Chaiken, I. M. (1979) *Anal. Biochem. 97*, 302–308.
Chaiken, I. M. (1981) *CRC Crit. Rev. Biochem. 11*, 255–301.
Chaiken, I. M., & Sanchez, G. R. (1972) *J. Biol. Chem. 247*, 6743–6747.
Chaiken, I. M., & Hough, C. J. (1980) *Anal. Biochem. 107*, 11–16.
Chaiken, I. M., Randolph, R. E., & Taylor, H. C. (1975) *Ann. N.Y. Acad. Sci. 248*, 422–450.
Chaiken, I. M., Fischer, E. A., Giudice, L. C., & Hough, C. B. (1982) in *Hormonally Active Brain Peptides: Structure and Function* (McKerns, K. W., & Pantič, V., Eds.) pp 327–347, Plenum Press, New York.
Chauvet, M. T., Codogno, P., Chauvet, J., & Acher, R. (1979) *FEBS Lett. 98*, 37–40.
Crestfield, A. M., Moore, S., & Stein, W. H. (1963) *J. Biol. Chem. 238*, 622–627.
Dreifuss, J. J. (1975) *Ann. N.Y. Acad. Sci. 248*, 184–201.
Fischer, E. A., Curd, J. G., & Chaiken, I. M. (1977) *Immunochemistry 14*, 595–602.
Gainer, H., Sarne, Y., & Brownstein, M. J. (1977) *J. Cell Biol. 73*, 366–381.
Glasel, J. A., McKelvy, J. F., Hruby, V. J., & Spatola, A. F. (1976) *J. Biol. Chem. 251*, 2929–2937.
Griffin, J. H., Alazard, R., & Cohen, P. (1973) *J. Biol. Chem. 248*, 7975–7978.
Hollenberg, M. D., & Hope, D. B. (1968) *Biochem. J. 106*, 557–564.
Land, H., Schütz, G., Schmale, H., & Richter, D. (1982) *Nature (London) 295*, 299–303.
Livett, B. G. (1978) *Int. Rev. Cytol., Suppl. No. 7*, 53–237.
Lord, S. T., & Breslow, E. (1980) *Biochemistry 19*, 5593–5602.
Midgley, A. R., Niswender, G. D., & Rebar, R. W. (1969) *Acta Endocrinol. (Copenhagen), Suppl. No. 142*, 163–184.
Nicolas, P., Camier, M., Dessen, P., & Cohen, P. (1976) *J. Biol. Chem. 251*, 3965–3971.
Nicolas, P., Wolff, J., Camier, M., DiBello, C., & Cohen, P. (1978) *J. Biol. Chem. 253*, 2633–2639.
Nicolas, P., Batelier, G., Rholam, M., & Cohen, P. (1980) *Biochemistry 19*, 3565–3573.
Pearlmutter, A. F., & Dalton, E. J. (1980) *Biochemistry 19*, 3550–3556.
Pickering, B. (1978) *Essays Biochem. 14*, 45–81.
Rabbani, L. D., Pagnozzi, M., Chang, P., & Breslow, E. (1982) *Biochemistry 21*, 817–826.
Sachs, H., Fawcett, Y., Takabatake, Y., & Portanova, R. (1969) *Recent Prog. Horm. Res. 25*, 447–491.
Schlesinger, D. H., Audhya, T. K., & Walter, R. (1978) *J. Biol. Chem. 253*, 5019–5024.
Schlesinger, D. H., Audhya, T. K., Hoffman, P. L., & Walter, R. (1979) *Proc. Int. Congr. Biochem., 11th, Abstr. 603* (Abstr. 11-3-S5).
Sur, S. S., Rabbani, L. D., Libman, L., & Breslow, E. (1979) *Biochemistry 18*, 1026–1036.
Tellam, R., & Winzor, D. J. (1980) *Arch. Biochem. Biophys. 201*, 20–24.
Walter, R., & Hoffman, P. L. (1973) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 32*, 1974.
Wuu, T., & Crumm, S. E. (1976) *Biochem. Biophys. Res. Commun. 68*, 634–639.

Conformational States of Tubulin Liganded to Colchicine, Tropolone Methyl Ether, and Podophyllotoxin†

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ABSTRACT: The conformational effects of colchicine, podophyllotoxin, and tropolone methyl ether binding to tubulin have been studied. Conditions for the stability of the purified calf brain protein were established, and the effects of binding were examined by means of difference absorption spectroscopy, circular dichroism, fluorescence, activation of tubulin GTPase, and tubulin self-association reactions. The tubulin–colchicine complex was isolated and characterized. It displays quenched intrinsic protein fluorescence, ligand fluorescence, and GTPase activity, probably accompanied by minor perturbations in the secondary structure. The conformation of the tubulin–colchicine complex appears to be nonidentical with that of the unliganded protein. Podophyllotoxin was not found to induce any of the mentioned changes. This ligand seems to bind through a hydrophobic interaction of its trimethoxybenzene ring with tubulin, as does colchicine. Binding of tropolone methyl ether, which is the analogue of the other part of the colchicine molecule that binds to tubulin, produced effects consistent with a ligand-linked conformational change. The small perturbation by tropolone methyl ether of the circular dichroism spectrum of tubulin resembles changes induced by colchicine.

The antimitotic drugs colchicine and podophyllotoxin are known to bind strongly to soluble tubulin (Cortese et al., 1977; Garland, 1978). The liganded protein can be incorporated into microtubules with the consequence that the formation of these tubulin polymers is inhibited by substoichiometric amounts of the drugs (Olmsted & Borisy, 1973; Margolis & Wilson, 1977; Sternlicht & Ringel, 1979; Margolis et al., 1980; Lambeir & Engelborghs, 1980). The binding of colchicine to tubulin is slow, but the high apparent affinity is difficult to measure quantitatively by equilibrium techniques due to the instability of the protein (Wilson & Bryan, 1974). Garland (1978) carried out a kinetic study of the binding and fitted the results by a binding scheme consisting of a fast reversible association, followed by a slow ligand-induced conformational

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change, with an overall binding equilibrium constant of (1–2) $\times 10^7$ M^{-1}. Furthermore, colchicine has been shown to elicit in tubulin a weak GTPase activity (David-Pfeuty et al., 1979; Andreu & Timasheff, 1981a) which is consistent with the induction or enhancement of a conformational change by this drug. Podophyllotoxin, which contains a trimethoxybenzene ring as a structural feature in common with colchicine, competes with colchicine for the binding to tubulin, but it binds with a slightly lower apparent affinity (Cortese et al., 1977). No protein conformational change has been reported to accompany the binding of podophyllotoxin to tubulin. Recently, we have demonstrated the interaction of simple trimethoxybenzene and tropolone compounds with the colchicine binding site of tubulin, in agreement with the concept that colchicine is a bifunctional ligand that binds principally through the trimethoxybenzene and tropolone moieties of its structure (Andreu & Timasheff, 1982a,b). We have proposed that one of the loci of podophyllotoxin binding to tubulin is its trimethoxybenzene ring and that the postulated conformational change linked to colchicine binding is related to the tropolone portion of that drug. We have proposed furthermore that colchicine binds first through its tropolone end, inducing or enhancing a conformational change in the protein which permits the subsequent easy binding of the colchicine trimethoxybenzene ring.

In their circular dichroism study of microtubule protein, Ventilla et al. (1972) reported a slow, partially irreversible change at temperatures above 15 °C which led to protein denaturation. Ligands, such as nucleotides, colchicine, and vinblastine, were shown to protect the native protein structure, but no other conformational effects were found at that time. Lee et al. (1978), on the other hand, did not report any effect of temperature in their conformational study of tubulin, although the decay of the colchicine binding activity was known to become faster with an increase in temperature (Frigon & Lee, 1972). We have now reexamined carefully the stability of purified calf brain tubulin, and we have probed the conformational effects of colchicine, tropolone methyl ether, and podophyllotoxin under conditions in which denaturation was rigorously minimized. The results of these studies are described in this paper.

Materials and Methods

Chemicals. Colchicine, podophyllotoxin (lot no. 01987 and 022757), and trimethoxybenzene were from Aldrich. Tropolone methyl ether (2-methoxy-2,4,6-cycloheptatrienone) was obtained by methylation of tropolone as described previously (Andreu & Timasheff, 1982a). Mescaline hydrochloride was from Sigma. Lumicolchicine was prepared by irradiation of dilute solutions of colchicine (Wilson & Friedkin, 1965) with long-wavelength ultraviolet light (UVSL 25 Mineralight lamp; UV Products, Inc.). The extent of transformation was more than 99% as assessed from the change in the absorption spectrum (Grewe & Wulf, 1951). [*methoxy*-^{3}H]Colchicine, 23 Ci/mmol, was from New England Nuclear (lot no. 1339-061); it was found to be more than 97% radiochromatographically homogeneous on silica gel sheets (Eastman Kodak) developed with pyridine–concentrated ammonia (9:1) or chloroform–acetone–diethylamine (7:2:1). [^{3}H]Lumicolchicine, prepared from labeled colchicine, retained the isotopic label and was more than 95% radiochromatographically homogeneous. GTP (type II) was from Sigma. [γ-^{32}P]GTP, 20–40 Ci/mmol, was from New England Nuclear. The following extinction coefficients (10 mM sodium phosphate, pH 7.0) were used: colchicine, E_{353} = 15 950 and E_{276} = 5260 M^{-1} cm^{-1}; lumicolchicine, E_{267} = 23 450 and E_{276} = 15 800 M^{-1} cm^{-1}; tropolone methyl ether, E_{345} = 6900 M^{-1} cm^{-1}; podophyllotoxin, E_{290} = 3700 M^{-1} cm^{-1}; GTP, E_{252} = 13 700 and E_{276} = 9700 M^{-1} cm^{-1}. Sephadex G25 and Sephacryl S300 were from Pharmacia. Sucrose (ultrapure) was from Schwarz/Mann. Guanidine hydrochloride was from Heico and dithiothreitol from Calbiochem. All other chemicals were of reagent grade.

