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Changes in the Circular Dichroic Spectrum of Colchicine Associated with Its Binding to Tubulin†

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ABSTRACT: Circular dichroism has been used to study the interaction of colchicine with the tubulin $\alpha\beta$ dimer at 26 °C. Tubulin purified from bovine brain microtubule protein exhibits negligible circular dichroism at wavelengths above 310 nm. Free colchicine exhibits a negative circular dichroic band at 340 nm characterized by an extremum in molar ellipticity $[\theta]$ of $-3.35\ (\pm 0.27) \times 10^4$ deg·cm^2/dmol. This negative band either vanished or was greatly reduced, i.e., $[\theta] = 0\ (\pm 0.57) \times 10^4$ deg·cm^2/dmol, when colchicine was bound to tubulin. This was demonstrated by circular dichroic studies on stable [^{3}H]colchicine–tubulin complexes which were separated from all unbound colchicine by means of gel filtration. Also, tubulin was titrated with colchicine, and at low colchicine concentrations, the observed ellipticity at 340 nm could be extrapolated to zero at 0 M colchicine; saturation occurred at a molar ratio of colchicine to tubulin of about 1:1. The association constant characterizing the drug–protein interaction was estimated to be about 0.9 μM^{-1}. As controls, three other acidic proteins were studied at a molar ratio of colchicine to protein of 2:1, and their presence had no effect on the circular dichroic properties of colchicine. These results are consistent with the idea that a conformational change in colchicine accompanies its binding to tubulin. The spectrum of the complex between 250 and 300 nm was quite similar to that expected from simple additivity of the spectra of drug and protein except between 255 and 265 nm. The technique described herein should be applicable to other protein–drug systems.

Colchicine, the active antimitotic agent of the meadow saffron *Colchicum autumnale*, binds specifically and with high affinity to the tubulin dimer, the major protein subunit of microtubules (Shelanski & Taylor, 1968; Weisenberg et al., 1968; Wilson, 1970; Wilson & Meza, 1973). Approximately 1 mol of colchicine binds per mol of tubulin (Owellen et al., 1972; Wilson et al., 1974; Bhattacharyya & Wolff, 1976) with an affinity constant (for vertebrate tubulins) that ranges from 1 to 40 μM^{-1}, depending upon the source of tubulin and the conditions of measurement (Owellen et al., 1972, 1974; Wilson et al., 1974; Bhattacharyya & Wolff, 1974, 1976; Sherline et al., 1975; McClure & Paulson, 1977). Colchicine binding requires native tubulin. Binding activity is abolished by denaturants (Wilson, 1970) or by extensive treatment of tubulin with sulfhydryl reagents (Kuriyama & Sakai, 1974; Ikeda & Steiner, 1978). The colchicine binding activity of tubulin is also unstable in the buffers routinely used, decaying according to first-order kinetics (Weisenberg et al., 1968; Wilson, 1970; Wilson & Meza, 1973; Sherline et al., 1975; Cortese et al., 1977). The rate of decay is a sensitive function of pH and temperature, with optimal stability of binding activity observed at pH 6.75 and 0 °C (Wilson, 1970). Furthermore, the colchicine binding activity of tubulin is stabilized by vin-

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blastine, GTP, sucrose, glycerol, and colchicine itself (Borisy & Taylor, 1967; Frigon & Lee, 1972; Solomon et al., 1973; Wilson et al., 1974; Sherline et al., 1975; McClure & Paulson, 1977). The binding of colchicine to tubulin is competitively inhibited by podophyllotoxin (Wilson, 1975; Cortese et al., 1977) and by tropolone (Bhattacharyya & Wolff, 1974; Cortese et al., 1977). However, each of the latter two compounds has no effect on the binding of the other. Therefore, it seems likely that the colchicine binding site is composed of a trimethoxyphenyl binding subdomain (shared with podophyllotoxin) and a tropolone subdomain. Finally, the colchicine binding site is distinct from the two GTP sites and the two vinblastine binding sites (Weisenberg et al., 1968; Wilson, 1970) and may be distinct from the site that controls polymerization (Kuriyama & Sakai, 1974; Lee et al., 1976; Ikeda & Steiner, 1978). Schmitt & Atlas (1976) have suggested that the high-affinity binding site is located on the α chain of the tubulin dimer.

Several features of the colchicine-binding reaction are unusual. The rates of association and dissociation of colchicine and tubulin are slow (Borisy & Taylor, 1967; Wilson, 1970; Sherline et al., 1975; Cortese et al., 1977; McClure & Paulson, 1977): the activation energy for the binding reaction is approximately 20 kcal/mol (Cortese et al., 1977). Garland (1978) has proposed that the slow rate of colchicine binding and the nearly irreversible nature of the binding reflect a ligand-induced conformational change in tubulin. Ventilla et al. (1972) have suggested, on the basis of circular dichroic (CD) studies of free tubulin and colchicine–tubulin complexes, that colchicine binds to a protein conformer favored at high temperature. Both mechanisms attribute the conformational change in the colchicine–tubulin system to the protein moiety.

