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Interaction of Tubulin with Bifunctional Colchicine Analogues: An Equilibrium Study†

Jose M. Andreu, Marina J. Gorbunoff, James C. Lee, and Serge N. Timasheff*

ABSTRACT: The interaction of tubulin with simple analogues of colchicine that contain both its tropolone and trimethoxyphenyl rings has been characterized, and the results were analyzed in terms of the simple bifunctional ligand model developed for the binding of colchicine [Andreu, J. M., & Timasheff, S. N. (1982) *Biochemistry 21*, 534–543] on the basis of interactions of tubulin with single-ring analogues. The compound 2-methoxy-5-(2,3,4-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one has been found to bind reversibly to 0.86 ± 0.06 site of purified calf brain tubulin with an equilibrium constant of $(4.9 \pm 0.3) \times 10^5$ M^{-1} (25 °C), $\Delta H°_{app} = -1.6 \pm 0.7$ kcal mol^{-1}, and $\Delta S°_{app} = 20.5 \pm 2.5$ eu. The binding appears specific for the colchicine site. The closely related compound 2-methoxy-5-[[3-(3,4,5-trimethoxyphenyl)-propionyl]amino]-2,4,6-cycloheptatrien-1-one interacts weakly with tubulin. Binding of the first analogue is accompanied by ligand fluorescence appearance, quenching of protein fluorescence, perturbation of the far-ultraviolet circular dichroism of tubulin, and induction of the tubulin GTPase activity, similarly to colchicine binding. Substoichiometric concentrations of the analogue inhibit microtubule assembly in vitro. Excess analogue concentration under microtubule-promoting conditions induces an abnormal cooperative polymerization of tubulin, similar to that of the tubulin–colchicine complex.

The alkaloid colchicine arrests mitosis and binds slowly with high apparent affinity to its principal cellular receptor, tubulin. The colchicine binding activity of tubulin is labile, and the tubulin–colchicine complex dissociates very slowly, (Wilson & Bryan, 1974; Ludueña, 1979), rendering difficult the measurement of the colchicine–tubulin interaction by equilibrium techniques. The kinetic properties of colchicine binding to tubulin conform to a two-step mechanism consisting of a fast and reversible bimolecular binding reaction, followed by a slow monomolecular reaction that has been interpreted as a conformational change (Garland, 1978; Lambeir & Engelborghs, 1981). The tubulin–colchicine complex actually differs from the unliganded protein in several properties, namely, GTPase activity, quenched protein fluorescence, and perturbed circular dichroism, which suggests a different conformational state (Andreu & Timasheff, 1982c). On the other hand, colchicine itself also appears to undergo an isomerization upon binding to tubulin (Detrich et al., 1981). Tubulin liganded to colchicine inhibits substoichiometrically the self-assembly of unliganded protein into microtubules by binding to the ends of these polymers (Margolis & Wilson, 1977; Lambeir & Engelborgs, 1983). The precise molecular mechanism of this inhibition is not known and may involve a ligand-induced distortion of the correct protomer bonding geometry (Andreu & Timasheff, 1982b, 1983a).

The colchicine binding site of tubulin has been probed with simple single-ring analogues of the trimethoxyphenyl and tropolone active parts of the colchicine molecule. These bind to tubulin reversibly and specifically, although with small apparent standard free-energy changes, of −3 to −4 kcal mol^{-1} (Andreu & Timasheff, 1982a). These observations have led to the description of the high-affinity binding of colchicine to tubulin ($\Delta G°_{app} = -10.3 \pm 0.3$ kcal mol^{-1}, estimated from the published rate measurements at 37 °C) in terms of a simple thermodynamic model of the binding of a bifunctional ligand to two subsites on the protein binding site. In this model (Andreu & Timasheff, 1982a), the relatively weak interactions of the monofunctional ligands, i.e., the separate parts of the large one, can account quantitatively for the tighter binding of the bifunctional ligand if the entropic advantage of the latter is taken into account. The high-affinity binding of colchicine (see compound **1** in Chart I) appears, then, to be due to the simultaneous binding of its trimethoxyphenyl and tropolone rings, whereas the middle connecting ring may be the source of the peculiar kinetic characteristics of the interaction. According to this model, colchicine binds to tubulin first through its tropolone ring by means of hydrogen bonding or ring stacking (Lambeir & Engelborghs, 1981; Andreu & Timasheff, 1982a,c). This first event causes a structural change in tubulin that brings into proper position the trimethoxyphenyl ring binding subsite, permitting that ring to fall into place on the protein surface with which it interacts hydrophobically (Andreu & Timasheff, 1982a,c). The podophyllotoxin (Kelleher, 1977) and steganacin (Schiff et al., 1978) series of drugs have the trimethoxyphenyl ring in common with colchicine and probably bind to partially overlapping sites by sharing the trimethoxyphenyl binding subsite.

Colchicine has been used extensively as a tool for the in vivo study of microtubule-dependent processes, in spite of its slow binding to and dissociation from tubulin and, hence, its slow and poorly reversible microtubule-related effects. The bindings and dissociations of colcemide (Banerjee & Bhattacharyya, 1979) and podophyllotoxin (Cortesse et al., 1977) are also relatively slow. The therapeutic use of colchicine and podophyllotoxin is hampered by their high toxicity, a problem that has led to the study of a variety of complex derivatives [e.g., Brewer et al. (1979)]. On the other hand, the relatively simple compound nocodazole, which has no obvious structural re-

† From the Unidad de Biomembranas, Instituta Immunologia, C.S.I.C., Velazquez 144, Madrid-6, Spain (J.M.A.), the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254 (M.J.G. and S.N.T.), and the Department of Biochemistry, St. Louis University, St. Louis, Missouri 63104 (J.C.L.). *Received November 8, 1983.* This work was supported by grants from the National Institutes of Health, CA 16707 and GM 14603 (to S.N.T.) and NS 14269 and AM 21489 (to J.C.L.), and from the Fondo de Investigaciones Sanitarias y Comision Asesora de Investigaciones (to Program No. 42032 of C.S.I.C. research, 1981–1983). This is Publication No. 1473 from the Graduate Department of Biochemistry, Brandeis University. Preliminary reports of this work were presented at the International Symposium of Thermodynamics of Proteins (Granada, Spain, 1982), the 13th International Congress of Chemotherapy (Andreu & Timasheff, 1983b), and the 2nd Meeting of the European Cytoskeletal Club (Siena, Italy) (Andreu & Timasheff, 1983c).

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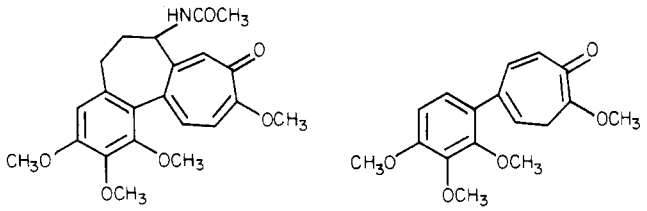
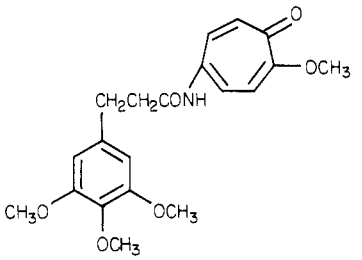
Chart I



(1) colchicine

(2) MTC



(3) MTPC

semblance to colchicine, binds rapidly and reversibly to tubulin in an apparently competitive manner with colchicine (Hoebke et al., 1976). This potentially useful antitumor agent (De Brabander et al., 1976) has proven itself as an excellent tool for investigating the role of microtuble organizing centers in vivo (De Brabander, 1982). The availability of relatively simple colchicine analogues that bind reversibly and with high affinity seems, therefore, to be of great interest.

With this aim in mind, we have undertaken the characterization of the interaction with purified tubulin of simple bifunctional ligands that contain only the trimethoxyphenyl (TMP)[1] and tropolone methyl ether (TME) rings of colchicine, connected by different arms. The compound 2-methoxy-5-(2,3,4-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one (MTC, compound **2** in Chart I), which contains the two rings joined by a carbon–carbon single bond, has been synthesized by Fitzgerald (1976), who has shown it to be nearly as powerful as colchicine in the inhibition of mitosis and in in vitro microtubule assembly assays. This compound has also been reported to bind rapidly to tubulin and to inhibit colchicine binding and microtubule assembly in a partially reversible manner (Ray et al., 1981). The compound 2-methoxy-5-[[3-(3,4,5-trimethoxyphenyl)propionyl]amino]-2,4,6-cycloheptatrien-1-one (MTPC, compound **3** in Chart I) consists of the same trimethoxyphenyl and tropolone moieties, but connected in a different manner by a propionamide spacer. The purpose of this study was to provide an experimental test of the bifunctional ligand model, to characterize further the colchicine binding site of tubulin, and to explore the effects of simple bifunctional analogues of colchicine on the structure of purified tubulin and its self-associations in vitro, since these ligands could be potentially useful tools for the reversible inhibition of microtubule-dependent processes in vivo.