Preparation of Tubulin and the Stable Tubulin–Colchicine Complex. Soluble purified calf brain tubulin was prepared as described before (Lee et al., 1973; Andreu & Timasheff, 1982a). The tubulin–colchicine complex was prepared by incubation for 10 min of 0.40 ± 0.05 mM tubulin with 1 mM [^{3}H]colchicine (at a specific activity of 20 Ci/mol) in 10 mM sodium phosphate, 0.1 mM GTP, 0.25 mM $MgCl_2$, and 1 M sucrose buffer, pH 7.0 at 25 °C. Under our conditions, this time was found to be more than sufficient to attain equilibrium, in agreement with the kinetic study of Lambeir & Engelborghs (1981). The protein–ligand complex was then separated from the excess ligand by means of rapid (5 min) Sephadex G25 (0.9 × 20 cm bed) chromatography in the desired buffer at 0 °C. After a second chromatography, the ligand concentration was measured by liquid scintillation counting and the protein by its light absorption at 276 nm, correcting for the small contribution of the ligand. In a control experiment, in which the colchicine was incubated with a large excess of tubulin, 95% of the tritium label was bound by the protein. When the raw data are corrected for this value, the binding stoichiometry obtained was $\bar{\nu}$ = 0.97 ± 0.05 mol of [^{3}H]-colchicine/10^5 g of tubulin (seven independent determinations performed on two different tubulin preparations). Preparations with $\bar{\nu} < 1$ were obtained by incubating the protein with the ligand at nonsaturating concentrations. From the extinction coefficient of tubulin, the extinction coefficient of colchicine at 276 nm, and the increment of absorption due to binding (see Results), the extinction coefficient of the tubulin–colchicine complex was calculated to be E_{276} = 1.23 L g^{-1} cm^{-1} (PG[1] buffer, pH 7.0 at 25 °C). Less than 2% of the complex dissociated during 2 h in PG buffer at room temperature. More than 99.8% of the complex dissociated in a 10 mM sodium phosphate, 6 M guanidine hydrochloride, and 5 mM dithiothreitol, pH 7.0, medium. Irradiation of the complex for 15 min in a quartz cell ($A_{353} \leq 0.1$) placed 1 cm away from the ultraviolet lamp (see above) resulted in the release of 93.4 ± 1.2% of the radioactive ligand.

Protein Concentration Determination. The tubulin concentration was determined spectrophotometrically, using a Cary 118 instrument. Since tubulin binds nucleotides, their contribution to absorption had to be taken into account. This was done as follows. Using the amino acid composition of calf brain tubulin (Lee et al., 1973) and the sequences of chick and pig brain tubulins (Valenzuela et al., 1981; Postingl et al., 1981), we calculated the refractive index increment of native tubulin, $\Delta n/c$, following the accurate procedure of McMeekin et al. (1964). The value obtained was $\Delta n/c$ = 0.191 ± 0.001 mL g^{-1} at 436 nm. Similarly, the partial specific volume was calculated by applying the procedure of Lee & Timasheff (1974) to the chick and pig brain tubulin sequences. The resulting value, $\bar{v}_2$ = 0.735 ± 0.001 mL^- g^{-1}, was in good agreement with the experimentally determined values ($\bar{v}_2$ = 0.736 ± 0.002 mL g^{-1}) for calf brain tubulin (Lee et al., 1973; Na & Timasheff, 1981). Tubulin samples were equilibrated in PG–20 mM NaCl buffer, pH 7.0, by rapid chromatography

[1] Abbreviations: EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PG buffer, 10 mM sodium phosphate–0.1 mM GTP, pH 7.0, buffer; CD, circular dichroism.

on G25 Sephadex columns, followed by 2 h of dialysis against the same buffer at 4 °C. The protein concentration was then determined by differential refractometry (Pittz et al., 1973), using $\Delta n/c = 0.191$ mL g^{-1}, and the absorption spectra of the clear samples were recorded and corrected for scattering (Leach & Scheraga, 1960). The scattering corrections were typically <5% of the absorbance at 276 nm. This procedure resulted in a native tubulin absorbance maximum at 276.5 ± 0.5 nm, with $E = 1.6$ L g^{-1} cm^{-1}. This value, which takes into account the nucleotide bound to the native tubulin under our experimental conditions, was then used for protein concentration determination. The ratio of maximal absorbances in neutral aqueous buffer and neutral 6 M guanidine hydrochloride was determined by means of gravimetric dilution and comparative absorption measurements. The result was E_{276}(buffer)/E_{275}(guanidine hydrochloride) = 1.066, giving an extinction coefficient of $E_{275} = 1.09$ L g^{-1} cm^{-1} for tubulin in our buffer diluted into 6 M guanidine hydrochloride. Na & Timasheff (1981) had previously reported an extinction coefficient $E_{275} = 1.03$ L g^{-1} cm^{-1} in 6 M guanidine hydrochloride, based on careful dry weight measurements. When their conditions (22 h of dialysis at 4 °C, followed by 2–3 h at 22 °C, using the same buffer as above) were reproduced and the present measurement procedure was employed, the extinction coefficients obtained were $E_{276} = 1.09$ L g^{-1} cm^{-1} in neutral buffer and $E_{275} = 1.02$ L g^{-1} cm^{-1} in 6 M guanidine hydrochloride, in agreement with their results. The decrease in extinction coefficient which followed the long dialysis time was found to be accounted for, within experimental error, by the release during dialysis of 0.63 ± 0.10 mol of guanine nucleotide per 10^5 g of protein. The nucleotide contents were determined spectrophotometrically after extraction of the tubulin and the buffer samples with 5% perchloric acid (Carlier & Pantaloni, 1978).

Optical Properties. The absorption spectra were obtained on a Cary 118 spectrophotometer using 1-cm cells. In order to obtain difference spectra, (0.4 + 0.4)-cm tandem cells were filled gravimetrically, and the spectra were recorded in the autogain mode with a 0.3-mm slit at 27 ± 0.5 °C. The spectra shown have been corrected for base-line deviations. The fluorescence measurements were made on Hitachi Perkin-Elmer MPF 3 and 650-40 spectrofluorometers operating in the ratio mode, using 1 × 1 cm cells at the given temperatures controlled to ±0.5 °C. The excitation bandwidth was 2 nm, and the emission bandwidth was 5 nm. The spectra shown are corrected (using the Hitachi 650-0178 data processor) for solvent contributions (stored solvent spectrum), excitation (stored correction factors generated with rhodamine B), and emission (stored correction factors generated with a white scatterer). Fluorescence polarization was measured with the 650-40 instrument equipped with the polarization accessory, analyzing the emission at 350 nm. The circular dichroism (CD) spectra were obtained with a Cary 60 spectropolarimeter equipped with the 6001 CD accessory, using solutions of absorbance smaller than 2.0 in 0.1-cm unjacketed (27 °C) or jacketed cells at the desired temperature ± 0.2 °C. The spectra shown have been corrected for the buffer base line and frequently are the result of averaging several individual spectra. The instrument was calibrated with camphorsulfonic acid.

GTPase Assay. The tubulin or the tubulin–colchicine complex was freed from the colchicine-independent contaminant GTPase activity found in tubulin preparations by passing through Sephacryl S300 columns equilibrated with the labeled substrate (Andreu & Timasheff, 1981a). It was then verified from the elution profile of the label that the protein was in

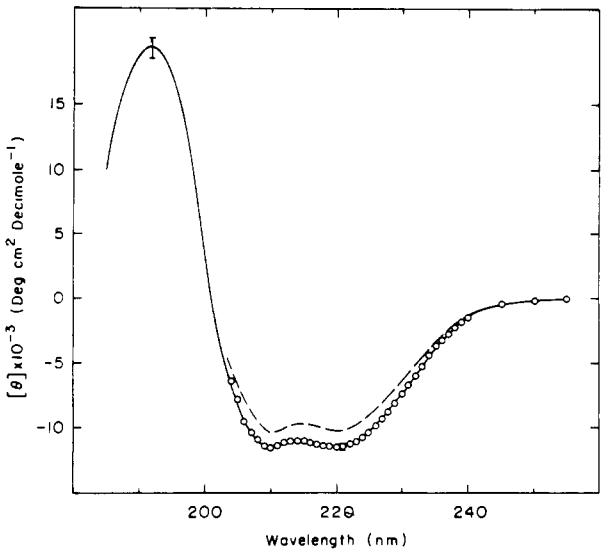


FIGURE 1: Circular dichroism spectrum of tubulin in PG buffer, 5 mM sucrose, and 2.5 μM $MgCl_2$, pH 7.0, after an average of 20 min at 27 °C (—). Samples of 0.5 mg/mL tubulin were prepared at zero time by a 200-fold dilution into PG buffer of a tubulin stock solution kept at 0 °C. The open circles are CD measurements under the same conditions except that 1 M sucrose was present in the medium. The dashed line shows the effect of aging of ca. 2 h in the absence of 1 M sucrose.

equilibrium with the known free [γ-^{32}P]GTP concentration and that it contained ≥0.8 mol of exchangeable [γ-^{32}P]GTP per 10^5 g of tubulin. Hydrolysis of the substrate was measured as described previously (Andreu & Timasheff, 1981a).

Protein Self-Associations. Sedimentation velocity experiments were performed on protein solutions of identical concentrations with and without ligand, run simultaneously in double-sector cells in an An-D rotor at 60 000 rpm in a Beckman Model E analytical ultracentrifuge equipped with an electronic speed control and an RTIC unit. Microtubule assembly in vitro was performed in 10 mM sodium phosphate, 0.1 mM GTP, 1 mM EGTA, 16 mM $MgCl_2$, and 3.4 M glycerol, pH 7.0, assembly buffer, and the reaction was followed turbidimetrically in a thermostated spectrophotometer cuvette at 37 °C (Lee & Timasheff, 1977).

Results

Tubulin Stability. The stability of tubulin was carefully scrutinized prior to probing the effects of the ligands on the conformation of the protein. The far-UV circular dichroism spectrum of tubulin in PG buffer at pH 7.0, 27 °C, is shown in Figure 1. This spectral range was covered by scanning individual samples starting at several wavelengths, since successive scans of the same sample over the entire range were not coincident. In fact, after 2 h, the negative ellipticity maxima at 210 and 220 nm had undergone a change (dashed line in Figure 1) equivalent to a decrease of approximately 4% in the apparent α-helical contents of the protein (Sears & Beychok, 1973). The protein did not seem to have aggregated significantly, since A^{1cm}_{350nm} remained practically constant at a value <0.01. As shown in Figure 2, the decay of the ellipticity at 220 nm followed apparent first-order kinetics with a half-life of 95 min. The presence of 1 M sucrose produced a clear stabilizing effect, as shown by the triangles of Figure 2, but had no effect on the initial spectrum (see Figure 1, open circles). Similar effects of spectral decay and its inhibition by 1 M sucrose were found in the protein fluorescence under identical conditions. As shown in Table I, the rate of decay increased at higher temperatures. There was no significant

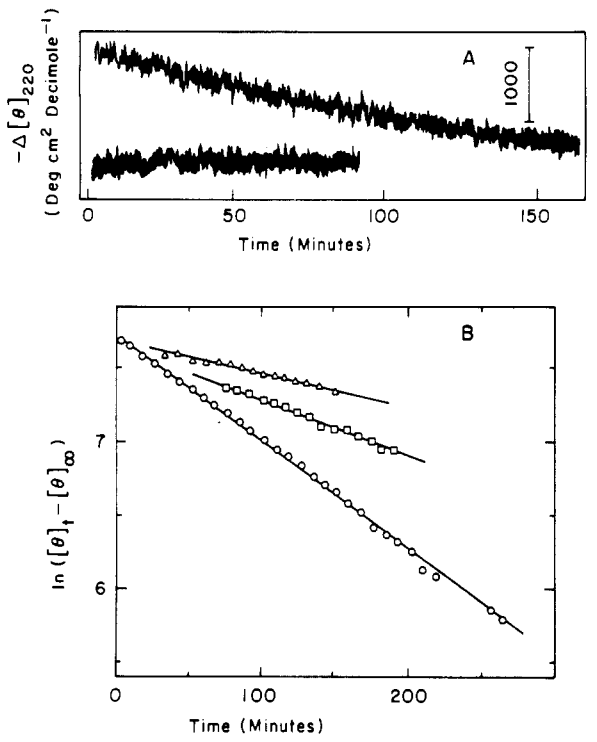


FIGURE 2: Effect of standing at 27 °C on the CD spectrum of tubulin. (A) The upper tracing is a recording of the ellipticity at 220 nm as a function of time in PG buffer, 5 mM sucrose, and 2.5 μM $MgCl_2$, pH 7.0. The lower tracing is a check of instrumental stability obtained with the same solution which had been rendered 0.1% in sodium dodecyl sulfate. (B) First-order plots of the change of $[\theta]_{220}$ for tubulin in PG buffer (○), in PG buffer with 1 M sucrose (△), and in the PG buffer in the presence of 0.5 mM tropolone methyl ether (□).

