Effect of Colchicine Binding on the Reversible Dissociation of the Tubulin Dimer†

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ABSTRACT: The reversible subunit-dissociation equilibria of the tubulin $\alpha\beta$ dimer and of the colchicine–tubulin dimer complex have been examined by equilibrium ultracentrifugation at 4.6 °C. The dissociation constants (K_D) of tubulin from bovine brain and from flagellar outer-doublet microtubules of the sea urchin *Strongylocentrotus purpuratus* were 7.4×10^{-7} M and 15.2×10^{-7} M, respectively. In contrast, both brain and outer-doublet colchicine–tubulin complexes dissociated less readily into their α- and β-tubulin monomers; $K_D = 2.7 \times 10^{-7}$ M for the brain complex and $K_D = 5.0 \times 10^{-7}$ M for the outer-doublet colchicine–tubulin species. Podophyllotoxin (2×10^{-4} M), tropolone (10^{-4} M), or both podophyllotoxin and tropolone (2×10^{-4} and 5×10^{-4} M, respectively) had no effect on the dissociation constant of brain tubulin. Under these experimental conditions, the initial colchicine-binding capacities of brain and flagellar tubulins were 0.87 ± 0.05 and 0.70 ± 0.07 mol/mol, respectively. The colchicine-binding activities of free tubulins decayed at 4 °C according to first-order kinetics with half-times of 37 h for brain tubulin and 26 h for flagellar tubulin. However, colchicine–tubulin complexes (brain or flagellar) showed no decay of binding activity when stored at 4 °C for periods up to 4 days. These results strongly support the following conclusions: (1) colchicine binding induces or stabilizes a conformational isomer of tubulin that dissociates into its α and β monomers less readily than free tubulin; (2) the conformational change in tubulin is specific for binding of the intact colchicine molecule and does not occur when ligands specific for the trimethoxyphenyl subdomain (i.e., podophyllotoxin) or tropolone subdomain of the colchicine-binding site bind singly or simultaneously to tubulin; and (3) bound colchicine stabilizes tubulin against loss of colchicine-binding activity at 4 °C. This altered conformation of tubulin may be involved in the substoichiometric poisoning of microtubule assembly produced by the addition of colchicine–tubulin complexes to the ends of microtubules.

Microtubule assembly in vitro has the characteristics of a condensation polymerization mechanism with discrete nucleation and elongation phases (Johnson & Borisy, 1977; Murphy et al., 1977). Although the nucleation phase is poorly understood, elongation of assembling microtubules proceeds through the biased polar addition of tubulin subunits to the ends of microtubules (Allen & Borisy, 1974; Dentler et al., 1974; Binder et al., 1975; Johnson & Borisy, 1977). A steady state is attained in which net tubulin addition continues at one end of the microtubule while net tubulin loss occurs at the opposite end (Margolis & Wilson, 1978; Farrell et al., 1979; Bergen & Borisy, 1980). This opposite-end assembly and disassembly gives rise to a unidirectional flux of tubulin subunits through microtubules under steady-state conditions in vitro (Margolis & Wilson, 1978).

Colchicine inhibits microtubule polymerization in vitro and poisons microtubule-dependent functions in vivo at concentrations that are small in comparison to the concentration of free tubulin (Olmsted & Borisy, 1973; Margolis & Wilson, 1977). Taylor (1965) has estimated that mitosis is blocked in KB cells when only 3–5% of the tubulin is complexed with colchicine. Margolis & Wilson (1977) have shown that under certain conditions colchicine inhibits microtubule polymerization through the addition of colchicine–tubulin complexes, but not free colchicine, to microtubule assembly ends.

Indirect evidence suggests that the colchicine-binding site of tubulin is not located in the region of contact between tubulin dimers in the microtubule (Kuriyama & Sakai, 1974; Ikeda & Steiner, 1978). Therefore, the inhibition of assembly produced by the incorporation of colchicine–tubulin complexes into microtubules may not be due to direct steric hindrance produced by colchicine. It is likely that the binding of colchicine to tubulin induces or stabilizes a conformational isomer of the protein that inhibits assembly. Thus, incorporation of colchicine–tubulin complexes into microtubules may perturb the microtubule lattice, thereby lowering the affinity of the ends for subsequent addition of drug-free tubulin (Sternlicht & Ringel, 1979; Farrell & Wilson, 1980).

The apparent rates of association and dissociation of tubulin and colchicine are unusually slow (Wilson, 1970; Sherline et al., 1975; Cortese et al., 1977). Garland (1978) and Lambeir & Engelborghs (1981) found the reaction to be biphasic and described it by a two-step mechanism. They assigned the slow apparent forward rate to a slow conformational change of a tubulin–colchicine complex. Recently, Detrich et al. (1981) have presented circular dichroic measurements indicating that colchicine itself undergoes a change in conformation upon binding to tubulin.

We have shown (Detrich & Williams, 1978) that the tubulin dimer isolated from bovine brain dissociates reversibly into its subunits at 4.6 °C under near-physiological conditions of pH and ionic strength. In this study we analyze the effect of the binding of colchicine, podophyllotoxin, and tropolone on the dissociation constant of tubulin from bovine brain and from flagellar outer-doublet microtubules of *Strongylocentrotus purpuratus*. The results suggest that the conformation of tubulin in the colchicine–tubulin complex is distinct from that of free tubulin and that the conformational change is specifically induced by the binding of the complete colchicine molecule. A preliminary report of this work has appeared

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(Detrich et al., 1980).

Materials and Methods

Reagents. Colchicine (CLC),[1] Pipes, EGTA, DTE, and GTP (type II-S) were obtained from Sigma Chemical Co. Podophyllotoxin (PLN) and tropolone (TRO) were products of Aldrich Chemical Co. Colchicine (ring C, *methoxy*-^{3}H), 16.05 Ci/mmol, was purchased from New England Nuclear, Inc.

Experimental Conditions. All experiments were performed in 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) with the addition of drugs as indicated. The ionic strength of this buffer was approximately 0.236, and its density was 1.017 g/mL at 4.95 °C (Detrich & Williams, 1978). Temperatures are detailed below.

Preparation of Brain and Flagellar Outer-Doublet Tubulins. Microtubule protein[2] (3XMT) was purified from bovine brain tissue through three cycles of in vitro assembly and disassembly by the method of Shelanski et al. (1973) as modified by Berkowitz et al. (1977). Brain tubulin was separated from the microtubule-associated proteins (MAPs) by chromatography of 3XMT on phosphocellulose (Whatman P11) (Detrich & Williams, 1978). The tubulin prepared by this protocol was routinely greater than 96% pure when analyzed by quantitative densitometry of 7.5% $NaDodSO_4$–polyacrylamide gels (Laemmli, 1970) stained with Fast Green (Detrich & Williams, 1978). Phosphocellulose-purified brain tubulin was stored at −196 °C by dropwise freezing in liquid nitrogen (Detrich & Williams, 1978). Immediately prior to ultracentrifugation, material stored in liquid nitrogen was thawed rapidly, centrifuged at 5000*g* for 10 min (4 °C) to remove small amounts of denatured protein, and then chromatographed on a 0.9 × 25 cm column of Sephadex G-25 (Pharmacia) equilibrated with PM buffer supplemented with drug(s) as indicated. Podophyllotoxin was dissolved in Me_2SO and added to PM buffer such that the final concentration of Me_2SO was 0.2% (w/v). Samples were then incubated for 90 min at 37 °C except where noted.

