Mason, R. P., & Polnaszek, C. F. (1978) *Biochemistry 17*, 1758.
McConnell, H. M., & McFarland, B. G. (1972) *Ann. N.Y. Acad. Sci. 195*, 207.
Meirovitch, E., & Freed, J. H. (1980a) *J. Phys. Chem. 84*, 3281.
Meirovitch, E., & Freed, J. H. (1980b) *J. Phys. Chem. 84*, 3295.
Norris, J. R., & Weissman, S. I. (1969) *J. Phys. Chem. 73*, 3119.
Powers, L., & Clark, N. A. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 840.
Ranck, J. L., Mateu, L., Sadler, D. M., Tardieu, A., Gulik-Krzywicki, T., & Luzzati, V. (1974) *J. Mol. Biol. 85*, 249.
Rand, R. P., Chapman, D., & Larsson, K. (1975) *Biophys. J. 15*, 1117.
Rao, K. V. S., Polnaszek, C. F., & Freed, J. H. (1977) *J. Phys. Chem. 81*, 449.
Redfield, A. G. (1965) *Adv. Magn. Reson. 1*, 1.
Reitz, D. C., & Weissman, V. I. (1960) *Z. Chem. Phys. 33*, 700.
Robinson, H. B., & Dalton, L. R. (1980) *J. Chem. Phys. 72*, 1312.
Saupe, A. (1964) *Z. Naturforsch. A 19A*, 161.
Seelig, A., & Seelig, J. (1974) *Biochemistry 13*, 4839.
Shimizu, H. (1962) *J. Chem. Phys. 37*, 705.
Tardieu, A., Luzzati, V., & Reman, F. C. (1973) *J. Mol. Biol. 75*, 711.
Van, S. P., Birrell, G. P., & Griffith, O. H. (1974) *J. Magn. Reson. 15*, 444.
Verkleij, A. J., Ververgaert, P. H. J., van Deenen, L. L. M., & Elbers, P. F. (1972) *Biochim. Biophys. Acta 288*, 326.

Interaction of Tubulin with Single Ring Analogues of Colchicine†

Jose Manuel Andreu and Serge N. Timasheff*

ABSTRACT: Simple analogues of the tropolone and trimethoxyphenyl moieties of colchicine have been used as probes for the colchicine binding site of purified calf brain tubulin. [^{3}H]Tropolone methyl ether was found to bind to one site per tubulin molecule with an equilibrium constant of (2.2 ± 0.2) × 10^3 M^{-1} at 0 °C, with the interaction having $\Delta H°_{app}$ = −8.3 ± 1.0 kcal mol^{-1} and $\Delta S°_{app}$ = −15.2 ± 3.6 eu. The binding of tropolone methyl ether and colchicine was inhibited by each other. Both tropolone and its methyl ether inhibited tubulin polymerization into microtubules in vitro. *N*-[^{3}H]Acetylmescaline bound to tubulin with a $K \simeq 4 \times 10^2$ M^{-1} at 37 °C. This interaction was inhibited by colchicine and at lower temperatures was below the sensitivity of the measuring method employed. [^{14}C]Mescaline interacted with higher affinity site(s) not related to the colchicine site. Both mescaline and *N*-acetylmescaline inhibited partially the microtubule assembly at 10^{-3} M concentrations. No linkage was observed between the binding of tropolone methyl ether and *N*-acetylmescaline. The relatively weak interactions of both the two separate parts of colchicine can account quantitatively for the much tighter binding of the complete drug to tubulin within a proposed model which takes into account the entropic advantage of colchicine as a bifunctional ligand.

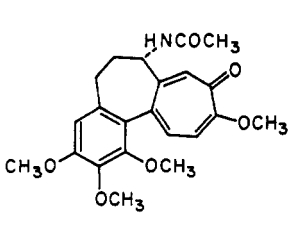
Colchicine and podophyllotoxin inhibit mitosis by interacting with the microtubule protein tubulin (Wilson & Bryan, 1974). These alkaloids have been used extensively for the inhibition of microtubule-mediated processes in vivo and have become important tools in the study of the mechanism of tubulin assembly into microtubules in vitro (Margolis & Wilson, 1977, 1978). In vitro, the assembly of pure tubulin into microtubules is known to conform thermodynamically to the Oosawa & Kasai (1971) model of nucleated helical polymerization (Lee & Timasheff, 1977). The actual kinetic pathway of microtubule assembly is probably much more complicated, but the final state is a steady state resulting from the incorporation and release of protomers at the ends of the microtubules. This can lead to an apparent movement of tubulin subunits from one end of the organelle to the other without changing its size, as described by the treadmilling mechanism of Margolis & Wilson (1978) (Karr & Purich, 1979; Bergen & Borisy, 1980). Colchicine and podophyllotoxin bind to soluble tubulin and inhibit microtubule assembly substoichiometrically, as incorporation of liganded protein at microtubule ends inhibits further polymer growth (Margolis & Wilson, 1977; Sternlicht & Ringel, 1979).

The interaction of colchicine with soluble tubulin is a complex and poorly understood phenomenon. The binding is slow and not easily reversed. The stoichiometry is close to one site per tubulin dimer. The binding site denatures rapidly, hampering equilibrium studies of the process (Wilson & Bryan, 1974). Nevertheless, different studies of the binding affinity of colchicine for brain tubulins of various origins, either purified or containing microtubule associated proteins, carried out by different techniques and under a variety of conditions (nature of buffer anions, presence of sucrose, Mg^{2+}, etc.) (Owellen et al., 1972; Wilson & Bryan, 1974; Bhattacharyya & Wolff, 1974; Sherline et al., 1975; Garland, 1978; Nunez et al., 1979) have given results not very different from each other. Indeed if the standard free energies of the binding reaction are averaged, the colchicine–tubulin interaction at pH 6.5–7.0, 37 °C, would have a $\Delta G°_{app}$ of −9.0 ± 0.2 kcal mol^{-1}. If, instead of equilibrium measurements, kinetic measurements are used to calculate the equilibrium constant, the numbers that come out give $\Delta G°_{app}$ = −10.3 ± 0.3 kcal mol^{-1}. This analysis strongly suggests that (i) the colchicine–tubulin interaction affinity is not significantly dependent on the origin of the brain tubulin, the small amounts of mi-

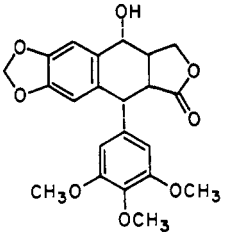
† From the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254. *Received June 3, 1981.* This work is Publication No. 1387. This research was supported by Grants CA 16707 and GM 14603 from the National Institutes of Health, a fellowship from the U.S.A.–Spain Joint Committee for Scientific and Technological Cooperation (J.M.A.), and a Fogarty International Fellowship from the National Institutes of Health (J.M.A.). A preliminary report of this work has been presented elsewhere (Andreu & Timasheff, 1981a).

0006-2960/82/0421-0534$01.25/0

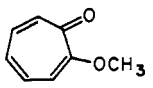
Chart I



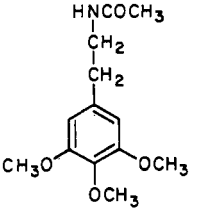
Colchicine



Podophyllotoxin



Tropolone Methyl Ether



N-Acetyl Mescaline

crotubule associated proteins, and the different buffer compositions used, and (ii) most equilibrium measurements may have been biased due to unliganded protein denaturation, as has been suggested previously (Sherline et al., 1975; Garland, 1978). One can expect that the equilibrium constant calculated from carefully made kinetic measurements should give a more reliable value, if all the steps along the reaction pathway have been properly identified. Garland (1978) proposed the colchicine–tubulin interaction to be a two-step process, namely, a fast reversible binding followed by a slow ligand-induced conformational change. The calculated overall equilibrium constants give $\Delta G°_{app} = -10.2 \pm 0.2$ kcal mol^{-1} at 32 °C. The colchicine–tubulin interaction is known to be strongly temperature dependent (Wilson & Bryan, 1974), with the standard enthalpy change deduced from equilibrium binding measurements ($\Delta H°_{app}$) being 10–16 kcal mol^{-1} and the standard entropy change ($\Delta S°_{app}$) being 60–80 eu (Bryan, 1972; Bhattacharyya & Wolff, 1974). Podophyllotoxin binding, which is competitive with colchicine, seems to be much faster and reversible (Wilson & Bryan, 1974; Cortese et al., 1977). So far, there is no reported evidence of any conformational change induced by podophyllotoxin. Furthermore, colchicine binding has been reported to induce a weak GTPase activity in soluble tubulin, while podophyllotoxin does not have such an effect (David-Pfeuty et al., 1979). Finally, colchicine induces changes in the divalent cation–tubulin interactions (L. M. Grisham and S. N. Timasheff, unpublished results).