Table I: Rate of Change of Tubulin Optical Properties[a]

temp (°C)	10^3 (initial rate of change)/(initial signal) (min^{-1}) fluorescence (excitation 276 nm, emission 330 nm)	circular dichroism ($[\theta]_{220\ nm}$)
6	⩾−0.2	
27	−0.9 ± 0.2	0.8 ± 0.1 (∿0.1)
37	−1.7 ± 0.3	
42	−2.8 ± 0.5 (−0.3)	

[a] The fluorescence and circular dichroism spectra were measured on 4.5 μM protein in PG buffer, pH 7.0, except for the numbers in parentheses that were obtained in the presence of additional 1 M sucrose.

effect on temperature (10–30 °C) over 2 h on the sedimentation coefficient, $s_{20,w}$, of 4 mg mL^{-1} tubulin in PG buffer, pH 7.0. Following these observations, all the ligand interaction studies were performed under critically defined conditions where the effects of liganding can be separated from those of denaturation. Whenever feasible, the experiments were carried out in the presence of 1 M sucrose, or else they were designed to take short times and were performed at low temperatures. The stabilities of the properties examined were checked on controls subjected to identical conditions, except for the presence of ligand. The effects of ligand binding on tubulin conformation were then probed by differential UV spectroscopy, circular dichroism, fluorescence, generation of GTPase activity, and the effect on tubulin self-associations, and the results are presented in turn.

Differential Absorption Spectroscopy. The difference spectra generated by the interaction of tubulin with colchicine and podophyllotoxin are shown in Figure 3. The spectra were obtained between 260 and 400 nm, the increased noise level preventing accurate measurements at lower wavelengths. The interaction of the protein with colchicine resulted in a difference spectrum with maxima at 271, 350, 363, and 388 nm (Figure 3a). In order to identify the nature of the interactions which gave rise to this perturbation of the colchicine spectrum, we obtained difference spectra of colchicine in media of different polarities and hydrophobicities. The difference spectra of colchicine in methanol or formamide vs. an aqueous solution were qualitatively similar to that of colchicine–tubulin, with maxima at 340–344, 363–365, and 386 nm, superimposed on a broad negative differential absorption between 280 and 370 nm. Chloroform gave somewhat more different perturbations, as shown in Figure 3b. The absorption in the 300–400-nm range must be due principally to the tropolone part of the drug since trimethoxybenzene absorbs at lower wavelengths. This is supported by the direct and difference spectra of tropolone methyl ether, both of which are equally shifted to lower wavelengths and display more fine structure than the colchicine spectra, as shown in Figure 3c.

The difference spectrum generated by tubulin in the presence of podophyllotoxin vs. the uncomplexed components of the system was characterized by sharper maxima, located at 249, 282, 288, 295, and 302 nm, as shown in Figure 3d. The methanol vs. aqueous solution difference spectrum of the ligand, shown in Figure 3e, was remarkably similar with maxima at 249, 282, 288, 294, and 302 nm. These spectral perturbation bands can be best assigned to the trimethoxybenzene moiety of podophyllotoxin, since the perturbation by methanol of the absorption spectra of mescaline (trimethoxyphenylethylamine) and trimethoxybenzene displayed features similar to those of the podophyllotoxin difference spectrum, although both the direct and difference spectra of these compounds were equally shifted to shorter wavelengths with respect to podophyllotoxin, as shown in Figure 3f.

Circular Dichroism. Colchicine at a saturating level produced a small and consistently reproducible change in the circular dichroism spectrum of tubulin in PG buffer–1 M sucrose, pH 7.0 at 27 °C. As shown in Figure 4A, the 210-nm negative band underwent a small decrease in intensity and a shift to 211 nm, whereas the 22.05-nm band shifted to 219.5 nm. These changes are clearly demonstrated by the calculated difference spectrum, shown in the inset of Figure 4A, characterized by a negative $\Delta[\theta]$ at 217.5 nm, a positive change below 212 nm, and no difference in the 212–214- and 222–224-nm regions. When the colchicine in the system was photoconverted to lumicolchicine, the shape of the spectrum returned close to that of the original unliganded one, although the intensity was lower. The statistical significance of these changes was firmly established by measuring $[\theta]_{220}/[\theta]_{210}$ of several samples and averaging for each set of experimental conditions. These numbers are presented in Table II. Podophyllotoxin at 50 μM had no significant effects on the circular dichroism of tubulin, and a similar result was obtained with tropolone methyl ether at 27 °C (Figure 4A and Table II), which displayed only a very small change in $[\theta]_{220}$ (ca. 150 deg cm^2 $dmol^{-1}$). While it was not possible to increase any further the concentration of this ligand due to its light absorption which would perturb the CD measurements, its binding to tubulin could be enhanced by working at lower temperature, since it is known that tropolone methyl ether binds better in the cold (Andreu & Timasheff, 1982a), to the contrary of colchicine and podophyllotoxin (Cortese et al., 1977). Therefore, the effect of temperature on tubulin circular dichroism was examined carefully, and the experiments with tropolone methyl ether were performed at 0 °C. The results

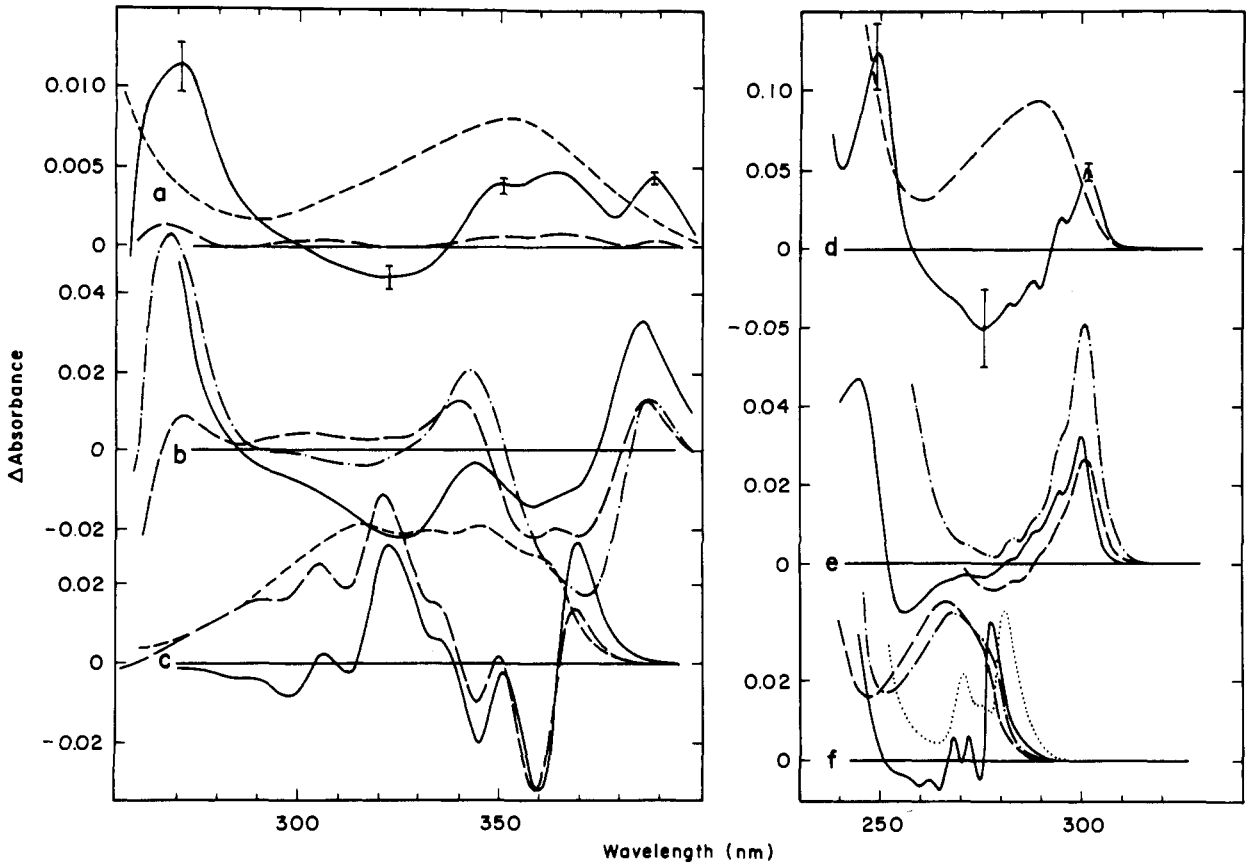


FIGURE 3: Difference absorption spectra generated by the interactions of tubulin with colchicine and podophyllotoxin in PG buffer–1 M sucrose, pH 7.0 at 27 °C. (a) (—) Equilibrium difference spectrum of a 16 μM tubulin–10 μM colchicine solution vs. 16 μM tubulin and 10 μM colchicine in separate solutions; the bars indicate the experimental error. (– –) after mixing. (– · –) Absorption spectrum of the ligand alone displayed on an 8× reduced scale. (b) Difference spectra of 100 μM colchicine in various solvents: (– –) methanol vs. aqueous solution (10 mM sodium phosphate, pH 7.0); (– · –) chloroform vs. aqueous solution; (—) formamide vs. aqueous solution. (c) Difference spectra of 100 μM tropolone methyl ether in various solvents: (– –) methanol vs. aqueous solution (10 mM sodium phosphate, pH 7.0); (—) formamide vs. aqueous solution; (– · –) absorption spectrum of the ligand alone displayed on an 8× reduced scale. (d) (—) Equilibrium difference spectrum of a 16 μM tubulin–50 μM podophyllotoxin solution vs. 16 μM tubulin and 50 μM podophyllotoxin in separate solutions. (– –) Absorption spectrum of the ligand alone displayed on an 8× reduced scale. (e) Difference spectra of 100 μM podophyllotoxin in various solvents: (—) methanol vs. aqueous solution (10 mM sodium phosphate, pH 7.0); (– · –) chloroform vs. aqueous solution; (– –) formamide vs. aqueous solution. (f) Methanol vs. aqueous solution (10 mM sodium phosphate, pH 7.0, containing 1% methanol) spectra of 1 mM trimethoxybenzene (—) and 1 mM mescaline (···). (– –) and (– · –) are the absorption spectra of trimethoxybenzene and mescaline, respectively, displayed on an 8× reduced scale.

