Interactions of 2-Methoxyestradiol, an Endogenous Mammalian Metabolite, with Unpolymerized Tubulin and with Tubulin Polymers

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ABSTRACT: 2-Methoxyestradiol (2ME) is an endogenous mammalian catabolite of estradiol with antimitotic activity. Although it is a competitive inhibitor of the binding of colchicine to tubulin, it has unusual effects on glutamate-induced tubulin polymerization. Polymer that was little changed in morphology assembled at a reduced rate and was relatively cold stable. We have now examined interactions of [4-^{3}H]-2ME with unpolymerized tubulin and polymer. The [^{3}H]2ME binds avidly to tubulin even on ice, and it is readily displaced by other colchicine site drugs. An association rate constant on ice of 1.9×10^2 $M^{-1}s^{-1}$ was obtained. Scatchard analysis indicated a single class of binding site and an association equilibrium constant of 5.7×10^5 M^{-1}. These values lead to a calculated dissociation rate constant of 3.3×10^{-4} s^{-1}. In glutamate-induced tubulin assembly, a reaction that requires GTP and leads to the formation of sheets of parallel protofilaments, increasing amounts of [^{3}H]2ME were incorporated into polymer, reaching near-stoichiometry with tubulin at 100 μM 2ME. Equivalent binding of [^{3}H]2ME occurred when the drug was added to preformed polymer, but binding of [^{3}H]2ME to polymer was not readily inhibited by colchicine site drugs. Significant amounts of [^{3}H]2ME were also incorporated into microtubule polymer formed with microtubule-associated proteins, glycerol, or 4-morpholineethanesulfonate buffer, but the stoichiometry was substantially lower than that in the sheet polymer induced by either glutamate or l,4-piperazineethanesulfonate buffer. The structural differences between the microtubule and sheet polymers leading to these differences in apparent affinity for 2ME are unknown, but presumably interaction of the estrogen metabolite with cellular microtubules has functional significance related to the antimitotic properties of the compound.

Microtubules have many important roles in cellular physiology, among which are support of cell shape and involvement in intracellular transport phenomena, functions of the interphase cytoskeleton, and promotion of cell division, the function of the mitotic spindle [see Hyams and Lloyd (1994) for a series of reviews]. Despite increasing understanding of microtubule dynamics and factors that control them in biochemical systems, cellular controls involved in the regulation of microtubule assembly, disassembly, and function remain obscure. The major component of microtubules, the heterodimeric protein tubulin, interacts with a large number of natural products and synthetic compounds at diverse binding sites (Hamel, 1990, 1996). These agents have a variety of effects on tubulin polymerization. Most of them inhibit assembly, sometimes promoting formation, generally at higher concentrations, of polymeric forms of highly aberrant morphology. A few compounds, most notably the taxoids, but also the epothilones (Bollag et al., 1995) and discodermolide (ter Haar et al., 1996), promote assembly of polymer of grossly normal morphology but enhanced stability. It has long been postulated that the drug binding sites of tubulin may play important roles in the cellular regulation of microtubules. In particular, interactions of protein components at the colchicine site have been reported (Lockwood, 1979; Sherline et al., 1979).

While studying the inhibitory effects on angiogenesis (D'Amato et al., 1994; Fotsis et al., 1994; Klauber et al., 1995) of 2-methoxyestradiol (2ME),[1] the major mammalian metabolite of estradiol (Gelbke & Knuppen, 1976; Berg et al., 1983), we became aware of its antimitotic properties (Seegers et al., 1989), which we have confirmed (Cushman et al., 1995). We demonstrated that the estrogen metabolite was a relatively weak competitive inhibitor of the binding of [^{3}H]colchicine to tubulin (apparent K_i of 22 μM versus 0.5 μM for podophyllotoxin). Effects of 2ME on tubulin assembly were more complicated. Polymerization dependent on microtubule-associated proteins (MAPs) was not strongly inhibited by the agent, at least under the reaction conditions we examined. In 1 M glutamate/1.0 mM $MgCl_2$ at 37 °C, the rate of assembly was progressively inhibited at superstoichiometric (relative to tubulin) concentrations of 2ME, but the polymerization plateau was unaltered even at 10-fold molar excess of drug. Although polymer morphology in the presence of 2ME did not change, as the 2ME concentration increased the polymer became progressively more stable to cold temperatures. Finally, at 25−30 °C

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[1] Abbreviations: 2ME, 2-methoxyestradiol; [^{3}H]2ME, [4-^{3}H]-2-methoxyestradiol; MAPs, microtubule-associated proteins; Mes, 4-morpholineethanesulfonate; Pipes, l,4-piperazineethanesulfonate; CS-A4, combretastatin A-4; SD, standard deviation.

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reaction temperatures in the absence of exogenous Mg^{2+}, 2ME appeared to act as a pure inhibitor of assembly, with total inhibition occurring at substoichiometric drug concentrations (D'Amato et al., 1994).