A number of studies have been described relating the structure, conformation, colchicine binding competition, and microtuble inhibitory activity of a variety of colchicine and podophyllotoxin analogues (Fitzgerald, 1976; McClure & Paulson, 1977; Brewer et al., 1979; Kelleher, 1977; Cortese et al., 1977, among others). There is strong evidence for the notion that colchicine and podophyllotoxin share a trimethoxybenzene binding zone of the protein binding site. Colchicine probably binds also through its tropolone ring and podophyllotoxin through its lactone ring, with these two sites being independent on the protein molecule. The structures of the ligands pertinent to this study are given in Chart I.

The microtubule inhibitory effect of 2-methoxy-5-(2,3,4-trimethoxyphenyl)tropone is nearly as strong as that of colchicine (Fitzgerald, 1976). This suggests that this compound may contain the features necessary for binding within its simpler structure of two rings joined by a single carbon–carbon bond. Colchicine and podophyllotoxin thus constitute relatively complex, probably bifunctional ligands. No significant interaction of analogous single ring structures with tubulin has been documented, except for tropolone and tropolone methyl ether, which were reported by Bhattacharyya & Wolff (1974) to inhibit competitively colchicine binding. Mescaline, an analogue of the trimethoxyphenyl moiety of colchicine, is an inhibitor of fast axoplasmic transport in nerve, a process believed to be microtubule dependent (Paulson & McClure, 1973). Harrison et al. (1976) reported mescaline to be a mitotic spindle inhibitor and mentioned that it binds to tubulin. In an attempt to resolve the complex colchicine–tubulin interaction and its consequences into individual simpler ones, we have used single ring ligands, namely, tropolone and mescaline derivatives, as probes of the colchicine binding site on tubulin. Their interactions with nonassociated, 5.8S purified calf brain tubulin and their effects on microtubule assembly in vitro have been examined with the hope of gaining a better understanding of the total interaction and of the mechanism of microtubule assembly inhibition by antimitotic drugs.

Materials and Methods

Ligands. Tropolone, colchicine, and podophyllotoxin were from Aldrich Chemical Co. (lot no. 062777, 010987, and 022757, respectively). Mescaline hydrochloride (lot no. 102C-1710) and GTP (type II-S) were obtained from Sigma. 8-Anilino-1-naphthalenesulfonic acid (ANS)[1] magnesium salt was from Eastman Kodak Co.

Tropolone methyl ether (TME, 2-methoxy-2,4,6-cycloheptatrienone) was synthesized by methylation of tropolone (2-hydroxy-2,4,6-cycloheptatrienone) with diazomethane in ether solution (Nozoe, et al., 1951). Diazomethane was generated by the action of aqueous alkali on *N*-methyl-*N*-nitroso-*N'*-nitroguanidine (Aldrich) in a millimole-scale closed apparatus (Fales et al., 1973). The hemihydrate of TME, a yellow solid that formed upon exposure to room humidity (Nozoe et al., 1951), was subjected to elementary analyses. Anal. Calcd for $C_8H_8O_2 \cdot 0.5H_2O$: C, 66.20; H, 6.25; O, 27.55%. Found: C, 66.17; H, 6.38; O, 27.34 (Galbraith Labs., Inc., Knoxville, TN). The product was further characterized by its absorption spectrum which was not significantly affected by H^+ and metal cations, as is true with the tropolone spectrum due to its free OH group (Andrew & Timasheff, 1981a,b). The principal peaks and extinction coefficients of TME in aqueous solution were found to be $E_{236} = 25\,900$ M^{-1} cm^{-1}, $E_{315} = 7040$ M^{-1} cm^{-1}, and $E_{345} = 6960$ M^{-1} cm^{-1}. The product gave a single spot in thin-layer chromatography on silica gel plates (with pyridine–concentrated ammonia, 9:1, R_f was 0.59; with chloroform–acetone-diethylamine, 7:2:1, R_f was 0.57). Finally, when the green chelate formed by tropolone in alcoholic $FeCl_3$ (Cook et al., 1951) and the fluorescent tropolone·Mg^{2+} chelate (Andreu & Timasheff, 1981b) were measured, the TME preparation used was found to contain less than 0.5% (w/w) of the starting tropolone material.

N-Acetylmescaline was obtained by reacting mescaline with acetic anhydride in alkaline aqueous solution (Lettré & Fernholtz, 1973). It was purified by means of passage through the AG501-X8 Bio-Rad mixed-bed resin. The product was found to have an absorption spectrum similar to that of the starting material, except for the absence of a small perturbation induced by alkali on the mescaline spectrum. In neutral aqueous solution, λ_{max} was 267.5 nm, and $E = 760$ M^{-1} cm^{-1}.

[1] Abbreviations: ANS, 8-anilino-1-naphthalenesulfonic acid; EDTA, (ethylenedinitrilo)tetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PG, 10 mM sodium phosphate–0.1 mM GTP; TME, tropolone methyl ether; NAM, *N*-acetylmescaline.

The contents of the starting material in the *N*-acetylmescaline batch used were found to be less than 0.3% (w/w), as measured by the reaction of the free amino group of mescaline with 2,4,6-trinitrobenzenesulfonic acid (Eastman Kodak Co.) followed spectrophotometrically at 420 nm (Snyder & Sobocinski, 1975).

[*methyl*-^{3}H]Tropolone methyl ether, 6.66 Ci/mol, was obtained by taking advantage of the exchangeability of diazomethane hydrogens in alkali (DeMore et al., 1959); 0.5 Ci of tritiated water (18 Ci/mol, New England Nuclear) was substituted for unlabeled water, and essentially the same operations as for the synthesis of cold TME were performed. [^{3}H]TME was found to have the same spectral properties and purity as the unlabeled product; more than 98.5% of the radioactivity applied was found in the TME spot in the two thin-layer chromatographic systems mentioned above.

[8-^{14}C]Mescaline, 22.8 Ci/mol, was obtained from New England Nuclear (lot no. 979-141). *N*-[^{3}H]Acetylmescaline, 26.3 Ci/mol, was produced by the same procedure as the unlabeled acetyl derivative, this time using [^{3}H]acetic anhydride (nominally 50 Ci/mol; New England Nuclear). The labeled product showed the same characteristics as *N*-acetylmescaline and was more than 99% radiochromatographically pure in silica gel thin-layer chromatography. The solvent was pyridine–concentrated ammonia, 9:1, and R_f = 0.69; mescaline and [^{14}C]mescaline gave R_f = 0.38 [Lundstrom & Agurell (1967) reported R_f = 0.68 for *N*-acetylmescaline and R_f = 0.36 for mescaline].

Other Materials. Glycerol, $MgCl_2$, and EDTA were from Fisher. EGTA was obtained from J. T. Baker. The Mg^{2+} concentration in stock solutions was determined by titration with a reference EDTA solution and Eriochrome black T indicator (Fisher). Extreme purity grade guanidine hydrochloride was from Heico, Inc. Silica gel sheets were from Eastman Kodak. DEAE-Sephadex A-50, Sephadex G-25 and G-50, and Blue Dextran were from Pharmacia. [^{3}H]Water tritium standard, 2.95×10^6 dpm/mL, was from New England Nuclear. Other chemicals were of reagent grade.

Protein Purification and Determinations. Calf brain tubulin was purified as described by Lee et al. (1973) (Weisenberg et al., 1968) with minor modifications. $MgCl_2$ (0.5 mM) was used throughout the procedure, and DEAE-Sephadex batchwise chromatography was performed on a sintered glass filter. The purified protein was dialyzed overnight in the cold against 10 mM sodium phosphate, 1 M sucrose, 0.5 mM $MgCl_2$, and 0.1 mM GTP, pH 7.0, then clarified by centrifugation at 20000*g* for 20 min, and stored at 80–100 mg/mL in liquid nitrogen. Prior to use the protein was equilibrated in the desired buffer by means of fast Sephadex G-25 chromatography; it was then maintained on ice and used within 4 h of sucrose removal. The protein so prepared was systematically found to give a single symmetrical 5.8S peak in the analytical ultracentrifuge and was more than 98% homogeneous in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Weber et al., 1972) as previously reported (Lee & Timasheff, 1975; Frigon & Timasheff, 1975). The protein concentration was measured spectrophotometrically in 6 M guanidine hydrochloride at neutral pH, using an absorption coefficient E at 275 nm of 1.03 L g^{-1} cm^{-1} (Na & Timasheff, 1981). Alternately the scattering corrected absorbance (Leach & Scheraga, 1960) of clear native protein solutions was measured and an extinction coefficient E at 276 nm of 1.10 L g^{-1} cm^{-1} was used.