Bovine brain microtubule protein (3XMT) was also prepared in the absence of glycerol by the method of Asnes & Wilson (1979). Glycerol-free tubulin was purified, stored, and prepared for ultracentrifugation exactly as described in the preceding paragraph.

Flagellar outer-doublet tubulin was purified from sperm tails of the sea urchin *Strongylocentrotus purpuratus* by one or two cycles of assembly and disassembly as described by Farrell & Wilson (1978). The purity of this material was greater than 95% (Farrell & Wilson, 1978) when analyzed in the electrophoretic gel system of Yang & Criddle (1970). Pellets of recycled outer-doublet microtubules were stored at −196 °C. Prior to use, pellets were thawed rapidly, resuspended in 1–1.5 mL of PM buffer, incubated on ice for 30 min, and centrifuged at 100000*g* for 30 min (4 °C) to remove undissociated material. The supernatant (which contained the tubulin) was recovered and prepared for ultracentrifugation by chromatography on a Sephadex G-25 column (0.9 × 25 cm) equilibrated with the appropriate buffer. Following Sephadex G-25 chromatography, samples were incubated for 90 min at 37 °C except where noted.

Brain and outer-doublet tubulins were stored in liquid nitrogen without detectable loss in colchicine-binding activity over the course of several months. In the experiments shown in Figures 1 and 2 and summarized in Table I, several different preparations of brain and outer-doublet tubulins were employed, with identical results.

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

Colchicine Binding Assays. The decay of the colchicine-binding activity of drug-free brain and outer-doublet tubulins was monitored as a function of storage time at 4 °C by the gel-filtration method of Detrich & Williams (1978). Aliquots of tubulin (0.5–1.0 mL at 1.5–3 mg/mL) in PM buffer were incubated with 50–100 μL of ^{3}H-labeled 1 mM colchicine (10 μCi/mL) for 1.5 h at 37 °C, applied to a PM-buffer-equilibrated column of Sephadex G-25 (0.9 × 25 cm), and eluted with PM buffer. Fractions (1 mL) were collected and protein concentrations were determined as described previously. Radioactivity was assayed by liquid scintillation counting in a Beckman Model LS-7000 counter. Aliquots (0.5 mL) of each fraction were counted in 10 mL of ACS (Amersham/Searle). Count rates were reduced to disintegration rates by means of external standardization quench correction.

To determine the decay of the colchicine-binding activity of brain or flagellar colchicine–tubulin complexes, we incubated tubulin samples with ^{3}H-labeled colchicine as described above and then stored them at 4 °C in ^{3}H-labeled colchicine of the same concentration and specific activity. Aliquots were withdrawn at appropriate time intervals and assayed for protein and bound colchicine. This method yields the rate of irreversible loss of colchicine-binding activity of the colchicine–tubulin complex due to inactivation of the tubulin in the complex (Garland, 1978).

Analytical Ultracentrifugation. Sedimentation equilibrium studies were performed in a Beckman Model E analytical ultracentrifuge according to the high-speed method of Yphantis (1964) at 4.6 °C as described previously (Detrich & Williams, 1978). A helium–neon laser light source (Williams, 1972) was employed, and Rayleigh interference patterns were recorded. Local apparent weight-average molecular weights, $M_{wa}(r)$, were calculated from microcomparator readings of the interference patterns by means of a computer program similar to those of Roark & Yphantis (1969) and of Teller (1973). Samples were run at three different initial concentrations in each experiment to test for heterogeneity within a protein sample (Roark & Yphantis, 1969; Teller, 1973).

Data Treatment. Bovine brain tubulin (at 4.7 °C in PM buffer) contains a 6S species (the tubulin monomer–dimer system) in rapidly reversible equilibrium with a 19–20S species (Detrich & Williams, 1978). Due to the formation of very small amounts of these higher aggregates of tubulin, values of $M_{wa}(r)$ are observed to exceed the molecular weight of the tubulin dimer (110000) at high protein concentration. In order to allow for this effect in the fitting of data, we postulated the formation of a tetrameric species ($\alpha_2\beta_2$). Association constants of the tubulin $\alpha\beta$ dimer were determined by fitting observed

[1] Abbreviations: CLC, colchicine; DTE, dithioerythritol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; GTP, guanosine 5′-triphosphate; MAPs, microtubule-associated proteins; Me_2SO, dimethyl sulfoxide; $M_{wa}(r)$, apparent radial weight-average molecular weight; $NaDodSO_4$, sodium dodecyl sulfate; *n*XMT, microtubule protein purified through *n* cycles of in vitro assembly and disassembly; Pipes, 1,4-piperazinediethanesulfonic acid; PLN, podophyllotoxin; Tb, tubulin; TRO, tropolone.

[2] In this paper microtubule protein denotes tubulin plus all the microtubule-associated proteins which copurify with tubulin through cycles of in vitro assembly and disassembly. Tubulin refers specifically to the $\alpha\beta$ dimer of the major microtubule protein.

Table I: Influence of Colchicine and Other Alkaloids on the Monomer-Dimer-Tetramer Equilibrium of Brain and Flagellar Tubulins

tubulin sample description	$K_1 \times 10^{-6}$ (M^{-1})[a]	$K_2 \times 10^{-4}$ (M^{-1})[a]	$K_D \times 10^7$ (M)[b]	
brain, run 1[c]	1.0 (±0.25)	0.43	10.0 (±2.5)	
brain, run 3[c]	1.0 (±0.25)	0.77	10.0 (±2.5)	
brain[d]	1.3 (±0.32)	1.3	7.7 (±1.9)	7.4 (±0.4)
brain, incubated at 37 °C for 1.5 h	1.4 (±0.35)	1.2	7.1 (±1.8)	
brain + 10^{-4} M CLC, run 1[d]	3.4 (±0.85)	1.1	2.9 (±0.74)	2.7 (±0.4)
brain + 10^{-4} M CLC, run 2	4.2 (±1.0)	1.0	2.4 (±0.60)	
flagellar[e]	0.66 (±0.16)	2.3	15.2 (±3.8)	
flagellar + 10^{-4} M CLC, run 1[e]	2.0 (±0.50)	2.2	5.0 (±1.2)	5.0 (±0.1)
flagellar + 10^{-4} M CLC, run 2	2.0 (±0.50)	2.2	5.0 (±1.2)	
brain + 2×10^{-4} M PLN[f]	1.9 (±0.48)	1.1	5.3 (±1.3)	
brain + 10^{-4} M TRO[f]	1.6 (±0.40)	1.6	6.2 (±1.6)	
brain + 2×10^{-4} M PLN + 5×10^{-4} M TRO[f]	1.6 (±0.40)	1.1	6.2 (±1.6)	
brain, glycerol free[g]	0.65 (±0.16)	2.2	15.4 (±3.8)	

[a] Monomer–dimer (K_1) and dimer–tetramer (K_2) association constants determined by the fitting routine described under Materials and Methods. [b] $K_D = 1/K_1$. [c] Detrich & Williams (1978). [d] See Figure 1. [e] See Figure 2. [f] See Figure 3. [g] See Figure 4.