These observations led us to postulate that 2ME was being incorporated into the polymer formed in glutamate/$MgCl_2$, possibly by copolymerization of tubulin and tubulin−2ME complexes, as has been shown to occur to a limited extent with colchicine (Sternlicht & Ringel, 1979). We speculated that 2ME or other steroid derivatives might play a role in intracellular microtubule regulation or function through interactions at the colchicine site. As a first step toward investigating these possibilities, we obtained [4-^{3}H]-2-methoxyestradiol ([^{3}H]2ME) and investigated its interactions with tubulin. Our goals were to determine whether the altered stability properties of the glutamate/$MgCl_2$ polymer correlated with increasing amounts of 2ME in polymer and to determine whether evidence could be found for an interaction of 2ME with MAP-dependent polymer.

MATERIALS AND METHODS

Materials. Nonradiolabeled 2ME and podophyllotoxin were from Aldrich, nonradiolabeled colchicine was from Sigma, and [^{3}H]colchicine was from DuPont. [^{3}H]2ME (21.6 Ci/mmol) was obtained from Moravek Biochemicals. Electrophoretically homogeneous bovine brain tubulin and heat-treated MAPs were prepared as described previously (Hamel & Lin, 1984b). Combretastatin A-4 (CS-A4) was a generous gift of Dr. G. R. Pettit, Arizona State University.

Methods. Binding of [^{3}H]2ME to tubulin was measured by centrifugal gel filtration, using 1.0 mL columns of Sephadex G-50 (superfine) prepared in tuberculin syringes. The procedure was described in detail previously (Hamel & Lin, 1984a). The reaction mixture and assay column always contained the same buffer and ionic components. Reaction mixtures incubated on ice were processed on columns kept in tubes on ice and centrifuged in a cold room. Reaction mixtures incubated at room temperature were processed on columns kept at room temperature, with centrifugation at room temperature. Reaction volume was 0.32 mL, and three 0.1 mL aliquots were placed on triplicate syringe columns. The protein content and radioactivity of column filtrates were determined, respectively, by the Lowry procedure with bovine serum albumin as standard and by liquid scintillation spectrometry.

Incorporation of [^{3}H]2ME into tubulin polymers was determined by harvesting polymer by centrifugation, as described for the individual experiments. Pellets were washed twice with a solution at 37 °C having the same buffer and ionic composition as the reaction mixture. Pellets were dissolved in 100 μL of 8 M urea, and the protein content and radioactivity of the resulting urea solutions were determined by the Lowry procedure and by liquid scintillation spectrometry.

Electron microscopy was performed on aliquots of reaction mixtures removed prior to centrifugation. Each aliquot was placed on a 200 mesh carbon-coated, Formavar-treated copper grid, and after a few seconds, the grid was washed with 6−10 drops of 0.5% (w/v) uranyl acetate. The grids were examined in a Zeiss Model 10CA electron microscope.

Table 1: Binding of 2ME Is Specific to Tubulin and Is Inhibited by Colchicine Site Drugs[a]

Experiment 1	
reaction components	pmol of 2ME bound/μg of protein
0.5 mg/mL tubulin/5 μM 2ME	1.53
1.0 mg/mL MAPs/10 μM 2ME	0.04
1.0 mg/mL albumin/10 μM 2ME	0.02

	Experiment 2 % inhibition of 2ME bound to tubulin	
inhibitor	no preincubation	preincubation
colchicine	6	96
combretastatin A-4	98	99
podophyllotoxin	83	99

[a] In experiment 1, reaction mixtures contained 0.1 M Mes (pH 6.6), 0.5 mM $MgCl_2$, and proteins and [^{3}H]2ME as indicated. Incubation was for 30 min on ice. In experiment 2, all reaction mixtures contained 1.0 M monosodium glutamate (pH 6.6), 5 μM (0.5 mg/mL) tubulin, 5.0 μM [^{3}H]2ME, and 50 μM inhibitor if present. In the nonpreincubated samples, 2ME and inhibitor were mixed in the reaction mixture prior to tubulin addition. Incubation was for 30 min on ice. In the preincubated samples, inhibitor and tubulin were mixed and incubated at 37 °C for 15 min. Reaction mixtures were place on ice, [^{3}H]2ME was added, and incubation was continued for 30 min on ice. Control values, 0.24−0.25 mol of 2ME bound per mole of tubulin.

RESULTS

Binding of 2ME to Unpolymerized Tubulin and Its Inhibition by Colchicine Site Drugs. Table 1 presents experiments that demonstrate the specificity of binding of the [^{3}H]2ME to tubulin and the expected inhibition of the binding reaction by colchicine site drugs. As demonstrated by experiment 1, there was recovery of [^{3}H]2ME bound to protein following centrifugal gel filtration when tubulin was a component of the reaction mixture, but there was no significant binding to either MAPs or albumin. Experiment 2 documents the near-total inhibition of binding that occurred when the tubulin was preincubated with podophyllotoxin or CS-A4 on ice or at 37 °C, while inhibition by colchicine required the 37 °C preincubation, consistent with the poor binding of colchicine to tubulin at lower temperatures. Although a high concentration of inhibitors was used in the Table 1 study, extensive inhibition of binding occurred with lower concentrations of these agents, as shown in Figure 1 for CS-A4, a particularly potent inhibitor of colchicine binding to tubulin (Lin et al., 1989).

