

THE MECHANISM OF ACTION OF COLCHICINE

Colchicine Binding Properties of Sea

Urchin Sperm Tail Outer Doublet Tubulin

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ABSTRACT

The thermal depolymerization procedure of Stephens (1970. *J. Mol. Biol.* 47:353) has been employed for solubilization of *Strongylocentrotus purpuratus* sperm tail outer doublet microtubules with the use of a buffer during solubilization which is of optimal pH and ionic strength for the preservation of colchicine binding activity of chick embryo brain tubulin. Colchicine binding values were corrected for first-order decay during heat solubilization at 50°C ($t_{1/2} = 5.4$ min) and incubation with colchicine at 37°C in the presence of vinblastine sulfate ($t_{1/2} = 485$ min). The colchicine binding properties of heat-solubilized outer doublet tubulin were qualitatively identical with those of other soluble forms of tubulin. The solubilized tubulin (mol wt, 115,000) bound 0.9 ± 0.2 mol of colchicine per mol of tubulin, with a binding constant of 6.3×10^5 liters/mol at 37°C. The colchicine binding reaction was both time and temperature dependent, and the binding of colchicine was prevented in a competitive manner by podophyllotoxin ($K_i = 1.3 \times 10^{-6}$ M). The first-order decay of colchicine binding activity was substantially decreased by the addition of the vinca alkaloids, vinblastine sulfate or vincristine sulfate, thus demonstrating the presence of a vinca alkaloid binding site(s) on the outer doublet tubulin. Tubulin contained within the assembled microtubules did not decay. Intact outer doublet microtubules bound less than 0.001 mol of colchicine per mol of tubulin contained in the microtubules, under conditions where soluble tubulin would have bound 1 mol of colchicine per mol of tubulin (saturating concentration of colchicine, no decay of colchicine binding activity). The presence of colchicine had no effect on the rate of solubilization of outer doublet microtubules during incubation at 37°C. Therefore, the colchicine binding site on tubulin is blocked (not available to bind colchicine) when the tubulin is in the assembled outer doublet microtubules.

INTRODUCTION

Microtubules can arbitrarily be classified into two general categories, labile, and stable. Microtubules found in the axons and dendrites of the central nervous system, the mitotic apparatus of dividing cells, and those found in the cytoplasm both of animal and plant cells fall into the labile

category. It is generally believed that labile microtubules are in a state of "dynamic equilibrium" (see Inoué and Sato, 1967). The essential feature of this model is that tubulin¹ is in a

¹ The term tubulin is used in this paper to describe

cytoplasmic pool which is in equilibrium with assembled microtubules. The model implies that the cell has the ability to control the association and dissociation of tubulin units. The characteristics of labile microtubules fit this model rather well; they are quickly destroyed (depolymerized) at low temperatures, under high hydrostatic pressure, and by the action of antimetabolic drugs (see reviews by Porter, 1966; Tilney, 1971). In contrast, stable microtubules do not appear to be in equilibrium with a tubulin pool. They can be isolated intact from cilia and flagella, they do not depolymerize at low temperature or under high pressure, and are not affected by any of the antimetabolic chemical agents which destroy labile microtubules (see Stephens, 1971; Behnke and Forer, 1967; Tilney and Gibbons, 1968).

One incompletely resolved question in the literature concerns the colchicine binding properties of outer doublet tubulin. Because of the stability of outer doublet microtubules, it has been necessary to use relatively harsh methods (e.g. detergents such as Sarkosyl; high pH; high temperature) to obtain the protein in soluble form (Shelanski and Taylor, 1968; Stephens, 1970). However, the colchicine binding activity of tubulin is markedly unstable, and decays rapidly even under the least adverse conditions (Weisenberg et al., 1968; Wilson, 1970; Bamburg et al., 1973 *a, b*). Thus as noted by Shelanski and Taylor (1968), the inability to detect colchicine binding activity in *S. purpuratus* sperm tail outer doublet tubulin solubilized by dialysis for 24 h at high pH was to be expected.

The question of whether or not solubilized tubulin from stable outer doublet microtubules possesses a high affinity binding site for colchicine which is similar to that described for other tubulins is important for two reasons. If the solubilized tubulin could be shown to possess a colchicine binding site, we could then ask whether that site was on the external surface of the microtubule or buried within it. Evidence could then be obtained in favor or against the hypothesis that colchicine prevents microtubule assembly, and does not directly disrupt a previously assembled microtubule through an interaction with the intact microtubule. Perhaps more importantly, the studies may lead to a more com-

the 6S 115,000-120,000 molecular weight protein complex which is considered to be the "building block" unit of the microtubule.

plete understanding of the chemical and functional similarities and differences between these classes of microtubules.