Ligand Binding. The gel chromatography equilibrium technique of Hummel & Dreyer (1962) was applied as follows. Samples containing 10–40 nmol of tubulin and a known total concentration of ligand in a given buffer (final volume ≤1 mL) were made and immediately applied to 0.9 × 25 ± 1 cm Sephadex G-25 columns equilibrated with the same buffer of identical ligand concentration. The temperature was controlled to ±0.2 °C by means of water jackets and a Neslab RTE-4 circulating bath. The column flow was kept constant during the experiment by means of LKB peristaltic pumps. The binding time (taken as the mean chromatographic elution time of the protein) could be varied among different experiments between 5 and 100 min with an accuracy of ±5% by simply changing the pump setting. Fractions of 1.05 ± 0.05 mL were collected, and the protein was determined spectrophotometrically.[2]

The radioactive ligand concentration was measured throughout the column eluate by means of carefully taken aliquots (typically with an 0.5-mL delivery pipet that afforded a reproducibility of ±0.25%, as determined by weighing buffer and protein solution aliquots) added to 10 mL of aqueous counting scintillant (ACS Amersham) and counted twice to a statistical counting error smaller than 0.3% (95% confidence) in a Beckman L 100 liquid scintillation spectrometer. Duplicate aliquots were taken in the peak region. The base line counts per minute were determined from the regions outside the peak and trough typically to a standard deviation ≤ 0.5% of the absolute value. Experiments without a stable base line in the vicinity of the protein peak were discarded. The specific activity of the ligand was calculated from the radioactivity and light absorption of the base line. The amount of bound ligand was calculated from the measured increment in eluate radioactivity coupled to protein elution; the standard deviation of the base line was taken as an estimate of the standard deviation of measurements of bound ligand.

When only limited amounts of ligand were available, the batch gel partition procedure (Fasella et al., 1965) was applied with several modifications in order to obtain approximate binding measurements at high ligand concentrations. Carefully weighed amounts of Sephadex G-50 (typically 20.0 ± 0.1 mg of prewashed and dried gel) were swollen in a ligand–buffer solution for several hours in a water bath at the experimental temperature. Then the protein was added, and the mixture (final volume ≤0.5 mL) was incubated with occasional shaking for a sufficiently long time to reach binding and partition equilibrium, with the latter being achieved much faster (Fasella et al., 1965) than in conventional equilibrium dialysis binding experiments, here technically excluded due to tubulin instability (Frigon & Lee, 1972). The concentrations of protein and ligand were measured on carefully taken aliquots of the outer phase. The outer volume was operationally defined within each experiment as the volume accessible to the macromolecule and the total volume as the volume occupied by the labeled ligand or [^{3}H]water. The amount of ligand bound was estimated from the increment in outer phase ligand concentration due to the presence of the macromolecule, with the appropriate volumetric corrections.

Optical Properties. Visible and ultraviolet absorption spectra were obtained with a Cary 118 spectrophotometer in

[2] Binding stoichiometries were calculated in this study on the basis of a molecular weight of 110000 for calf brain tubulin (Lee et al., 1973). However, recent nucleotide sequence studies on chick brain tubulin mRNAs (Valenzuela et al., 1981) and protein sequence studies on porcine brain α-tubulin (Postingl et al., 1981) indicate molecular weights of the $\alpha\beta$ heterodimers very close to M_r 100000. Using this value of the molecular weight would introduce a correction that falls within the experimental error of most of our stoichiometry measurements and does not affect the interaction free-energy changes reported.

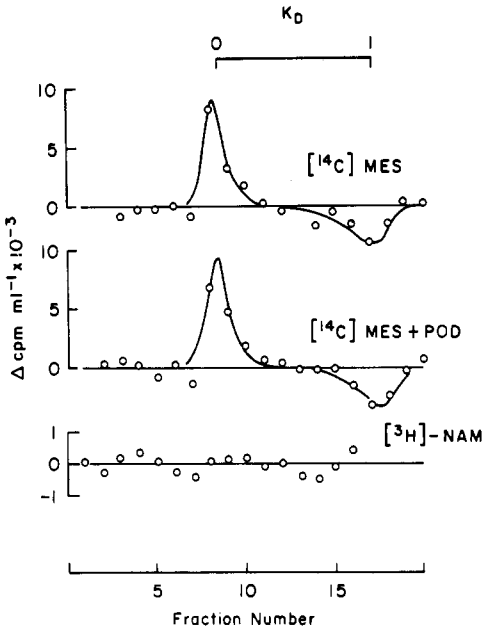


FIGURE 1: Interaction of mescaline with tubulin; labeled ligand elution profiles in gel chromatography; Hummel and Dreyer experiments performed in PG buffer, pH 7.0, at 25 °C. (Upper profile) Variation of [^{14}C] mescaline (MES) (9.3×10^4 cpm mL^{-1}) concentration as counts per minute when 38 nmol of tubulin was chromatographed in a column equilibrated with 52 μm MES. (Middle profile) Similar run in the presence of 50 μM podophyllotoxin (POD). (Lower profile) Variation of *N*-[^{3}H]acetylmescaline (5.5×10^5 cpm mL^{-1}) (NAM) concentration when 26 nmol of tubulin was chromatographed in a column equilibrated with 48 μM NAM under the same conditions. The rate of flow was 22 mL h^{-1} in all cases, and the protein eluted at $K_d = 0$ (not shown).

1-cm cells at 25 ± 2 °C. Difference spectra were obtained by using 0.4 + 0.4 cm tandem cells and corrected for any base-line deviations. Fluorescence measurements were made with a Hitachi–Perkin-Elmer MPF-3 spectrofluorometer stabilized in the ratio mode of operation, using 1 × 1 cm cells thermostated to the desired temperature to ±0.5 °C. The excitation and emission bandwidths were 2.5 and 5 nm, respectively. Whenever an inner filter effect was to be avoided, wavelengths and sample concentrations were chosen to make the absorbance <0.05 (1 cm). The emission intensity was corrected for the small solvent contribution of the solution that had been centrifuged prior to the experiment.

Sedimentation Velocity. Samples with and without ligands were run simultaneously in an An-D rotor with double-sector cells at 60 000 rpm, 20 °C, using a Beckman Model E analytical ultracentrifuge equipped with electronic speed control and RTIC temperature control.

Microtubule Assembly. The in vitro reconstitution of microtubules was performed in 10 mM sodium phosphate, 0.1 mM GTP, 1 mM EGTA, 16 mM $MgCl_2$, 3.4 M glycerol, pH 7.0, and assembly buffer at 37 °C in a thermostated cuvette (Lee & Timasheff, 1977). The mass of polymer formed was monitored turbidimetrically (Gaskin et al., 1974) by using a Cary Model 14 spectrophotometer.

Results

Interactions of Mescaline and N-Acetylmescaline with Tubulin. The interaction of [^{14}C]mescaline with tubulin was measured by the equilibrium gel chromatography technique, and typical elution profiles in the absence and presence of podophyllotoxin are shown in Figure 1. At low ligand concentrations, the binding was to less than one site, with an apparent equilibrium constant of (1.0–1.5) × 10^4 M^{-1} (Figure 2). There was also an undetermined number of lower affinity sites (Figure 3). The first, moderate-affinity interaction, however, seemed not to be directed to the trimethoxyphenyl binding region of the podophyllotoxin–colchicine site on the tubulin molecule, since podophyllotoxin had no detectable inhibitory effect on mescaline binding (Figures 1 and 2) and mescaline concentrations up to 10^{-3} M had an almost undetectable effect on the binding of 5×10^{-6} M colchicine to tubulin, as monitored by fluorescence of the colchicine–tubulin complex (see Figure 9). These results suggested that the observed interaction of mescaline with tubulin was related to the positive charge on the protonated amino group of the ligand at neutral pH. This possibility was tested by examining the interaction of *N*-[^{3}H]acetylmescaline with the protein under identical conditions. This latter ligand was found not to inhibit mescaline binding, and its own interaction was almost undetectable under the conditions used for mescaline (Figure 1).

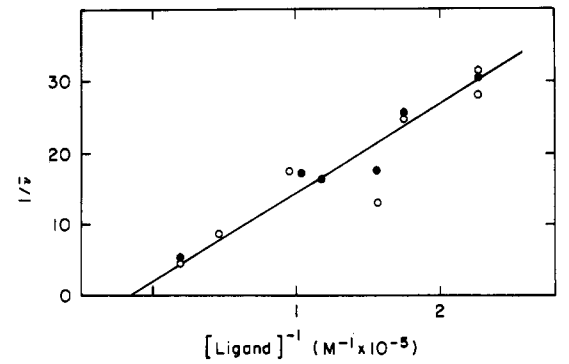


FIGURE 2: Double-reciprocal plot of the binding of [^{14}C]mescaline in the absence (open circles) and the presence (filled circles) of 5 μM podophyllotoxin. The point at highest mescaline concentration was obtained with 500 μM podophylloxtoxin. Conditions are the same as in Figure 1.