Materials and Methods

Ligands and Other Materials. Colchicine and podophyllotoxin were from Aldrich Chemical Co. (lot nos. 16788 and 022757, respectively). Mescaline hydrochloride (lot no. 102C-1710) was from Sigma. GTP, dilithium salt, was from Boeringher-Manheim. Tropolone methyl ether was prepared as described previously (Nozoe et al., 1951; Andreu & Timasheff, 1982a). MTC (compound **2**) was prepared according to Fitzgerald (1976). It was subjected to thin-layer chromatography on 0.2 mm thick silica gel sheets (Alufolien, Merck) developed with pyridine–concentrated ammonia (9:1) or chloroform–acetone–diethylamine (7:2:1) and found to give a single spot under ultraviolet light. Overloading (40 μg of sample) allowed one to see a faint contaminant trace spot of lower relative mobility in the second solvent system. The concentration of MTC was measured spectrophotometrically. The extinction coefficient, ϵ, was determined by dissolving in PG buffer crystals of the compound dried in a vacuum oven and taking the UV spectrum. Four independent determinations gave $\epsilon_{343nm} = (1.76 \pm 0.01) \times 10^4$ M^{-1} cm^{-1} and $\epsilon_{238nm} = (3.38 \pm 0.04) \times 10^4$ M^{-1} cm^{-1}. MTPC (compound **3**) was synthesized by coupling (trimethoxyphenyl)propionyl chloride with 5-aminotropolone, followed by methylation of the OH group (M. J. Gorbunoff, unpublished results). Its concentration was determined spectrophotometrically with $\epsilon_{325nm} = 1.39 \times 10^4$ M^{-1} cm^{-1} (neutral aqueous buffer). [^{3}H]Colchicine was from New England Nuclear (37.2 Ci/mmol, lot no. 1433-276) and was found to be more than 93–95% radiochromathographically homogeneous in the thin-layer systems mentioned above. [γ-^{32}P]GTP, triethylammonium salt, was from Amersham (15–25 Ci/mmol by the time of use). Sephadex G-25 was from Pharmacia. [Ethylenebis(oxyethylenenitrilo)]tetraacetic acid was from Sigma. Ultrapure guanidine hydrochloride was obtained from Schwarz/Mann. Glycerol was from Merck, analytical grade, and all other chemicals were of reagent grade.

Protein. Calf brain tubulin was purified, stored in liquid nitrogen, and prepared for use as described previously (Weisenberg et al., 1968; Lee et al., 1973; Andreu & Timasheff, 1982a). Its concentration was measured spectrophotometrically with the extinction coefficients $\epsilon_{276} = 1.16$ L g^{-1} cm^{-1} (scattering-corrected absorbance in neutral aqueous buffer), $\epsilon_{275} = 1.09$ L g^{-1} cm^{-1} (6 M guanidine hydrochloride) (Andreu & Timasheff, 1982c), and $\epsilon_{275} = 1.07$ L g^{-1} cm^{-1} (0.4% sodium dodecyl sulfate in neutral aqueous buffer). The last value was obtained by comparison of the absorbance of solutions of identical protein concentration in the detergent and guanidine hydrochloride. The tubulin–colchicine complex was prepared as described previously (Andreu & Timasheff, 1982c).

Spectroscopic Measurements. Visible and ultraviolet absorption spectra were obtained with a Cary 16 spectrophotometer, with adjustment of the photomultiplier gain to give band widths typically smaller than 1 nm with the automatic-slit mechanism. Difference absorption spectra were obtained by using a pair of matched mixing tandem cells of 0.438 + 0.438 cm light path (Hellma) mounted in thermostated cell holders at 25 ± 0.5 °C. The base line was recorded with protein and ligand in separate cell compartments; then, the interaction spectrum was recorded after the solutions in the sample beam were mixed. At the end of the experiment, the solutions in the reference beam were also mixed, and the base line was checked again.

Fluorescence measurements were made in a Fica MK II double-beam spectrofluorometer that gives corrected excitation and emission spectra. Fluorescence cells (0.5 × 0.5 cm) were mounted on holders thermostated with a Lauda K2RD water bath, and the temperatures of the solutions inside the cells were measured with an Omega copper–constantan thermocouple connected to a Yew digital thermometer. The inner filter

[1] Abbreviations: PG, 10 mM sodium phosphate–0.1 mM GTP, pH 7.0; TME, tropolone methyl ether; TMP, trimethoxyphenyl; MTC, 2-methoxy-5-(2,3,4-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one; MTPC, 2-methoxy-5-[[3-(3,4,5-trimethoxyphenyl)propionyl]amino]-2,4,6-cycloheptatrien-1-one.

effect was minimized by using sample absorbances <0.05 or otherwise corrected by the graphical procedure of Mertens & Kägi (1979). Fluorescence is expressed in arbitrary units that correspond throughout this paper to the spectrofluorometer output (millivolts) with the reference potential set at 600 V and a gain of 50.

Circular dichroism spectra were obtained with a Rousell-Jouan Dicrograph II, in 0.1- and 1.0-cm cells at 25 ± 0.5 °C. The difference dichroic absorption ($A_L - A_R$) was employed to calculate the mean residue molar ellipticity, [θ], from the relation $[\theta] = 3300(A_L - A_R)c^{-1}d^{-1}$, where c is the mean residue concentration and d the light path (Adler et al., 1973). A mean residue weight of 109 was used (Lee et al., 1978).

Binding Measurements. Binding Measurements were done by the equilibrium gel chromatography technique of Hummel & Dreyer (1962), as carefully applied previously to tubulin (Andreu & Timasheff, 1982a). Here, the ligand concentration in the column effluent was measured spectrophotometrically. Such a procedure relies frequently on the measurement of small absorbance increments due to bound ligand and is, therefore, subject to several potential sources of error: interference of light absorption by the protein, absorption increments due to protein effects on bound ligand, significant light scattering by the protein solution, and instrumental noise. In our case, the ligand concentration measurements were made at 335–342 nm, where tubulin does not absorb light. The second effect was not large and could be easily overcome with MTC, if needed, by measuring the ligand absorbance at 340 nm, that is, an isosbestic point of the interaction difference spectrum (see Results and Figure 1). The third source of error is a systematic one and can be important in proteins with a tendency to aggregate, like tubulin. The second and third effects were easily avoided by addition of a small volume of concentrated sodium dodecyl sulfate to each effluent fraction, giving a final concentration of 0.4% detergent; this displaced the protein–ligand interaction and solubilized any aggregated protein (giving typically $A_{350} < 0.001$ in the absence of ligand; see Figure 7). Finally, noise was reduced by carefully repeated concentration measurements through the whole column effluent, leading to a good statistical estimation of the base-line concentration. The absorption increments due to bound ligand were estimated by simple subtraction as before (Andreu & Timasheff, 1982a) or, better, by difference spectrophotometric measurements of the protein-containing fractions vs. the noncontaining ones (see Figure 7).

Fluorometric binding titrations were performed as follows. The fluorescence of free ligand was measured, and its value, practically negligible for MTC, was subtracted from any further results. Then a concentration of ligand giving no appreciable inner filter effect was titrated with tubulin until the fluorescence due to bound ligand reached saturation. This procedure yielded the fluorescence intensity of bound ligand (i.e., 9.7 ± 0.3 units/10^{-6} M bound MTC in PG buffer at pH 7.0 at 25 °C with excitation at 350 nm and emission at 423 nm; two different tubulin preparations were used). Aliquots of a protein solution were then titrated with known total concentrations of ligand, the amount of bound ligand was measured by its inner filter corrected fluorescence, and the free-ligand concentration was taken as the difference between total and bound concentrations. Above 10^{-5} M MTC, the inner filter correction became larger than 5% of the fluorescence values. Since these conditions were not far from binding saturation, this correction is believed not to have caused any large errors. The values of the binding equilibrium constant and the number of sites were obtained from Scatchard plots of the saturation data. Approximate stoichiometries were estimated by the continuous-variation method (Asmus, 1961).

Quenching of the intrinsic protein fluorescence by ligand was also employed to estimate the binding affinity. The maximal fluorescence quenching by excess ligand was measured, correcting for the large inner filter effect by the procedure of Mertens & Kägi (1981). Then, the fraction of sites occupied, θ, was taken as equal to the fraction of the maximal quenching effect at a given total ligand concentration. The binding equilibrium constant was determined by employing the relationship $\theta/(1 - \theta) = K_b[A]$ (Lehrer & Fasman, 1966), where [A] is the free-ligand concentration calculated from the total ligand and protein concentrations and θ, on the assumption of a binding stoichiometry of one.

Difference spectrophotometric titrations were performed at wavelengths at which only ligand absorbed light. The increment in the ligand extinction coefficient due to binding, $\Delta\epsilon = \epsilon$(bound) − ϵ(free), was determined from extrapolation of a titration with excess protein. Then aliquots of a protein solution were titrated with known total concentrations of ligand, the bound ligand concentration was determined as Δabsorbance/$\Delta\epsilon$, and the free-ligand concentration was estimated from the difference. The binding equilibrium constant and stoichiometry were obtained from Scatchard plots of the data.