Initial studies to determine the best reaction condition for evaluating the binding of [^{3}H]2ME to tubulin showed wide variation depending on reaction components and temperature (Table 2). $MgCl_2$ in the reaction mixture and incubation at room temperature as opposed to incubation on ice caused substantial reduction in the amount of [^{3}H]2ME in the column filtrates. We believe that most of this variability was caused by drug dissociation in the microcolumns, because when room temperature samples were processed on cold columns the results were closer to those obtained with samples always kept cold. Moreover, samples incubated at room temperature, placed on ice, and processed in a cold room yielded data indistinguishable from those shown in Table 2 for the cold samples.

We found that the time course of binding under the "optimal" condition shown in Table 2 (1 M glutamate, no $MgCl_2$, incubation on ice with processing in a cold room) could be readily followed, although the reaction was about

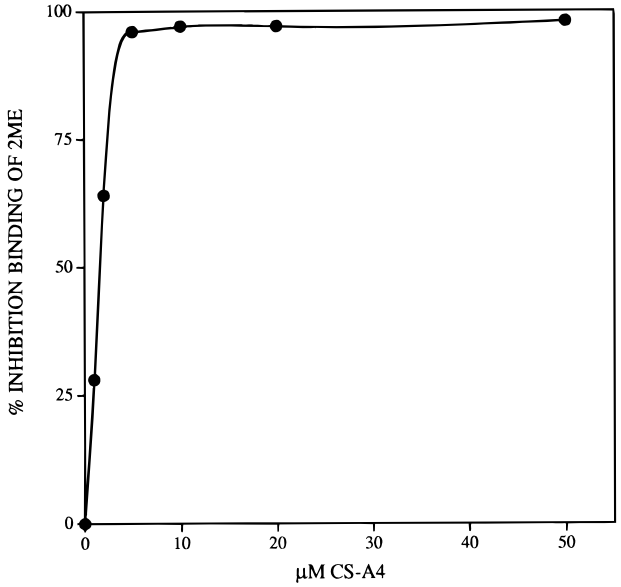

FIGURE 1: Inhibition of binding of [^{3}H]2ME to tubulin by low concentrations of CS-A4. Reaction mixtures contained 5.0 μM (0.5 mg/mL) tubulin, 5.0 μM [^{3}H]2ME, 1.0 M monosodium glutamate (pH 6.6), 2.5% (v/v) dimethyl sulfoxide, and the indicated concentration of CS-A4. The 2ME was the last component added to each reaction mixture. Incubation was for 30 min on ice, with gel filtration in a cold room. Without CS-A4, 0.40 mol of 2ME was bound per mole of tubulin.

Table 2: Recovery of 2ME Bound to Tubulin Varies with Reaction Conditions[a]

	incubation (mol of 2ME bound/mol of tubulin ± SD)	
buffer	on ice	at room temperature
1 M glutamate (pH 6.6)	0.34 ± 0.04	0.082 ± 0.03
1 M glutamate (pH 6.6) + 1 mM $MgCl_2$	0.24 ± 0.01	0.023 ± 0.007
0.1 M Mes (pH 6.6)	0.27 ± 0.04	0.067 ± 0.02
0.1 M Mes (pH 6.6) + 1 mM $MgCl_2$	0.11 ± 0.01	0.014 ± 0.007
0.1 M Mes (pH 6.9)	0.23 ± 0.03	0.020 ± 0.006
0.1 M Mes (pH 6.9) + 1 mM $MgCl_2$	0.11 ± 0.009	0.008 ± 0.002

[a] Reaction mixtures contained the indicated components, 10 μM (1.0 mg/mL) tubulin, and 10 μM [^{3}H]2ME. Incubation was at the indicated temperature for 10 min. Average values from three experiments are presented.

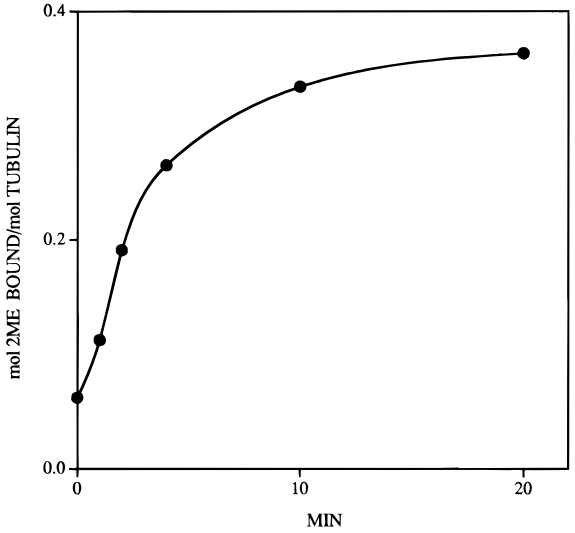

FIGURE 2: Time course of [^{3}H]2ME binding to tubulin on ice. Each reaction mixture contained 1.0 M monosodium glutamate (pH 6.6), 10 μM [^{3}H]2ME, and 10 μM (1.0 mg/mL) tubulin. Incubation was for the indicated times on ice, with each time point processed individually.