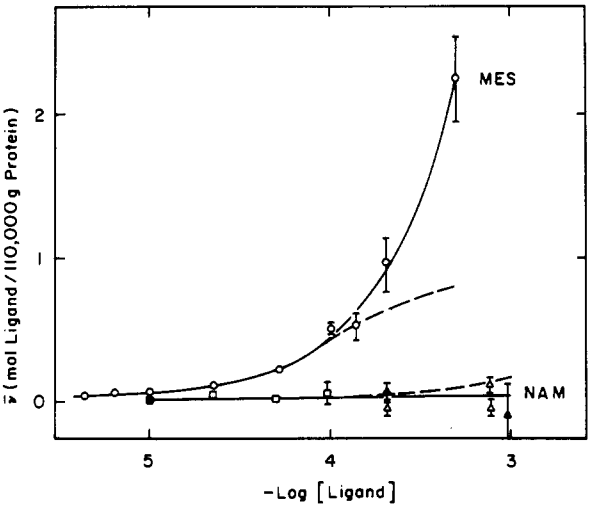


FIGURE 3: Binding isotherms of mescaline (MES) and *N*-acetylmescaline (NAM) at 25 °C. Mescaline binding measurements (○) were performed as in Figure 1. A theoretical correction of these values for the Donnan effect, assuming tubulin to have a net charge of approximately −28 at pH 7.0 (Lee et al., 1973), gave slightly smaller numbers, but well within experimental error (vertical bars). The *N*-acetylmescaline binding measurements were made under the same conditions (□), in the presence of 0.5 mM tropolone methyl ether (■), or by means of the equilibrium batch gel partition procedure (see Materials and Methods) in PG buffer, pH 7.0 (△), and in PG–16 mM $MgCl_2$–1 mM $CaCl_2$–3.4 M glycerol buffer, pH 7.0 (▲). The partition procedure was validated by showing that it gave values close to those obtained by the Hummel and Dreyer method with mescaline (not shown). The solid lines are the experimental isotherms, and the dashed lines are theoretical ones for $n = 1$ $K = 8 \times 10^3$ M^{-1} (mescaline), and $n = 1$, $K = 3 \times 10^2$ M^{-1} (*N*-acetylmescaline).

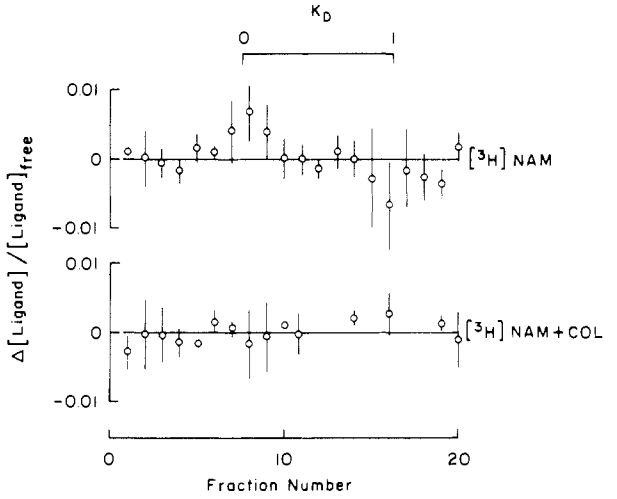


FIGURE 4: Interaction of *N*-acetylmescaline (NAM) with tubulin at 37 °C. The upper profile is the result of averaging five Hummel and Dreyer runs (see Materials and Methods) on 36 ± 2 nmol of tubulin in PG buffer, pH 7.0; the ligand concentration varied between 0.9 and 11 μM (eluate radioactivity between 11 600 and 64 000 cpm mL^{-1}). The lower profile is the result of averaging three runs under identical conditions but with the addition of 10 μM colchicine (COL). The ordinate scale was calculated as the increment of radioactivity divided by the base-line radioactivity for each run. Note that since $[protein]_{free} \simeq [protein]_{total}$ (there is very little binding), the parameter measured in the peak is very close to the product of the protein concentration and the equilibrium constant, permitting it to be averaged over the interval of free ligand concentrations. The chromatographic flow was 40 mL/h; the protein eluted at K_d = 0.0 ± 0.1.

Varying ligand concentration and conditions, e.g., the presence of tropolone methyl ether or of Mg^{2+} ions in the buffer, resulted in numbers within the experimental error of the equilibrium gel chromatography and batch partition techniques employed (Figure 3). This sets an upper value of 300 M^{-1} at 25 °C to the equilibrium binding constant of *N*-acetylmescaline to tubulin under these conditions. In view of the predicted characteristics of the *N*-acetylmescaline–tubulin interaction (see Discussion), the binding was explored more carefully at 37 °C in repeated and averaged Hummel and Dreyer experiments. The results, shown in Figure 4, indicate a statistically detectable interaction which was inhibited by colchicine. Assuming the binding to be to one site, an equilibrium constant, K_b, of 430 ± 250 M^{-1} could be estimated from the data.

The binding of mescaline had no significant effect on either the circular dichroism of tubulin, the fluorescence of ANS bound to tubulin (Lee et al., 1975), the sedimentation velocity patterns of tubulin in PG buffer, or the Mg^{2+}-induced tubulin self-association (Frigon & Timasheff, 1975). On the other hand, mescaline produced a partial but significant inhibition of microtubule assembly, which seemed not to be a charge-mediated effect since a similar inhibition was obtained with *N*-acetylmescaline. As shown in Figures 5 and 6, both mescaline and its *N*-acetyl derivative lowered significantly the turbidity plateau values in reconstitution experiments. Empirical fits of the data by simple inhibition curves show that this effect occurs in a ligand concentration range consistent with the reciprocal of the estimated binding constant. It is not clear why the turbidity was not fully repressed even at the highest ligand concentrations used. The present data, however, do not allow one to select among various possible explanations, with the simplest one being that the only effect of binding of the ligand is to weaken the self-assembly reaction, raising the critical concentration to a value somewhat higher than that of the unliganded tubulin.

Interactions of Tropolone and Tropolone Methyl Ether with Tubulin. Tubulin produces a small perturbation in the absorption spectrum of tropolone in PG buffer and 1 mM EDTA, pH 7.0, as shown in Figure 7. No tubulin-induced fluorescence of tropolone was observed under the same conditions. Similarly there was no fluorescence of TME in PG buffer in the presence of tubulin. On the other hand, when tubulin was added to tropolone solutions in 16 mM Mg^{2+}, pH 7.0, a slight increase in the fluorescence of the tropolone–Mg^{2+} complex (Andreu & Timasheff, 1981b) was observed. This effect was observed repeatedly, and although it was probably due to a tropolone–Mg^{2+}–tubulin ternary complex, its small amplitude precluded its use in a quantitative study. The interactions of tropolone and tropolone methyl ether with tubulin were subjected to a quantitative study by means of the Hummel and Dreyer technique (see Materials and Methods) in PG buffer, pH 7.0, with and without 16 mM Mg^{2+}. The binding was found to be not immediate. To ensure attainment of equilibrium required between 10 min at 37 °C and 120 min at 0 °C. At 25 °C the binding time course indicated an apparent bimolecular forward rate constant in the order of 10^2 M^{-1} min^{-1}. The ligand concentration was first followed by scat-

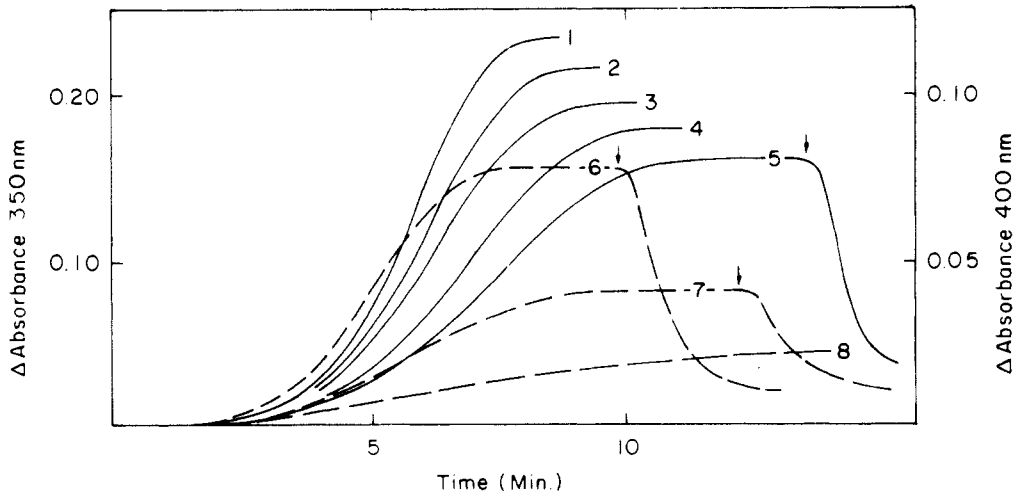


FIGURE 5: Effects of *N*-acetylmescaline and tropolone methyl ether on microtubule assembly in vitro. Solid tracings are the time course (absorbance at 350 nm) of the polymerization reaction of 17 μM tubulin (performed as described under Materials and Methods) in the presence of none (1), 0.25 mM (2), 0.61 mM (3), 1.21 mM (4), and 2.78 mM (5) *N*-acetylmescaline. Discontinuous tracings are time course (absorbance at 400 nm) of the polymerization reaction of 19 μM tubulin with none (6), 0.16 mM (7), and 1.25 mM (8) tropolone methyl ether. The protein solutions were incubated with ligands for 2 h at 0 °C, and assembly was started by warming to 37 °C; arrows indicate cooling to 10 °C.