Table II: Effects of Ligands and Temperature on the Circular Dichroism of Tubulin[a]

ligand added	$[\theta]_{220}/[\theta]_{210}$ at 0 °C	10 °C	20 °C	27 °C	37 °C
none	1.015 ± 0.008 (3)	1.018 ± 0.003 (3)	0.983 (1)	0.992 ± 0.005 (5)	0.975 (1)
colchicine			1.027 (1)	1.029 ± 0.0016 (4)	
colchicine, *hν* (lumicolchicine)				0.984 ± 0.003 (2)	
tropolone methyl ether	1.039 ± 0.008 (3)			0.996 ± 0.002 (2)	
podophyllotoxin				0.987 ± 0.002 (2)	

[a] The circular dichroism was measured on 4 μM tubulin in PG buffer–1 M sucrose, pH 7.0. The values are average ± standard deviation with the number of experiments performed in parentheses. The conditions are as in Figure 4, except for the colchicine concentration at 20 °C, which was 100 μM.

are shown in Figure 4B and Table II. In pure tubulin, there seems to be a significant trend to more negative ellipticities between 215 and 226 nm with decreasing temperatures. This effect is clearly evidenced in the calculated thermal difference CD spectrum, shown in the inset of Figure 4B. At 0 °C, tropolone methyl ether produced a significant perturbation of the circular dichroism of tubulin (Figure 4B, inset, and Table II) that strongly resembles that induced by colchicine at 27 °C (see inset of Figure 4A), with a negative maximum at 218 nm and a shoulder at 212–214 nm. The near-ultraviolet circular dichroism spectrum of a solution containing 10 μM tubulin and 40 μM colchicine in PG–1 M sucrose buffer, pH 7.0 at 27 °C, was not significantly different (within experimental error) from the algebraic sum of the separate spectra of the protein and the ligand.[2]

Fluorescence. The tubulin–colchicine stoichiometric complex was prepared rapidly and under mild conditions by using high concentrations of the reactants (see Materials and Methods). This complex contained 1 mol of ligand/mol of protein (tubulin dimer) and was stable during the time of the

[2] Subsequent to submission of this paper, we became aware of the results of Detrich et al. (1981), who reported a perturbation of the near-UV CD spectrum of colchicine when working with isolated tubulin–colchicine complex. Our failure to observe this effect can be ascribed to the large excess of free colchicine in our experimental solution.

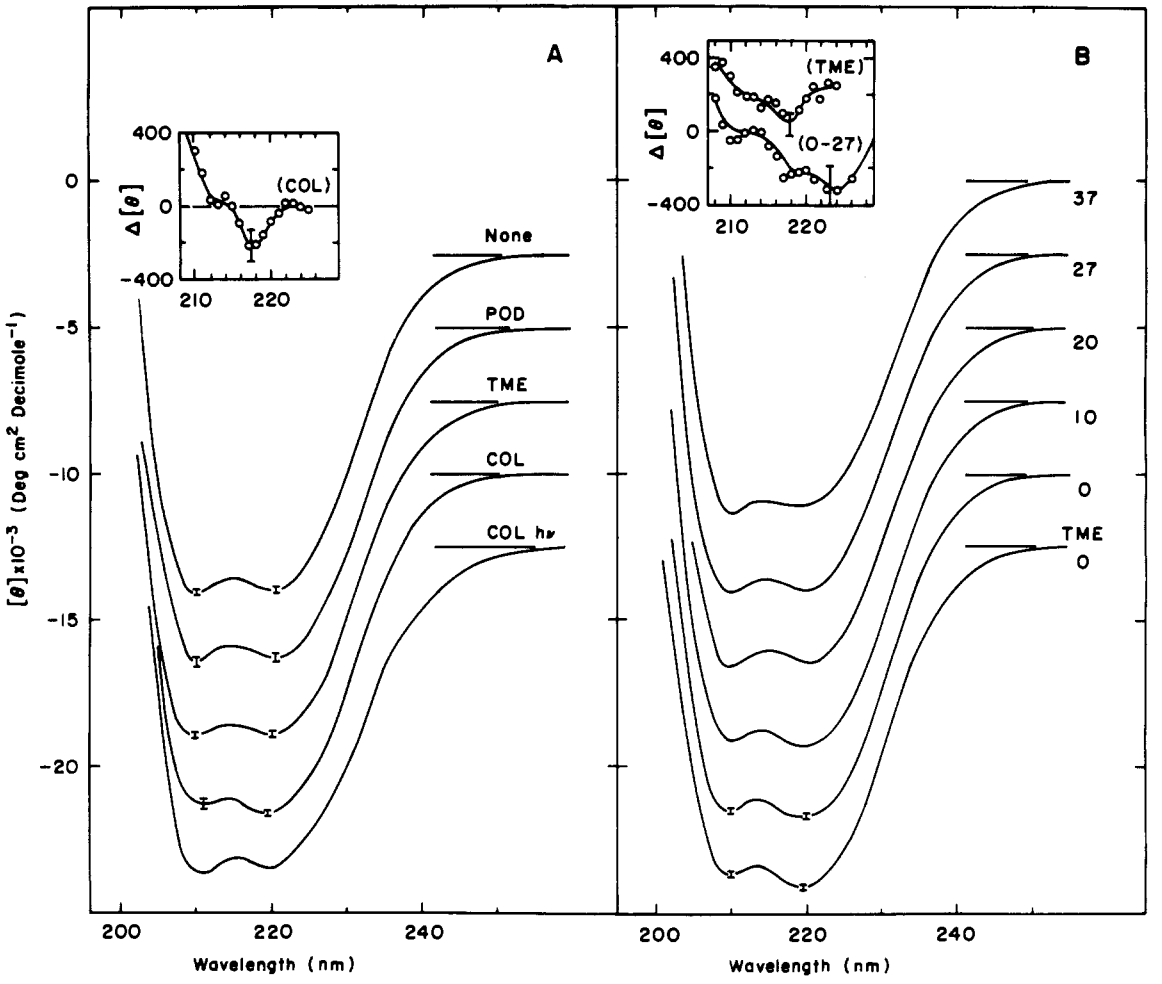


FIGURE 4: Effects of ligands and temperature on the circular dichroism spectrum of 4 μM tubulin in PG buffer–1 M sucrose, pH 7.0. (A) Effect of ligands at 27 °C. The following spectra are shown: in the absence of ligand (None, average of five samples); 50 μM podophyllotoxin (POD, average of two samples); 0.5 mM tropolone methyl ether (TME, average of two samples); 50 μM colchicine (COL, average of three samples); 50 μM colchicine after photoconversion to lumicolchicine (see Materials and Methods) (COL $h\nu$, one sample). Irradiation of unliganded tubulin produced no significant effects on its CD spectrum. The inset [$\Delta[\theta]$, (COL)] is the difference obtained by subtracting the None spectrum from the COL spectrum. (B) Effects of temperature and tropolone methyl ether on the initial circular dichroism spectrum. The spectra were obtained at close to zero time at 37, 27, 20, 10, and 0 °C (average of three samples) and at 0 °C with 0.5 mM tropolone methyl ether (TME, 0) (average of three samples). The difference CD spectra in the inset show the results of subtracting the 0 °C tubulin spectrum from the TME 0 °C spectrum [$\Delta[\theta]$, (TME)] and the 27 °C tubulin spectrum from the 0 °C tubulin spectrum [$\Delta[\theta]$, (0–27)]. All spectra were corrected for the base line and for any small ligand contributions to the circular dichroism. All spectra were coincident at 255 nm; they have been displaced vertically by increments of 2500 deg cm^2 dmol^{-1} for comparison. The 37 °C spectrum of Figure 4B is correctly set on the coordinates; the 27 °C spectrum of pure tubulin (None) of Figure 4A is displaced by −2500 deg cm^2 dmol^{-1} to match the corresponding spectrum of Figure 4B.

experiment in the absence of free ligand. The protein fluorescence spectra of tubulin and the tubulin–colchicine complex appear in Figure 5A. The binding of colchicine to tubulin quenched the protein fluorescence. The quenching was proportional to the occupancy of the colchicine binding site, as shown in the inset of Figure 5A, the protein fluorescence decreasing by one-half in the saturated complex. Shining long-wavelength ultraviolet light on the tubulin–colchicine complex dissociated the complex (see Materials and Methods) and increased the protein fluorescence, although not up to its original intensity. Such irradiation had no effect on the fluorescence of unliganded protein. Since tubulin is known to undergo readily disulfide interchange reactions (Timasheff & Grisham, 1980), these experiments were repeated in the presence of 5 mM dithiothreitol with no effect on the results, indicating that sulfhydryl reactions were not the cause of the observations. The protein fluorescence polarization excitation spectra of tubulin (emission analyzed at 350 nm), tubulin–colchicine, and the dissociated complex were very similar to each other, indicating the absence of significant differences in the rotational freedom of the protein fluorophores in these states. Tubulin, from which colchicine had been dissociated by light, retained colchicine-binding activity, although the extent of the binding was decreased. When exposed to 200 μM colchicine, it bound 60–80% of the ligand bound by previously nonliganded, but equally irradiated tubulin controls. Similarly, when the irradiated tubulin–colchicine complex was rapidly chromatographed on Sephadex G25 (Materials and Methods) equilibrated with 10 mM phosphate buffer, pH 7.0, at 0 °C, it was found to contain only 75 ± 10% of the amount of bound nucleotide found in the controls. Tropolone methyl ether, lumicolchicine, and podophyllotoxin were not found to have any significant effects on the fluorescence of tubulin.