Colchicine is optically active and exhibits a double-negative Cotton effect in its optical rotatory dispersion spectrum (Hrbek et al., 1964). One negative trough occurs at about 330 nm and appears to result from the methoxytropone system [this ring is also responsible for the fluorescence of colchicine bound to tubulin (Bhattacharyya & Wolff, 1974)], while the second, at 280 nm, has been assigned to the K band of the biaryl system. We report here studies of the circular dichroism of free colchicine and of colchicine bound to tubulin. We have found that the negative CD band of the colchicine chromophore at 340 nm either vanishes or is greatly reduced in magnitude when colchicine is bound to tubulin. These results suggest either that the colchicine binding site on tubulin cancels the inherent asymmetry of the colchicine chromophore or that a conformational change in the colchicine molecule accompanies the binding of colchicine to tubulin.

Materials and Methods

Materials. Colchicine, Pipes,[1] EGTA, DTE, and GTP (type II-S) were obtained from Sigma. Colchicine (ring C, methoxy-^{3}H-labeled), 18.45 mCi/mmol, was purchased from New England Nuclear. Phosphocellulose (Whatman P-11, control no. 2111 62) was precycled according to the manufacturer's instructions.

Protein Determinations. Protein concentrations were determined by the method of Bradford (1976). The assay was calibrated for tubulin as described by Detrich & Williams (1978).

Microtubule Protein and Tubulin. Microtubule protein[2] was purified from bovine brain by three cycles of in vitro assembly and disassembly as described previously (Berkowitz et al., 1977; Detrich & Williams, 1978). Tubulin was separated from the microtubule-associated proteins by chromatography of microtubule proteins on phosphocellulose (Weingarten et al., 1975; Detrich & Williams, 1978) equilibrated with PM buffer (0.1 M Pipes–NaOH, pH 6.9, 2 mM EGTA, 1 mM $MgSO_4$, 0.1 mM GTP, and 2 mM DTE). $MgSO_4$ was added to the Mg^{2+}-depleted tubulin fractions to a final concentration of 1 mM (Williams & Detrich, 1979) to enhance the stability of the tubulin purified by PC chromatography. PC–tubulin was stored in liquid nitrogen (Detrich & Williams, 1978) without detectable loss in colchicine-binding activity over the course of several months. The tubulin prepared by this technique was routinely 96% pure when analyzed on sodium dodecyl sulfate–polyacrylamide gels (Laemmli, 1970) stained with Fast Green (Gorovsky et al., 1970; Berkowitz et al., 1977; Detrich & Williams, 1978).

Prior to use in the CD experiments described below, material stored in liquid nitrogen was thawed rapidly, centrifuged at 5000g for 10 min to remove small amounts of denatured protein, and then gel filtered at 4 °C on a 0.9 × 25 cm column of Sephadex G-25 (Pharmacia) equilibrated with PMCD buffer (0.1 M Pipes–NaOH, pH 6.9, containing 2 mM EGTA, 1 mM $MgSO_4$, 30 μM GTP, and 0.2 mM DTE). The concentrations of GTP and DTE in PMCD were chosen to minimize absorption by the buffer in the near-ultraviolet while maintaining excess free nucleotide and reductant with respect to the tubulin concentration (1–3 mg/mL = 9–27 μM). The absorption spectrum of PMCD from 240 to 500 nm shows a local maximum at 250 nm with A = 0.466. All subsequent sample preparation and handling were performed at 25–26 °C except where noted.

Preparation of Tubulin Complexed with Colchicine. Samples of tubulin partially or fully saturated with colchicine were prepared by incubation of PC–tubulin with ^{3}H-labeled colchicine, followed by gel filtration to separate free colchicine from the drug–protein complex (Wilson et al., 1974; Detrich & Williams, 1978). Aliquots (2 mL) of a PC–tubulin stock solution in PMCD (3.1 mg/mL) were incubated for 90 min at 25 °C with ^{3}H-labeled colchicine (2.40×10^{10} dpm/mmol) at final colchicine concentrations of either 30 or 100 μM. Each samples was then gel filtered at 25 °C on a PMCD-equilibrated column of Sephadex G-25 (0.9 × 25 cm). The void volume (containing the drug–protein complex) was collected from each column and adjusted with PMCD to a final protein concentration of approximately 1 mg/mL. Radioactivity was assayed by liquid scintillation counting in a Beckman Model LS-7000 counter. Aliquots (0.5 mL) of each preparation were counted in 10 mL of ACS (Amersham/Searle). Observed counts per minute were reduced to disintegrations per minute by means of external standardization quench correction (H-number method), and they were employed together with the protein concentration to calculate the ratio of bound colchicine to tubulin.