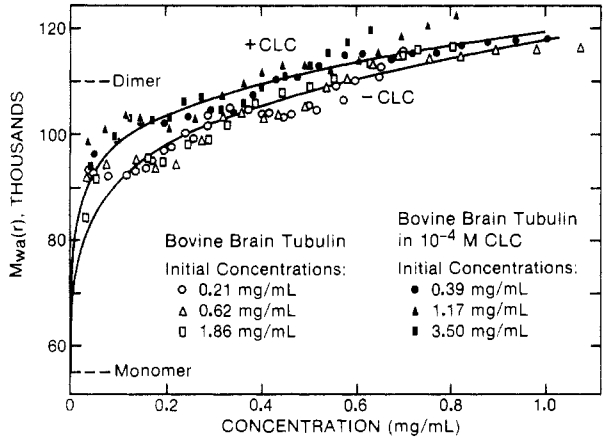


FIGURE 1: Weight-average molecular weight [$M_{wa}(r)$] of bovine brain tubulin as a function of its concentration in PM buffer in the presence and absence of 10^{-4} M colchicine (CLC). Initial protein concentrations are as indicated. Centrifugation was performed at 4.6 °C and interference patterns were recorded between 15 and 30 h after the start of each run. Lines were calculated from the best-fitting values of K_1 and K_2 (see Table I). (Open symbols) Tubulin centrifuged to equilibrium at 20000 rpm in the absence of CLC. The dissociation constant of the tubulin dimer (K_D) determined by the fitting routine described under Materials and Methods was $(7.7 \pm 1.9) \times 10^{-7}$ M. A sample of tubulin (in PM buffer) incubated at 37 °C for 1.5 h prior to centrifugation gave identical results (Table I). (Closed symbols) Tubulin was chromatographed on a Sephadex G-25 column (0.9 × 25 cm) equilibrated with PM buffer + 10^{-4} M CLC. The void-volume peak was incubated at 37 °C for 1.5 h and centrifuged to equilibrium at 18000 rpm and 4.6 °C. K_D was found to be $(2.9 \pm 0.74) \times 10^{-7}$ M.

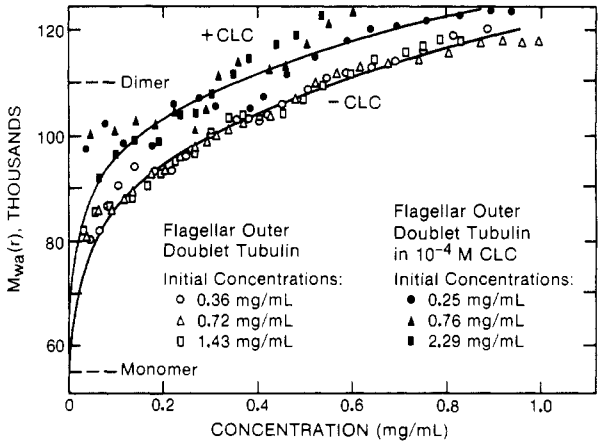


FIGURE 2: Weight-average molecular weight of flagellar outer-doublet tubulin as a function of its concentration in PM buffer in the presence and absence of 10^{-4} M colchicine (CLC). Initial concentrations are given in the figure. Centrifugation was performed at 4.6 °C and the rotor speed was 20000 rpm for both runs. Other details are described in the legend to Figure 1. (Open symbols) Outer-doublet tubulin in the absence of CLC. $K_D = (1.52 \pm 0.38) \times 10^{-6}$ M. (Closed symbols) Outer-doublet tubulin incubated in PM buffer + 10^{-4} M CLC at 37 °C for 1.5 h. $K_D = (5.0 \pm 1.2) \times 10^{-7}$ M.

distributions of $M_{wa}(r)$ vs. concentration to the following monomer–dimer–tetramer scheme:

$$\alpha + \beta \underset{}{\overset{K_1}{\rightleftharpoons}} \alpha\beta$$

$$2\alpha\beta \overset{K_2}{\rightleftharpoons} \alpha_2\beta_2$$

where K_1 is the monomer–dimer association constant ($=1/K_D$, where K_D is the dissociation constant of the $\alpha\beta$ dimer) and K_2 is the dimer–tetramer association constant. Given this model, and assuming that the protein behaves ideally, the following relations must hold for each radial concentration:

$$c(r) = c_1(r) + K_1[c_1(r)]^2 + K_3[c_1(r)]^4 \quad (1)$$

and

$$c(r)M_{wa}(r)/M_1 = c_1(r) + 2K_1[c_1(r)]^2 + 4K_3[c_1(r)]^4 \quad (2)$$

where $c(r)$ is the total concentration (w/v) at radius r, $c_1(r)$ is the concentration (w/v) of monomer at r, $K_3 = K_2K_1^2$, M_1 is the molecular weight of the tubulin monomer (55000), and $M_{wa}(r)$ is the experimentally observed apparent radial weight-average molecular weight at radius r. The fitting routine begins with initial guesses for K_1 and K_3 and solves eq 1 for $c_1(r)$ at each value of $c(r)$ by Newton's method. With these approximate values of $c_1(r)$, the best-fitting values of K_1 and K_3 are determined for all the data of $M_{wa}(r)$ and $c(r)$ from eq 2 by the method of least squares. With the new values for K_1 and K_3 the program reenters eq 1 and calculates a set of new approximations to $c_1(r)$. Reiteration continues until the sum of squares of the deviations of the observed data from the calculated curve is minimized. The fitting program converged rapidly to a solution from widely disparate initial guesses. No statistical weighting was applied to the data, which did not vary greatly in precision. Estimates of uncertainty attached to the values of K_D represent an approximation to the standard error of the mean, estimated from the deviation of fits to individual channels of data (i.e., association constants calculated from the data for each initial concentration alone) from the effective mean (the association constant derived from simultaneous analysis of the total pooled data for each run). The maximal standard error observed was ±25% of the association constant, and this figure has been used throughout as the *estimate* of uncertainty. This figure probably overes-