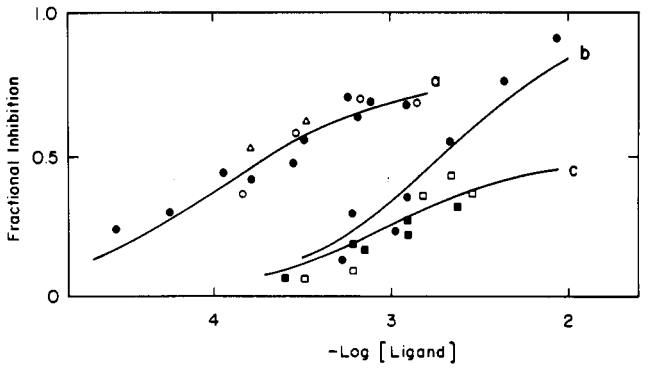


FIGURE 6: Inhibition of microtubule assembly (calculated from values of the absorbance plateaus) by tropolone (○), tropolone in the presence of 1.6 mM mescaline (△), tropolone methyl ether (●), mescaline (□), and *N*-acetylmescaline (■). In (a) and (c), ligands were bound to tubulin at 0 °C, and assembly was started by a jump to 37 °C, whereas in (b), tropolone methyl ether was bound to protein at 37 °C in an assembly buffer containing 25 μM $CaCl_2$ and no EGTA and the polymerization reaction started by adding EGTA to 1 mM. These results were obtained with different tubulin preparations at a 19 ± 2 μM protein concentration. The solid lines are empirically fitted inhibition curves for (a) maximal inhibition of 0.75 and a half-effect reciprocal concentration of 10^4 M^{-1}, (b) a maximal inhibition of 1.0 and a half-effect reciprocal concentration of 5×10^2 M^{-1}, and (c) a maximal inhibition of 0.5 and a half-effect reciprocal concentration of 10^3 M^{-1}. It must be noted that since the maximal inhibition may be dependent on total protein concentration, these numbers should be considered only as semiquantitative indications. On the other hand, the total ligand concentrations used contain more than 95% unbound ligand.

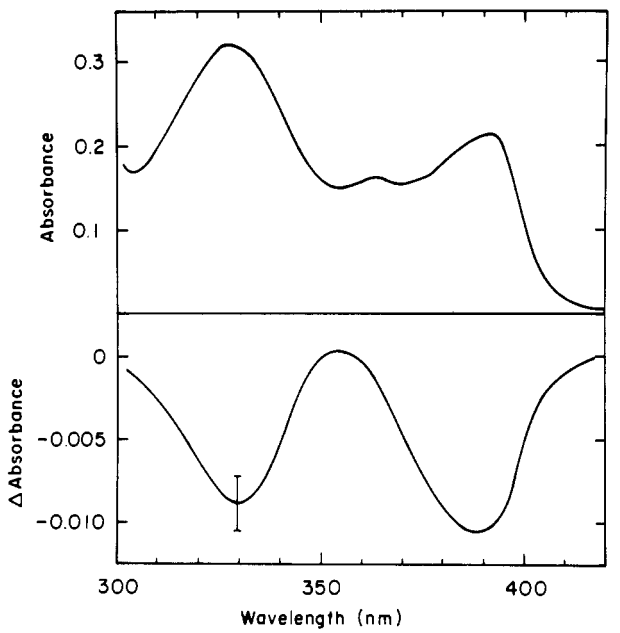


FIGURE 7: Difference absorption spectrum of tropolone–tubulin. The upper portion shows the spectrum of 77 μM tropolone in PG buffer and 1 mM EDTA, pH 7.0, at 25 °C (0.4 cm path); the lower portion is the difference spectrum between the above concentration of tropolone in the presence and absence of 31 μM tubulin, and the vertical bar is an indication of the experimetnal error.

tering-corrected absorbance, a procedure that could yield only an estimate of the binding equilibrium constant, $K_b \simeq 10^3$ M^{-1} at 25 °C for both ligands. More exact data were obtained by a careful application of the same technique using tritium-labeled tropolone methyl ether (see Materials and Methods), with a typical elution profile shown in Figure 8. Addition of colchicine abolished the peak and trough (Figure 8), suggesting that the two ligands were competing for the same site; this was confirmed by the observation that TME partially inhibited the binding of colchicine to tubulin, as shown in

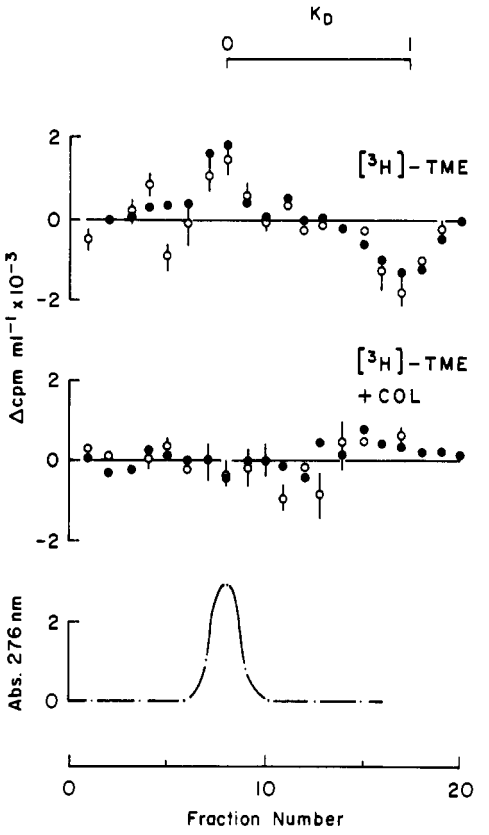


FIGURE 8: Interaction of tropolone methyl ether (TME) with tubulin. The results of a typical equilibrium binding gel chromatography experiment with 105 μM [³H]TME (1.4×10^5 cpm mL^{-1}) in PG buffer, pH 7.0, at 18 °C are shown by the open circles. The changes in ligand concentration of the upper profile were produced by chromatography of 32 nmol of tubulin through the column, while the middle profile was obtained under identical conditions after the addition of 100 μM colchicine (COL) to the column and sample; the vertical bars show the standard deviation of the measurements. The filled circles are the average of three (upper profile) and two (middle profile) runs, which reduce the noise and show unequivocally the positions of the peak and trough. The lower profile corresponds to protein elution. The flow was 10 mL h^{-1}.

Figure 9. Mescaline, 1×10^{-3} M, on the other hand, had a possibly marginal effect on the binding of colchicine to tubulin which seemed to be additive to the inhibitory action of TME. Addition of Mg^{2+} has no significant effect on the TME–tubulin interaction. The binding isotherm of TME to tubulin in PG buffer, pH 7.0, 0 °C, is shown in Figure 10. These data correspond to 0.95 ± 0.20 binding site per tubulin dimer with an apparent equilibrium constant of $(2.2 \pm 0.2) \times 10^3$ M^{-1}. Further binding to lower affinity sites cannot be excluded. Examination of the binding as a function of temperature, presented in a van't Hoff plot in Figure 11, indicated that the interaction was favored by lower temperatures, being characterized by $\Delta H^\circ_{app} = -8.3 \pm 1.0$ kcal mol^{-1}, $\Delta S^\circ_{app} = -15.2 \pm 3.6$ eu, $\Delta C_p \simeq 0$ cal $(deg \cdot mol)^{-1}$, and ΔG°_{app} varying between −4.2 and −3.5 kcal mol^{-1} within the temperature range studied.

No changes were observed in the fluorescence either of the protein or of the protein–ANS complex in the presence of tropolone or tropolone methyl ether. Furthermore, neither ligand at a level of 1.5×10^{-3} M produced any marked changes in the sedimentation of tubulin in PG buffer, pH 7.0, 20 °C, except for a slight sharpening of the peak that could result from a possible stabilization of the protein (V. Prakash and S. N. Timasheff, umpublished results), nor did they affect significantly the Mg^{2+}-induced tubulin self-association in PG buffer and 16 mM Mg^{2+}, pH 7.0, 20 °C (Frigon & Timasheff, 1975).