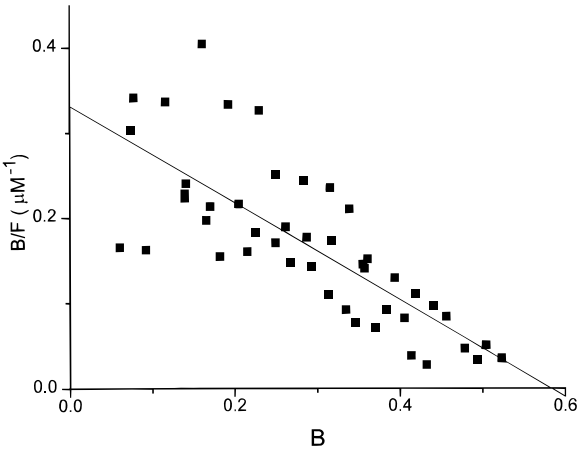

FIGURE 3: Scatchard analysis of the binding of [^{3}H]2ME to tubulin on ice in the absence of exogenous Mg^{2+}. Reaction mixtures contained the components described in legend for Figure 2, with varying concentrations of [^{3}H]2ME, and were incubated for 30 min on ice before being processed in a cold room. Data of three independent experiments are presented in the figure with linear regression analysis of the data (slope = −0.569 ± 0.069 SD).

50% complete within 2 min (Figure 2). Ignoring the dissociation reaction, these data imply an association rate constant of about 1.9×10^2 $M^{-1}s^{-1}$.

As will be discussed below, the binding reaction of [^{3}H]-2ME to polymer displayed properties that raised the question of whether more than one binding site exists. The binding of 2ME to tubulin was therefore examined by Scatchard analysis under several different reaction conditions, but no evidence for a biphasic curve was obtained. Figure 3 presents data summarizing three independent experiments performed under the same reaction conditions as were used in the time course study of Figure 2. These data indicate a single class of binding site (maximum stoichiometry about 0.6), and the slope of the curve yields an apparent K_D value of 1.8 μM (or K_A of 5.7×10^5 M^{-1}). Combining this value with the association rate constant above yields a dissociation rate constant on ice of 3.3×10^{-4} s^{-1}. The other reaction condition studied most thoroughly was that in which high stoichiometry of binding of drug to polymer occurred (glutamate + $MgCl_2$ + GTP; 37 °C incubation; see below). For the Scatchard studies summarized in Figure 4, however, GTP was omitted so that assembly would not occur. Moreover, centrifugal gel filtration at higher temperatures resulted in low levels of radiolabeled drug in the filtrates (data not shown, but see above, Table 2). When samples were chilled on ice prior to processing, however, ample radiolabel was recovered in the filtrates that appeared similar to amounts found in samples only incubated on ice. Thus, for the Scatchard studies of Figure 4, samples were successively incubated at 37 °C, to mimic the assembly condition, and on ice, to obtain data that could be analyzed. Again, a single class of binding site was observed (maximum stoichiometry about 0.4), and the slope of the curve yields an apparent K_D value of 5.6 μM (or K_A of 1.8×10^5 M^{-1}). If this represents a valid measure of the equilibrium constant on ice, and if Mg^{2+} does not greatly affect the association

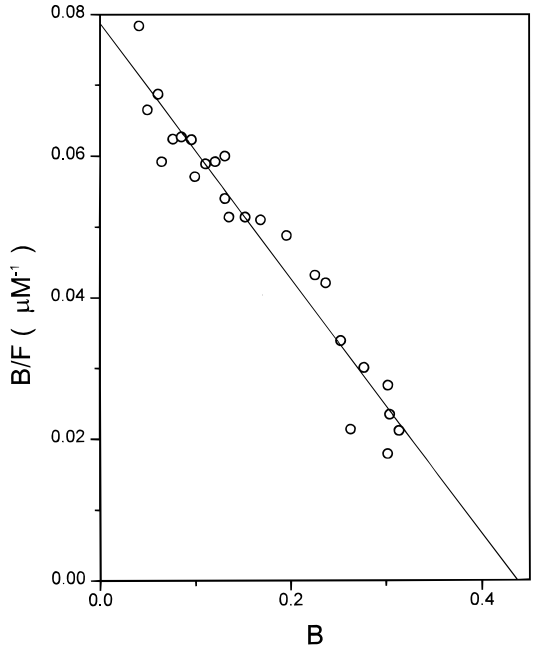

FIGURE 4: Scatchard analysis of the binding of [^{3}H]2ME to tubulin in the presence of 1 mM $MgCl_2$. Reaction mixtures contained varying concentrations of [^{3}H]2ME, 10 μM (1.0 mg/mL) tubulin, 1.0 M monosodium glutamate (pH 6.6), and 1.0 mM $MgCl_2$. Incubation was for 15 min at 37 °C, followed by 5 min on ice, with gel filtration in a cold room. Data of four independent experiments are presented in the figure with linear regression analysis of the data (slope = -0.180 ± 0.0097 SD).

rate constant, then Mg^{2+} would appear to cause a significant increase in the dissociation rate constant to 1.1×10^{-3} s^{-1}, consistent with the relatively rapid dissociation reaction postulated above. For comparison, we have previously obtained dissociation rate constants in glutamate/$MgCl_2$ for colchicine (known for its slow dissociation) at 23 and 37 °C of 1.4×10^{-6} s^{-1} and 7.1×10^{-6} s^{-1}, respectively (Kang et al., 1990); and the dissociation rate constant of 2-methoxy-5-(2,3,4-trimethoxyphenyl)tropone (known to dissociate rapidly) at 37 °C is 6×10^{-2} s^{-1} (Hastie, 1991).