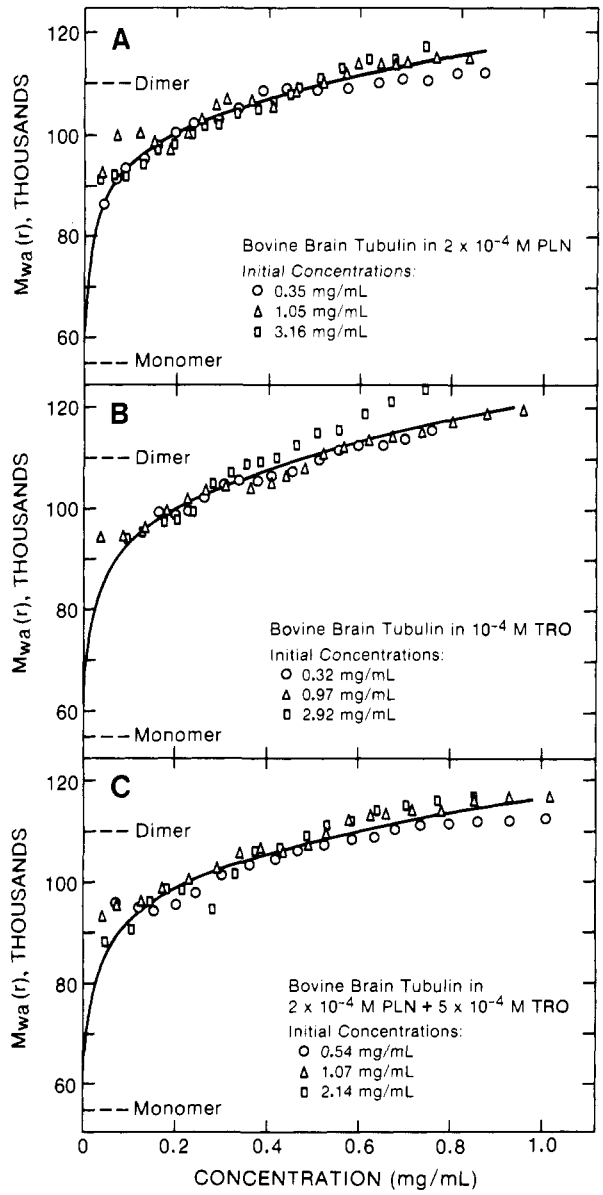


FIGURE 3: Weight-average molecular weight of brain tubulin (Tb) as a function of its concentration in PM buffer + podophyllotoxin (PLN) and/or tropolone (TRO). T = 4.6 °C and rotor speed = 20 000 rpm for each experiment. Other details are given in the legend to Figure 1. (A) Brain Tb in PM buffer + 2 × 10^{-4} M PLN. K_D = (5.3 ± 1.3) × 10^{-7} M. This sample was not incubated at 37 °C prior to centrifugation. (B) Brain Tb in PM buffer + 10^{-4} M TRO. K_D = (6.2 ± 1.5) × 10^{-7} M. This sample was incubated at 37 °C for 1.5 h prior to centrifugation. (C) Brain Tb in PM buffer + 2 × 10^{-4} M PLN + 5 × 10^{-4} M TRO. K_D = (6.2 ± 1.6) × 10^{-7} M. This sample was incubated at 37 °C for 1.5 h prior to incubation.

timates the true uncertainties.

Results

Sedimentation Equilibrium Studies. Results of the sedimentation equilibrium experiments with brain and flagellar tubulins are presented in Figures 1–4 and Table I. Values of apparent weight-average molecular weight are plotted as the function of the local concentration $c(r)$ in the ultracentrifuge cell. Two general features of these results are important. First, within any given experiment the data from each of three initial concentrations superimpose within error, as expected for a homogeneous solute that participates in a rapidly reversible association equilibrium (Roark & Yphantis, 1969; Teller, 1973; Detrich & Williams, 1978). Thus, no significant amount of slowly equilibrating or irreversibly aggregated material is present in these solutions, and eq 1 and

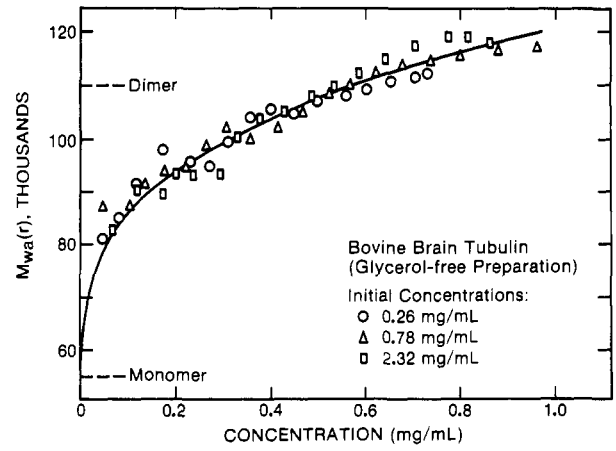


FIGURE 4: Weight-average molecular weight of glycerol-free brain tubulin as a function of its concentration in PM buffer at 4.6 °C. Rotor speed = 20 000 rpm. Bovine brain microtubule protein was purified in the absence of glycerol by the method of Asnes & Wilson (1979). Glycerol-free tubulin was purified from 3XMT by chromatography on phosphocellulose as described under Materials and Methods. K_D = (1.54 ± 0.38) × 10^{-6} M.

2 apply directly. Second, weight-average molecular weights in excess of the molecular weight of the tubulin dimer (110 000) are observed at protein concentrations above approximately 0.5–0.7 mg/mL. This deviation from the hyperbolic dependence of molecular weight on concentration that would be predicted for dimerization of two 55 000 molecular weight monomers reflects the contribution of small amounts of higher aggregates of tubulin that are in rapid equilibrium with the tubulin monomer–dimer system (Detrich & Williams, 1978).

Monomer–dimer and dimer–tetramer association and dissociation constants obtained from the fitting routine described under Materials and Methods are given in Table I for the tubulin samples of Figures 1–4. The lines in Figures 1–4 are drawn from the values of K_1 and K_2.

Figure 1 presents the weight-average molecular weight, $M_{wa}(r)$, of bovine brain tubulin as a function of its concentration in PM buffer, in the presence and absence of 10^{-4} M colchicine. Similar data for flagellar outer-doublet tubulin are shown in Figure 2. It is clear from these results that colchicine–tubulin complexes (brain or outer doublet) dissociate into their monomeric chains less readily than the respective drug-free proteins do. The monomer–dimer association constant of both brain and flagellar tubulins is approximately 3-fold greater in the presence of colchicine than in its absence (Table I). This difference was not due to modification of tubulin by exposure to elevated temperature during incubation with the drug: when brain tubulin was incubated for 90 min at 37 °C in the absence of colchicine, then cooled and centrifuged to equilibrium as described previously, the concentration dependence of molecular weight was identical with that shown in Figure 1 for colchicine-free tubulin (see Table I). These observations are consistent with the idea that the binding of colchicine to tubulin is accompanied by a conformational change in the dimer that affects the free energy of association at the monomer–monomer interface (see Discussion).