In a recent review, Stephens (1971) noted that thermally depolymerized tubulin from *Strongylocentrotus droebachiensis* outer doublets possessed colchicine binding activity. We have employed the high temperature solubilization procedure of Stephens with an important modification; the substitution of a buffer during heating which provides optimal maintenance of colchicine binding activity (Wilson, 1970). With the use of this buffer, inclusion of vinblastine sulfate as a stabilizing agent, and by use of appropriate corrections for decay of binding activity during heating and incubation with colchicine, the colchicine binding properties of solubilized outer doublet tubulin have been characterized.

MATERIALS AND METHODS

Isolation of Purified Outer

Doublet Microtubules

Purified outer doublet microtubules from *Strongylocentrotus purpuratus* sperm tails were isolated essentially by the method of Stephens (1970). Freshly shed packed sperm (6-10 ml) were suspended in 60-80 ml of filtered sea water containing 10^{-4} M EDTA. Sperm tails were sheared from the heads with a motor-driven Teflon glass tissue homogenizer and isolated by differential centrifugation as described by Stephens (1970). Flagella membranes were removed with either 1% Triton X-100, or 1% Nonidet. Central pair microtubules, dynein, and other contaminating proteins were removed by extraction with 0.6 M KCl. Outer doublet microtubules were subsequently washed, and stored as a suspension (approximately 10 mg/ml) in 10 mM Tris-HCl, pH 8.0, at 0°C (ice bath).

Gel electrophoresis of reduced and carboxymethylated purified outer doublet microtubule proteins was carried out in 8 M urea on 5% polyacrylamide gels as described by Meza et al. (1972). A typical stained gel and gel scan of reduced and carboxymethylated proteins from a suspension of purified outer doublet microtubules appear in Fig. 1. The α - and β -subunits are present in equal concentration (Meza et al., 1972), with little or no contaminating proteins.

Heat Solubilization of Outer

Doublet Proteins

An aliquot of the outer doublet suspension containing the desired quantity of microtubule protein (usually 3-5 mg) was diluted to a volume of 10 ml

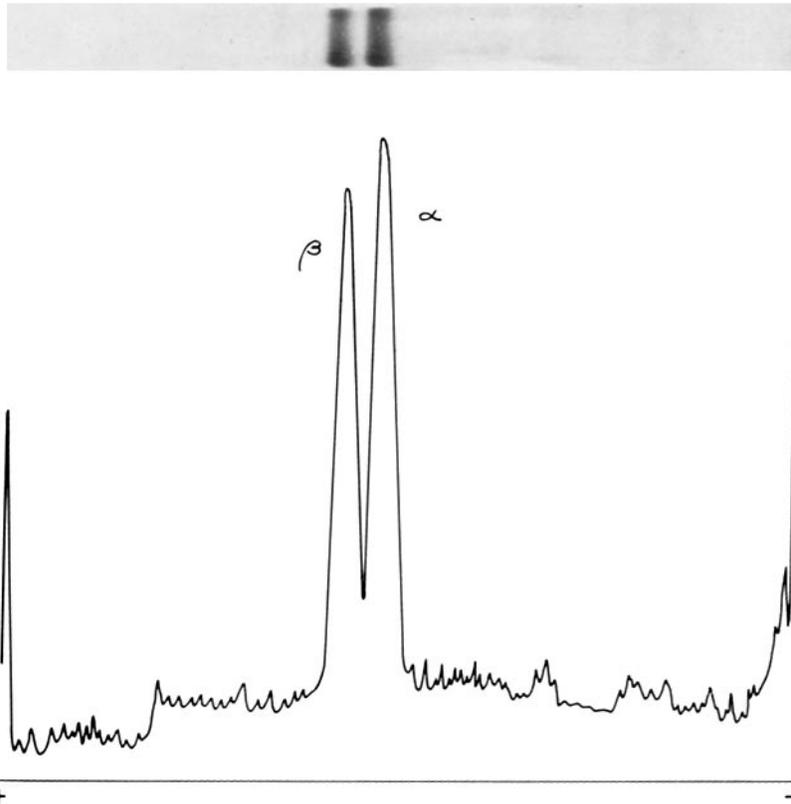


FIGURE 1 Urea polyacrylamide gel and corresponding densitometer scan of urea-dissolved, reduced and carboxymethylated proteins from a purified outer doublet microtubule suspension. (Stained with Coomassie blue.)

with 10 mM Tris-HCl (pH 8.0, 0°C). The microtubules were sedimented by centrifugation at 27,000 *g* for 15 min, and resuspended by homogenization in 2-3 ml of 20 mM sodium phosphate, 100 mM sodium glutamate, pH 6.8 at 0°C (phosphate-glutamate buffer). This step served as a wash to remove protein which had become soluble during storage, and also permitted transfer of the outer doublet microtubules to a buffer which was of optimal pH and ionic strength for maintenance of colchicine binding activity. Colchicine binding experiments were carried out utilizing heat-solubilized tubulin obtained from washed outer doublet microtubules which were stored in the low ionic strength Tris buffer for a minimum of 1 day after their isolation, to ensure complete absence of contaminating central pair microtubules. The suspension of outer doublet microtubules was heated at 50°C with constant mixing for the desired length of time, then quickly cooled to 0°C by immersion in an ice bath. Heat-solubilized tubulin was obtained in the supernatant fraction after centrifugation of the heated suspension at 27,000 *g* for 15 min at 4°C.