The CD spectra of these samples were recorded within 2 h of sample preparation. The degree of dissociation of colchicine from tubulin during the CD studies was assessed after measurement of spectra by rechromatography of the samples at 25 °C on Sephadex G-25 columns (0.9 × 25 cm) equilibrated with PMCD. Radioactivity and protein were deter-

[1] Abbreviations used: DTE, dithioerythritol; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; NMR, nuclear magnetic resonance; PC, phosphocellulose; Pipes, piperazine-N,N'-bis-(2-ethanesulfonic acid); CD, circular dichroic; Tris tris(hydroxymethyl)aminomethane.

[2] Microtubule protein denotes tubulin plus all microtubule-associated proteins that copurify with tubulin through cycles of in vitro assembly and disassembly. Tubulin denotes specifically the $\alpha\beta$ dimer of the microtubule.

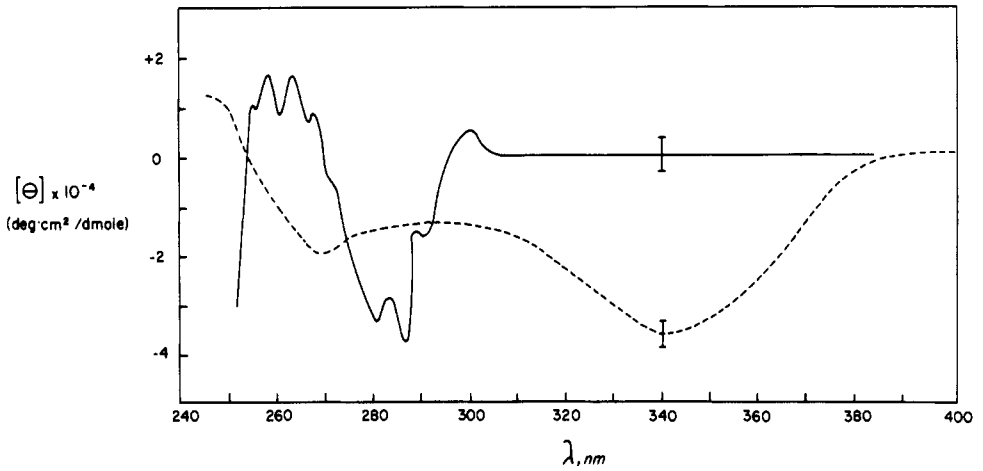

FIGURE 1: CD spectra of tubulin (—) and colchicine (---) over the wavelength range 250–400 nm. The spectra are reported as molar ellipticity of each compound, and the molecular weight of the tubulin dimer was taken as 110 000. The spectrum for tubulin represents an average of three independent spectra on two protein preparations at 1 mg/mL. The spectrum for colchicine between 290 and 400 nm is an average obtained on eight spectra at various drug concentrations (10–40 μM); the spectrum below 290 nm is from a 9 μM solution. Estimated standard errors are indicated by the bars on both spectra.

mined as described previously.

A control sample of drug-free tubulin was prepared from the original stock solution by dilution with PMCD to a final concentration of approximately 1 mg/mL. The turbidity of such a sample, monitored as apparent A_{350}, was very low when measured against a PMCD blank (A_{350} = 0.015) and did not change during a 1-h incubation at 25 °C. Therefore, the CD spectra of the control tubulin were not complicated by turbidity artifacts introduced by microtubule assembly. Colchicine controls were prepared by dilution of 1 mM colchicine in PMCD with PMCD buffer to final concentrations identical with the concentrations of bound colchicine in the drug–protein complexes described above.

CD Spectroscopy. CD spectra were obtained on a Cary 60 spectropolarimeter equipped with a CD attachment. All spectra were taken at 26 °C with the use of a 1-cm cell, a full-scale sensitivity of 40 × 10^{-3} deg, and a time constant of 3 s. Duplicate or triplicate scans were made of all solutions, and reproducibility was invariably within ±1 × 10^{-3} deg. Tubulin concentrations varied from 7 to 14 μM (conditions which did not favor polymerization even in colchicine-free solutions), and PMCD buffer was used for all spectral studies.

Titrations to obtain colchicine/tubulin ratios in the range 0–3 mol/mol were accomplished by adding aliquots of colchicine (1 mM in PMCD buffer) to 3.0 mL of tubulin (13.5 μM) in the CD cell. The ellipticity at 340 nm was followed with time, and after apparent equilibrium was reached, scans were taken. Under these conditions, the CD signal stabilized after 15–30 min. Correction was introduced for the changes of a few percent in tubulin concentration that were produced by the addition of the colchicine stock.

Control CD spectra between 300 and 400 nm were also obtained for mixtures of colchicine (30 μM) with each of the following acidic proteins (15 μM): chicken ovalbumin (Worthington Biochemicals, Freehold, NJ), human choriogonadotropin purified as described elsewhere (Puett et al., 1978), and G-actin prepared from rabbit skeletal muscle and kindly provided by Dr. Dixie L. Frederiksen. The first two proteins were in PMCD buffer; G-actin was in a low ionic strength buffer (2 mM Tris-HCl, 0.2 mM $CaCl_2$, 0.2 mM ATP, and 0.5 mM mercaptoethanol, pH 8.0) to prevent association.