Miscellaneous Procedures. Colchicine binding to tubulin was followed by the fluorescence of bound colchicine (Bhattacharyya & Wolf, 1974; Andreu & Timasheff, 1982a,c). The rates of binding of [^{3}H]colchicine to tubulin were measured as follows. The labeled ligand and protein were incubated in a final volume of 0.1 mL of PG buffer at 37 °C for given times, and the binding reaction was stopped by the addition of cold buffer and immediate chromatography on 10 × 0.9 cm Sephadex G-25 columns, run at approximately 100 mL/h PG buffer with the aid of a peristaltic pump. This procedure gave in a few minutes a reasonable separation of free ligand from bound ligand and protein, concentrations of which were measured by liquid scintillation and absorbance at 276 nm, respectively. Ligand-induced tubulin GTPase activity was assayed by the release of [^{32}P]phosphate, as described previously (Andreu & Timasheff, 1981) in 10 mM sodium phosphate–2 mM $MgCl_2$ buffer, pH 7.0, containing 0.1 mM [γ-^{32}P]GTP, at 37 °C.

Sedimentation velocity experiments were performed in a Beckman Model E analytical ultracentrifuge equipped with electronic speed control. Identical samples, with and without ligand, were run simultaneously in double-sector cells in an An-D rotor at 56 000 rpm. The in vitro assembly of microtubules was performed in 10 mM sodium phosphate, 0.1 mM GTP, 1 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid, 16 mM $MgCl_2$, and 3.4 M glycerol, pH 7.0 buffer (assembly buffer) at 37 °C in a thermostated cuvette, and the mass of polymer formed was followed turbidimetrically (Lee & Timasheff, 1977) with a Varian 635 spectrophotometer. The polymers formed were fixed with 0.5% glutaraldehyde, adsorbed to carbon-coated grids, negatively stained with 2% uranyl acetate, and examined under a Jeol 100 B electron microscope.

Results

Determination of the Interaction of Bifunctional Colchicine Analogues with Tubulin by Difference Absorption and Fluorescence Spectroscopy. The interaction of tubulin with compounds **2** and **3** (Chart I) was first examined by difference absorption spectroscopy, and the results are displayed in Figure 1. A solution of 3.1 × 10^{-5} M MTC and 4.0 × 10^{-6} M tubulin

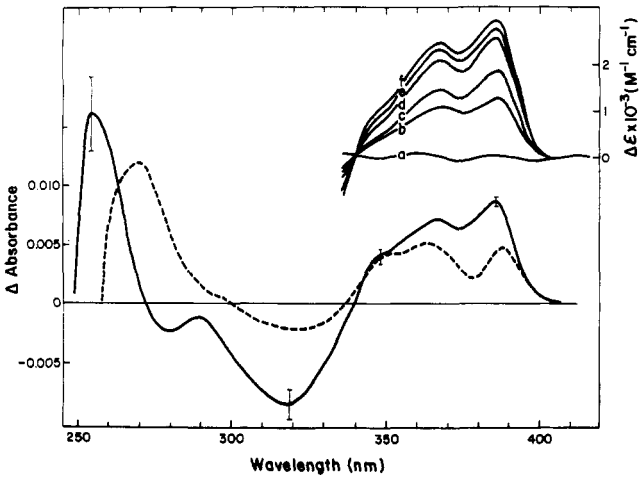


FIGURE 1: Difference absorption spectra generated by the interaction of tubulin with MTC in PG buffer, pH 7.0 at 25 °C: (—) difference spectrum of 3.1×10^{-5} M MTC and 4.0×10^{-6} M tubulin vs. ligand and protein in separate solutions. The difference spectrum (– –) generated by 1.6×10^{-5} M colchicine and 1×10^{-5} M tubulin (Andreu & Timasheff, 1982c) is shown for comparison. The 350–400-nm spectra in the upper right-hand corner were generated by 9.5×10^{-6} M MTC with (a) no tubulin, (b) 6.9×10^{-6} M tubulin, (c) 1.37×10^{-5} M tubulin, (d) 2.72×10^{-5} M tubulin, (e) 4.02×10^{-5} M tubulin, and (f) 6.55×10^{-5} M tubulin.

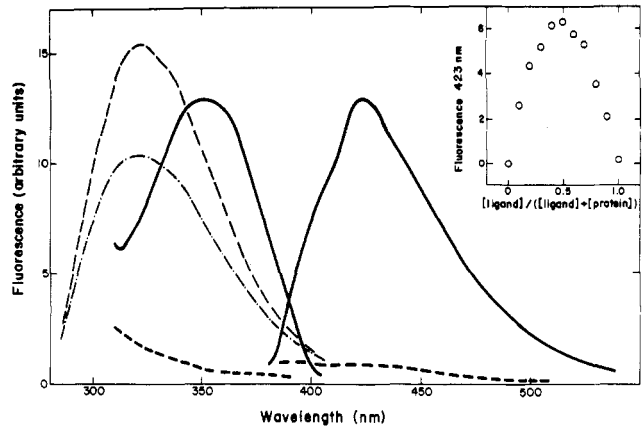


FIGURE 2: Fluorescence changes produced by the tubulin–MTC interaction: (—) fluorescence excitation (left, emission analyzed at 423 nm) and emission (right, excitation at 350 nm) spectra of a solution containing 4×10^{-6} M tubulin equilibrated with 1×10^{-5} M free MTC by means of gel chromatography (see Materials and Methods). (---) Blank of 4×10^{-6} M tubulin without ligand. Ligand alone gave negligible fluorescence. (– –) Fluorescence emission spectrum of 4×10^{-6} M tubulin (excitation at 278 nm) displayed on a 25×-reduced scale. (–·–) Emission spectrum of 4×10^{-6} M tubulin equilibrated with 1×10^{-5} M MTC. The inset shows the bound ligand fluorescence of solutions in which the sum of the total concentrations of ligand and protein was maintained at 9.9×10^{-6} M while varying their proportion.

vs. ligand and protein in separate solutions generated the spectrum shown by the solid line. This is characterized by maxima at 256, 291, 366, and 387 nm, a shoulder at 345 nm, minima at 278 and 319 nm, and an isosbestic point in the vicinity of 340 nm. This spectrum is closely related to the colchicine–tubulin interaction spectrum, shown by the dashed line (Andreu & Timasheff, 1982c), in which the perturbations in the 300–400-nm region were attributed to the binding of the tropolone ring of the ligand to tubulin. The upper right-hand part of Figure 1 displays a titration of 9.5×10^{-6} M MTC with increasing concentrations of tubulin, namely, 0 (tracing a), 6.90×10^{-6} (b), 1.37×10^{-5} (c), 2.72×10^{-5} (d), 4.02×10^{-5} (e), and 6.55×10^{-5} M (f). Extrapolation of these measurements to infinite protein concentration gave the extinction coefficient increment, $\Delta\epsilon_{387} = 3460 \pm 100$ M^{-1} cm^{-1}, due to the binding of this compound to tubulin. There is an approximately 2-fold increase in absorption from free to bound ligand at this wavelength. On the other hand, 10^{-4} M MTPC (compound **3**, chart I) and tubulin did not generate any difference spectrum significantly distinct from the base line under the same experimental conditions.

Examination of the fluorescence of tubulin, MTC, and mixtures of the two, presented in Figure 2, showed that the free ligand (10^{-5} M) exhibited negligible fluorescence, while bound ligand was fluorescent in the presence of 4×10^{-6} M tubulin, with excitation and emission maxima at 350 and 423 nm, respectively, as shown by the solid lines. This effect is similar to the fluorescence of colchicine bound to tubulin (Bhattacharyya & Wolf, 1974; Andreu & Timasheff, 1982c). In the present case, however, the binding time course could not be followed in a conventional fluorometer, since the emission intensity reached more than 90% of its maximal value in less than 1 min. Similarly, attempts to follow the dissociation of ligand by simple dilution experiments were unsuccessfull due to the practical completion of the reaction within 1 min. These observations imply an apparent bimolecular association rate constant greater than 3×10^3 M^{-1} s^{-1} and a dissociation rate constant greater than 0.2 s^{-1}. The upper right-hand insert of Figure 2 depicts a continuous-variation experiment. It is seen that the maximum in the fluorescence occurs at a [ligand] to ([ligand] + [protein]) ratio very close to 0.5, indicating the formation of a one-to-one complex (Asmus, 1961) between tubulin and MTC. The interaction of this compound with tubulin produced also quenching of the intrinsic fluorescence of the protein, as shown clearly by comparison of the unliganded and liganded protein emission spectra (the long dashed line and the dot–dash line, respectively, in Figure 2). This quenching effect is similar to the quenching of protein fluorescence in the tubulin–colchicine complex (Andreu & Timasheff, 1982c). When the fluorescence of 10^{-4} M MTPC in the presence of tubulin was examined, no ligand fluorescence enhancement due to binding could be observed.