Determination of [*acetyl-³H]Colchicine-Tubulin Complex*

Unless otherwise noted, incubation of solubilized outer doublet tubulin with colchicine was carried out in phosphate-glutamate buffer, for 2 h at 37°C. Colchicine-tubulin complex formation was determined by gel filtration on 1 × 18 cm columns of Bio-Gel P10 (see Wilson, 1970, Bamberg et al., 1973 *a, b*) or by ionic absorption on 2.5 cm squares of DEAE-impregnated filter paper utilizing a wash procedure to remove unbound colchicine (Wilson, 1970). Determination of half-times for loss of colchicine binding activity was carried out as described previously for chick embryo brain microtubule protein (Wilson, 1970, Bamberg et al., 1973 *a, b*).

Thin section electron microscopy was performed with a Siemens-Elmiskop I as described previously (Meza et al., 1972). Protein concentrations were determined by the method of Lowry et al. (1951) with standard solutions of purified outer doublet microtubule protein. Tritium was determined in a

Nuclear-Chicago scintillation counter, and tritiated water used as an internal standard (efficiency = 17%).

Chemicals

[Acetyl-³H]colchicine (155 mCi/mmol) was prepared as described by Wilson and Friedkin (1966). Podophyllotoxin was obtained from the Aldrich Chemical Co. (Milwaukee, Wis.), (mp 112–114°C, λ_{\max} 290 nm in 95% ethanol, $\epsilon = 3750$). Vinblastine sulfate (99.8% pure) and vincristine sulfate (97.2% pure) were gifts from the Eli Lilly & Co. (Indianapolis, Ind.). A mixture of β - and γ -lumi-colchicine was prepared as described by Mizel and Wilson (1972). Triton X-100 (Grade B) was obtained from Calbiochem (San Diego, Calif.). Nonidet was a product of the Shell Oil Co. (New York). Bio-Gel P10 was obtained from Bio-Rad Laboratories (Richmond, Calif.). Squares (side = 2.5 cm) of Whatman DE81 Chromedia paper were cut from 46 × 57 cm sheets obtained from the H.

Reeve Angel & Co., Inc. (Clifton, N. J.). All other chemicals were analytical grade.

RESULTS

Absence of Central Pair Microtubules

Solubilized tubulin from central pair microtubules bind colchicine (Shelanski and Taylor, 1967, 1968). Thus it was critical to ensure that the central pair microtubules had been completely removed from the outer doublet microtubules during their isolation. An electron micrograph showing a portion of a typical 1 day old outer doublet microtubule suspension is shown in Fig. 2. In this and in all other preparations, no more than 2% of the axonemes ever showed any indication of the presence of central pair microtubules.

Further evidence that the outer doublet microtubule suspensions utilized in this study were