Colchicine bound to tubulin displayed fluorescence at 435 nm, with excitation maxima at approximately 228, 260–270, and 357 nm, as shown in Figure 5B. Unbound colchicine had very weak fluorescence, with maxima around 227, 260, and 320 nm, as shown by the dashed line of Figure 5B. Figure 6A depicts the time courses of ligand fluorescence (tracing a), protein fluorescence (tracing b), and ligand fluorescence

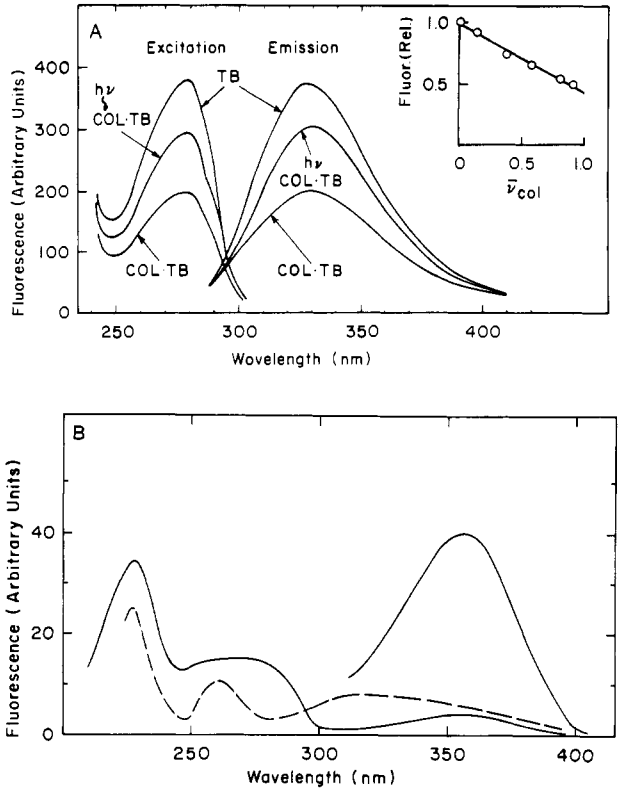


FIGURE 5: (A) Protein fluorescence of tubulin and of the tubulin–colchicine complex. The spectra were obtained immediately after the dilution of 0.3 μM protein in 10 mM sodium phosphate buffer–5 μM GTP, pH 7.0 at 25 °C. Tubulin (TB) had excitation and emission maxima at 278 ± 1 and 329 ± 1 nm, respectively. The tubulin–colchicine complex (COL·TB) displayed the same maxima with intensities quenched to 50 ± 2% of the unliganded protein values. When the ligand was dissociated from the complex by the action of light (see Materials and Methods) ($h\nu \rightarrow$ COL·TB), the fluorescence intensity was 80 ± 3% of that of the unliganded protein, and the emission maximum was 331 ± 1 nm. Irradiation of unliganded protein produced no significant effects. The inset shows the relationship between the fluorescence (unliganded protein ≡ 1.0) and the saturation of colchicine binding. (B) Ligand fluorescence excitation spectrum of the tubulin–colchicine complex. The colchicine emission intensity of a solution of 0.3 μM tubulin–colchicine, prepared as in Figure 5A, was analyzed at 435 nm and the excitation spectrum stored. This spectrum (not shown) contained a significant contribution of the tail of protein emission at 435 nm. An unliganded tubulin solution was diluted to match the protein fluorescence (Figure 5A) of the tubulin–colchicine complex. Its excitation spectrum (emission at 435 nm) was then digitally subtracted from the spectrum of the tubulin–colchicine complex, and the results are displayed by the solid line; above 300 nm, this spectrum is also shown amplified 10×. The dashed line is the approximate excitation spectrum of 1 μM free colchicine under the same conditions, corrected for solvent fluorescence, and displayed on a 100× amplified scale.

excited at 290 nm (tracing c) of a solution containing a large excess of colchicine over protein in PG buffer–1 M sucrose, pH 7.0 at 25 °C. The three processes are similarly slow and non pseudo first order, in qualitative agreement with the bound ligand fluorescence time course described by Garland (1978). Since we had demonstrated earlier that tropolone methyl ether is a weak inhibitor of colchicine binding (Andreu & Timasheff, 1982a), detailed experiments were carried out to characterize this phenomenon. In experiments aimed to follow the displacement from tubulin of tropolone methyl ether by colchicine, the protein was preincubated with a saturating concentration of tropolone methyl ether in the absence of colchicine. This solution was then diluted into the buffer containing an excess of colchicine, so that the residual concentration of tropolone methyl ether was small, and the time course of

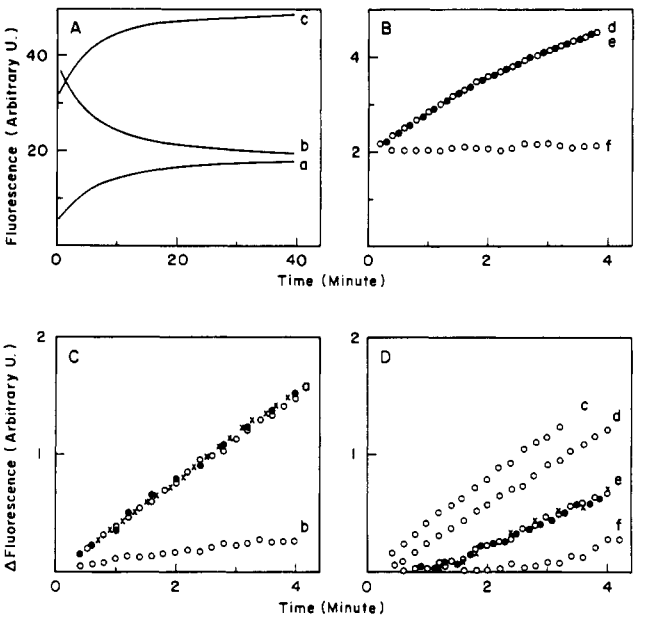


FIGURE 6: Time course of fluorescence change during complexing of ligands to tubulin. (A and B) Ligand and protein fluorescence time courses during colchicine binding. The solution contained 100 μM colchicine and 4 μM tubulin (protein added at time 0) in PG–1 M sucrose buffer, pH 7.0 at 25 °C. (a) Excitation at 360 nm and emission at 435 nm (intensity amplified 2×); (b) excitation at 276 nm and emission at 330 nm (intesity reduced 6×); (c) excitation at 290 nm and emission at 435 nm. The first stages of the reaction of 3.2 μM tubulin with colchicine under the same conditions have been followed (excitation at 390 nm and emission at 435 nm) in (d) (○); the protein of sample e (●) had been previously incubated with 5 mM tropolone methyl ether for 1 h at 0 °C (conditions for saturation; Andreu & Timasheff, 1982a) and then added to the reaction mixture [the final composition was as in (d) plus 25 μM tropolone methyl ether]. Sample f contained protein preincubated with 1 mM podophyllotoxin for 5 min at 25 °C (the final concentration of podophyllotoxin was 5 μM). (C and D) Effects of inhibitors on the binding of 5 μM colchicine to 8 μM tubulin in PG–1 M sucrose, pH 7.0 at 37 °C. Excitation was at 390 nm, emission was analyzed at 435 nm, and the background fluorescence at time 0 was subtracted. (a) No inhibitor added, samples run in triplicate (○, ●, ×). (b) Effect of 10 μM podophyllotoxin; [c–e (triplicate) and f] effects of 0.25, 0.95, 2.4, and 4.8 mM tropolone methyl ether, respectively.

fluorescence change was followed. As shown in Figure 6B, tracings d and e, the effect of the tropolone methyl ether on fluorescence development was insignificant, indicating that the dissociation of tropolone methyl ether was essentially complete in less than 0.1 min. A similar preincubation performed with podophyllotoxin (tracing f) resulted in nearly complete inhibition of colchicine binding during the course of the observation. Figure 6C,D shows a different experiment, designed to detect the inhibition of colchicine binding by tropolone methyl ether. Tracing a of Figure 6C shows the fluorescence time course of a solution containing 5 μM colchicine and 8 μM tubulin at 37 °C, while curves c–f of Figure 6D present the effects of incorporating increasing amounts of tropolone methyl ether into aliquots of the same solution. At all concentrations of tropolone methyl ether, there is a lag in the appearance of colchicine fluorescence, after which the fluorescence develops, but at a reduced rate. Both the lag and the rate of fluorescence development were dependent on the concentration of tropolone methyl ether. Under identical conditions, podophyllotoxin produced a reduction of the slope but no detectable lag, as shown by curve b of Figure 6C.

Enzyme Activity. The GTPase activity of the tubulin–colchicine complex was characterized kinetically (see Materials and Methods), and the results are presented in the form of a double-reciprocal plot in Figure 7. The straight line plots

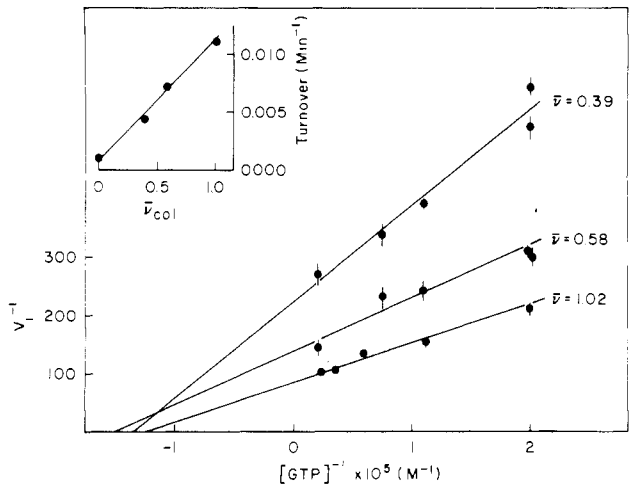


FIGURE 7: GTPase activity of the tubulin–colchicine complex. Tubulin with various amounts of colchicine bound was prepared and equilibrated with substrate, and its GTPase activity was measured, as described under Materials and Methods, in 10 mM sodium morpholineethanesulfonate buffer–1 mM $MgCl_2$, pH 7.0 at 37 °C. The protein concentration was 3.2 μM, and the time of the assay was 20 min (the reaction was linear to 12 μM protein or 30 min). Initial velocities (V_i) are expressed as moles of [^{32}P]phosphate released per minute per 100 000 g of tubulin. The inset shows the dependence of the experimentally determined turnover number (assuming a single hydrolysis site) on the saturation of colchicine binding.

indicate that the activity can be analyzed most simply in terms of a Michaelis–Menten dependence, with $K_m = 7 \pm 2$ μM and a turnover number of 0.012 ± 0.001 min^{-1}, as determined from the abscissa and ordinate intercepts, respectively. The enzyme activity of several samples of unliganded tubulin was found to be smaller than the sensitivity of the assay (i.e., <0.0005 min^{-1}). As shown in the inset of Figure 7, the turnover increased linearly with saturation of the colchicine site, while K_m remained constant within experimental error. Neither podophyllotoxin nor tropolone methyl ether induced any measurable GTPase activity, as has already been reported (Andreu & Timasheff, 1981a).

