immediately below.

Colchicine–Dimer Complex as an Assembly-End Probe. With the assumption that the microtubule is composed of heterodimeric tubulin subunits (Ludueña et al., 1977; Crepeau et al., 1978), its dimeric subunits are simultaneously arrayed

into a 13-start protofilamentous, an 8-start helical, and a 5-start helical lattice (Amos & Klug, 1974; Snyder & McIntosh, 1976). This means that, for instance, for the 8-start helical array, there are eight unique positions, or pockets, exposed for dimer addition at the microtubule assembly ends. Each represents the terminus of an independent dimeric helix in the microtubule. Similarly, the 5-start helical pattern exposes five unique assembly sites. Alternatively, dimer addition could possibly occur on each of the 13 longitudinal protofilaments of which the microtubule is composed.

At saturation, there were approximately nine colchicine–dimer complexes bound to the microtubule assembly end. We believe this value approaches as close as can reasonably be expected to a value of eight, given the sensitivity to error of the assay. Eight is well within the limits of variation at the 95% level of confidence (Student's t test). These data are consistent with the possibility that colchicine–dimer complex addition occurs on each of eight helical starts. Microtubule assembly may thus be pictured as proceeding by the sequential addition of dimers on each of the eight independent right-handed helices exposed at the microtubule assembly end.

Mammalian brain tubulin forms oligomeric rings at cold temperature or in the presence of calcium (Kirschner et al., 1974). These 20–36S rings (size varies with conditions) have been modeled as intermediates in the microtubule assembly reaction, in the manner of coiled protofilaments that unroll and add protofilament rows at the microtubule assembly end (Krischner et al., 1974; Erickson, 1974). At least three systems are known in which microtubule assembly proceeds in the apparent absence of oligomeric rings: dogfish brain tubulin (Langford, 1978), cell culture tubulin (Nagle et al., 1977), and sea urchin sperm tail outer doublet tubulin (Farrell & Wilson, 1978). Oligomeric rings are, further, not strictly obligatory for mammalian brain tubulin assembly in vitro, and conditions are known in which assembly proceeds in the absence of rings (Bryan, 1976; Lee & Timasheff, 1977; Herzog & Weber, 1977).

Nonetheless, it has been difficult to assess whether rings, when present, represent physiological intermediates in assembly and are therefore, perhaps, initiators of assembly in the cell. The helical addition of dimers at the microtubule assembly end that we believe may occur further weakens an argument for protofilament ring mediated elongation. Further, it is unlikely that rings represent helical arrays that add as a unit, since such addition would bury the free ends of adjacent underlying helices. We therefore tentatively suggest that oligomeric rings are alternate tubulin–MAP protein aggregates, stable at low temperature, which must disperse during microtubule assembly.

Initiation of the condensation polymerization reaction must proceed by means of some structure intermediate between the dimer and a completed cylinder, probably proceeding by helical bond formation in the manner of elongation. Lateral dimer–dimer associations (helical starts) could possibly occur during initiation and then proceed by coalescence with other helical starts (via longitudinal bonds) toward completion of a tubular base. Microtubule-associated proteins, by changing the dissociation constant of dimer–dimer bonds (Murphy et al., 1977), could make the formation of stable initiation intermediates more probable.

If our picture of helical assembly is correct, the nature of the microtubule assembly mechanism would place restrictions on the molecular structure of assembly templates in the cell, such as basal bodies, spindle pole bodies and plaques, and kinetochores. It can be imagined that the templates contain a structure that is permissive for assembly to initiate upon eight helical start positions.

Microtubules in the cell can conceivably be maintained by any of a few equilibrium mechanisms. They may maintain a steady state arising from opposite end assembly–disassembly kinetics (Margolis & Wilson, 1978) or they may establish an assembly-end equilibrium, if the disassembly end is attached to some structure or otherwise unavailable for equilibrium exchange. A similar disassembly-end equilibrium is possible, but kinetically unlikely. If in cells, colchicine adds to microtubules at the assembly end by an assembly mechanism and if it caps and blocks dimer dissociation at that end in cells, we might begin to classify microtubules in the cell with respect to their assembly mechanism and their orientation by observing their response to colchicine. If a microtubule is maintained by an assembly-end equilibrium, with an attached or blocked disassembly end, colchicine at low concentration will "freeze" the structure, preventing its further assembly or disassembly. If, on the other hand, the microtubule exists in a steady state maintained by an opposite end assembly–disassembly mechanism, it will respond to low concentrations of colchicine by disassembling at a rate determined by its disassembly-end dissociation rate.

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Solution Conformation of Thymopoietin$_{32-36}$: A Proton Nuclear Magnetic Resonance Study†

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ABSTRACT: The aqueous solution conformation of Arg-Lys-Asp-Val-Tyr (TP5), corresponding to positions 32–36 of the thymic hormone thymopoietin has been investigated by proton nuclear magnetic resonance (NMR). This pentapeptide fragment retains the biological activity of the parent protein, viz., induction of selective differentiation of T lymphocytes. All the observed NH and CH resonances of TP5 have been assigned, and the solution conformation of this peptide has been investigated by analysis of chemical shift variations with pH, vicinal NH–C$^{\alpha}$H coupling constant data, and amide hydrogen exchange rates. The latter were measured in H_2O by using a combination technique consisting of the transfer of solvent saturation and saturation recovery NMR experiments. The data are compatible with the assumption of a highly motile dynamic equilibrium among different conformations for TP5. A comparison of the amide hydrogen exchange rates of the pentapeptide with that of solvated model compounds shows that Val^4-NH is significantly shielded from the solvent. In addition, the chemical shift variations with pH suggest that the guanidino-N$^{\epsilon}$H of arginine is associated with one of the carboxylate groups. These observations provide specific boundary conditions for the construction of molecular models of the conformation(s) of TP5 in aqueous solution.

Thymopoietin is a polypeptide hormone of the thymus. The 49 amino acid sequence of this hormone has been determined (Schlesinger & Goldstein, 1975). Thymopoietin has a number of biological activities. Initially, it was detected and isolated by its effects on neuromuscular transmission (Goldstein & Hofmann, 1969; Goldstein, 1974). Subsequently, thymopoietin was shown to induce early T cell differentiation (Basch & Goldstein, 1974), and this action was quite selective in that thymopoietin inhibited early B cell differentiations (Scheid et al., 1978). In addition to generating lymphocyte populations by induction of early T cell differentiation, thymopoietin also appears to regulate more mature populations (Sunshine et al., 1978; Weksler et al., 1978). Two synthetic fragments were shown to be biologically active—first a tridecapeptide corresponding to positions 29–41 (Schlesinger et al., 1975) and, subsequently, a pentapeptide corresponding to positions 32–36 (Goldstein et al., 1979). Thus, the synthetic pentapeptide arginyllysylaspartylvalyltyrosine (TP5) appears to correspond to a biologically active site of the parent thymopoietin molecule. Information concerning its solution conformation would be of interest in relation to the activity of the parent hormone.

Retention of the thymopoietic activity by TP5 suggests that this fragment may be capable of assuming a conformation similar to the active site of thymopoietin. We have, therefore, concentrated on defining the conformation–activity relationship in this smaller molecule which is more amenable to study by physicochemical techniques. Toward this end, we have employed NMR[1] to characterize the free solution conformation

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