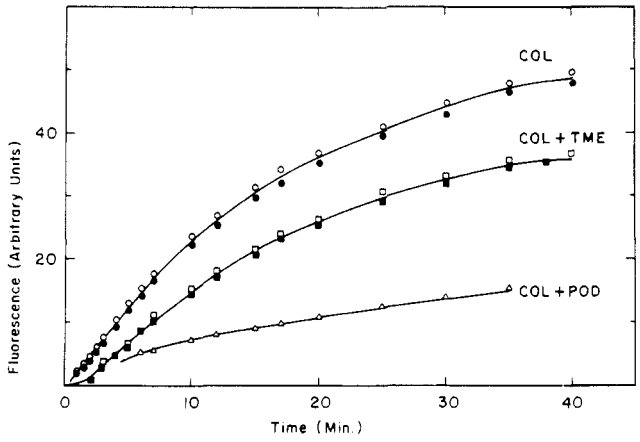


FIGURE 9: Effects of tropolone methyl ether and mescaline on colchicine binding to tubulin. The binding of 4.7 μM colchicine to 3.8 μM tubulin in PG buffer and 10 mM $MgCl_2$, pH 7.0, at 37 °C was monitored by fluorescence of the tubulin–colchicine complex (excitation 380 nm; emission 435 nm). (○) No other ligand added to the system. (□) Same experiment with addition of 0.47 mM tropolone methyl ether. (■) Addition of 0.93 mM mescaline to the system containing 4.7 μM colchicine and 0.47 mM TME. (●) Addition of 0.93 mM mescaline to the system containing 4.7 mM colchicine. (△) Inhibition of the binding of 4.7 μM colchicine by 4.7 μM podophyllotoxin. Similar results were obtained in the absence of Mg^{2+}.

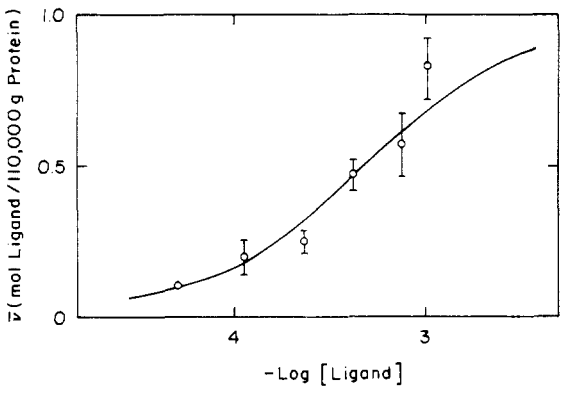


FIGURE 10: Binding isotherm of tropolone methyl ethher to tubulin in PG buffer, pH 7.0, at 0 °C. The bars indicate the standard deviation of the measurements, and the solid line is the binding isotherm for $K = 2.2 \times 10^3$ M^{-1}, $n = 1$, parameters obtained from a Scatchard plot of the data.

Tropolone and its methyl ether inhibited microtubule assembly to identical extents, as shown in Figures 5 and 6. The inhibition experiments were carried out in two ways. When, following the binding results, the ligand was first bound to tubulin in the cold and then assembly was triggered by raising the temperature to 37 °C, inhibition occurred in a ligand concentration range of $\sim 10^{-4}$ M. On the other hand, when the binding was performed at 37 °C in the presence of 2.5×10^{-5} M Ca^{2+} and the assembly was started at the same temperature by injection of 10^{-3} M EGTA, a weaker, although complete, inhibitory effect was observed with a reciprocal half-inhibitory concentration of $(5 \pm 2) \times 10^2$ M^{-1}, as shown in Figure 6. Again, just as in the case of NAM, interpretation of the relative extent of the inhibition of terms of specific models is not warranted at present.

Discussion

Trimethoxyphenyl Binding Region of the Colchicine Site. The above-described experiments with mescaline and *N*-acetylmescaline, aimed at exploring the interaction of these simple trimethoxyphenyl-containing probes with the corresponding part of the colchicine–podophyllotoxin binding site

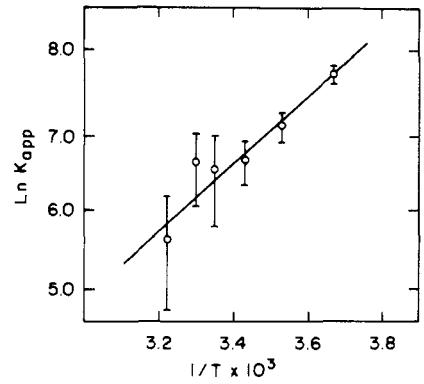


FIGURE 11: van't Hoff plot of the tropolone methyl ether–tubulin interaction. The determinations shown in Figure 10 were repeated at several temperatures. Typical binding times needed to ensure attainment of equilibrium were 120 (0 °C), 70 (10 °C), 40 (18 °C), 26 (25 °C), 20 (30 °C), and 10 min (37 °C). The bars indicate experimental error. The binding stoichiometry could be measured at 0, 10, and 18 °C, and it was assumed to remain at 1 mol of ligand per mol of tubulin at 25, 30, and 37 °C where the weakness of the interaction precluded accurate measurements.

of tubulin, have demonstrated a weak but statistically significant interaction of NAM with tubulin, which was inhibited by colchicine. Mescaline presented a higher affinity interaction with tubulin that was probably charge mediated and not directed to the colchicine binding site. Further evidence that both probes are capable of the weak specific interaction with tubulin is afforded by the fact that both ligands inhibited significantly microtubule assembly at concentrations close to the reciprocal equilibrium constants estimated for the weak binding interactions. If these ligands are indeed good analogues of the corresponding moiety of colchicine and podophyllotoxin, these observations lead to the conclusion that the interaction with tubulin of this shared trimethoxyphenyl part is a relatively weak one, in contrast with the high affinity binding of the complete drugs. This apparent paradox can be resolved, as will be shown below, by a proper thermodynamic analysis of the binding of bifunctional ligands such as colchicine and the resulting understanding that such a weak interaction may indeed make a major contribution to their binding strengths. These results may also furnish the explanation for the very weak binding of lumicolchicine to tubulin [$K_b \simeq 640$ M^{-1} (McClure & Paulson, 1977)], since that molecule possesses only the trimethoxyphenyl ring of colchicine but not the tropolone ring. On the other hand, our results with purified tubulin in vitro do not help explain the antimitotic effect of mescaline at low concentrations, reported by Harrisson et al. (1976), although we have obviously not taken into account any of the possible modulating factors that could shift the free energy of the mescaline–tubulin interaction in vivo toward more favorable values.

Tropolone Binding Region of the Colchicine Site. Both tropolone and its methoxy derivative, TME, have been shown to interact with tubulin in the presence and absence of Mg^{2+}, to inhibit colchicine binding to tubulin, and to interfere with in vitro microtubule assembly at concentrations consistent with those necessary for binding at any given temperature. Since tropolone methyl ether has the exact structure of the corresponding part of colchicine and since furthermore it has the advantage of not interacting with Mg^{2+}, it was selected as a more significant probe.

The binding of [^{3}H]TME to tubulin was to one site on the dimeric protein molecule; it was inhibited by colchicine and was not affected by Mg^{2+}. The time necessary to attain equilibrium at any temperature was well below the reported

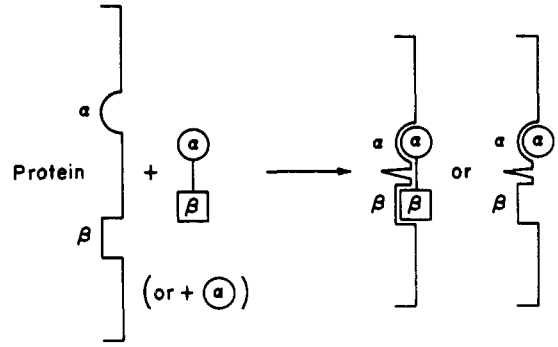


FIGURE 12: Model reaction for the binding of a bifunctional ligand (α–β) to two subsites on the protein. The binding of the α moiety induces in the protein a conformational change that brings into proper geometric alignment the β binding locus, but it does not affect the conformation of the β locus itself.

half-life values of the colchicine binding site (Wilson & Bryan, 1974), so that no significant denaturation that would bias the results could take place. The binding parameters measured suggest a moderate interaction ($\Delta G°_{app}$ near −4 kcal mol^{-1} for the temperature range of 0 to 37 °C), favored by lower temperatures and probably not mediated by hydrophobic effects ($\Delta H°_{app}$ = −8.3 kcal mol^{-1}; $\Delta S°_{app}$ = −15 eu). In this regard, it should be noted that TME spontaneously forms a hydrogen-bonded hemihydrate (Nozoe et al., 1951). The binding time course (between 10 and 120 min depending on temperature) suggests that this reaction is accompanied by a conformational change in the protein. This is supported by circular dichroism and microtuble inhibition studies which suggest a possible TME-induced conformational change in tubulin (J. M. Andreu and S. N. Timasheff, unpublished results). In cases where such a linkage exists between binding and conformational changes, the thermodynamic parameters, such as those reported here, are evidently only apparent ones, since they contain the contributions of any linked conformational changes, as well as that of the contact-making reaction, a situation akin to that reported with colchicine (Garland, 1978).