Incorporation of Radiolabeled 2ME into Glutamate-Induced Tubulin Polymer. The key observation that led us to propose that polymer formed in glutamate/$MgCl_2$ must contain tubulin-bound 2ME was that the temperature stability of polymer increased substantially as the 2ME concentration in the reaction mixture rose. Tubulin was therefore incubated under this reaction condition with varying concentrations of [^{3}H]2ME, and, following centrifugation, protein and 2ME in polymer were quantitated (Figure 5). Net assembly was only inhibited at the highest 2ME concentrations, but radiolabel in the pellet climbed steadily between 10 and 75 μM 2ME. Stoichiometry of binding increased as a linear function of the 2ME concentration, almost reaching parity with the tubulin at 100 μM drug. (Higher concentrations could not be readily examined, as 2ME began to precipitate at concentrations greater than 100 μM.) The prediction based on the observation of increasing polymer stability as the 2ME concentration increased was thus validated. Significant radiolabel and protein were only recovered in pellets following centrifugation when GTP was included in the reaction mixture, demonstrating that drug was bound to polymer.

Although there are only a few points, the data of Figure 5 can be readily analyzed by the Scatchard method in terms of binding sites on the tubulin dimer (based on the stoichiometry values) and derived values for free drug concentration (based on the amount of polymer and the stoichiometry values). This analysis is presented in Figure 6 and suggests that a single class of binding site exists. However, unlike the substoichiometric intercepts obtained when the binding of 2ME to $\alpha-\beta$ dimer was evaluated (Figures 3 and 4), the glutamate polymer appears to have about two 2ME binding sites per tubulin molecule. These conclusions should be viewed as tentative since only a few data points are available and higher drug concentrations cannot be readily evaluated due to the limited solubility of 2ME.

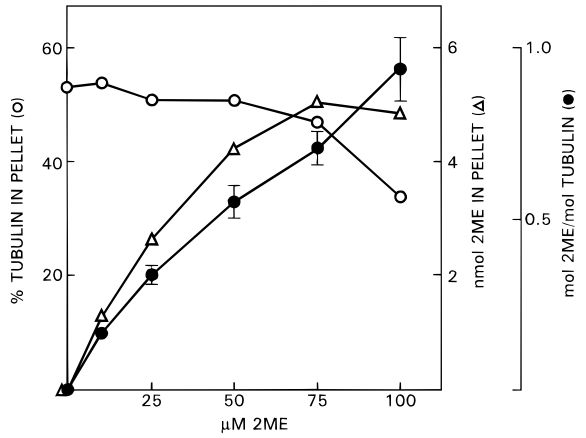

FIGURE 5: Incorporation of [^{3}H]2ME into glutamate polymer as a function of 2ME concentration. Reaction mixtures (0.4 mL) contained 15 μM (1.5 mg/mL) tubulin, 1.0 M monosodium glutamate (pH 6.6), 1.0 mM $MgCl_2$, 0.1 mM GTP, and the indicated concentration of [^{3}H]2ME. Incubation was for 15 min at 37 °C. Centrifugation was for 10 min at 14 000 rpm at room temperature in an Eppendorf microcentrifuge. Pellets were washed twice with a 37 °C solution containing 1 M monosodium glutamate and 1 mM $MgCl_2$. Pellets were dissolved in 8 M urea, and protein and radioactivity in the urea solutions were determined. Standard deviations larger than the symbol size are presented for the stoichiometry values (●).

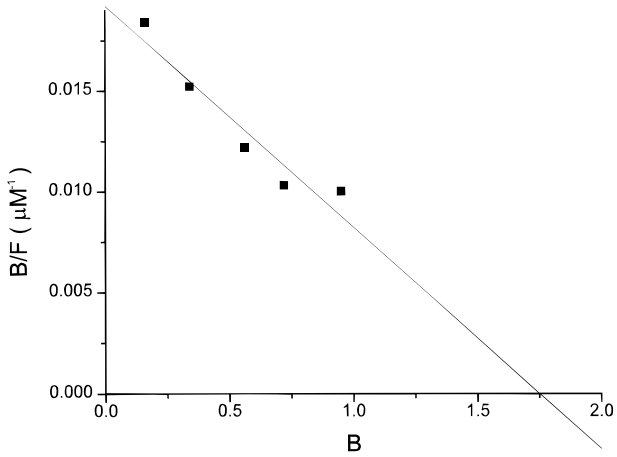

FIGURE 6: Scatchard analysis of the binding of [^{3}H]2ME to glutamate-induced tubulin polymer. The data of Figure 5 are presented in a Scatchard format (linear regression analysis of the data; slope = -0.0110 ± 0.0019 SD).

Based on the slope of the curve in Figure 6, the affinity of 2ME for tubulin in glutamate polymer is much reduced relative to unpolymerized tubulin. The apparent K_D is about 91 μM (equivalent to a K_A value of 1.1×10^4 M^{-1}). Consistent with this relatively low affinity of 2ME for polymer, little drug was recovered bound to polymer if centrifugation was through a sucrose or glycerol cushion (data not presented).