Results

The CD spectra of tubulin and of colchicine between 250 and 400 nm are shown in Figure 1. (Spectra were also obtained between 400 and 500 nm, and no CD bands were observed.) As expected, tubulin exhibits no ellipticity above 310 nm. The small positive band at about 300 nm and the shoulder (negative band) at 292 nm can be assigned to tryptophan, the double minima at 281 and 287 nm are assigned to tyrosine, and the bands between 255 and 270 nm probably result from phenylalanyl residues; these latter bands may be superimposed on bands from other aromatics and disulfides (Sears & Beychok, 1973; Strickland, 1974). The CD spectrum of colchicine is characterized by a major negative band at 340 nm which was assigned to the methoxytropone ring system; the other negative band at 269 nm probably arises from the biaryl system (Hrbek et al., 1964).

Colchicine–tubulin complexes at two different concentrations exhibit the CD spectra shown in Figure 2B,C; the spectra of equivalent concentrations of free colchicine are also shown in the same panels, and a comparable spectrum of tubulin is also given (Figure 2A). (The spectra are reported in the measured units of degrees since with two optically active species there is no a priori reason to express the data as molar ellipticity based on the concentration of one compound and not the other.) The notable feature of the CD spectrum of the drug–protein complex is the absence of the 340-nm band of colchicine. The additive spectra of free colchicine and tubulin at their respective concentrations in the complex are shown in Figure 2D. In the wavelength range from 270 to 300 nm, these simulated spectra are similar to those of the complexes. Differences, however, clearly exist at both lower and higher wavelengths. (The 292-nm negative band observed in tubulin in Figure 1 is not as obvious in Figure 2. Presumably this is due to the slightly lower protein concentrations used for the latter studies.)

CD spectra of colchicine in the presence and absence of tubulin (drug to protein molar ratio of 2.7) showed that the addition of tubulin clearly reduced the rotational strength of the solution (data not shown). This result confirms our findings that formation of the colchicine–tubulin complex leads to a reduction (essentially to zero) of the rotational strength of the 340-nm band of colchicine.

The molar ellipticity at 340 nm was determined at various colchicine concentrations between 3.5 and 43 μM, in the presence and absence of a fixed concentration of tubulin (Figure 3). A slight dependence of the ellipticity on drug concentration is noted in the absence of tubulin. The ellipticity

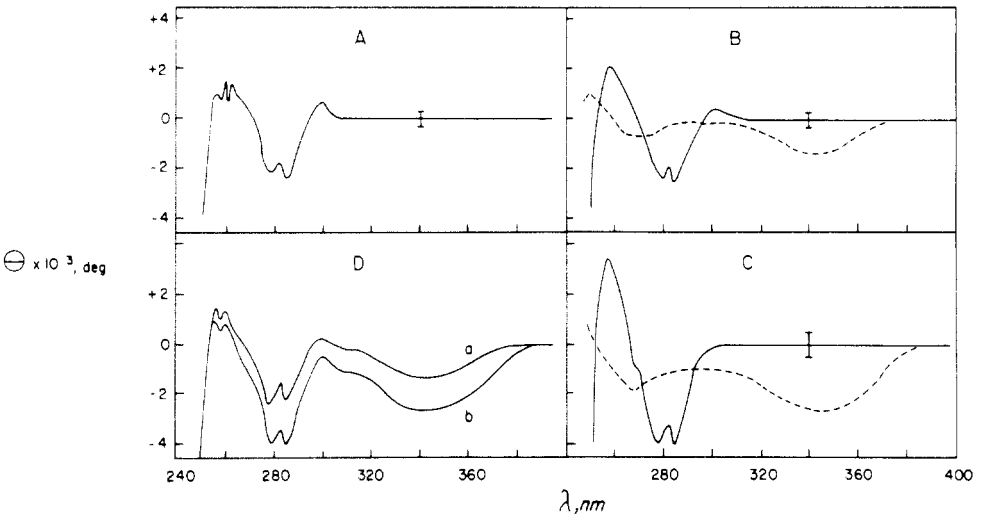

FIGURE 2: CD spectra of tubulin, colchicine, and preformed tubulin–colchicine complexes prepared as described in the text. The data are plotted as measured ellipticity (in degrees) in a 1-cm cell. (A) Free tubulin at a concentration of 8.9 μM. (B) A tubulin–colchicine complex which is 7.7 μM protein and 5.2 μM drug (—); free colchicine at a concentration of 5 μM (---). (C) A tubulin–colchicine complex which is 10.5 μM protein and 9 μM drug (—); free colchicine at a concentration of 9 μM (---). (D) Additive spectra for free tubulin and free colchicine at concentrations equivalent to those in the protein–drug complexes. Curve a is the spectrum that was calculated on the assumption of additivity of the spectra of the free compounds for tubulin and colchicine at respective concentrations of 7.7 and 5.2 μM; this is to be compared to the actual spectrum of the complex in panel B. Curve b represents the added spectra of free tubulin and free colchicine at concentrations of 10.5 μM and 9 μM, respectively; this should be compared with the spectrum of the complex shown in panel C. All spectra were recorded in replicate (three to four times), and ellipticities were measured in 2.5-nm intervals from the original tracings.