Contributions to Colchicine Binding of Its Trimethoxyphenyl- and Methoxytropolone Moieties. A Model of Bifunctional Ligand Binding. Both its structure and an analysis of its binding behavior (see introduction) give strong indications that colchicine is a bifunctional ligand that interacts with tubulin through two parts, most probably the tropolone and trimethoxyphenyl rings. It seems of interest at present to analyze how the binding of each contributes to the binding of the whole. Let us take a bifunctional ligand, α–β, capable of interacting with a bifocal binding site on the protein, illustrated in Figure 12. Let us construct a simple model of the binding thermodynamics based on a set of three assumptions: (1) the bindings of α and β are independent; (2) the bindings of α and β to the protein are not perturbed by the covalent attachment α–β; (3) the changes in external and internal mobility (rotational, translational, and vibrational), conformation, and solvation of the ligands and the protein do not differ significantly for the binding of α and α–β to the protein.

Let us now decompose the apparent (experimentally measured) binding standard free energy change, $\Delta G°_{obsd} = -RT \ln K_b^{app}$, into two contributions, $\Delta G°_{int}$, for the intrinsic standard free energy change of the formation of the protein–ligand bond which is additive when the ligand is part of a larger molecule that contains several interacting parts, and $\Delta G°_{na}$, the difference between $\Delta G°_{obsd}$ and $\Delta G°_{int}$, which is nonadditive for the parts of the ligand. Writing $\Delta G°$ for the binding of species i as ΔG^i, we have

$$\Delta G^{\alpha}_{obsd} = \Delta G^{\alpha}_{int} + \Delta G^{\alpha}_{na} \quad (1)$$

assumptions 1 and 2:

$$\Delta G^{\alpha-\beta}_{obsd} = \Delta G^{\alpha}_{int} + \Delta G^{\beta}_{int} + \Delta G^{\alpha-\beta}_{na} \quad (2)$$

assumption 3:

$$\Delta G^{\alpha-\beta}_{na} = \Delta G^{\alpha}_{na} \quad (3)$$

Combining these statements, we obtain

$$\Delta G^{\beta}_{int} = \Delta G^{\alpha-\beta}_{obsd} - \Delta G^{\alpha}_{obsd} \quad (4)$$

and

$$\Delta G^{\beta}_{na} = \Delta G^{\alpha}_{obsd} + \Delta G^{\beta}_{obsd} - \Delta G^{\alpha-\beta}_{obsd} \quad (5)$$

Knowledge of the apparent free energy changes of binding of α, β, and α–β to the protein permits us then to calculate the intrinsic and nonadditive portions of the free-energy change of binding of species β. What should be the expected value of ΔG^{β}_{na}? By definition, this term should contain the contribution from the change of the entropy of mixing and contributions from the changes in mobility, conformation, etc., of the ligand on formation of the protein–species β contact. The last term should be unfavorable. When the binding is noncovalent, however, and considerable freedom of motion is maintained, as may be in the formation of hydrophobic contacts, these contributions may be small (Steinberg & Scheraga, 1962). The immutable contribution to ΔG^i_{na} is that of the cratic free energy change, $\Delta G°_c$, for the formation of a bimolecular complex in dilute solution (Gurney, 1962; Kauzmann, 1959):

$$\Delta G°_c = -T\delta\Delta S_{mix} = -RT \ln \frac{X_{pl}}{X_p X_l} \quad (6)$$

where ΔS_{mix} is the entropy of mixing and X_i is the mole fraction of specifies i. The subscripts p, pl, and l refer to unliganded protein, liganded protein, and ligand l, respectively. Expressing the concentration in molal units and using a 1.0 m standard state and an aqueous medium, we obtain $\Delta G°_c$ = −RT ln 58.55 ≃ 2.4 kcal mol^{-1} at room temperature.

Let us now apply this model to the tubulin–colchicine interaction in terms of the measured binding constants of colchicine (α–β), tropolone methyl ether (α), and N-acetylmescaline (β).

Assumption 1 (independence of α and β binding) seems to be generally satisfied, since there was no detectable cooperativity between the binding of the two small ligands nor between their effects on colchicine binding. Assumption 2 (no effect on binding of the α–β covalent attachment) may also not be far from reality, since the microtubule inhibitory effect of 2-methoxy-5-(2,3,4-trimethoxyphenyl)tropolone was reported to be nearly as good as that of colchicine (Fitzgerald, 1976), with α and β being in a more rigid tilted conformation in colchicine (Margulis, 1975) while they should have rotational freedom in the analogue. Assumption 3 is more difficult to assess. Two arguments may be advanced in its favor. First, this assumption is consistent with the slow time course of both colchicine and TME binding, as well as the observation (J. M. Andreu and S. N. Timasheff, unpublished results) that the binding of TME to tubulin induces small conformational changes in the protein which may be similar, at least in part, to those induced by colchicine binding. Second, it seems to be supported by estimates in the literature of the contribution of various entropic effects to noncovalent liganding (Steinberg & Scheraga, 1962; Erickson & Pantaloni, 1981).

Keeping cognizance of these uncertainties, $\Delta G°_{int}$ and $\Delta G°_{na}$ were calculated for N-acetylmescaline from the experimental

Table I: Contributions of the Tropolone and Mescaline Rings to the Binding of Colchicine

ligand	free-energy change: nature of contribution[a]	value (kcal mol^{-1}) 25 °C	37 °C
colchicine[b]	$\Delta G^{\alpha-\beta}_{obsd}$	−9.4 ± 0.5	−10.2 ± 0.2
tropolone methyl ether	ΔG^{α}_{obsd}	−3.9 ± 0.4	−3.6 ± 0.6
N-acetylmescaline	ΔG^{β}_{int}	−5.5 ± 0.5	−6.6 ± 0.4
N-acetylmescaline	ΔG^{β}_{obsd}	≥ -3.4	−3.7 ± 0.5
N-acetylmescaline	ΔG^{β}_{na}	$\geq +2.1$	+2.9 ± 0.5

[a] See the text for explanation. [b] Values from a critical inspection of the literature (see introduction). Namely, the value at 37 °C is from the data of Garland (1978), and the value at 25 °C derives from same data assuming that the ΔH°_{app} estimates (Bryan, 1972; Bhattacharyya & Wolff, 1974) are within a factor of 2 of the correct value.

values of the observed standard free energy changes of binding of colchicine, tropolone methyl ether, and *N*-acetylmescaline. The results are summarized in Table I. The conclusions of this analysis are the following:

(I) Even though the binding of NAM to tubulin was experimentally detectable with difficulty, nevertheless it is characterized by a sizeable intrinsic standard free energy change (−5 to −7 kcal mol^{-1}). The trimethoxyphenyl region shared by colchicine and podophyllotoxin appears then to contribute significantly to the binding and may be decisive in determining the strength of the interaction. The difficulty of measuring its small interaction affinity when separated from the other ring can serve as a good illustration of the entropic advantage of bifunctional ligands over their monofunctional moieties. This thermodynamic fact may also explain the apparent discrepancy between the presently reported binding results and the pharmacological study which showed no significant effects when the single ring compounds were used at levels similar to that of colchicine (Fitzgerald, 1976). The possibility remains that some effects of the binding of the bifunctional ligand are not present when the two moieties are bound individually. There could be a colchicine-induced conformational change in the protein not induced separately by the tropolone and mescaline moieties, an interaction between sites, or a distortion of the individual binding contacts. Any such effects would render our simple assumptions incorrect, requiring the introduction of additional free-energy terms into our calculation. Since at present there is no evidence for such complexity, incorporation of such terms would only complicate the model without giving any new insight into the nature of the phenomenon.

(II) The nonadditive portion of the binding standard free energy change for NAM (2–3.5 kcal mol^{-1} can be accounted for almost totally by the cratic contribution (2.4 kcal mol^{-1}).