Tubulin Self-Association Reactions. Neither colchicine (3.2×10^{-4} M) nor podophyllotoxin (1.6×10^{-4} M) had any significant effects on the sedimentation velocity behavior of 3–6 mg mL^{-1} tubulin in PG buffer, pH 7.0 at 20 °C. Similarly, 3×10^{-3} M tropolone methyl ether produced no changes, both under the same conditions and at 12 or 30 °C. The effects of the three ligands on the Mg^{2+}-induced tubulin self-association with the formation of 42S double rings (Frigon & Timasheff, 1975) were examined on 6–10 mg mL^{-1} tubulin solutions in PG–16 mM $MgCl_2$ buffer, pH 7.0 at 20 °C. In the presence of colchicine or podophyllotoxin, the area under the slow peak of the bimodal Gilbert-type schlieren profile decreased b 5–10%, indicating a slight enhancement of the association. Tropolone methyl ether had no significant effect on the Mg^{2+}-induced self-association. Weisenberg & Timasheff (1970) had reported that colchicine increased association considerably. This may be due to the facts that, in the early study, a different and slightly impure tubulin preparation had been used and that the protein solutions were incubated at 37 °C to bind colchicine. When the ligand was dissociated from the tubulin–colchicine complex by ultraviolet irradiation (see above) in this buffer, 40–60% of the dissociated protein precipitated.

All three ligands inhibited microtubule assembly in vitro (see Materials and Methods). Podophyllotoxin (2.5×10^{-7} M) and 5×10^{-7} M colchicine or podophyllotoxin produced, respectively, 50% and 80% inhibitions of the steady-state turbidity of (1.6–1.8) $\times 10^{-5}$ M tubulin solutions. Considering the association constants of the two ligands to tubulin (see the introduction), it follows that at these levels most of the ligand was bound by the large excess of protein. Therefore, the inhibition of microtubule assembly must have been caused by only 1–2% of liganded tubulin molecules. Tubulin from which colchicine had been dissociated by ultraviolet light did not assemble at concentrations of 2.1×10^{-5} M, whereas irradiation had no effect on controls. Tropolone methyl ether inhibited microtubule assembly at higher concentrations than the other two ligands, namely, at levels comparable to its dissocation constant (Andreu & Timasheff, 1982a). The extent of the inhibition, however, had a complex dependence on the time of protein incubation with ligand prior to assembly, as shown in Figure 8. Thus, preincubation at 27 °C of 1.8×10^{-5} M tubulin with 6×10^{-4} M tropolone methyl ether in assembly buffer for up to 10 min followed by a temperature jump to 37 °C resulted in a 25–30% decrease of the plateau turbidity, as shown by the filled circles of Figure 8A. The level of inhibition, which appeared to be independent of the preincubation time up to 10 min, increased with further preincubation and reached 60% of the plateau turbidity at 20 min, as shown by the filled squares of Figure 8B. It leveled off then at a new steady state of turbidity.

Discussion

The present conformational examination of the effect of colchicine and related ligands on tubulin has shown that tubulin stabilized by sucrose is affected by the examined ligands in a consistent manner. In the absence of sucrose, the circular dichroism and fluorescence spectra of purified calf brain tubulin undergo a relaxation which speeds up at higher temperatures. Since the protein concentration employed was well above that at which the tubulin dimer dissociates into its α and β subunits (Detrich & Williams, 1978), these changes can be attributed best to a partial unfolding of the protein and are in qualitative agreement with the results of Ventilla et al. (1972). The magnitude of the change in ellipticity reported here (Figure 4B, inset) is considerably smaller than that found by Ventilla et al. (1972). Our spectra were obtained at early times in the presence of sucrose and represent essentially zero time data (see Figure 2). Those of Ventilla et al. (1972) are final equilibrium values obtained in the absence of a stabilizer and are of similar magnitude to those reported in this paper for long times in the absence of sucrose (see Figure 1). Lee et al. (1978), who did not report any effect of temperature, worked at close to zero time, and a change in ellipticity of 400 deg cm^2 dmol^{-1} (see inset of Figure 4B) falls within the experimental error of their early study. The CD spectral changes with time are probably related to the decay of the microtubule assembly ability of tubulin (Borisy et al., 1975; Prakash & Timasheff, 1982), the decay of its colchicine binding ability (Frigon & Lee, 1972), and the spontaneous slow aggregation of the protein (Prakash & Timasheff, 1982), since it is reasonable to expect that the relaxation of the protein secondary structure would precede the other changes (Prakash & Timasheff, 1982). Just as the colchicine binding activity (Frigon & Lee, 1972), the secondary structure of tubulin was not affected by 1 M sucrose, but it was stabilized considerably by that cosolvent, which is known to favor thermodynamically the native folded state of proteins (Lee et al., 1975; Lee & Timasheff, 1981; Na & Timasheff, 1981).

Properties of the Tubulin–Colchicine Complex: Evidence of a Conformational Change in the Presence of Colchicine. The absorption spectrum of colchicine was perturbed by binding to tubulin, in general agreement with the results of

Arai & Okuyama (1975) and Bhattacharyya & Wolff (1974), but shown here at a better resolution. The perturbations between 300 and 400 nm seem to be contributed by the tropolone ring. The 350- and 388-nm peaks were also produced by organic solvent perturbation of the spectrum of colchicine (Figure 3B), while the peak at 364 nm seems to be related to a similar peak in the formamide and methanol difference spectra of colchicine, but not seen in the chloroform spectrum. It is interesting to note that a perturbation at 387 nm accompanies the dimerization of colchicine at relatively high concentration in aqueous solution (Engelborghs, 1981). This, together with the qualitative resemblance of the formamide and methanol vs. water spectra of colchicine and the one obtained upon interaction of the drug with tubulin, suggests the participation of hydrogen bonding at the carbonyl group of the tropolone ring in the binding of colchicine to the protein. This is consistent with the proposal of Arai & Okuyama (1975) and in agreement with the thermodynamic characteristics of the interaction of tubulin with tropolone methyl ether (Andreu & Timasheff, 1982a). The peak at 271 nm in the complex difference spectrum cannot be ascribed unequivocally to the ligand, although a similar peak in the colchicine difference spectrum and the maximum at approximately 260 nm in the fluorescence excitation spectrum of free colchicine (Figure 5B) would tend to suggest this.

The very weak fluorescence of colchicine was dramatically increased upon binding to tubulin (more than 200-fold) as shown in Figure 5B in agreement with Arai & Okuyama (1975), but only in partial agreement with Bhattacharyya & Wolff (1974), who reported that (1) colchicine was not fluorescent by itself, but became fluorescent only when bound to tubulin, and that (2) the ligand did not quench the intrinsic fluorescence of the protein. We have observed both the time-dependent quenching of protein fluorescence in solutions prepared by mixing free tubulin and colchicine and the quenched fluorescence of the isolated, stable, tubulin–colchicine complex (see Figure 5A). Furthermore, the appearance of bound ligand fluorescence emission by excitation of the complex at 290 nm (Figure 5B and Figure 6A, tracing c) suggests fluorescence energy transfer from protein tryptophans to colchicine, since the free ligand has absorption and excitation minima in this region, although possible overlapping with the excitation of bound ligand at this wavelength cannot be rigorously excluded at present. The strong quenching of protein fluorescence induced by ligand binding (see Figure 5B) suggests that the colchicine binds to tubulin close to a fluorophore, most probably a tryptophan. The kinetic characteristics of this phenomenon [Figure 6 and Garland (1978)] indicate, however, that the binding reaction is not a simple bimolecular association but that it involves other rate-limiting steps, such as a ligand-induced or stabilized conformational change. The changes generated in the circular dichroism spectrum of tubulin by interaction with colchicine support this notion. They could be consistent, for example, with a small increase in β-structure contents (ca. 1%) at the expense of unordered regions (Sears & Beychok, 1973), although no such conclusions can be drawn reasonably from such small changes in circular dichroism (Timasheff, 1970).

Conversion of colchicine to lumicolchicine by light with release of the ligand from the protein resulted in fluorescence and circular dichroism spectra similar in shape to those of native unliganded tubulin, but of lower intensity. The impairment of the tubulin–colchicine and nucleotide binding activities and of the ability of tubulin to assemble into microtubules indicates that the process of binding colchicine to this protein and releasing it by conversion to lumicolchicine had resulted in some structural alterations. That this denaturation is not simply a result of irradiation is clear from the observation that irradiation in the absence of bound colchicine did not lead to any changes either in the spectral properties of the protein or in its ability to assemble into microtubules.

Finally, the GTPase activity of tubulin when bound to colchicine also supports the concept of a conformational alteration. The maximal rate of GTP hydrolysis by the tubulin–colchicine complex was 1 mol of substrate per 10^5 g of protein per 87 ± 7 min. Either the unliganded protein did not hydrolyze GTP at all or, if it did, the rate had to be more than 20 times slower. This rather inefficient enzyme activity compares reasonably with the numbers reported by David-Pfeuty et al. (1979), who used a different preparation of tubulin. It is 1 order of magnitude slower than the ATPase activity induced in actin by cytochalasin D, a system similar to tubulin–colchicine in many respects (Brenner & Korn, 1980). Furthermore, since an excess of colchicine did not affect the sedimentation properties of tubulin, and the specific GTPase activity of the tubulin–colchicine complex was independent of protein concentration (Andreu & Timasheff, 1982a,b), the colchicine-induced GTPase activation must be the consequence of a conformational change of the soluble tubulin heterodimer rather than of a self-association reaction. Finally, preliminary results from our laboratory, obtained with our tubulin preparation, are in agreement with the observation of David-Pfeuty et al. (1979) that griseofulvin and daunorubomycin also induce GTPase activity. These drugs bind to tubulin (Wheland et al., 1977; Na & Timasheff, 1977), but most probably at sites different from colchicine.

Effects of Podophyllotoxin Binding. Podophyllotoxin competes with colchicing binding, probably through the trimethoxybenzene ring common to the two alkaloids (see the introduction). The tubulin–podophyllotoxin difference spectrum was strikingly mimicked by difference spectra of the alkaloid in methanol and chloroform vs. aqueous solution, and less so in formamide. This perturbation is most likely related to the trimethoxybenzene ring (Figure 3f). These results indicate (i) that podophyllotoxin binds to the protein through the trimethoxybenzene ring and (ii) that the tubulin trimethoxybenzene binding site provides an environment more similar to methanol and chloroform than to water, supporting the hydrophobic character of the trimethoxybenzene ligands–tubulin interaction proposed previously on strictly thermodynamic grounds (Andreu & Timasheff, 1982a). None of the effects of colchicine binding were observed with saturating amounts of podophyllotoxin, indicating that its binding does not produce the colchicine-linked conformational change in the protein. Since the binding of mescaline also had no significant effects on the fluorescence and circular dichroism of tubulin (Andreu & Timasheff, 1982a), the observed conformational effect must be related to parts of the colchicine molecule other than the trimethoxybenzene ring.