Specificity of the Interactions. Once it had been established that MTC interacts with tubulin generating difference absorption and fluorescence spectra, while no such spectra were obtained with the similar compound MTPC, it was of interest to ask whether (i) the binding of MTC was specifically directed to the colchicine site and (ii) MTPC was capable of a low-affinity binding to tubulin, similar to that of the monofunctional colchicine analogues (Andreu & Timasheff, 1982a). The first question was approached by examining the binding of MTC to the tubulin–colchicine complex. A difference fluorescence experiment was performed in which aliquots of a tubulin solution were placed into the reference and sample positions of the double-beam spectrofluorometer and MTC was added to the sample solution. As shown in Figure 3, tracings a and b, the expected fluorescence developed when the unliganded tubulin was employed, while no fluorescence increment was detected with the tubulin–colchicine complex. Since colchicine bound to tubulin is released by photoconversion to lumicolchicine and the deliganded protein retains a partial colchicine binding activity (Andreu & Timasheff, 1983a), a tubulin–colchicine solution (spectrum c in Figure 3) was irradiated, and MTC was added to the system. It is seen that the fluorescence, which had decreased to spectrum d on irradiation, increased again on addition of the ligand to a spectrum characteristic of the tubulin–MTC complex (spectrum e). The simplest interpretation of these results is

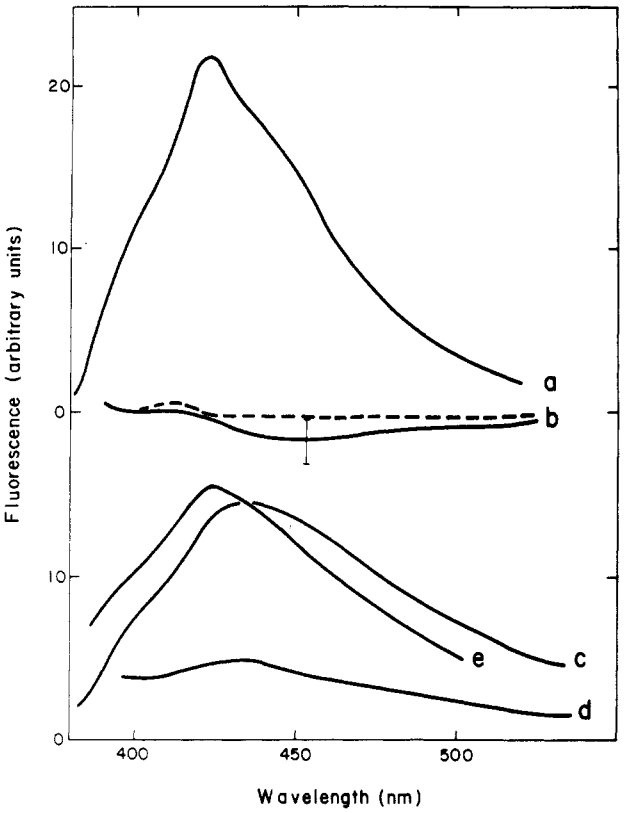


FIGURE 3: Inhibition by colchicine of the binding of MTC. (a) Difference emission spectrum (excitation at 350 nm) of 1.1×10^{-5} M tubulin and 5×10^{-6} M MTC vs. 1.1×10^{-5} M tubulin. (b) Difference emission spectra of 1.1×10^{-5} M tubulin–colchicine complex and 5×10^{-6} M MTC vs. 1.1×10^{-5} M tubulin–colchicine complex; the dashed line corresponds to the latter solutions without MTC addition. (c) Direct emission spectrum of 1.1×10^{-5} M tubulin–colchicine complex. (d) Spectrum of the same solution after irradiation with long-wavelength ultraviolet light. (e) Same solution as (d) after addition of 5×10^{-6} M MTC.

that MTC does not bind to the tubulin–colchicine complex but does bind to the sites left vacant when colchicine is converted to lumicolchicine.

The effects of MTC on the fluorescence time course of colchicine binding were also examined. The results are presented in Figure 4, where tracing a is the fluorescence time course generated by the binding of 2×10^{-4}M colchicine to 1.1×10^{-5} M tubulin at 25 °C. Increasing concentrations of MTC in the solution (tracings b–d correspond to 2.5×10^{-6}, 1.24×10^{-5}, and 2.48×10^{-5} M analogue, respectively) produced progressively higher initial fluorescence values and reduced slopes, most probably due to the fast binding of this compound to the colchicine site. At later times, the time courses converged to practically the same fluorescence value, probably by displacement of the analogue by the kinetically irreversible binding of colchicine, which was present in excess concentration. Tracing e was obtained with 2.48×10^{-4} M MTC without colchicine. At the point indicated by the arrow, 4×10^{-4} M podophyllotoxin was added, and the displacement of MTC by podophyllotoxin was followed. In the sample recorded as f, tubulin was incubated for 3 min with 2×10^{-4} M podophyllotoxin prior to the addition of 5×10^{-6} M MTC.

To establish further the inhibition of colchicine binding by MTC and to answer the second question, the effects of inhibitors on the initial binding rate of 5×10^{-6} M [^{3}H]colchicine to tubulin at 37 °C were examined by the fast exclusion chromatography binding assay (see Materials and Methods). The binding reaction was found to be linear with time up to 3 min and with tubulin concentration higher than 1.5×10^{-5}

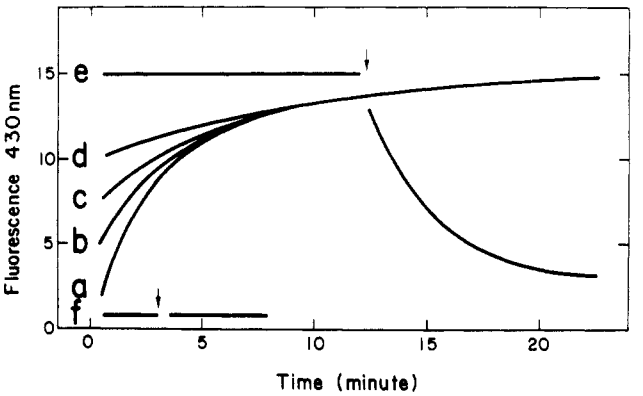


FIGURE 4: Fluorescence time course of 2×10^{-4} M colchicine binding to 1.1×10^{-5} M tubulin at 25 °C (excitation at 360 nm): (a) no other addition; (b) 2.5×10^{-6} M MTC; (c) 1.24×10^{-5} M MTC; (d) 2.48×10^{-5} M MTC; (e) contained no colchicine but 2.48×10^{-4} M MTC, and 4×10^{-4} M podophyllotoxin was added at the time indicated by the arrow; (f) contained no colchicine but 2×10^{-4} M podophyllotoxin, and 5×10^{-6} M MTC was added at the time indicated by the arrow.

M, in agreement with Garland (1979). This made possible the use of the inhibition of the initial colchicine binding rate to estimate the occupancy of the colchicine site by competing compounds (Andreu & Timasheff, 1982c). Figure 5A shows the inhibition of the initial stages of colchicine binding by MTC at micromolar concentration and by MTPT and TME at millimolar concentrations. Figure 5B displays the inhibition of the initial colchicine binding rate as a function of the concentration of the low-affinity competitors TME, MTPC, and mescaline. The concentrations plotted are to a good approximation free-ligand concentrations, since ligands are in a ≥300 times molar excess over protein. The three compounds are shown to inhibit in a roughly similar manner within the experimental error of the measurements, which were limited also by the solubility of MTPC. The solid line is a theoretical curve for an inhibitor binding with an equilibrium constant of 50 M^{-1} to the colchicine site. This approximate value is about 6 times smaller than the equilibrium constants previously estimated for the tropolone and mescaline ligands (Andreu & Timasheff, 1982a,c). Similarly, in Figure 5A, the inhibitory effect of MTC is weaker than that on the initial slope of the colchicine fluorescence time course of Figure 4. Thus, while the [^{3}H]colchicine competition binding assay was somewhat less sensitive to the competitors than the other procedures, its results confirmed the low-affinity inhibition of colchicine binding by the monofunctional ligands tropolone methyl ether and mescaline (Andreu & Timasheff, 1982a) and indicated that MTPC interacts with tubulin and may bind to the colchicine site in a similarly weak manner.

Binding Equilibrium Parameters. The quantitative characterization of the binding equilibrium of MTC to tubulin was performed by the careful application of the equilibrium gel chromatography technique of Hummel & Dreyer (1962) (see Materials and Methods). Figure 6 shows typical elution profiles obtained in the chromatography of tubulin on columns equilibrated with 1.24×10^{-6} (profile a) and 1.63×10^{-5} M (profile b, circles) MTC at 25 °C, pH 7.0. The good separation of the peak and trough was taken as an indication of the attainment of equilibrium. The elution of ligand bound to tubulin could be followed also by its fluorescence as shown by the squares in profile b. When an experiment identical with b was performed in the presence of an excess of podophyllotoxin, no binding of MTC was detected, as shown by profile c. This is in agreement with the fluorescence competition results of Figure 4. Similarly, when the same experiment (b)