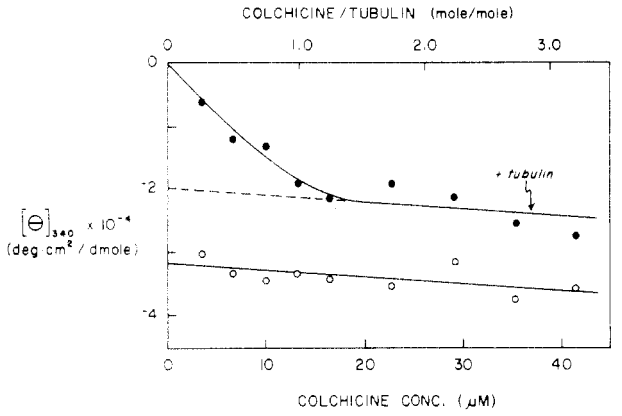

FIGURE 3: The molar ellipticity of colchicine at 340 nm is given as a function of colchicine (CLC) concentration in the absence (○) and presence (●) of 13 μM tubulin. The lower fitted line represents the linear relation described in the text.

in degree centimeter squared per decimole is given from linear regression by

$$[\theta]_{340} = (-3.18 \times 10^4) - (1.25 \times 10^2)[\mathrm{CLC}]$$

where [CLC] refers to the colchicine concentration in micromolar concentration. In the presence of tubulin, the ellipticity at low colchicine concentrations can be extrapolated to zero in agreement with the findings reported in Figure 2. At molar ratios of colchicine to tubulin exceeding ~1.2, there is a fairly constant difference at any colchicine concentration between the solutions ± tubulin of $|1.2 \times 10^4|$ deg·cm^2/dmol. When combined with our finding that the ellipticity of bound colchicine is zero or nearly so at 340 nm, this fact indicates that 70% of the tubulin is capable of binding colchicine under these conditions. This finding is consistent with results of independent binding studies which have demonstrated stoichiometries of 0.7–0.85 mol of colchicine per mol of dimer (Owellen et al., 1972; Wilson & Meza, 1973; Wilson et al., 1974; H. W. Detrich, III, R. C. Williams, Jr., and L. Wilson, unpublished data). Although our CD data extend to colchicine/tubulin ratios of only 3.4, it is obvious that at much higher ratios the ellipticity even in the presence of tubulin will approach that expected for free colchicine.

The CD data on the colchicine–tubulin solutions in Figure 3 were analyzed via a Scatchard plot in which the amount of colchicine bound was estimated from the ellipticities determined in the presence of colchicine and tubulin. For the equilibrium

$$\alpha\beta + \mathrm{CLC} \rightleftharpoons \alpha\beta\cdot\mathrm{CLC}$$

the association constant K is given by $f/[(1-f)[\mathrm{CLC}]]$ where f is the fraction of occupied colchicine binding sites (taken to be 1.0 at colchicine/tubulin ratios greater than 1.2), and [CLC] is the concentration of free colchicine. The latter is readily obtained from f and the known concentrations of total (t) colchicine and tubulin dimers, i.e., $[\mathrm{CLC}] = [\mathrm{CLC}]_t - f[\alpha\beta]_t$. Due to the inherent experimental error in measurements at low colchicine concentrations in the presence of tubulin where the signals are close to base line and to the limited number of data points, the association constant of 0.9 μM^{-1} obtained from the Scatchard plot can be considered only a rough estimate.

Tubulin is an acidic protein; the reduced and denatured subunits exhibit an apparent isoelectric pH between 5.3 and 5.4 on isoelectric focusing gels (Bekowitz et al., 1977). Several other acidic proteins were investigated by CD spectroscopy at colchicine:protein molar ratios of 2:1 in order to see whether nonspecific electrostatic and protein asymmetric effects might be responsible for the decrease in ellipticity concomitant with colchicine binding to tubulin. The proteins used were G-actin, ovalbumin, and choriogonadotropin, and they were found to have no effect on the colchicine ellipticity at 340 nm (results not shown). In contrast, under these conditions, tubulin reduces the magnitude of the ellipticity by nearly 40%.

Discussion

The data presented above demonstrate that the negative CD band of the colchicine chromophore at 340 nm, which is related to the methoxytropone ring system, either vanishes or is greatly reduced in magnitude when colchicine is bound to tubulin. This result was observed in two independent experiments.