Effects of Tropolone Methyl Ether on Tubulin Conformation. Tropolone methyl ether, which has the structure of a part of the colchicine molecule, binds to tubulin competitively with colchicine, although with a considerably weaker affinity (Andreu & Timasheff, 1982a) (see Figure 6). Three types of observations indicate that the binding of this ligand to tubulin does not proceed by a reversible one-step bimolecular association mechanism: (i) The dependence of the extent of the inhibition of microtubule assembly on the duration of tubulin incubation with the tropolone methyl ether, described in Figure 8, is clearly inconsistent with such a simple binding

and assembly inhibition mechanism. (ii) If the binding of tropolone methyl ether was a simple diffusion-controlled bimolecular reaction, it would attain equilibrium in a time (Eigen & Hammes, 1963) insignificant with respect to the time scale of the present experiments. From our previous binding studies, it is known that the attainment of equilibrium is slow, the apparent bimolecular forward rate constant being of the order of 10^2 M^{-1} min^{-1} at 25 °C (Andreu & Timasheff, 1982a). The colchicine binding competition experiments, reported in the present paper, indicate an apparent dissociation rate constant of the tubulin–tropolone methyl ether complex of ≥ 10 min^{-1}. Thus, the resulting calculated equilibrium binding constant for a simple bimolecular reaction would be ~ 10 M^{-1}, in clear disagreement with the value derived under the same conditions from the equilibrium binding studies, i.e., 280 ± 200 M^{-1} (Andreu & Timasheff, 1982a). (iii) For the slow binding of colchicine, the rate of binding is a function of the concentration of free tubulin binding sites.[3] Therefore, the extent of binding of tropolone methyl ether to the colchicine site can be determined from the tropolone methyl ether concentration dependence of the reduction in the slope of the linear portion of the fluorescence time course, shown in Figure 6D. Application of a simple treatment[3] to the experimental slopes (tracings a and c–f of Figure 6C,D), while neglecting the lag, showed binding of tropolone methyl ether to 85 ± 20% of the colchicine binding sites with an apparent equilibrium constant of 350 ± 100 M^{-1}, in good agreement with the numbers previously determined by means of direct equilibrium techniques (Andreu & Timasheff, 1982a). This analysis, however, is insufficient, since it does not account for the lag in colchicine binding in the presence of tropolone methyl ether. The simplest explanation of this lag, namely, an initial transient occupation of all colchicine binding sites by tropolone methyl ether[3] at concentrations known to be nonsaturating at equilibrium (Andreu & Timasheff, 1982a), is inconsistent with a simple binding reaction mechanism. The process must, therefore, be more complex, and the results require the existence of steps other than the simple formation of contacts between tropolone methyl ether and tubulin. What are these steps?

Mechanism of Colchicine Binding to Tubulin. A conformational change linked to the binding of tropolone methyl ether can account for all the idiosyncrasies of the interaction of this ligand with tubulin. Is this change related to the conformational change observed during colchicine binding? The similarity of the changes of the protein circular dichroism observed in the presence of the two ligands under appropriate binding conditions suggests that this is so. Let us assume first for the purposes of this discussion that the equilibria involved are simply those described in the Garland (1978) model of the colchicine binding kinetics:

$$\mathrm{TB} + \mathrm{COL} \overset{K_1}{\rightleftharpoons} \mathrm{TB{\cdot}COL} \overset{K_2}{\rightleftharpoons} \mathrm{TB^*{\cdot}COL} \qquad (1)$$

$$\mathrm{TB} + \mathrm{TME} \overset{K_3}{\rightleftharpoons} \mathrm{TB{\cdot}TME} \overset{K_4}{\rightleftharpoons} \mathrm{TB^*{\cdot}TME} \qquad (2)$$

where TB is tubulin, COL is colchicine, TME is tropolone methyl ether, and the asterisks indicate an altered conformation of the protein. The failure of tropolone methyl ether to fluoresce or to quench the protein fluorescence when it binds to tubulin may be related to the fact that, in colchicine, the tropolone ring is fused to the rest of the drug molecule, with resulting photochemical characteristics absent in the small tropolone molecule.[4] The lack of any detected tubulin GTPase activity in the presence of tropolone methyl ether, however, suggests that the conformations of tubulin in TB*·COL and TB*·TME may not be identical or that induction of the enzymic activity requires the binding of both moieties of colchicine to tubulin. Alternately, it could be related to the reversibility of equilibrium 4 (eq 2) being more rapid than the very slow enzymic reaction.

The relation between the mechanisms of binding of colchicine and tropolone methyl ether, proposed here, is strongly

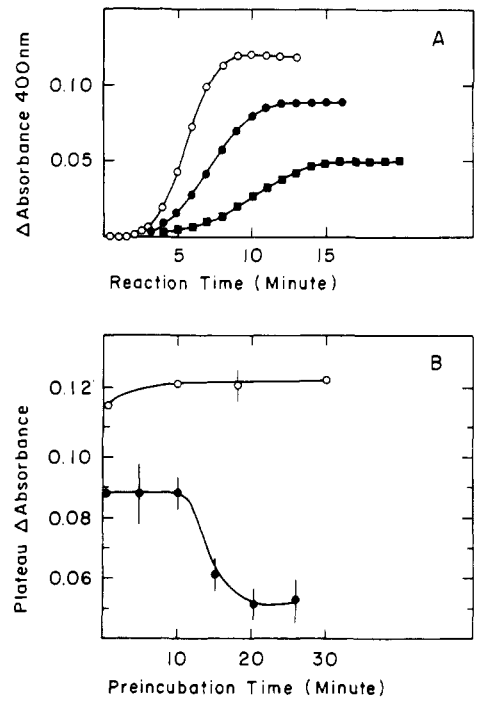


FIGURE 8: Effects of tropolone methyl ether on the in vitro microtubule assembly. (A) 1.8 μM tubulin was preincubated for 10 or 20 min at 27 °C in assembly buffer (see Materials and Methods), and the polymerization was started by a jump to 37 °C (○). The same experiment was performed in the presence of 0.6 mM tropolone methyl ether with 10 (●) and 20 min (■) of preincubation at 27 °C. (B) 37 °C plateau turbidity values of 18 μM tubulin solutions as a function of the preincubation time at 27 °C in the absence (○) and presence (●) of 6×10^{-4} M tropolone methyl ether. The vertical bars indicate experimental errors in duplicate samples.

[3] According to Garland (1978), the fluorescence (F) is proportional to the concentration of the tubulin–colchicine complex formed. Under conditions similar to ours, the initial rate of formation of this species is proportional to the concentration of free tubulin, [TB] (Garland, 1979):

$$(dF/dt)_{t\approx 0} \propto [\mathrm{TB}] \simeq [\mathrm{TB}]_0 \qquad (I)$$

In the presence of tropolone methyl ether (TME) already in binding equilibrium at $t \approx 0$ on the time scale of colchicine binding

$$[\mathrm{TB}] = [\mathrm{TB}]_0 - [\mathrm{TB{\cdot}TME}] \qquad (II)$$

and

$$(dF/dt)_{t\approx 0,\mathrm{TME}} \propto [\mathrm{TB}]_0 - [\mathrm{TB{\cdot}TME}] \qquad (III)$$

Dividing eq III by eq I and rearranging

$$\bar{\nu}_{\mathrm{TME}} = \frac{[\mathrm{TB{\cdot}TME}]}{[\mathrm{TB}]_0} = 1 - \frac{(dF/dt)_{t\approx 0,\mathrm{TME}}}{(dF/dt)_{t\approx 0}} \qquad (IV)$$

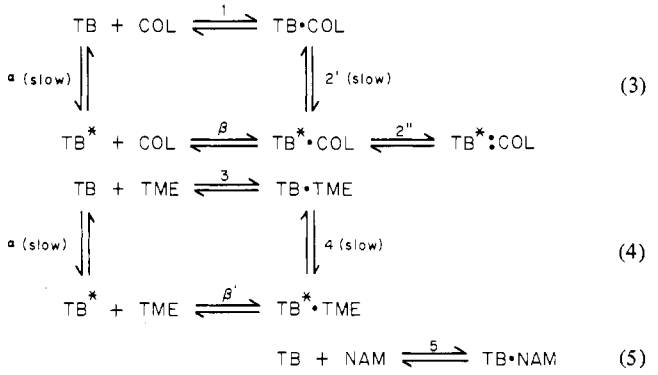
As the binding of TME is weak and $[\mathrm{TME}]_0 \gg [\mathrm{TB}]_0$, $[\mathrm{TME}] \simeq [\mathrm{TME}]_0$ (Andreu & Timasheff, 1981b). When eq IV was applied to our data, the results were directly fitted by the binding isotherm

$$\bar{\nu}_{\mathrm{TME}} = nK_{\mathrm{app}}[\mathrm{TME}]/(1 + K_{\mathrm{app}}[\mathrm{TME}]) \qquad (V)$$

giving $n = 0.85 \pm 0.15$ and $K_{\mathrm{app}} = 350 \pm 100$ M^{-1}, where n is the fraction of colchicine binding sites occupied by TME at saturation.

[4] It must be recalled that the tropolone molecule does not fluoresce by itself (Andreu & Timasheff, 1982b), while colchicine displays weak fluorescence (see Figure 5B).

supported by the recent results of Lambeir & Engelborghs (1981), who have analyzed the kinetics of the binding of colchicine to tubulin. The thermodynamic parameters, $\Delta H°$ and $\Delta S°$ ($\Delta H° = -7.9 \pm 2.9$ kcal mol^{-1}, $\Delta S° = -15.1 \pm 9.6$ eu), that they have calculated for the first step of colchicine binding to tubulin (equilibrium 1 in eq 1) are strikingly coincident with our measured values for the interaction of tropolone methyl ether with tubulin ($\Delta H° = -8.3 \pm 1.0$ kcal mol^{-1}, $\Delta S° = -15.2 \pm 3.6$ eu) (Andreu & Timasheff, 1981b). This is fully consistent with the model which we have proposed recently (Andreu & Timasheff, 1982a) in which the binding to tubulin of colchicine, a bifunctional ligand, proceeds through three linked reactions, namely, first the binding of colchicine through its tropolone ring, followed by a conformational change, and finally by the binding of the trimethoxybenzene ring of the drug. If this is true, then the simplest hypothesis is that equilibrium 3 (eq 2) is essentially equal to equilibrium 1 (eq 1). Equilibrium 4 would have, then, a small free energy change (K_4 not much different from unity) and would not contribute significantly to the overall apparent standard free energy change, $\Delta G°_{app,TME}$, measured for the binding of tropolone methyl ether to tubulin, although equilibrium 4 (eq 2) could involve changes in protein structure related to those of equilibrium 2 (eq 1). Equilibrium 2, on the other hand, encompasses both the conformational change with an apparent standard free energy change close to zero and the binding of the trimethoxybenzene ring of colchicine to tubulin in a manner consistent with the known thermodynamic parameters of the binding of these ligands to tubulin. The resulting complete scheme for the various binding and conformational equilibria involved would be



(3)

(4)

(5)

Equations 3 and 4 are equivalent to eq 1 and 2, except that now they include the ligand-facilitated pathway, in which the conformational change precedes ligand binding (reactions α, β, and β'). The complexes TB*•COL and TB*:COL represent tubulin–colchicine complexes in which only one and both tubulin–ligand contacts are made, respectively. Proceeding along the ligand-mediated pathway,[5] equilibrium 1 corresponds to the binding of the tropolone ring of colchicine to tubulin, equilibrium 2′ is the conformational change of liganded tubulin, and equilibrium 2″ is the intramolecular binding of the trimethoxybenzene ring of colchicine to the altered tubulin. These reactions have been probed with the monofunctional analogues (Andreu & Timasheff, 1982a,b) tropolone methyl ether (TME, eq 4) and *N*-acetylmescaline (trimethoxyphenethylamine, NAM, eq 5). In the case of the binding of tropolone methyl ether to tubulin, the complete scheme of eq 4 gives for $K_{app,TME}$, the equilibrium binding constant previously measured (Andreu & Timasheff, 1982a) by the Hummel & Dreyer (1962) procedure:

$$K_{app,TME} = \frac{K_3(1 + K_4)}{1 + K_\alpha} = \exp[-\Delta G°_{app}(3,4,\alpha)/(RT)] \quad (6)$$

The best available data for these equilibria are at 37 °C: $\Delta G°(1) + \Delta G°(2') + \Delta G°(2'') = -10.2$ kcal mol^{-1} (Garland, 1978); $\Delta G°(1) = -3.2$ kcal mol^{-1} (Lambeir & Engelborghs, 1981); $\Delta G°_{app}(3,4,\alpha) = -3.6$ kcal mol^{-1} (Andreu & Timasheff, 1982a); $\Delta G°(5) = -3.7$ kcal mol^{-1} (Andreu & Timasheff, 1982a).