The colchicine-binding site on tubulin appears to consist of a trimethoxyphenyl-binding subdomain (shared with podophyllotoxin) and a tropolone-binding subdomain (Bhattacharyya & Wolff, 1974; Wilson, 1975; Cortese et al., 1977). Therefore, it was of interest to determine whether the change in dissociation behavior of the tubulin dimer in the presence of colchicine was attributable to binding of ligands at either

Table II: First-Order Decay of the Colchicine-Binding Activity (CBA) of Free Tubulin and of the Colchicine–Tubulin Complex at 4 °C

sample description	initial CLC-binding capacity[a] (mol/mol)	first-order decay constant at 4 °C (h^{-1})	half-time[b] for decay of CBA at 4 °C (h)
brain Tb	0.87 ± 0.05[c]	0.0187 ± 0.0016[c]	37.2 ± 3.1[c]
glycerol-free brain Tb[d]	0.86	0.0207	33.5
flagellar Tb[d]	0.70 ± 0.07[c]	0.0267	26.0
brain Tb–CLC complex[d]	0.83	0	∞
flagellar Tb–CLC complex[d]	0.75	0	∞

[a] Values are uncorrected for the loss in CBA during the 1.5-h incubation at 37 °C. [b] $T_{1/2} = (\ln 2)/k$, where k is the first-order decay constant. [c] Values are averages obtained from two independent experiments. [d] See Figure 5.

or both of these subdomains. Parts A–C of Figure 3 present the concentration dependence of $M_{wa}(r)$ for bovine brain tubulin in 2×10^{-4} M podophyllotoxin, 10^{-4} M tropolone, and 2×10^{-4} M podophyllotoxin + 5×10^{-4} M tropolone, respectively. Dissociation constants are given in Table I. The dissociation behavior of the tubulin dimer in the presence of each of these drugs separately, and of both of them together, was not distinguishable within experimental uncertainty from that of colchicine-free brain tubulin. These observations suggest that the conformational change in tubulin results from the binding of the intact colchicine molecule.

The dissociation behavior of a sample of phosphocellulose-purified bovine brain tubulin isolated from 3XMT prepared by the glycerol-free protocol of Asnes & Wilson (1979) is presented in Figure 4. The dissociation constant, 15.4×10^{-7} M, was approximately twice as great as the value [$(7.4 \pm 0.4) \times 10^{-7}$ M] obtained for tubulin derived from 3XMT purified by the method of Berkowitz et al. (1977) (Figure 1 and Table I). Indeed, the K_D for this brain tubulin sample was identical with that of outer-doublet tubulin. This difference between the two brain tubulin preparations may be attributable to exposure of one preparation to glycerol during purification of microtubule protein (see Discussion).

Table I also reports values of K_2, the dimer–tetramer association constant incorporated in the model of tubulin association. The small magnitude of K_2 (ca. $0.01K_1$) indicates that brain and outer-doublet tubulins in the presence and absence of the drugs investigated have a slight tendency to associate into higher aggregates at 4.6 °C. The weight fraction of putative $\alpha_2\beta_2$ was always less than 10% in these experiments. We wish to emphasize that the true stoichiometry of the 19S species and the nature of the association reaction that produces it remain unknown and are not addressed by these results.

Colchicine Binding Studies. The first-order decay of the colchicine-binding activity of brain and outer-doublet tubulins and of preformed colchicine–tubulin complexes at 4 °C is presented in Figure 5. First-order decay parameters were calculated from these data and are summarized in Table II. Under these experimental conditions, the initial colchicine-binding capacities of brain and flagellar tubulins ranged from 0.70 to 0.87 mol/mol, with consistently higher ratios observed for the brain preparations. The half-time for decay of the colchicine-binding activity at 4 °C of the standard brain preparation was approximately 37 h, while that of flagellar outer-doublet tubulin was 26 h; glycerol-free tubulin decayed with a half-time (33.5 h) comparable to that of the standard brain tubulin. In contrast, colchicine–tubulin complexes (brain or flagellar) showed no decay of binding activity when stored at 4 °C in PM buffer + 10^{-4} M colchicine for periods up to 4 days. This observation supports the conclusion that bound colchicine stabilizes tubulin against loss of colchicine-binding activity as reported previously (Borisy & Taylor, 1967; Weisenberg et al., 1968; Sherline et al., 1975; Garland, 1978). However, the magnitude of the stabilization observed is substantially greater at 4 °C than at 37 °C.

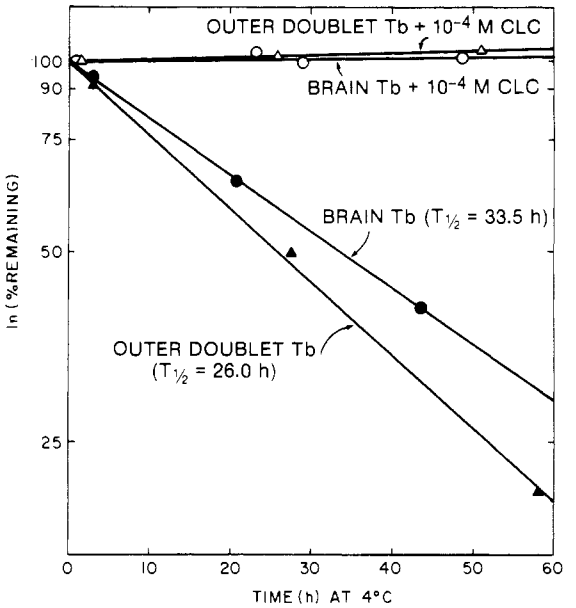


FIGURE 5: First-order decay of the colchicine-binding activity of free tubulin [brain (glycerol-free preparation) and flagellar] and of the colchicine–tubulin complex (brain and flagellar) at 4 °C. Colchicine-binding assays were performed as described under Materials and Methods. First-order decay parameters for colchicine binding are summarized in Table II.