Table 3: 2ME, but Not Colchicine, Binds to Preformed Polymer[a]

drug	when added	mol of drug bound/ mol of tubulin ± SD
10 μM 2ME	initially	0.15 ± 0.03
10 μM 2ME	at plateau	0.16 ± 0.03
50 μM 2ME	initially	0.56 ± 0.1
50 μM 2ME	at plateau	0.64 ± 0.03
10 μM colchicine	at plateau	0.05
50 μM colchicine	at plateau	0.15 ± 0.006

[a] Reaction mixtures (0.4 mL) contained 15 μM (1.5 mg/mL) tubulin, 1.0 M monosodium glutamate (pH 6.6), 1.0 mM $MgCl_2$, 0.1 mM GTP, and, if indicated "initially", [^{3}H]2ME. Incubation was for 15 min at 37 °C. If indicated "at plateau", [^{3}H]2ME or [^{3}H]colchicine was added to the reaction mixture, and incubation at 37 °C was continued for an additional 5 min. Centrifugation was for 10 min at 14 000 rpm in an Eppendorf microcentrifuge at room temperature. Pellets were washed twice with a 37 °C solution containing 1 M monosodium glutamate and 1 mM $MgCl_2$. Pellets were dissolved in 8 M urea, and protein and radioactivity in the urea solutions were determined. Except for the 10 μM colchicine value, the experiment was performed 3 times, and average values are presented.

Table 4: Colchicine Site Drugs Poorly Inhibit Binding of 2ME to Polymer[a]

inhibitor	% of control (2ME bound to polymer) ± SD
50 μM podophyllotoxin	104 ± 13
50 μM colchicine	103 ± 8
100 μM 2-methoxy-5-(2,3,4-trimethoxyphenyl)tropone	77 ± 2
50 μM combretastatin A-4	67 ± 3
100 μM combretastatin A-4	57 ± 2

[a] Reaction mixtures (0.4 mL) contained 15 μM (1.5 mg/mL) tubulin, 1.0 M monosodium glutamate (pH 6.6), 1.0 mM $MgCl_2$, and 0.1 mM GTP and were incubated for 10 min at 37 °C. The inhibitors were added (little disassembly occurred except with podophyllotoxin), and incubation was continued for 10 min at 37 °C. At this point, 10 μM [^{3}H]2ME was added, and the incubation was continued for another 15 min at 37 °C. Centrifugation and processing of pellets were as described in the legend of Table 3. Percent of control is in terms of moles of 2ME bound per mole of tubulin in the absence of inhibitor. With podophyllotoxin, the pellet was about 60% smaller than with 2ME only, but the stoichiometry of 2ME in the surviving polymer was little changed.

Since it was shown many years ago that [^{3}H]colchicine did not bind in substantial amounts to microtubules (Wilson & Meza, 1973; Lee et al., 1974), implying that the site was masked in polymer, we explored whether [^{3}H]2ME would bind differently to glutamate/$MgCl_2$ polymer depending on whether the drug was added prior to incubation or after assembly had occurred. As shown in Table 3, there was little difference in radiolabeled 2ME associated with polymer as a function of time of addition to the reaction mixture. Moreover, we found that the cold stability of the glutamate/$MgCl_2$ polymer differed little whether 2ME was added prior to or after assembly (data not presented).

These findings raised the question of whether access to the colchicine site was different in the glutamate/$MgCl_2$ polymer as compared with microtubules. Since colchicine strongly inhibits the glutamate-induced reaction but does not cause rapid disassembly of preformed polymer, [^{3}H]colchicine was added to reaction mixtures following polymerization. Only small amounts of radiolabeled drug were recovered in the polymer pellets (Table 3), indicating that the binding site for colchicine is largely masked in the glutamate/$MgCl_2$ polymer as well as in microtubules.

This major difference between the interactions of 2ME and colchicine with preformed glutamate/$MgCl_2$ polymer raised the issue of whether other colchicine site drugs would bind to the polymer. We explored this question by determining whether such agents would inhibit binding of [^{3}H]-2ME to tubulin polymers, and we were unable to identify any compound that strongly inhibited this binding reaction. Several examples are shown in Table 4. The greatest inhibition we observed was with CS-A4, but the extent of this inhibition was much reduced compared to the potent inhibition by this agent of binding to unpolymerized tubulin, as shown above in Figure 1.

Binding of 2ME to Polymers Formed with Other Inducers of Polymerization. Previously we had observed only minimal effects of 2ME on polymerization induced by MAPs. There was only slight inhibition of the rate or extent of assembly at high (25−100 μM) 2ME concentrations, and the temperature stability and morphology of the polymer (predominantly microtubules) were unaffected by 2ME in the reaction mixture. Nevertheless, in initial experiments in which we measured the incorporation of [^{3}H]2ME into microtubule pellets formed with MAPs, we obtained essentially the same results as observed with glutamate.