If the identity of equilibria 1 and 3 is accepted and $K_\alpha < 0.1$ L mol^{-1}, as suggested by the absence of GTPase activity in free tubulin, the following relationships result from the present analysis:[6]

$$\Delta G°(1) = \Delta G°(3) = -3.2 \pm 0.4 \text{ kcal mol}^{-1} \quad (7)$$

$$\Delta G°(2') \simeq \Delta G°(4) = -0.2 \pm 0.5 \text{ kcal mol}^{-1} \quad (8)$$

$$\Delta G°(2'') = \Delta G°(5,\text{intrinsic}) = -6.8 \pm 0.5 \text{ kcal mol}^{-1} \quad (9)$$

where $\Delta G°$(5,intrinsic) is the free energy change for the binding of *N*-acetylmescaline to tubulin in a hypothetical unimolecular reaction that would involve insignificant changes in the translational, rotational, and vibrational freedoms of the ligand (Andreu & Timasheff, 1982a). The difference between $\Delta G°$(5,intrinsic) and $\Delta G°(5)$ is, in fact, close to the cratic free energy change which must be taken into account in a bimolecular reaction but makes no contribution in a unimolecular reaction (Gurney, 1962; Andreu & Timasheff, 1982a,b).

The spectroscopic and functional evidence in favor of a conformational change in tubulin either induced or stabilized by colchicine, described in this paper, is in full agreement with the kinetic studies of Garland (1978) and of Lambeir & Engelborghs (1981). These changes are not induced by podophyllotoxin, meaning that they are probably not related to the binding of the trimethoxybenzene ring. They do appear to be linked to the liganding of the tropolone methyl ether which either induces or stabilizes a conformational change in tubulin, in agreement with our model that colchicine, a bifunctional ligand, binds in sequence first through its tropolone and then its trimethoxybenzene rings (Andreu & Timasheff, 1981b, 1982a). Examination of the standard free energy change which accompanies each step in the binding (eq 7–9) reveals that although the tropolone methyl ether end of colchicine binds first to tubulin and imparts some stability to the slowly forming isomer, Tb*, it is the subsequent rapid binding of the trimethoxybenzene ring, when it is covalently bonded to the tropolone methyl ether, which provides the strong free energy change that thermodynamically drives the reaction to completion, permitting to lock kinetically the liganded protein in the new conformation. In the absence of these chemical characteristics, both the binding reaction and the conforma-

[5] Although along this path (equilibria 1 and 2) liganding precedes the conformational change, this is no way implies that the colchicine binding induces it. Thermodynamically, it is equally plausible that the binding of the ligand stabilizes, or enhances, the conformatioal change through a simple linkage (Wyman, 1964), although the kinetic studies of Garland (1978) and Lambeir & Engelborghs (1981) favor the ligand-mediated pathway.

[6] Lambeir & Engelborghs (1981) suggest that a slow preequilibration of free tubulin may precede colchicine binding. This would introduce into eq 3 and 4 the preequilibrium

$$TB^+ \overset{K_\lambda}{\rightleftharpoons} TB$$

with $K_\lambda \simeq 1$. This would result in $\Delta G°(2') = \Delta G°(4) = -0.7 \pm 0.5$ kcal mol^{-1} and $\Delta G°(2'') = \Delta G°(5,\text{intrinsic}) = -6.3 \pm 0.5$ kcal mol^{-1}, i.e., within experimental error of the values of eq 8 and 9.

tional change are weak. Thus, for tropolone methyl ether alone, close to half of the complex with tubulin remains in the initial protein conformation, resulting in either a diminution or absence of properties linked to the stable locked complex. Furthermore, the rapidity of the dissociation of the tubulin–tropolone methyl ether complex, ≥ 10 min^{-1}, relative to the turnover of the GTPase activity of the tubulin–colchicine stable complex, 0.012 min^{-1}, may explain the lack of observed GTPase activity of tubulin in the presence of tropolone methyl ether, even though the protein conformation of TB*·TME is the same as that of the stable complex with colchicine.

References

Andreu, J. M., & Timasehff, S. N. (1981a) *Arch. Biochem. Biophys. 211*, 151–157.

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

Andreu, J. M., & Timasheff, S. N. (1982a) *Biochemistry 21*, 534–543.

Andreu, J. M., & Timasheff, S. N. (1982b) *Biochim. Biophys. Acta 714*, 373–377.

Arai, T., & Okuyama, T. (1975) *Anal. Biochem. 69*, 422–450.

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

Borisy, G. G., Marcum, J. M., Olmsted, J. B., Murphy, D. B., & Johnson, K. A. (1975) *Ann. N.Y. Acad. Sci. 253*, 107–132.

Brenner, S. L., & Korn, E. D. (1980) *J. Biol. Chem. 255*, 841–844.

Carlier, M. F., & Pantaloni, D. (1978) *Biochemistry 17*, 1908–1915.

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*, 11696–11702.

Detrich, H. W., & Williams, R. C., Jr. (1978) *Biochemistry 17*, 3900–3907.

Detrich, H. W., Williams, R. C., Jr., MacDonald, T. L., Wilson, L., & Puett, D. (1981) *Biochemistry 20*, 5999–6005.

Eigen, M., & Hammes, G. G. (1963) *Adv. Enzymol. Relat. Areas Mol. Biol. 25*, 1–38.

Engelborghs, Y. (1981) *J. Biol. Chem. 256*, 3276–3278.

Frigon, R. P., & Lee, J. C. (1972) *Arch. Biochem. Biophys. 153*, 587–589.

Frigon, R. P., & Timasheff, S. N. (1975) *Biochemistry 14*, 4559–4566.

Garland, D. L. (1978) *Biochemistry 17*, 4266–4272.

Garland, D. L. (1979) *Arch. Biochem. Biophys. 198*, 335–337.

Grewe, R., & Wulf, W. (1951) *Chem. Ber. 84*, 621–625.

Gurney, R. W. (1962) *Ionic Processes in Solution*, pp 90–104, Dover, New York.

Hummel, J. M. P., & Dreyer, W. J. (1962) *Biochim. Biophys. Acta 63*, 530–532.

Lambeir, A., & Engelborghs, Y. (1980) *Eur. J. Biochem. 109*, 619–624.

Lambeir, A., & Engelborghs, Y. (1981) *J. Biol. Chem. 256*, 3279–3292.

Leach, S. J., & Scheraga, H. A. (1960) *J. Am. Chem. Soc. 82*, 4790–4792.

Lee, J. C., & Timasheff, S. N. (1974) *Arch. Biochem. Biophys. 165*, 268–273.

Lee, J. C., & Timasheff, S. N. (1977) *Biochemistry 16*, 1754–1764.

Lee, J. C., & Timasheff, S. N. (1981) *J. Biol. Chem. 256*, 7193–7201.

Lee, J. C., Frigon, R. P., & Timasheff, S. N. (1973) *J. Biol. Chem. 248*, 7253–7262.

Lee, J. C., Frigon, R. P., & Timasheff, S. N. (1975) *Ann. N.Y. Acad. Sci. 253*, 284–291.

Lee, J. C., Corfman, D., Frigon, R. P., & Timasheff, S. N. (1978) *Arch. Biochem. Biophys. 185*, 4–14.

Margolis, R. L., & Wilson, L. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3466–3470.

Margolis, R. L., Rauch, C. T., & Wilson, L. (1980) *Biochemistry 19*, 5530–5557.

McMeekin, T. L., Groves, M. L., & Hipp, N. J. (1964) *Adv. Chem. Ser. No. 44*, 54–66.

Na, G. C., & Timasheff, S. N. (1977) *Arch. Biochem. Biophys. 182*, 147–154.

Na, G. C., & Timasheff, S. N. (1981) *J. Mol. Biol. 151*, 165–178.

Olmsted, J. B., & Borisy, G. G. (1973) *Biochemistry 12*, 4282–4289.

Pittz, E. P., Lee, J. C., Bablouzian, B., Townend, R., & Timasheff, S. N. (1973) *Methods Enzymol. 27*, 209–256.

Postingl, H., Kraus, E., Little, M., & Kempf, T. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2757–2761.

Prakash, V., & Timasheff, S. N. (1982) *J. Mol. Biol. 160*, 499–515.

Sears, D. W., & Beychok, S. (1973) in *Physical Principles and Techniques of Protein Chemistry, Part C* (Leach, S. J., Ed.) pp 445–593, Academic Press, New York.

Sternlicht, H., & Ringel, I. (1979) *J. Biol. Chem. 254*, 10540–10550.

Timasheff, S. N. (1970) *Enzymes, 3rd Ed. 2*, 371–443.

Timasheff, S. N., & Grisham, L. M. (1980) *Annu. Rev. Biochem. 49*, 565–591.

Valenzuela, P., Quiroga, M., Zaldivar, J., Rutter, W. J., Kirschner, M. W., & Cleveland, D. W. (1981) *Nature (London) 289*, 650–655.

Ventilla, M., Cantor, C. R., & Shelanski, M. (1972) *Biochemistry 11*, 1554–1567.

Weisenberg, R. L., & Timasheff, S. N. (1970) *Biochemistry 9*, 4110–4116.

Wheland, J., Herzog, W., & Weber, K. (1977) *J. Mol. Biol. 111*, 329–342.

Wilson, L., & Friedkin, M. (1966) *Biochemistry 5*, 2463–2468.

Wilson, L., & Bryan, J. (1974) *Adv. Cell Mol. Biol. 3*, 21–72.

Wyman, J. J., Jr. (1964) *Adv. Protein Chem. 19*, 223–286.