First, analysis of the CD spectra of [^{3}H]colchicine–tubulin complexes revealed the absence of the 340-nm band. Because more than 90% of the ^{3}H label remained associated with tubulin following CD spectroscopy, the observed decrease in ellipticity could not have resulted from production of lumicolchicines, light-induced isomers of colchicine which do not bind to tubulin (Wilson & Friedkin, 1967). Second, when tubulin was titrated with low concentrations of colchicine, the ellipticity at 340 nm could be extrapolated to zero at 0 M colchicine. Furthermore, the magnitude of the 340-nm band of colchicine was not reduced by acidic proteins that do not bind colchicine. These observations strongly indicate that the disappearance of the optical activity of colchicine at 340 nm is a consequence of specific binding of the drug to its high affinity site on tubulin. Furthermore, it is clear that although the CD spectra of the complexes between 250 and 300 nm do not result solely from summation of the drug and protein spectra, the similarities between the summed spectrum and that of the mixture are more striking than the differences.

Binding of an optically inactive chromophore to the asymmetric environment of a protein may give rise to characteristic extrinsic Cotton effects. For example, Glaser & Singer (1971) have shown that the binding of nitrophenyl haptens to a myeloma protein or to antihapten antibodies produces distinctive extrinsic CD spectra. A similar induction of extrinsic optical activity occurs when heme binds to globin (Harrison & Blout, 1965). In contrast, we have observed the disappearance of an optically active band in colchicine concomitant with its binding to tubulin. In addition, we have observed a partial decrease in the intensity of the 340-nm band when colchicine binds to a partially purified heterogeneous antibody preparation (Wolff et al., 1980) under conditions of large antibody excess (unpublished observations). The less stringent spatial requirements of the antibody site (compared to the colchicine binding site on tubulin) for the methoxytropone ring (ring C) of colchicine probably accounts both for the failure of the colchicine–antibody complex to fluoresce (Wolff et al., 1980) and for the incomplete abolition of the 340-nm CD band of colchicine.

The rotational strength R associated with a given transition in a noninteracting chromophore is given by the following equation, $R = \mathrm{Im}[\mu \cdot m]$, which reduces to $-i\mu \cdot m$ (Sears & Beychok, 1973). In this equation, Im denotes the imaginary component of the scalar product of the electric (μ) and magnetic (m) transition dipole moments (Sears & Beychok, 1973). On the basis of this relationship and considerations of the methoxytropone ring only, there are at least four potential mechanisms which could explain the tubulin-associated reduction in R of the 340-nm CD band of colchicine. (1) The intrinsic asymmetry of tubulin could cancel exactly, or nearly so, that of colchicine. (2) The formation of a Schiff-base linkage between the tropone carbonyl and a lysyl ϵ-amino group in the binding site of tubulin may lead either to a change in the orientation of the transition moments or to a reduction in m. (3) Protonation of the carbonyl group in the tropone ring may lead to a resonance-stabilized, planar tropolonium ion with CD parameters quite distinct from those of colchicine. (4) Binding of colchicine to tubulin may induce a conformational change in the drug such that μ and m either become orthogonal (or nearly so) or greatly reduced in magnitude. (We know from absorption measurements on the colchicine–tubulin complex that the magnitude of μ is not appreciably altered but m may be vastly reduced in magnitude.)

The first possibility seems unlikely since it requires a precise spatial orientation of the nonglycyl C_α's and helices of tubulin, and the lower wavelength CD bands of colchicine do not seem to be altered in the same manner. Also, under comparable experimental conditions to those described herein, deacetamidocolchicine was found to exhibit no appreciable ellipticity in the vicinity of 340 nm, as might be expected for induced optical activity (unpublished results with T. J. Fitzgerald). If formation of a Schiff base occurs, it must be rapidly reversible because chemically unaltered colchicine may be readily extracted from colchicine–tubulin complexes by a variety of procedures (Wilson & Friedkin, 1967; Bhattacharyya & Wolff, 1974; our own unpublished observations). So that the hypothesis that Schiff-base formation occurs during the binding process could be tested, a colchicine–tubulin complex (molar ratio of 0.8:1) was incubated with cyanoborohydride (44 mM) at 0 °C for 2 and 18 h. The cyanoborohydride did not strip the bound colchicine from the tubulin, and moreover, denaturation in 8 M urea, followed by exhaustive dialysis, led to the total removal of colchicine. Similar results were obtained with sodium borohydride. Thus, these findings also argue against the formation of a Schiff base between colchicine and tubulin. Preliminary data indicate that acidic pH does not lead to a large reduction in the 340-nm CD band of colchicine [unpublished results; see also Wilczok et al. (1979), who used other spectral techniques]. Thus, if a protonated species of colchicine is formed upon binding to tubulin, its molecular characteristics or microenvironment must differ appreciably from that produced by acidic conditions only.