However, in the course of these experiments, we noted that control reaction mixtures lacking only GTP yielded small pellets that were easily lost during the washing procedure. If care was taken to avoid dislodging these pellets, they were found to contain up to 75% as much radiolabel but significantly less protein than when GTP was included in the reaction. Further studies demonstrated that the pelleted radiolabel did not represent precipitated drug at concentrations up to 100 μM, that both tubulin and MAPs were required for this "background reaction," and that microtubules were not formed in the absence of GTP. We remain uncertain how to treat the data generated by this series of experiments. The addition of GTP could result in conversion of the precipitated protein with its bound 2ME into microtubules containing 2ME, or, alternatively, the material pelleted in the absence of GTP could coprecipitate with microtubules in samples incubated with GTP and represent a high background. In careful studies of MAP-dependent assembly with 40 μM tubulin and 100 μM 2ME in either 0.1 M Mes or 0.1 M Pipes, the stoichiometry without correcting for the radiolabel and protein in pellets obtained in the absence of GTP was about 0.5 mol of 2ME per mole of tubulin in polymer, while with a correction the stoichiometry was about 0.2. (In 1.0 M glutamate with 40 μM tubulin and 100 μM 2ME, the stoichiometries were 0.68 uncorrected and 0.63 corrected.)

If the corrected stoichiometries are more accurate, then MAP-dependent polymer (microtubules) binds substantially less 2ME than glutamate-dependent polymer (sheets), possibly as a consequence either of direct inhibition of drug binding by the MAPs or of the differing morphologies of the two polymers. It was feasible to explore these possibilities because (1) with glycerol tubulin will polymerize into microtubules both with (Shelanski et al., 1973) and without MAPs (Lee & Timasheff, 1975), and MAPs, when present, enter the polymer; and because (2) with high sulfonate buffer concentrations, Mes induces microtubule formation while Pipes induces formation of a mixture of sheets and microtubules, with sheets predominating (Waxman et al., 1981).

The sulfonate buffers at high concentrations, like glutamate and unlike glycerol, inhibit MAP binding to tubulin polymers.

In no other reaction condition examined was significant radiolabel or protein obtained in pellets when GTP was not in the reaction mixture (including 4 M glycerol + MAPs). In a series of studies with 40 μM tubulin and 100 μM [^{3}H]-2ME, we obtained the following stoichiometries (moles of 2ME per mole of tubulin in polymer pellet) when the predominant reaction product was microtubules: when assembly was induced with 1.6 M Mes, 0.1; when assembly was induced with 4 M glycerol, 0.2; when assembly was induced with 4 M glycerol + MAPs, 0.3. When assembly of a sheets/microtubules mixture was induced by 0.75 M Pipes, the stoichiometry obtained was 0.4.

Comparing the effects of high concentrations of Mes and Pipes, substantially more 2ME was recovered in polymer with Pipes, which induces a mixture of sheets, microtubules, and sheets attached to microtubules, than with Mes, which induces microtubule formation (Waxman et al., 1981). Further, the amount of 2ME in the Pipes polymer was not as great as that in the glutamate polymer, which consists almost entirely of sheets. In glycerol, both with and without MAPs, microtubules were the predominant polymer, and lower amounts of 2ME were associated with these polymers. Of particular importance, the MAPs did not reduce the amount of 2ME associated with the glycerol microtubules, implying that polymer morphology rather than the presence of MAPs is a more significant factor in the affinity of 2ME for polymer.

DISCUSSION

Although not an equilibrium method, since the reaction mixture is applied to a small column free of drug, the centrifugal gel filtration method has proved useful for the study of binding of a variety of ligands to tubulin (Caplow & Zeeberg, 1980; Hamel & Lin, 1984a; Bai et al., 1995). The method requires that dissociation be sufficiently slow so that the ligand remains associated with protein following rapid separation of the protein from free ligand. We have shown here that this technique can be used to study the binding of [^{3}H]2ME to tubulin, and it has allowed us to make preliminary estimates of association rate (by direct measurement) and equilibrium (by Scatchard analysis) constants. Drug recovery by gel filtration was significantly affected by reaction conditions, being reduced particularly by addition of Mg^{2+} to the reaction mixture and at a higher reaction and sample processing temperature. The latter finding suggests that drug dissociation may be disproportionately increased relative to drug association as the reaction temperature increases, and the reduced drug binding in the presence of Mg^{2+} may explain the more dramatic inhibition of glutamate-induced assembly in the absence of exogenous cation reported previously (D'Amato et al., 1994). If the reduced affinity of 2ME for unpolymerized tubulin in the presence of Mg^{2+} is the sole factor in the dramatic differences observed in inhibition of glutamate-induced polymerization, then there is a significant amplification effect, for there is only a 3-fold reduction in the drug affinity for tubulin with the cation (cf. K_A values presented above). The dissociation rate constant calculated from the association rate and equilibrium constants is consistent with rapid dissociation of the tubulin−2ME complex.

Using [^{3}H]2ME we were able to demonstrate increasing incorporation of the estrogen metabolite into tubulin polymer induced by glutamate/$MgCl_2$ as the 2ME concentration increased. The amount of drug bound to polymer was almost identical whether the 2ME was present initially in the reaction mixture or added when net assembly was essentially complete. The enhanced temperature stability of polymer observed at high 2ME concentrations was also independent of the time of drug addition. These observations, together with the binding properties just described, suggest the following overall model for the effects of 2ME on tubulin polymerization in glutamate/$MgCl_2$. The drug binds rapidly to tubulin at low temperatures, thus inhibiting the rate of assembly as the reaction mixture is warmed. Rapid drug dissociation from the tubulin heterodimer results in little overall effect on net assembly. The 2ME then binds to the polymer as it is formed, enhancing its temperature stability. Some copolymerization of the tubulin−2ME complex with unliganded tubulin may occur, but evoking such a reaction is not required to explain the effects of 2ME on the properties of glutamate-induced polymer.