Discussion

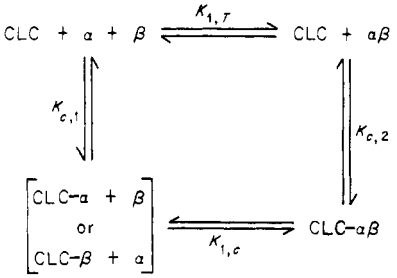
We have shown previously (Detrich & Williams, 1978) that the dimer of bovine brain tubulin undergoes a reversible, concentration-dependent dissociation into its monomeric subunits at 4.6 °C under conditions of near-physiological pH and ionic strength. This study confirms this finding and demonstrates that flagellar outer-doublet tubulin derived from sperm of the sea urchin *Strongylocentrotus purpuratus* dissociates in a similar fashion. The binding of colchicine to either brain or flagellar tubulin causes the dissociation constant of the dimer to decrease by a factor of approximately 3. Saturation of either the trimethoxyphenyl or tropolone subdomains, or both, with podophyllotoxin and/or tropolone has no effect on the magnitude of the dissociation constant. Both types of tubulin, in the presence or absence of various alkaloids, associate into structures larger than the dimer at 4.6 °C. Finally, bound colchicine stabilizes tubulin totally to loss of colchicine-binding activity at 4 °C.

Dissociation of Tubulins. The value of K_D observed for bovine brain tubulin prepared from microtubule protein isolated by the method of Berkowitz et al. (1977) was $(7.4 \pm 0.4) \times 10^{-7}$ M. This value agrees well with the dissociation constant (8×10^{-7} M) that we reported previously (Detrich & Williams, 1978), although reanalysis of the older data by the fitting routine described under Materials and Methods produced a

refined value for K_D of $(10 \pm 2.5) \times 10^{-7}$ M (Table I). These small differences in absolute values are within the limits of experimental uncertainty and are not considered to be important. In contrast, both flagellar outer-doublet tubulin and glycerol-free bovine brain tubulin isolated from microtubule protein prepared by the method of Asnes & Wilson (1979) were observed to dissociate with a $K_D \simeq 15 \times 10^{-7}$ M. It is likely that the identity of the values of K_D for outer-doublet tubulin of the sea urchin and glycerol-free brain tubulin indicates that tubulins derived from these phylogenetically distant organisms share conserved monomer–monomer contact surfaces. This conclusion is consistent with the apparent conservation of the primary sequences of α- and β-tubulins from embryonic chick brain and outer-doublet tubulin from sea urchin sperm (Ludueña & Woodward, 1973, 1975). Some difference in preparative conditions (perhaps exposure to glycerol) appears to be responsible for the difference in dissociation constants obtained for the two brain tubulin preparations. Scheele & Borisy (1976) have shown that the physicochemical properties and protein composition of microtubule protein prepared in the presence of glycerol differ from those of proteins prepared in its absence. Although these differences may not be due to the binding of glycerol per se (Zabrecky & Cole, 1979) as we suggested previously (Detrich et al., 1976), aldehyde and peroxide contaminants have been detected in reagent or "spectroquality" glycerol, particularly after exposure to air (Bello & Bello, 1976). Therefore, we disagree with the conclusion of Zabrecky & Cole (1979) that glycerol may be employed in microtubule protein preparations without consideration of the possibility of artifactual modification of the protein.

Dimer–Monomer Dissociation of Colchicine–Tubulin Complexes. The observation that the dimer–monomer dissociation constant of both brain and flagellar tubulins decreases 3-fold when colchicine is bound to the protein strongly suggests that the binding of colchicine to tubulin is accompanied by a conformational change in the dimer, which includes structural rearrangements at the interface between the α and β monomers (see below). Furthermore, it is possible to demonstrate from our data that one of the two tubulin monomers is capable of binding colchicine.

Energetics of Binding. Consider the two possible pathways between the following initial ($\alpha + \beta$ + CLC) and final ($\alpha\beta$–CLC) states of the colchicine–tubulin system:



We assume that only one of the two dissociated monomers is able to bind colchicine. If the rapidly reversible dissociation of the tubulin dimer exposed cryptic binding sites for colchicine, then one might expect to observe stoichiometries for binding in excess of 1 mol of colchicine/mol of tubulin dimer: the extent of binding would be governed by the relative magnitudes of the association constants for dimerization of the tubulin monomers and for association of colchicine with the hypothetical multiple binding sites. However, initial binding capacities in excess of 1 mol of colchicine/mol of tubulin dimer have not been reported.

The constants in this cycle of ligand binding and subunit association are given by

$$K_{1,T} = \frac{[\alpha\beta]}{[\alpha][\beta]}$$

$$K_{1,c} = \frac{[\text{CLC}{-}\alpha\beta]}{[\text{CLC}{-}\alpha][\beta]} \text{ or } \frac{[\text{CLC}{-}\alpha\beta]}{[\text{CLC}{-}\beta][\alpha]}$$

$$K_{c,2} = \frac{[\text{CLC}{-}\alpha\beta]}{[\text{CLC}][\alpha\beta]}$$

$$K_{c,1} = \frac{[\text{CLC}{-}\alpha]}{[\text{CLC}][\alpha]} \text{ or } \frac{[\text{CLC}{-}\beta]}{[\text{CLC}][\beta]}$$

Standard free energies corresponding to the four association constants are related by

$$\Delta G^\circ_{1,T} + \Delta G^\circ_{c,2} = \Delta G^\circ_{1,c} + \Delta G^\circ_{c,1} \qquad (3)$$

$K_{1,T}$ and $K_{1,c}$ have been determined in this study and are given in Table I. [It is important to note that the concentration of colchicine employed in these studies (10^{-4} M) was sufficient to saturate its binding site on tubulin under the experimental conditions (Table II). Thus, the value for $K_{1,c}$ that we have determined (Table I) represents the association constant of the fully liganded monomer–dimer system.] $K_{c,2}$ was estimated to be 4.0×10^5 M^{-1} at 4.6 °C from a linear extrapolation of the van't Hoff plot for colchicine binding to rat brain tubulin (Bhattacharyya & Wolff, 1974). The validity of this extrapolation is supported by the observed linear dependence of $\ln K$ vs. $1/T$ for colchicine binding to tubulin in sea urchin egg supernatant fractions between 8 and 37 °C (Pfeffer et al., 1976). Substituting the free-energy changes corresponding to these constants into eq 3 gives $\Delta G^\circ_{c,1} = -6.5$ kcal/mol. Therefore, $K_{c,1} \simeq 1.3 \times 10^5$ M^{-1} at 4.6 °C. If binding of colchicine to one of the free monomers did not occur (i.e., if the colchicine-binding site were to form only when the α and β monomers are associated), then the association constant of the liganded (CLC–) tubulin dimer would be approximately 14-fold greater than that of the unliganded dimer and, therefore, could not have been measured by our methods. It is unclear which monomer binds colchicine. On the basis of affinity labeling of tubulin with bromocolchicine, Schmitt & Atlas (1976) have suggested that the high-affinity site for colchicine is on the α chain. However, analysis of mutants of *Aspergillus nidulans* resistant to methyl *N*-(benzimidazol-2-yl)carbamate, a competitive inhibitor of colchicine binding to fungal tubulin (Davidse & Flach, 1977), has revealed that some resistant lines with a lower affinity for this benomyl derivative have an altered β-tubulin (Sheir-Neiss et al., 1978). Cabral et al. (1980) have reported that some CHO mutants resistant to colchicine, colcemid, or griseofulvin express a modified β-tubulin. Both studies tend to suggest that colchicine may bind to the β chain of tubulin. Further work, however, will be required to identify conclusively the monomer responsible for colchicine binding.