The remaining alternative invokes a conformational change in the colchicine molecule accompanying binding to tubulin. On the basis of studies of the crystal structure of colchicine and several of its derivatives, Margulis and co-workers have inferred that the colchicine molecule, with the exception of the methoxy groups, is quite rigid (Margulis, 1974; Koerntgen & Margulis, 1977; Bessinger & Margulis, 1978). Rapoport & Lavigne (1956) have reported that the optical rotation of chloroform solutions of isocolchicine, but not colchicine, changes with time, while neither compound shows mutarotation in ethanol. They suggested that the mutarotation of isocolchicine reflected a hindered rotation between rings A and C and that such a rotation was not possible in colchicine. Nevertheless, there exist at least two potential stable conformations of colchicine (Macdonald & Reagan, 1980), as illustrated in Figure 4. If the activation energy barrier between these two conformations is relatively low, then the free energy of binding of colchicine to tubulin (~ -10 kcal/mol) may be sufficient to permit the transition between the two conformations.

If colchicine rings A and C as well as the N-acetyl side chain are considered to form a set of potentially *interacting* chromophores, then the rotational strength of the 340-nm band cannot be adequately described by the simple expression $-i\mu \cdot m$. Several treatments are available for considering interacting chromophores (Tinoco, 1962; Schellman, 1968; Sears & Beychok, 1973), but the most likely approach to describe colchicine would appear to involve either a coupled oscillator or an electric–magnetic coupling mechanism (Sears & Beychok, 1973). In these cases, an alteration in the conformation of colchicine concomitant with binding could lead to changes in the relative orientations of the various μ's and m's which in turn could account for the reduction of the rotational strength of the 340-nm CD band of colchicine. It is possible that the chirality of the troponoid C ring chromophore in colchicine's ground state (Figure 4, conformer 1) is due to interaction with the adjacent phenyl moiety ($\theta \simeq 53°$). [Related biaryl systems are known to be intensely optically active

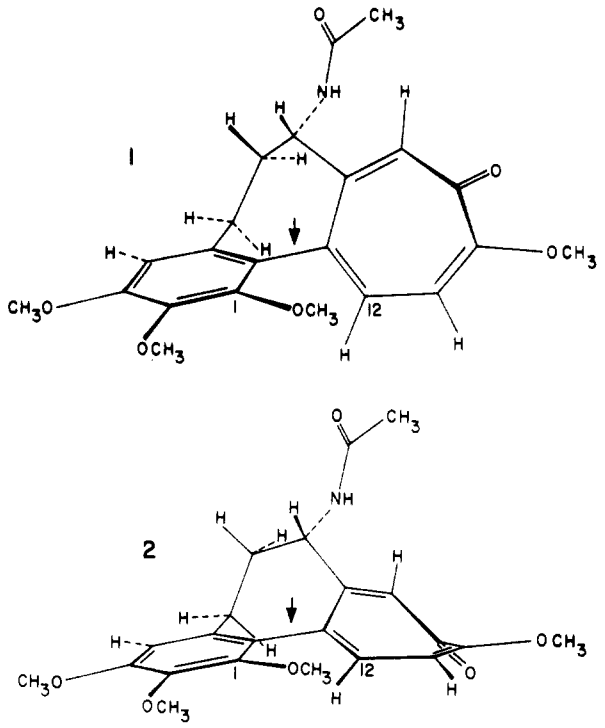

FIGURE 4: Possible multiple conformations of colchicine. Conformation 1 is that observed in the crystal by X-ray crystallography (Margulis, 1974) and established in solution by ^1H and ^{13}C NMR spectroscopy (Macdonald & Reagan, 1980). It has a dihedral angle of approximately 53° (*R*) between the plane defined by the planar aromatic A ring (left) and by the carbons C_{7a}, C_{11}, C_{12}, and C_{12a} of the "tub-shaped" methoxytropone C ring (right). Conformation 2 is related to conformation 1 by a boat–boat interconversion of the troponoid C ring and has a considerably smaller dihedral angle [≤19° (*R*) by inspection of Dreiding molecular models] between the A and C rings. The decreased dihedral angle in conformation 2 engenders an unfavorable steric interaction (~3 kcal/mol) between the C_1 methoxyl and the C_{12} hydrogen. Two additional conformations of colchicine may be energetically accessible. They are related to the two illustrated C ring boat–boat conformers by atropisomerism [about the biaryl bond(↓)]. Such an atropisomeric transition has the effect of changing the chirality of the biaryl moieties [however, with idential dihedral angles: from ~53° (*R*) for 1 and ≤19° (*R*) for 2 to ~53° (*S*) and ≤19° (*S*) for the two *S*-chirality isomers] and of placing the acetamide moiety in the pseudo-axial C_7 position. The *R* chirality of the biaryl junction appears to be the predominant molecular characteristic, permitting recognition by tubulin of colchicine and related agents (Zavala et al., 1980; Robin et al., 1980); consequently the *S*-chirality conformations are not illustrated.