(III) The temperature dependence of the trimethoxyphenyl ring–tubulin interaction can be predicted. Since colchicine binding is characterized by large positive standard enthalpy and entropy changes and tropolone methyl ether seems to contribute a negative enthalpy change and a weakly negative entropy change, the trimethoxyphenyl ring would be expected to contribute a large positive standard enthalpy change and a positive standard entropy change. The predicted temperature dependence of NAM binding is presented in Figure 13, showing agreement with the available experimental results. Furthermore, the calculated thermodynamic characteristics of the *N*-acetylmescaline interaction with tubulin are consistent with the expected values for a hydrophobic interaction involving the transfer of a nonpolar molecule from a polar environment to a less polar one (Tanford, 1973). In our case this could be envisaged as the binding in aqueous medium of the trimethoxyphenyl compounds to a hydrophobic crevice on the tubulin molecule.

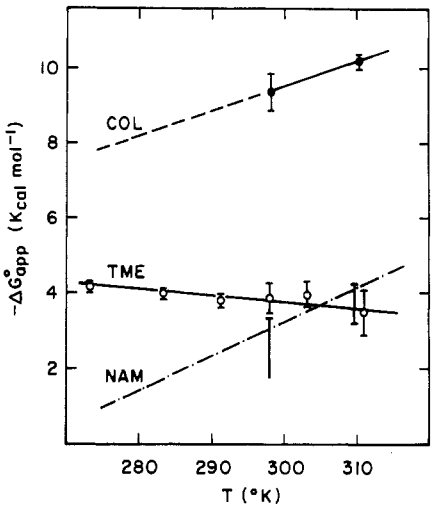


FIGURE 13: Temperature dependence of the colchicine, tropolone methyl ether, and *N*-acetylmescaline interactions with tubulin. TME data (○) are those determined experimentally, while the colchicine data (●) are those taken from the literature as discussed in the text. The line for *N*-acetylmescaline (- - -) was calculated according to eq 5, assuming $\Delta G_{na} \simeq 2.4$ kcal mol^{-1}, in agreement with Table I. The experimentally determined ranges for the *N*-acetylmescaline interaction are marked by the heavy vertical bars.

(IV) Further reflection suggests the order of binding of the two ends of colchicine to tubulin. The hypothesis most consistent with the various observations, namely, the inhibition of colchicine binding by TME, the lack of cooperativity between TME and NAM in this inhibition, as well as the noncooperative binding of the two ligands to tubulin, the inhibition of colchicine binding by podophyllotoxin, and the inability of podophyllotoxin to displace colchicine once it is tightly bound, is depicted schematically in Figure 12. According to this hypothesis, the tropolone end would bind first, and this binding would induce in the protein a conformational change, bringing the trimethoxyphenyl binding site into proper position for this ring to fall into place on the protein without any further changes in tubulin conformation; i.e., binding of TME moiety does not induce the formation of the second site but only brings it into the proper position. This hypothesis also gains support from the observation (J. M. Andreu and S. N. Timasheff, unpublished results) that tropolone methyl ether induces in tubulin a conformational change that seems to be related to that induced by colchicine, while NAM does not seem to affect the protein conformation within the limits of detection of techniques available to us.

References

Andreu, J. M., & Timasheff, S. N. (1981a) *Biophys. J. 33*, 247a.

Andreu, J. M., & Timasheff, S. N. (1981b) *Biochim. Biophys. Acta* (in press).

Bergen, L. G., & Borisy, G. G. (1980) *J. Cell Biol. 84*, 141–150.

Bhattacharyya, B., & Wolff, J. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2627–2631.

Brewer, C. F., Loike, J. D., Horwitz, S. B., Sternlicht, H., & Gensler, W. J. (1979) *J. Med. Chem. 22*, 215–221.

Bryan, J. (1972) *Biochemistry 11*, 2611–2616.

Cook, J. W., Gibbs, A. R., Raphael, R. A., & Somerville, A. R. (1951) *J. Chem. Soc.*, 503–511.

Cortese, F., Bhattacharyya, B., & Wolff, J. (1977) *J. Biol. Chem. 252*, 1134–1140.
David-Pfeuty, T., Simon, C., & Pantaloni, D. (1979) *J. Biol. Chem. 254*, 11696–11202.
DeMore, W. B., Pritchard, H. W., & Davidson, N. (1959) *J. Am. Chem. Soc. 81*, 5824–5829.
Erickson, H. P., & Pantaloni, D. (1981) *Biophys. J. 34*, 293–309.
Fales, H. M., Jaoumi, T. M., & Babashak, J. F. (1973) *Anal. Chem. 45*, 2302–2303.
Fasella, P., Hammes, G., & Schimmel, P. (1965) *Biochim. Biophys. Acta 103*, 708–710.
Fitzgerald, T. J. (1976) *Biochem. Pharmacol. 25*, 1383–1387.
Frigon, R. P., & Lee, J. C. (1972) *Arch. Biochem. Biophys. 153*, 587–589.
Frigon, R. P., & Timasheff, S. N. (1975) *Biochemistry 148* 4559–4566, 4567–4573.
Garland, D. L. (1978) *Biochemistry 178* 4266–4272.
Gaskin, F., Cantor, C. R., & Shelanski, M. L. (1974) *J. Mol. Biol. 89*, 737–758.
Gurney, R. W. (1962) *Ionic Processes in Solution*, pp 90–104, Dover Publications, New York.
Harrisson, C. M., Page, B. M., & Keir, H. M. (1976) *Nature (London) 250*, 138–139.
Hummel, J. P., & Dreyer, W. J. (1962) *Biochim. Biophys. Acta 63*, 530–532.
Karr, T. L., & Purich, D. L. (1979) *J. Biol. Chem. 259*, 10885–10888.
Kauzmann, W. (1959) *Adv. Protein Chem. 14*, 1–63.
Kelleher, J. K. (1977) *Mol. Pharmacol. 13*, 32–241.
Leach, S. J., & Scheraga, H. A. (1960) *J. Am. Chem. Soc. 82*, 4790–4792.
Lee, J. C., & Timasheff, S. N. (1975) *Biochemistry 14*, 5183–5187.
Lee, J. C., & Timasheff, S. N. (1977) *Biochemistry 16*, 1754–1764.
Lee, J. C., Frigon, R. P., & Timasheff, S. N. (1973) *J. Biol. Chem. 248*, 7253–7262.
Lee, J. C., Harrison, D., & Timasheff, S. N. (1975) *J. Biol. Chem. 250*, 9276–9282.
Lettré, H., & Fernholtz, M. (1943) *Hoppe-Seyler's Z. Physiol. Chem. 278*, 125.
Lundstrom, J., & Agurell, S. (1967) *J. Chromatogr. 30*, 271–272.
Margolis, R. L., & Wilson, L. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3466–3470.
Margolis, R. L., & Wilson, L. (1978) *Cell (Cambridge, Mass.) 13*, 1–8.
Margulis, T. N. (1975) in *Microtubules and Microtubule Inhibitors* (Borgers, M., & deBrabander, M., Eds.) pp 67–78, Elsevier/North-Holland, Amsterdam.
McClure, W. O., & Paulson, J. C. (1977) *Mol. Pharmacol. 13*, 560–575.
Na, G. C., & Timasheff, S. N. (1981) *J. Mol. Biol. 151*, 165–178.
Nozoe, T., Seto, S., Ikemi, T., & Arai, T. (1951) *Proc. Jpn. Acad. 27*, 102–106.
Nunez, J., Fellows, A., Francon, J., & Lennon, A. M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 86–90.
Oosawa, F., & Kasai, M. (1971) *Biol. Macromol. 5*, 261–322.
Owellen, R. J., Owens, A M., Jr., & Donnigen, D. W. (1972) *Biochem. Biophys. Res. Commun. 47*, 685–691.
Paulson, J. C., & McClure, W. O. (1973) *Mol. Pharmacol. 9*, 41–50.
Postingl, H., Kraus, E., Little, M., & Kempf, T. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2757–2761.
Sherline, P., Leung, J. T., & Kipnis, D. M. (1975) *J. Biol. Chem. 250*, 5481–5486.
Snyder, S. L., & Sobocinski, P. Z. (1975) *Anal. Biochem. 64*, 284–288.
Steinberg, I. Z., & Scheraga, H. A. (1962) *J. Biol. Chem. 238*, 172–181.
Sternlicht, N., & Ringel, I. (1979) *J. Biol. Chem. 254*, 10540–10550.
Tanford, C. (1973) *The Hydrophobic Effect*, pp 16–23, Wiley, New York.
Valenzuela, P., Quiroga, M., Zladivar, J., Rutter, W. J., Kirschner, M. W., & Cleveland, D. W. (1981) *Nature (London) 289*, 650–655.
Weber, K., Pringle, J. R., & Osborn, M. (1972) *Methods Enzymol. 26*, 3–27.
Weisenberg, R. C., Borisy, G. G., & Taylor, E. (1968) *Biochemistry 7*, 4466–4479.
Wilson, L., & Bryan, J. (1974) *Adv. Cell Mol. Biol. 3*, 21–72.