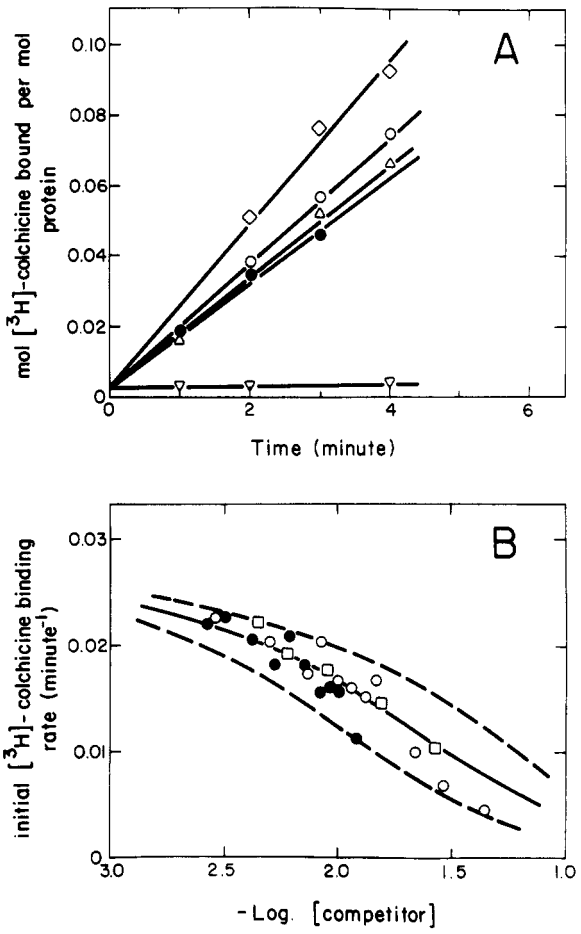


FIGURE 5: (A) Initial binding time course of 5×10^{-6} [^{3}H]colchicine to 10^{-5} M tubulin at 37 °C (see Materials and Methods): (◇) no other addition; (△) 2.48×10^{-5} M MTC; (●) 6.7×10^{-3} M MTPC; (○) 1×10^{-2} M TME; (▽) binding to 10^{-5} M tubulin–colchicine complex. (B) Inhibition of initial colchicine binding rate by TME (○), MTPC (●), and mescaline (□); measurements were made at 2 and 3 min of reaction and are expressed in moles of colchicine bound per mole of tubulin per minute. The solid line is the theoretical inhibition curve for a compound binding rapidly to the colchicine site with an equilibrium constant of 50 M^{-1}. The upper and lower dashed lines correspond to equilibrium constants of 25 and 100 M^{-1}, respectively.

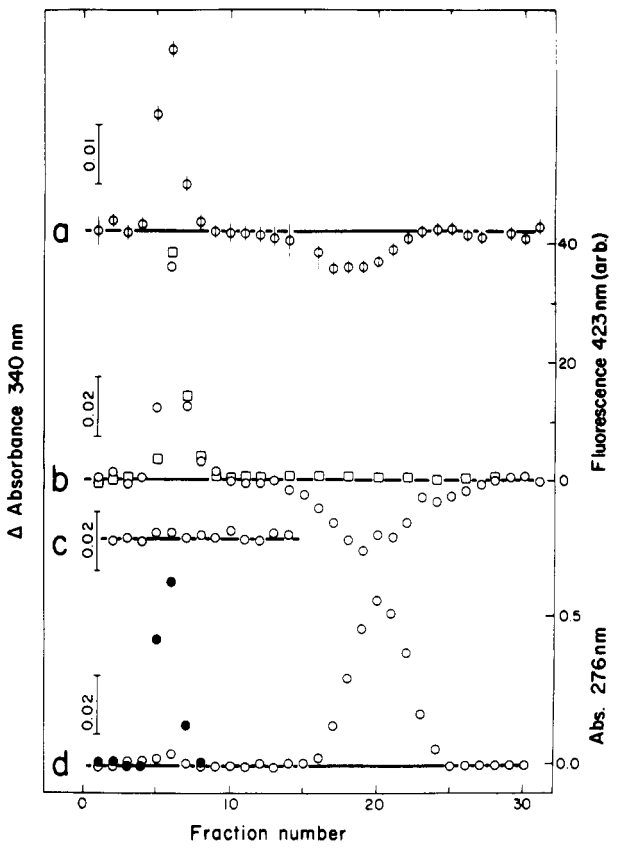


FIGURE 6: Gel chromatography of tubulin in columns equilibrated with MTC. (a) A total of 18 nmol tubulin was chromatographed in a column equilibrated with 1.24×10^{-6} M free MTC in PG buffer, pH 7.0, 25 °C; absorbance measurements were done in duplicate, and bars indicate experimental error. (b) A total of 18 nmol of tubulin in 1.63×10^{-5} M MTC; the circles are the absorbance increments, and the squares are the fluorescence of the fractions (right ordinate). (c) Same as (b) except that the elution buffer contained 1.63×10^{-5} M MTC and 2×10^{-4} M podophyllotoxin. (d) A total of 0.7 mL of solution containing 3.8×10^{-5} M tubulin and 8.8×10^{-5} M MTC was chromatographed in a column without ligand; the open circles correspond to the ligand elution profile and the filled circles to the protein elution (right ordinate). The column size was 0.9×24 cm, the flow rate was 40 mL/h, and fractions were approximately 1.5 mL in all cases (see Materials and Methods for binding measurements).

was performed with the tubulin–colchicine complex substituted for tubulin, the ligand absorption increment in the protein elution peak could be completely accounted for by the bound colchicine within experimental error. When tubulin was preincubated with an excess of MTC and run in a column without this ligand, no significant ligand absorption was detected in the void volume, as shown by profile d (open circles), indicating complete dissociation of the tubulin–MTC complex. This is in agreement with the fluorescence results, which involve shorter observation times. The use of the column technique with spectrophotometric ligand concentration measurements is subject to several possible errors (see Materials and Methods). It was, therefore, necessary to ascertain that the absorption increments measured were actually due to ligand. For this purpose, absorption spectra of the protein-containing fractions of the effluent were measured vs. noncontaining ones (i.e., fraction 6 vs. 3 in Figure 6). Typical results are displayed, without base-line corrections, in Figure 7. It can be seen that the absorption at 340 nm is due to MTC, the contribution of protein light scattering at this wavelength having been rendered insignificant by the appropriate experimental approach (see legend to Figure 7 and Materials and Methods). This procedure also affords a good

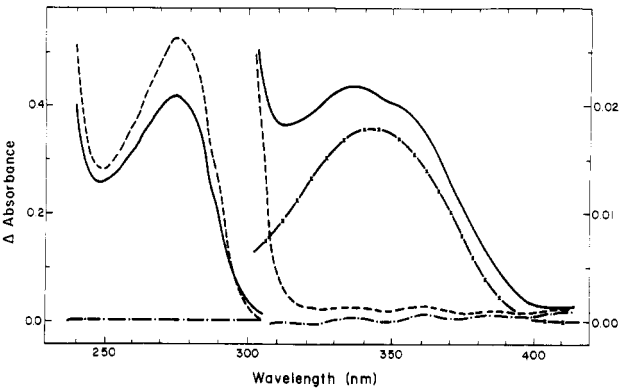


FIGURE 7: Absorption spectra of protein-containing column fractions vs. noncontaining ones after the addition of sodium dodecyl sulfate (see Materials and Methods). (—) Fraction 6 of run a (Figure 6; 1.24×10^{-6} M MTC) vs. fraction 3 of the same run; below 305 nm the absorbances are read on the left ordinate and above 305 nm on the right ordinate. (– –) Fraction 6 vs. fraction 3 of a similar run in a column without ligand. (–·–) Base line obtained by measuring fraction 3 vs. itself. (–×–) Direct base-line-corrected absorption spectrum of MTC in PG buffer, shown for comparison.

sensitivity, enabling the detection of relatively small binding effects. Application to the interaction of 10^{-4} M MTPC with

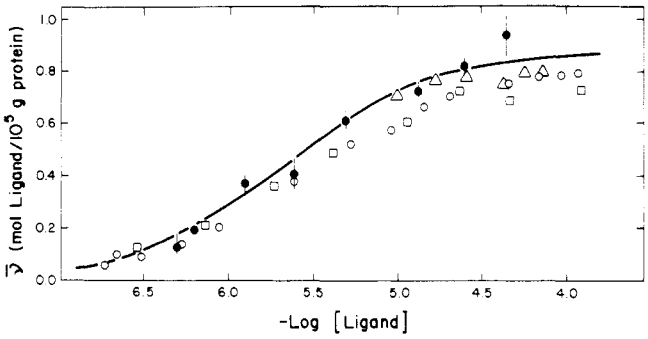


FIGURE 8: Binding isotherm of MTC to tubulin in PG buffer, pH 7.0, 25 °C. Solid circles (●) are column measurements (see Figures 6 and 7 and Materials and Methods) at a protein concentration of (0.5–1.2) × 10^{-5} M. Open symbols are binding measurements from ligand fluorescence at (see Material and Methods and Figure 2) (□) 1.8 × 10^{-6}, (○) 5.3 × 10^{-6}, and (△) 3.6 × 10^{-5} M protein. The solid line is a fit to the column measurements (K_b = 4.8 × 10^5 M^{-1}; n = 0.88) obtained from a Scatchard plot of these data.