(Hall, 1969).] If this is the case, then binding of colchicine conformer 2 to tubulin would be anticipated to reduce the chirality of the ultraviolet chromophore because of the smaller dihedral angle of this conformer ($\theta \simeq 19°$). In addition, the near planarity of the unsaturated moieties in conformer 2 might be anticipated to exhibit extended conjugation, leading to enhanced fluorescence. Thus, the possibility that the binding of colchicine by tubulin induces a change from conformation 1 to conformation 2 provides a potential explanation of both the CD results and the well-known enhancement of fluorescence. It should be noted, however, that deacetamidocolchicine exhibits little, if any, ellipticity in PMCD at the same concentrations used to study colchicine (unpublished results with T. J. Fitzgerald). These data suggest either that the possible ring interactions in this molecule do not lead to optical activity of the tropone moiety or that facile atropisomerism of the biaryl junction occurs, leading to an atropisomerically racemic mixture (e.g., [R atropisomer] = [S atropisomer]).

It is also conceivable that conformational isomerization of colchicine precedes binding to tubulin and that tubulin binds the relatively rare conformer that lacks the CD band at 340 nm. ^1H and ^{13}C NMR spectra of colchicine show no evidence for two distinct solution conformers (Severini-Ricca & Danielli, 1969; Blade-Font et al., 1979). However, these negative findings could indicate that the putative binding conformation is rare (~5%) or that the conformational equilibrium is so rapid that the NMR spectra represent time-averaged signals. It is interesting to note that the keto ester of steganone, a compound that shares certain structural similarities with colchicine, undergoes a conformational isomerization at elevated temperatures between two stereochemical forms by rotation about the biaryl bond (Kende et al., 1976). Whatever the mechanism, our results seem most consistent with the concept of a conformational change in the drug upon binding to tubulin.

Titration of tubulin with colchicine permitted an estimate of the association constant for the colchicine–tubulin reaction. Our value agrees well with that derived from other equilibrium protocols (Owellen et al., 1972, 1974; Bhattacharyya & Wolff, 1974, 1976; Wilson et al., 1974; Sherline et al., 1975; McClure & Paulson, 1977) and is smaller by an order of magnitude than values obtained by kinetic measurements (Sherline et al., 1975; Bhattacharyya & Wolff, 1976; McClure & Paulson, 1977). Sherline et al. (1975) and Garland (1978) have suggested that the discrepancy between equilibrium constants derived from kinetic and thermodynamic data results from the loss of colchicine-binding activity of tubulin during attainment of equilibrium. On the basis of our limited kinetic observations, it seems plausible that the decrease in ellipticity of colchicine at 340 nm monitors the same colchicine-induced conformational change in tubulin revealed by the enhancement of fluorescence which accompanies colchicine binding to tubulin (Garland, 1978). Such a possibility is supported by the association of both the fluorescence of the colchicine–tubulin complex (Bhattacharyya & Wolff, 1974) and the 340-nm CD band, i.e., the first Cotton effect at 330 nm reported by Hbrek et al. (1964), with the tropone moiety of colchicine.

The near ultraviolet CD spectrum obtained here for bovine brain tubulin in PMCD is very similar to that reported by Lee et al. (1978) for bovine brain tubulin in 10 mM sodium phosphate and 0.1 mM GTP, pH 7.0. However, our measured value of the rotational strength of the positive band(s) at 261 nm is significantly lower than that observed by Lee and coworkers at 265 nm. Because buffer identity and ionic strength have relatively little influence on the near- and far-ultraviolet CD spectra of tubulin (Lee & Timasheff, 1977), we suspect that the intensity of this band is sensitive to the degree of aggregation of tubulin. Whereas Lee et al. (1973, 1975) report a significant, but unspecified, degree of turbidity associated with tubulin purified by the procedure of Weisenberg et al. (1968), the light scattering of our tubulin preparation was quite low when measured as apparent absorbance at 350 nm. The CD spectra of colchicine–tubulin complexes show band positions characteristic of tubulin.

Garland (1978) has presented evidence that the slow kinetics of the colchicine-binding reaction result from a ligand-induced conformational change in tubulin. On the basis of CD studies of porcine brain tubulin in the wavelength region between 200 and 250 nm, Ventilla et al. (1972) concluded that colchicine binding required a conformational species of tubulin favored at high temperature, but Lee et al. (1978) were unable to confirm the temperature-dependent conformational change in tubulin isolated from bovine brain. We have observed a decrease in the dissociation constant of the tubulin $\alpha\beta$ dimer when colchicine is bound (Detrich et al., 1980); this observation

is consistent with the hypothesis that the conformation of tubulin is altered in the complex. The data presented in this report indicate the possiblity that a conformational change in colchicine itself may also occur in the reaction sequence leading to the formation of the colchicine–tubulin complex.

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