Two aspects of the binding of 2ME to glutamate polymer merit particular attention: first, the relative inability of colchicine site drugs to inhibit the reaction; second, the substantial differences in the Scatchard pattern obtained when polymer binding and dimer binding were analyzed.

The strongest inhibitory effect was seen with the potent colchicine site drug CS-A4. However, even 100 μM CS-A4 inhibited less than 50% of the binding of 10 μM [^{3}H]-2ME to polymer, as compared to the greater than 90% inhibition by 5 μM CS-A4 of 2ME binding to tubulin heterodimer. This may indicate that the polymer 2ME site differs from the classic colchicine site on tubulin, or, perhaps, that 2ME binds to the low-affinity colchicinoid site(s) described by several groups (Williams et al., 1983; Ray et al., 1984; Ringel & Sternlicht, 1984; Deinum & Lincoln, 1986; Hastie & Macdonald, 1990).

Scatchard analysis of the binding data of [^{3}H]2ME to glutamate polymer, however, indicated a single class of binding site, with a stoichiometry of 1.8 (Figure 6), in contrast to the substoichiometric stoichiometry of binding to the $\alpha-\beta$ dimer, both with and without Mg^{2+} (Figures 3 and 4). This can be interpreted in a number of ways, including, as the simplest possibilities, (i) as binding to both classes of colchicine site, both of which have low affinity in polymer versus dimer (K_D of 91 μM for polymer versus 6 μM for dimer in glutamate/$MgCl_2$), and (ii) as binding to entirely new low-affinity sites that develop as a consequence of conformational changes in tubulin that result from assembly. At present, we have no data that allow us to choose between these models. Thus, it is unclear whether the 2ME binding site on the $\alpha-\beta$ dimer is involved in the binding of 2ME to polymer, because of the major difference in the effects of colchicine site drugs as inhibitors of the two reactions. If binding both to heterodimer and to polymer is at the "colchicine site(s)" broadly defined, then the relative affinities of different drugs for the site must change when tubulin molecules enter polymer. Some evidence for this exists with colchicine derivatives. In contrast to the poor binding of [^{3}H]colchicine to microtubules (Wilson & Meza, 1973; Lee et al., 1974), two B-ring colchicine derivatives have been described that bind along the entire length of microtubules (Zimmermann & Doenges, 1981; Hiratsuka &

Kato, 1987). One derivative had peroxidase activity, permitting electron microscopic localization (Zimmermann & Doenges, 1981); the other was intensely fluorescent (Hiratsuka & Kato, 1987).

Since the effect of 2ME in enhancing polymer stability to cold was qualitatively similar to that of paclitaxel (Taxol) (paclitaxel, however, causes more rapid, not slower, assembly in vitro), we performed initial experiments examining potential cross-inhibition of radiolabeled 2ME and paclitaxel binding to polymer. We observed partial inhibition by paclitaxel of [^{3}H]2ME binding to tubulin (quantitatively similar to the effect of CS-A4), but no inhibitory effect of 2ME on [^{3}H]paclitaxel binding (data not presented). This might indicate some overlap in binding sites and is consistent with the much higher affinity of paclitaxel for polymer (apparent K_D value of 0.8 μM; Parness & Horwitz, 1981).

The difficulty of demonstrating unambiguous effects of 2ME in MAP-dependent polymerization systems has been frustrating, in view of the initial identification of 2ME as an estrogen metabolite causing abnormal spindle formation and mitotic aberrations in cells in culture (Seegers et al., 1989; Cushman et al., 1995). Our negative observations have included negligible effects on assembly rate and plateau, polymer temperature stability, and polymer morphology, including no marked effect on microtubule length distribution. We did find evidence for substantial incorporation of [^{3}H]2ME into MAP-induced microtubules, but the significance of this observation was made less certain by an apparent high background of [^{3}H]2ME that appears to be associated with pelleted protein in the absence of GTP, a reaction condition in which microtubules do not form. Results from examination of other reaction conditions (glycerol- and Mes-induced assembly) that lead to microtubule formation favor the view that 2ME does bind relatively poorly to microtubules. MAPs had little effect in the glycerol system, appearing to eliminate the possibility that these proteins interfere directly with the binding of 2ME to tubulin in polymer. Moreover, in Pipes-induced assembly, where a polymer of mixed morphology is formed, an intermediate amount of [^{3}H]2ME was bound to polymer.

Overall these observations strongly suggest that [^{3}H]2ME binds more readily to sheets than to microtubules. It is tempting to speculate that binding may occur preferentially to the internal surface of the protofilament, which would be exposed in sheets but not in microtubules. The lower levels of binding in the microtubule systems might indicate binding occurs on the internal surface at polymer ends. However, the stoichiometry relative to tubulin observed in the microtubule systems is too high for this simple explanation. More likely the difference in apparent binding affinities is caused by differences in interprotofilament contacts in the sheet polymer as compared with the microtubule polymer.

The interaction of 2ME with both unpolymerized tubulin and polymer and its effects on interactions between microtubule components are strongly affected by reaction conditions. We next plan to study effects of 2ME on a more functional aspect of microtubule biochemistry and determine whether the drug alters interactions of motor proteins with microtubules.

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