Table I: Binding of MTC to Tubulin at 25 °C

method and protein concn (M)	K_b (M^{-1})	$\bar{\nu}$ (mol of ligand/10^5 g of tubulin)
gel chromatography (5 × 10^{-6} to 1.2 × 10^{-5})	(4.9 ± 0.3) × 10^5	0.86 ± 0.06
ligand fluorescence (1.8 × 10^{-6} to 3.6 × 10^{-5})	(4.6 ± 0.6) × 10^5	0.77 ± 0.08
protein fluorescence quenching (1.8 × 10^{-7} and 1.8 × 10^{-6})	4.8 × 10^5	*a*
ligand difference absorption spectrophotometry (5.5 × 10^{-6})	3.9 × 10^5	0.61

[a] Information not afforded by this procedure; a stoichiometry of one was assumed.

tubulin at 0, 25, and 37 °C gave no significant deviations from the base line in the individual experiments, indicating that binding would be characterized by an equilibrium constant smaller than 10^3 M^{-1}.

The binding isotherm of MTC to tubulin at pH 7.0, 25 °C, is shown in Figure 8, where the filled circles are the individual column technique measurements and the solid line corresponds to a binding equilibrium constant, K_b = 4.9 × 10^{-5} M^{-1}, and 0.86 site/tubulin dimer, as estimated from a Scatchard plot of the data. The results of the ligand fluorescence titrations (see Materials and Methods), performed under the same conditions with 1.8 × 10^{-6} to 3.6 × 10^{-5} M tubulin, are shown by the open symbols in Figure 8. It is not known whether the small deviation suggested at high ligand concentrations (namely, ca. 0.1 mol of ligand/mol of protein) is real or a consequence of the fluorescence inner filter corrections applied (see Materials and Methods). The binding of MTC to tubulin was also followed by the protein fluorescence quenching procedure (see Materials and Methods) at low saturation and protein concentrations, and the results are shown in Figure 9A. The marked change in absorbance at 387 nm of MTC upon binding to tubulin (Figure 1) was employed in the spectrophotometric titration presented in Figure 9B in the form of a Scatchard plot. A summary of the results obtained by means of the four different procedures is presented in Table I. A good agreement between the different measurements can be seen, and there is no trend with protein concentration. Averaging the values of the apparent standard free-energy changes ($\Delta G°_{app} = -RT \ln K_b$) resulted in a value of −7.72 ± 0.05 kcal mol^{-1} for the interaction of MTC with tubulin at pH 7.0, 25 °C.

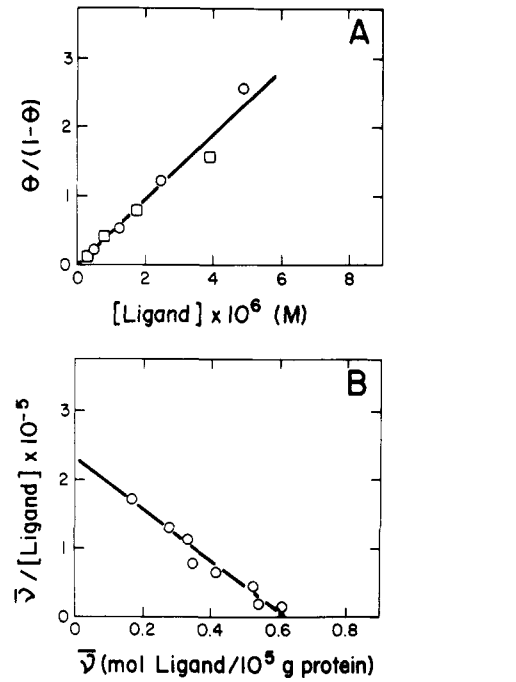


FIGURE 9: (A) Protein fluorescence quenching titration of the binding of MTC to (○) 1.8 × 10^{-7} and (□) 1.8 × 10^{-6} M tubulin at 25 °C (see Figure 2 and Materials and Methods). (B) Difference spectrophotometric titration of the binding of MTC to 5.5 × 10^{-6} M tubulin at 25 °C. The information contained in Figure 1 and differential absorbance measurements at 387 nm at various ligand concentrations were employed in this titration, presented as a Scatchard plot (see Materials and Methods).

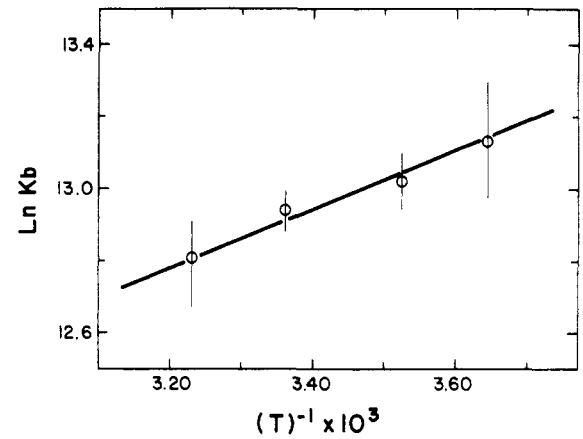


FIGURE 10: van't Hoff plot of MTC binding to tubulin. Apparent binding equilibrium constants were measured by the ligand fluorescence procedure at each temperature (see Material and Methods). Vertical bars indicate experimental error.

The binding of MTC to tubulin was found to be weakly dependent on temperature, as shown by the van't Hoff plot of Figure 10, which was obtained by ligand fluorescence titrations at different temperatures. The apparent thermodynamic parameters obtained were $\Delta H°_{app}$ = −1.6 ± 0.7 kcal mol^{-1}, $\Delta S°_{app}$ = 20.5 ± 2.5 eu, and $\Delta C_p°$ = 0 cal deg^{-1} mol^{-1}, giving apparent standard free-energy changes varying from −7.3 to −7.9 kcal mol^{-1} over the range of temperatures studied.[2]

Effects of MTC Binding on the Conformational Parameters and Self-Associations of Tubulin. In view of the similarities

[2] After submission of this paper, we learned of related work by Bane et al. (1984) on the binding of MTC to tubulin. They report an association constant similar to that reported here, but different values of $\Delta H°$ and $\Delta S°$. These seem best ascribable to the difference in buffer compositions.

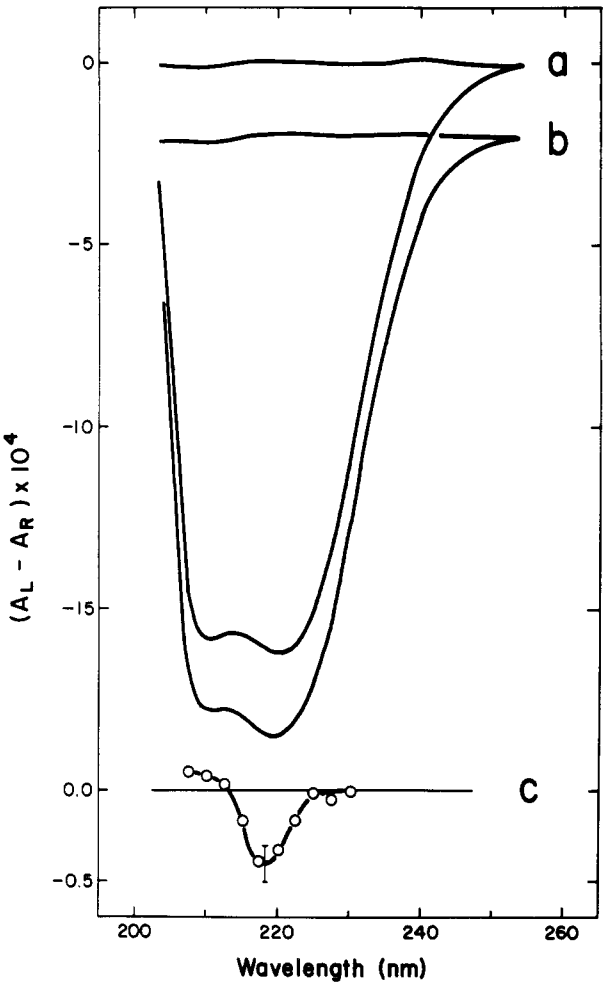


FIGURE 11: Circular dichroism spectra of 5.9×10^{-6} M tubulin at 25 °C: (a) no ligand added; (b) in the presence of 1.65×10^{-5} M MTC (left minus right circularly polarized light absorption spectra are shown with their corresponding base lines); (c) result of subtracting (a) from (b), amplified 5-fold.

between the interactions of colchicine and its analogue, MTC, with tubulin, and since colchicine is believed to induce a structural change in this protein manifested by a small perturbation of the protein circular dichroism at 217 nm and the appearance of GTPase activity (Andreu & Timasheff, 1982c), these properties were examined in tubulin liganded to MTC. Figure 11 shows the far-ultraviolet circular dichroism spectra of tubulin unliganded (a) and in the presence of MTC (b). There are small but significant differences between the two spectra, shown clearly by the calculated difference spectrum of profile c with an ellipticity increment of -250 ± 50 deg cm^2 dmol^{-1} at 217 nm. The ratio $[\theta]_{220}/[\theta]_{210}$ changed from 1.030 ± 0.005 for unliganded tubulin to 1.056 ± 0.006 in the presence of MTC. These changes are very similar to those previously reported for tubulin liganded to colchicine (Andreu & Timasheff, 1982c). No marked changes due to the tubulin–MTC interaction were observed in the near-ultraviolet circular dichroism. In particular, there was no prominent MTC circularly dichroic band in the 300–400-nm region, both in the absence and the presence of tubulin.