Binding to Subdomains. The binding of podophyllotoxin, tropolone, or both to brain tubulin had no effect on the dissociation equilibrium of the dimer. Therefore, the presumptive conformational change in tubulin requires binding of the intact colchicine molecule and does not occur when the subdomains of the colchicine-binding site are occupied either singly or simultaneously by separate molecules. These observations tend to rule out the possibility that colchicine binding increases the magnitude of the monomer–dimer association constant (i.e., causes ΔG° to become more negative) through intercalation at the region of subunit contact, thereby increasing the hy-

drophobicity of the monomer–monomer interface. If such were the case, one might expect the combination of podophyllotoxin and tropolone to be an equally effective thermodynamic "cement". They also are consistent with the demonstration by Fitzgerald (1976) that the tetramethyoxy bicyclic compound 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone possesses the minimal molecular features required for "colchicine-like" biological activity. Fitzgerald pointed out that the trimethoxybenzene and methoxytropone ring systems must be combined into a single molecular entity by at least the appropriate carbon–carbon single bond. Although our results do not directly demonstrate binding of podophyllotoxin and tropolone to tubulin at 4.6 °C, the concentrations employed were sufficient to saturate their respective binding sites on tubulin, according to existing data (Wilson, 1970, 1975; Bhattacharyya & Wolff, 1974; Cortese et al., 1977).

We have employed podophyllotoxin in combination with tropolone to saturate the trimethoxyphenyl- and tropolone-binding subdomains on tubulin. Podophyllotoxin contains a trimethoxyphenyl ring bridged to a tetrahydronaphthol moiety by a carbon–carbon single bond. Thus, it is conceivable that occupation of the binding subdomains by separate molecules would lead to a conformational change in tubulin but that the tetrahydronaphthol group of podophyllotoxin blocks the conformational change due to steric hindrance. Therefore, our interpretation of the effect of podophyllotoxin together with tropolone on the conformation of tubulin must remain tentative. One alternative approach to this problem would entail the use of trimethoxybenzene with tropolone to occupy the colchicine-binding subdomains on tubulin; however, this approach is not feasible because simple trimethoxyphenyl derivatives do not interact appreciably with tubulin (Cortese et al., 1977).

Podophyllotoxin poisons microtubule assembly by an end-dependent mechanism similar to that of colchicine (Margolis & Wilson, 1978) and competitively inhibits the binding of colchicine to tubulin (Wilson, 1975). However, the dimer–monomer dissociation constant of podophyllotoxin-complexed tubulin was not significantly different from that of free tubulin (Table I). Therefore, if binding of podophyllotoxin to tubulin induces a conformational change in the dimer, the alteration is not communicated to the monomer–monomer interface.

Larger Aggregates. The results summarized in Table I indicate that brain and flagellar tubulins have a weak tendency at 4.6 °C to associate into structures larger than the tubulin dimer. Although the precision associated with the values of K_2 (the dimer–tetramer association constant) is probably low, they do suggest that the alkaloids examined in this study (colchicine, podophyllotoxin, tropolone) have no large effect on the association of tubulin into higher aggregates at low temperature. Again, we emphasize that the true nature of the association reaction that gives rise to the higher aggregates is unknown and that K_2 should be considered only an estimate of the tendency for association to higher level polymers.

Decay of Colchicine Binding. The colchicine-binding activities of free brain and flagellar tubulins decayed slowly at 4 °C according to first-order kinetics (Figure 5, Table II). The initial colchicine-binding capacities of brain and flagellar tubulins (0.70–0.87 mol/mol) fell in the generally accepted range (Wilson et al., 1974; Wilson, 1975), but the rate of loss of colchicine-binding activity was substantially slower at 4 °C than at 37 °C. Under the conditions employed in this study, the initial colchicine-binding capacity of outer-doublet tubulin was lower, and its decay rate was greater than the corresponding values for brain tubulin (standard or glycerol-free preparations). These differences appear to be significant and cannot be attributed to the dependence of decay rate on protein concentration (Bamburg et al., 1973) since these decay studies were performed at tubulin concentrations greater than 1.5 mg/mL. It is possible that subtle differences in primary structure are responsible for these differences in pharmacological properties. In contrast to the observed decay of the colchicine-binding activity of free tubulins, colchicine–tubulin complexes showed no decay of binding activity when stored at 4 °C in PM buffer + 10^{-4} M colchicine. Similar, but less complete, protection of tubulin against loss of colchicine-binding activity has been reported for tubulins from a variety of sources (Borisy & Taylor, 1967; Weisenberg et al., 1968; Wilson, 1970; Sherline et al., 1975; Garland, 1978). Therefore, bound colchicine apparently stabilizes tubulin in a conformation that protects the colchicine-binding site against denaturation. [We assume that bound colchicine dissociates rapidly from denatured colchicine–tubulin complexes, a conclusion consistent with the results of Garland (1978).] In agreement with our interpretation, Ventilla et al. (1972) have shown by circular dichroic spectroscopy that bound colchicine protects tubulin against the denatured conformation induced by elevated temperature. It is interesting to note that dissociation of free dimeric tubulin was fully reversible and gave no evidence for generation of denatured protein. Therefore, the denaturation that presumably accompanies the loss of colchicine-binding activity may involve a local loss in tertiary structure of the protein and may not be linked to the monomer–dimer equilibrium.

Conformational Change. The data are consistent with the interpretation that the conformation of tubulin in the colchicine–tubulin complex differs from that of the unliganded protein and raise the possibility that simultaneous binding of podophyllotoxin and tropolone does not result in a similar structural perturbation. Garland (1978) and Lambeir & Engelborghs (1981) have presented evidence that the slow kinetics of the colchicine-binding reaction result from a ligand-induced conformational change in tubulin. On the basis of circular dichroic studies of porcine brain tubulin, Ventilla et al. (1972) reported that colchicine binding required a conformational isomer of tubulin favored at elevated temperatures; however, Lee et al. (1978) were unable to confirm this temperature-dependent conformational change in tubulin from bovine brain. We conclude that the majority of the available evidence supports the interpretation that structural changes in tubulin accompany the binding of colchicine.

We propose that the conformational change in tubulin is required to permit the trimethoxyphenyl-binding and tropolone-binding subdomains on the protein to interact with rings A and C of colchicine, respectively. In other words, we suggest that tubulin must fold around colchicine to produce the best-fitting (i.e., the most energetically favorable) binding site. Furthermore, once the colchicine–tubulin complex is formed, the colchicine-binding site is presumably protected from subsequent denaturation.