The ligand MTC was found to elicit a GTPase activity in tubulin, proceeding at a maximal rate of 0.0098 ± 0.0005 mol of GTP hydrolyzed (mol of tubulin)$^{-1}$ min^{-1}, while tubulin–colchicine had an activity of 0.0013 ± 0.0006 and MTPC was inactive. The enzyme activity induced by 2×10^{-4} M MTC was abolished by 2×10^{-4} M podophyllotoxin, and the tubulin–colchicine GTPase activity was not increased by the addition of MTC. These results suggest that the GTPase activity elicited by MTC is due to its binding to the colchicine site. Since the enzyme kinetic parameters of the colchicine-induced GTPase have been characterized previously (Andreu & Timasheff, 1982c), the GTP hydrolysis rate was titrated with varying concentrations of MTC, and the results are shown in Figure 12, where the solid line is a theoretical curve with the assumptions of an inactive unliganded protein, a fully liganded protein with a GTPase rate of 0.010 min^{-1} and a ligand (TMP–TME) binding constant $K_b = 1 \times 10^5$ M^{-1}. A fluorescence titration performed in parallel under identical conditions (2 mM $MgCl_2$, 37 °C) gave $K_b = (2.4 \pm 0.4) \times 10^5$ M^{-1}. The large experimental error in the measurements of the very slow hydrolysis rates does not allow one to ascertain whether this difference is significant.

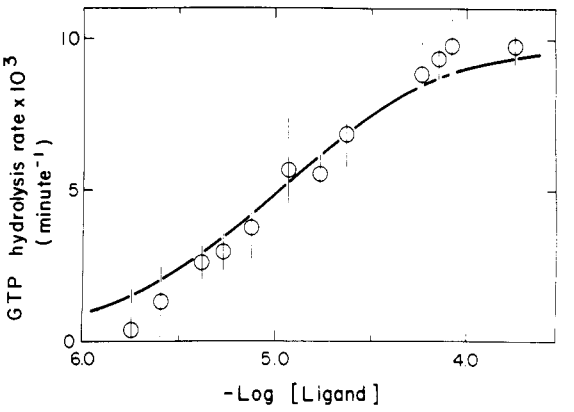


FIGURE 12: Titration of the tubulin GTPase activity induced by MTC. The ligand concentrations plotted are estimated free concentrations obtained from the total concentrations, protein concentrations, and fractional enzyme activations, assuming a binding stoichiometry of one. Protein concentrations were between 8.8×10^{-6} and 2.2×10^{-5} M; enzyme activity is expressed as moles of [^{32}P]phosphate released per mole of protein per minute (10^{-4} M GTP, 37 °C; see Materials and Methods). The solid line is a theoretical curve for a binding constant of MTC to tubulin of $K_b = 10^5$ M^{-1} and a maximal activity of 10^{-2} min^{-1} for the fully liganded protein.

Finally, the effects of MTC binding on tubulin self-association reactions were examined. MTC at 1.6×10^{-4} M did not induce any significant changes in the sedimentation velocity (see Materials and Methods) of tubulin (8.3 mg mL^{-1}) in PG buffer, pH 7.0 at 20 °C, indicating the absence of ligand-induced tubulin self-association under these conditions, in agreement with the observation that the binding results are independent of protein concentration. In the presence of Mg^{2+}, tubulin undergoes a self-association reaction with the formation of 42S double rings, characterized by a bimodal schlieren profile (Frigon & Timasheff, 1975). Under these conditions (PG buffer, 16 mM $MgCl_2$, pH 7.0, 20 °C, 7.5 mg mL^{-1} tubulin), an excess of MTC did not induce any noticeable changes, such as had been reported previously for colchicine (Andreu & Timasheff, 1983).

Substoichiometric amounts of MTC were found to inhibit the in vitro microtubule assembly (see Materials and Methods), as shown in Figure 13A, where tracing a is the turbidity time course of 1.86×10^{-5} M tubulin and b is that of a similar sample containing 1.24×10^{-6} M MTC. MTPC at 4.5×10^{-3} M produced a partial inhibition of assembly (tracing c). Under the same solution conditions and in the presence of an excess of MTC, 2.47×10^{-5} M tubulin developed a turbidity increase that was cold reversible (as shown by the dashed line d in Figure 13A), but no microtubules were observed under the electron microscope. This polymerization induced by MTC did not occur in the absence of GTP and was partially inhibited

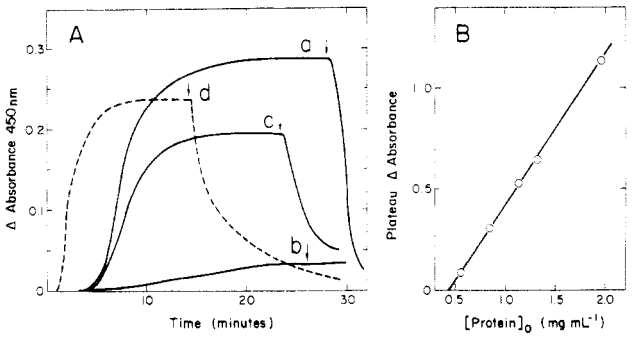


FIGURE 13: (A) Turbidity time course of in vitro microtubule assembly (see Materials and Methods). The reaction was started by warming the solution from 10 to 37 °C; the arrows indicate cooling of the samples: (a) 1.86×10^{-5} M tubulin; (b) same with 1.24×10^{-6} M MTC; (c) same with 4.5×10^{-3} M MTPC; (d) anomalous polymerization (see text) of 2.47×10^{-5} M tubulin with 2×10^{-4} M MTC displayed on a 4 times reduced vertical scale. (B) Plateau absorbance values of the polymerization of tubulin induced by 2×10^{-4} M MTC in PG–16 mM $MgCl_2$ buffer, pH 7.0, 37 °C, as a function of total ligand concentration.

by 10^{-4} M $CaCl_2$. The polymerization proceeded also in the absence of glycerol (PG buffer, 16 mM $MgCl_2$, pH 7.0, 37 °C). It exhibited a critical protein concentration of 0.44 mg mL^{-1}, as shown in Figure 13B. These characteristics are very similar to those of the polymerization of the tubulin–colchicine complex (Andreu & Timasheff, 1982b, 1983a).

Discussion

Binding to Tubulin of the Colchicine Analogues MTC and MTPC. The interaction of MTC (compound **2**) with tubulin has been firmly established by means of difference absorption spectroscopy, fluorescence, and equilibrium gel chromatography. MTC inhibits the binding of colchicine; colchicine and podophyllotoxin inhibit the binding of MTC. Just as with colchicine, the binding of MTC to tubulin causes characteristic perturbations in the ligand UV absorption that are attributable to the binding of the tropolone moiety and the appearance of ligand fluorescence (Andreu & Timasheff, 1982c). It is possible to conclude, therefore, that MTC binds most probably to the colchicine site of tubulin but the binding and dissociation are much faster than that with colchicine. This fact renders possible the study of the tubulin–MTC interaction by equilibrium techniques. MTC has the same chemical structure as colchicine except for three carbon atoms of the middle ring and its substituent. These must be involved, therefore, in determining the peculiar kinetic characteristics of colchicine binding, as has been suggested (Ray et al., 1981). Upon binding to tubulin, colchicine undergoes a conformational change manifested by a reduction in the 340-nm circular dichroic signal of the alkaloid (Detrich et al., 1981). Colchicine can assume four possible conformations related by a boat–boat interconversion of the tropolone ring and a partial rotation about the carbon–carbon bond connecting the trimethoxyphenyl and tropolone rings (Detrich et al., 1981). These motions are obviously restricted by the middle ring. The absence of this ring in MTC may allow this ligand to assume more rapidly the proper conformation for binding to tubulin. Therefore, the slow binding of colchicine to tubulin (Garland, 1978; Lambeir & Engelborghs, 1981) could be caused either by a ligand conformational change (Detrich et al., 1981), a protein conformational change (Andreu & Timasheff, 1982c), or both.

The interaction of MTPC (compound **3**) with tubulin was weak and could not be detected except for the inhibition of the binding of [^{3}H]colchicine and of microtubule assembly at high concentrations of the analogue (Figures 5 and 13). In the binding experiments, MTPC inhibited the initial rate of colchicine binding in a manner similar, within the limitations of the assay (see Results), to that of the previously characterized monofunctional ligands tropolone methyl ether and mescaline (Andreu & Timasheff, 1982a). Since neither the direct interaction of MTPC with tubulin nor its inhibition by colchicine has been observed, the specificity of the inhibitory effect on colchicine binding cannot be asserted unequivocally. Nevertheless, it is most plausible that MTPC binds weakly to the colchicine site. This analogue has the same chemical structure as MTC but with a different spacer between the trimethoxyphenyl and tropolone rings (see Chart I). Several possible explanations may be offered for its poor activity. These include the following: (i) the propionamide spacer of this ligand may cause a steric hindrance, rendering impossible the simultaneous binding of the two rings; (ii) the relative spacial positions of the two rings may not allow the binding of both simultaneously to the two colchicine subsites, as a result of, for example, the distance between the two rings being too large; (iii) the configurational entropy change required to immobilize one ring with respect to the other at the binding site may be highly negative. The first explanation may be possible, although substitutions in the middle ring of colchicine do not seem to affect markedly its activity [e.g., see Kelleher (1977) and Clark & Garland (1978)]. The second possibility appears likely, although the tolerance of the colchicine binding site to the distance between the two rings is not known nor is there any information on its flexibility. Examination of space-filling molecular models of colchicine, MTC, and MTPC has shown, however, that the two rings of MTPC cannot assume relative positions similar to any of the conformations of colchicine. The third effect may certainly contribute, although the unfavorable entropic contributions to the binding of the monofunctional ligand *N*-acetylmescaline were estimated to be 2–3 kcal mol^{-1}, or close to the cratic free-energy contribution (Andreu & Timasheff, 1982a), which indicates that the hydrophobic binding of the trimethoxyphenyl ring of colchicine (and its bifunctional analogues) to tubulin may not imply a strong immobilization. Therefore, the compound MTPC may be considered as an analogue of colchicine and MTC, partially inactivated conformationally by a combination of the second and third factors listed above, which make it into a weak ligand, capable of binding through one of its rings only.

Does the Bifunctional Ligand Model Hold? The binding of colchicine to tubulin has been described in terms of a simple model of the binding of a bifunctional ligand to two subsites of the protein (Andreu & Timasheff, 1982a). This general thermodynamic model is based on the numerical decomposition of the apparent binding standard free-energy change for a ligand, $\Delta G°_{obsd}$, into two contributions, an intrinsic contribution, $\Delta G°_{int}$, which is defined as additive when the ligand becomes part of one with additional interacting parts, and a nonadditive contribution, defined as $\Delta G°_{na} = \Delta G°_{obsd} - \Delta G°_{int}$. This model contains three assumptions: (i) independent binding of the parts of a bifunctional ligand, (ii) no perturbation of the binding of each part by covalent attachment between them, and (iii) equality of the solvation changes, conformational changes, and $\Delta G°_{na}$ for the binding of the bifunctional ligand and one of its parts. Application of this model to the existing data on the binding of colchicine and its monofunctional analogues gave self-consistent results and proved useful in the description of the colchicine site (Andreu & Timasheff, 1982a,c). Let us turn now to the binding of MTC. This ligand binds with $\Delta G°_{app} = -7.90 \pm 0.05$ kcal

mol^{-1} at 37 °C (Results), which is weaker than colchicine by ca. 2.5 kcal/mol. On the other hand, the binding of MTC to tubulin is much stronger than that of its separate constituent parts, which, under the same conditions, bind with apparent standard free-energy changes of -3.6 ± 0.6 kcal mol^{-1} (tropolone methyl ether) and -3.7 ± 0.5 kcal mol^{-1} (*N*-acetylmescaline) (Andreu & Timasheff, 1982a). This result is in qualitative agreement with the model. A closer look at this analysis, however, shows that the third assumption above (equality of $\Delta G°_{na}$), which was consistent with the colchicine results, is not strictly valid for MTC, in view of the following considerations. In the case of colchicine binding, once the tropolone ring is bound to tubulin, the hydrophobic binding of the trimethoxyphenyl moiety may not require any energetically significant immobilization (Andreu & Timasheff, 1982a), this ring being in the proper spacial orientation in one of the colchicine conformers (Detrich et al., 1981). On the other hand, in MTC, the trimethoxyphenyl ring is at least partially free to rotate about the bond connecting it to the tropolone ring (see Chart I). As a result, bifunctional binding of MTC to tubulin may involve a significant restriction of its internal rotation.

This factor can be accounted for by introducing an additional term, $\Delta G_{i.rot}{}^{\alpha-\beta}$, into the thermodynamic binding equation [eq 3 of Andreu & Timasheff (1982a)], namely

$$\Delta G_{na}{}^{\alpha-\beta} = \Delta G_{na}{}^{\alpha} + \Delta G_{i.rot}{}^{\alpha-\beta} \quad (1)$$

Following the same reasoning as before (Andreu & Timasheff, 1982a), this leads to

$$\Delta G_{int}{}^{\beta} = \Delta G_{obsd}{}^{\alpha-\beta} - \Delta G_{obsd}{}^{\alpha} - \Delta G_{i.rot}{}^{\alpha-\beta} \quad (2)$$

and

$$\Delta G_{na}{}^{\beta} = \Delta G_{obsd}{}^{\alpha} + \Delta G_{obsd}{}^{\beta} - \Delta G_{obsd}{}^{\alpha-\beta} + \Delta G_{i.rot}{}^{\alpha-\beta} \quad (3)$$

The free-energy change resulting from the partial immobilization of the internal rotation of the TMP and TME rings in MTC can be estimated as being not large, i.e., $\Delta G°_{i.rot} = 1$–3 kcal mol^{-1} (Glasstone, 1940). Introduction into eq 3 of this value and the experimentally determined ΔG_{obsd} values [this work and Table I in Andreu & Timasheff (1982a)] leads to $\Delta G_{na}{}^{\beta} = 1.5$–3.5 kcal mol^{-1}, which, within experimental error, is close to the previously deduced value as well as to the cratic free-energy change for the formation of a bimolecular complex in dilute aqueous solution (Gurney, 1962). It would appear, therefore, that the cratic entropy advantage of this bifunctional ligand over its monofunctional parts may be overcome, at least in part, by the decrease of the rotational entropy brought about by binding. Experimentally, this manifests itself in an apparent binding equilibrium constant of MTC to tubulin that is about 30 times smaller than that deduced for colchicine from kinetic measurements.

Another aspect of the bifunctional binding model is the additivity of the enthalpy and entropy of binding of the constituent parts of the ligand. The binding of colchicine to tubulin is believed to involve apparent positive enthalpy and entropy changes (Bryan, 1972; Bhattacharyya & Wolf, 1976), although attainment of equilibrium with this ligand is difficult and any measurement of equilibrum thermodynamic parameters may be biased by unwanted kinetic or denaturation effects (Andreu & Timasheff, 1982a,c). Since the binding of tropolone methyl ether is characterized by negative apparent enthalpy and entropy changes, the trimethoxyphenyl ring–tubulin interaction could be predicted to contribute large positive enthalpy and entropy changes, characteristic of hydrophobic contacts (Andreu & Timasheff, 1982a). The binding of MTC to tubulin is characterized by a close to nil apparent enthalpy change, the reaction seemingly being driven by a positive entropy change (see Results). Application of the same reasoning to MTC and TME as has been done for colchicine and TME leads to a qualitatively similar prediction, namely, positive apparent entropy and enthalpy changes for the trimethoxyphenyl ring–tubulin interaction, in agreement with the available data (Andreu & Timasheff, 1982a,c).

Effects of MTC Binding on Tubulin Conformation and Microtubule Assembly. The application of the bifunctional ligand model to the binding of colchicine to tubulin implies that the colchicine-induced conformational change in tubulin should also be induced by either the tropolone ring or the trimethoxyphenyl ring alone (Andreu & Timasheff, 1982a). Since the change was not induced by podophyllotoxin, which shares the trimethoxyphenyl ring with colchicine, the tropolone ring was considered the best candidate. Consistent with this, the binding of tropolone methyl ether to tubulin was found not to be a simple bimolecular reaction, and it induced a perturbation of the circular dichroism spectrum of tubulin similar to that induced by colchicine (Andreu & Timasheff, 1982c). Nevertheless, the weak binding of this ligand made difficult appropriate direct experimental observations. In the present study, MTC, which contains exclusively the tropolone and trimethoxyphenyl rings, has been shown to induce effects very similar to those that accompany colchicine binding, namely, quenching of the intrinsic protein fluorescence, a weak perturbation of the protein circular dichroism at 217 nm, and the induction of GTPase activity. This indicates that the conformational state of tubulin liganded to MTC is probably similar to that of tubulin within the tubulin–colchicine complex and that, most likely, this state is a consequence of the binding of the tropolone moiety of the drug to the protein.

Just like colchicine, MTC did not induce any aggregation of tubulin under our standard solution conditions. Furthermore, it did not affect significantly the Mg^{2+}-induced tubulin self-association. This analogue has been found to be a substoichiometric inhibitor of microtubule assembly in vitro, indicating that the formation of a stable soluble tubulin–ligand complex, as formed in the case of colchicine, is not required for this type of inhibition of microtubule assembly. The stoichiometric binding of MTC to tubulin induced, under microtubule-promoting conditions, a reversible protein polymerization into a nonmicrotubule polymer of elusive morphological characterization. This polymerization has the characteristics of a nucleated condensation polymerization; it requires GTP and is sensitive to Ca^{2+}, in a manner very similar to the previously described polymerization of the tubulin–colchicine complex (Saltarelli & Pantaloni, 1982; Andreu & Timasheff, 1982b, 1983a). These effects of MTC on the tubulin self-association reactions suggest that the substoichiometric inhibition of microtubule assembly by this compound may be due to a ligand-induced distortion of the normal tubulin–tubulin bonding geometry in the microtubule wall, just as has been proposed for colchicine (Andreu & Timasheff, 1983a). Finally, the characteristics of the reversible interactions of purified tubulin with simple bifunctional analogues of colchicine, reported in this study, make this type of ligands attractive compounds for the testing of the fast and reversible inhibition of microtubules in vivo.

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