Margolis & Wilson (1978) have demonstrated that net assembly and disassembly of bovine brain microtubules occur at opposite ends of the microtubules under steady-state conditions in vitro. Recent evidence has shown that a similar opposite-end assembly–disassembly mechanism operates for singlet microtubules polymerized in vitro from flagellar outer-doublet tubulin (Farrell et al., 1979). Colchicine inhibits microtubule polymerization substoichiometrically through addition of colchicine–tubulin complexes to microtubule assembly ends (Margolis & Wilson, 1977). Under certain

conditions a single colchicine–tubulin complex bound at the primary assembly end of a microtubule appears to be sufficient to block further steady-state assembly (Margolis et al., 1980). However, data derived from flagellar outer-doublet microtubules reassembled to steady state (Farrell & Wilson, 1980) and from bovine brain microtubules polymerized de novo from tubulin solutions (Sternlicht & Ringel, 1979) support a poisoning mechanism in which copolymerization of colchicine–tubulin complexes and free tubulin into the assembly ends of microtubules lowers the affinity of the microtubules for subsequent addition of drug-free tubulin. Therefore, the critical concentration of tubulin required for assembly in the presence of colchicine–tubulin complexes is increased. We suggest that the conformational change in tubulin that accompanies binding of colchicine (Garland, 1978; Detrich et al., 1980; Lambeir & Engelborghs, 1981; this report) may be responsible both for the increased affinity of colchicine–tubulin complexes (compared to free tubulin) for microtubule assembly ends occupied by tubulin-lacking colchicine (Engelborghs & Lambeir, 1979) and for the decreased affinity of free tubulin for microtubule assembly ends containing colchicine–tubulin complexes (Sternlicht & Ringel, 1979; Farrell & Wilson, 1980). Further studies will be required to determine whether the putative endogenous colchicines (Lockwood, 1979; Sherline et al., 1979) poison microtubule assembly by a similar mechanism involving conformational alteration of tubulin.

Recently, we (Detrich et al., 1981) have presented evidence obtained by circular dichroic spectrophotometry that indicates that the conformation of colchicine itself is altered in the colchicine–tubulin complex. This observation suggests that the colchicine-binding reaction may be more complex than previously thought. Further experiments are in progress to determine the kinetic mechanism that incorporates conformational changes in both drug and protein moieties.

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Purification and Characterization of Androgen Binding Protein from Rabbit Epididymis†

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ABSTRACT: A method for the purification of androgen binding protein (ABP) from the rabbit epididymis is presented. Epididymal extracts were submitted to sequential ammonium sulfate precipitation, androgen affinity chromatography, concanavalin A (Con A) affinity chromatography, and preparative polyacrylamide gel electrophoresis. Since the blood protein testosterone–estradiol binding globulin (TeBG) was a possible component of the epididymal extract, ABP was differentiated and separated from TeBG by affinity chromatography on Con A–Sepharose since the latter protein was shown to be completely adsorbed by the lectin while the former was not. The final product was shown to be pure by polyacrylamide gel electrophoresis. Electrophoresis in the presence of sodium dodecyl sulfate revealed that ABP is comprised of subunits.

Androgen binding protein (ABP)[1] is present in the male reproductive tract of rat, rabbit, and other mammalian species [for a review, see Hansson et al. (1975a) and Bardin et al. (1981)]. ABP is synthesized and secreted by the Sertoli cells under the influence of FSH and testosterone, and as a consequence, it has been used as a marker protein to study the physiology of the seminiferous tubule. The first highly purified ABP was isolated from rat epididymides (Musto et al., 1977, 1980); this was possible because the rat does not have testosterone–estradiol binding globulin (TeBG), a serum protein of hepatic origin which binds sex steroids (Khan et al., 1981; Corval & Bardin, 1973) and has properties very similar to ABP (Hansson et al., 1975b). The isolation of ABP from the rabbit and man has been complicated by the presence of TeBG in epididymal extracts. For example, Weddington et al. (1975b) partially purified a small quantity of ABP (rbABP) from rabbit epididymis, and antiserum generated against this preparation cross-reacted with both rbTeBG and rbABP (Weddington et al., 1975a). Since the fractionation procedures did not separate these two proteins, it is therefore possible that the antiserum used in these experiments was prepared against a mixture of both TeBG and ABP. In view of the interest in studying ABP production in species which also make TeBG, we thought it pertinent to develop a procedure for preparing highly purified rbABP which was free of rbTeBG. Such a technique was an essential prerequisite for determining whether rbABP and rbTeBG are similar or identical proteins. In this paper, we present the purification and characterization of rbABP which is free of rbTeBG.

Materials and Methods

Materials. Epididymides from mature rabbits were purchased from Pel-Freez Biological, Inc.; 3-oxo-17β-hydroxy-5α-androstane-17α-(6-hexanoic acid) (DHT-HA) was synthesized according to the procedure published previously (Musto et al., 1977). 1,2-5α-[^{3}H]Dihydrotestosterone ([^{3}H]DHT), 40 Ci/mmol, was obtained from New England Nuclear Corp. and purified on silica gel thin-layer plates developed in chloroform–methanol (25:2). Radioinert DHT, Sepharose 4B, concanavalin A–Sepharose, and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDAC) were obtained from Sigma Chemical Co. Epichlorohydrin was from Aldrich Chemical Co.; ammonium hydroxide was from Mallinckrodt, Inc. Ampholines pH 4–6 were from Bio-Rad Laboratories, and gelamide was from Polysciences, Inc. All the chemicals were reagent grade. Double-distilled water was utilized throughout.

Preparation of Affinity Column Matrix (DHT–Sepharose). Sepharose substituted with primary amino group was prepared by the method of Nishikawa & Bailon (1976) by using epichlorohydrin as follows: Sepharose 4B washed with water was suspended in an equal volume of 0.6 M NaOH. Epichlorohydrin was added to a final concentration of 0.24 M and the mixture incubated at 30 °C for 4 h with shaking. At the end of incubation, the gel was transferred to a glass filter and washed with water to pH 7 and then suspended in an equal volume of 2 M ammonium hydroxide solution. The suspension was incubated at room temperature overnight with shaking. The gel was washed with water on a glass funnel until the wash was neutral. It was further washed with 0.5 volume each of 0.02 M NaH_2PO_4 and 1 M NaCl. The gel was then washed

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[1] Abbreviations: ABP, androgen binding protein; DHT, 5α-dihydrotestosterone; TeBG, testosterone–estradiol binding globulin; rbABP, rabbit ABP; DHT-HA, 3-oxo-17β-hydroxy-5α-androstane-17α-(6-hexanoic acid); Con A, concanavalin A; EDAC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride; TG buffer, 20 mM Tris, pH 7.4, and 10% glycerol; TDK buffer, 20 mM Tris, pH 7.4, 10% *N,N*-dimethylformamide, and 1 M KCl; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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