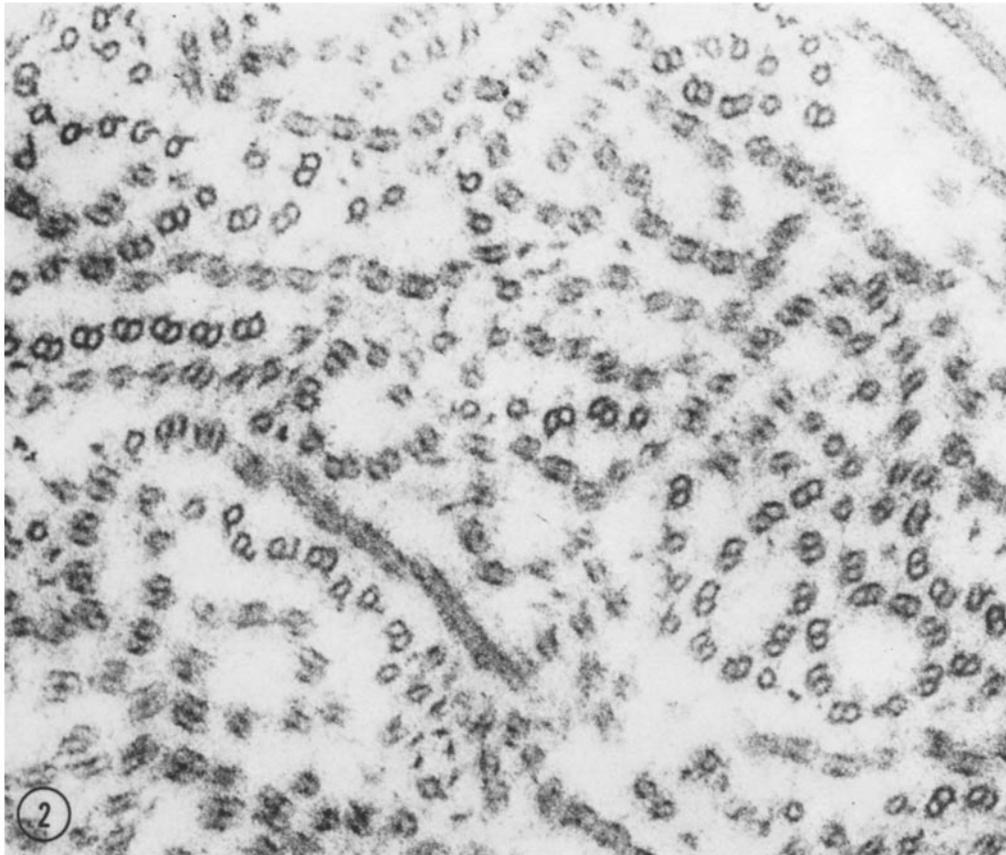


FIGURE 2 Electron micrograph showing a cross-section of purified outer doublet microtubules. Magnification, $\times 90,000$.

free of central pair microtubules was obtained through the finding that the proteins contained in the outer doublet microtubule suspensions dissolved slowly during storage at 0°C. Solubilization of outer doublet microtubules at 0°C has also been observed by Stephens (1970). Thus as seen in Fig. 2, after storage of the suspension at 0°C for 1 day, portions of the B subfibers were missing on several of the axonemes. The percentage of microtubule protein solubilized with time during storage at 0°C is shown in Fig. 3. The rate of solubilization was initially rapid, 20% of the total outer doublet protein being solubilized after only 1 day of storage. Solubilization continued at a slower but constant rate during the next 6 days. At the end of 7 days, approximately 40% of the total protein initially present

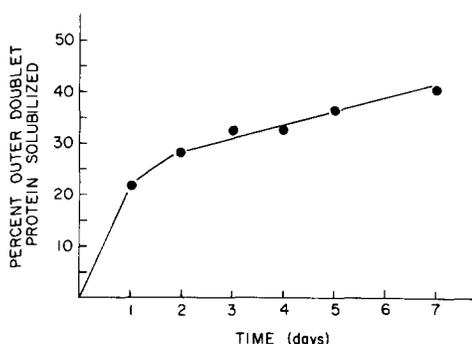


FIGURE 3 Percentage of total protein solubilized during storage of outer doublet microtubules as a suspension of 10 mM Tris-HCl, pH 8.0, 0°C (ice bath).

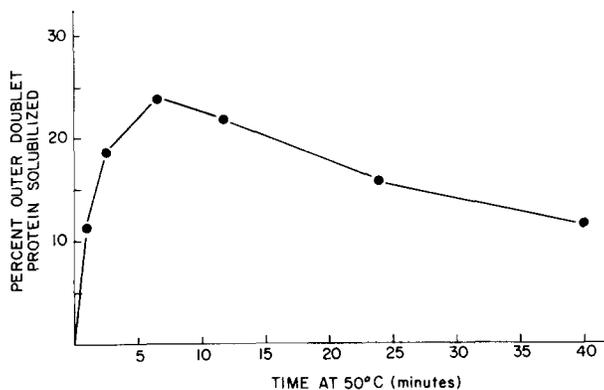


FIGURE 4 Heat solubilization (50°C) of outer doublet microtubules suspended in phosphate-glutamate buffer. Aliquots (0.5 ml, 1.5 mg of total protein/ml) of a washed outer doublet microtubule suspension in phosphate-glutamate buffer were heated at 50°C for the indicated lengths of time as described in the Methods section. Heated suspensions were cooled to 0°C, centrifuged at 16,000 *g* for 20 min, and total protein was determined in the resulting particulate and supernatant fraction.

in the outer doublet microtubule suspension was solubilized. Since the central pair microtubules are much more labile than the outer doublet microtubules at low ionic strength and low temperature (Shelanski and Taylor, 1967, 1968; Stephens, 1970, 1971) we can conclude that there was little or no contamination with central pair microtubules in these preparations.

Heat Solubilization of Outer Doublet Microtubules with Minimal Loss of Colchicine Binding Activity

Heating a suspension of outer doublet microtubules in 1 mM Tris-HCl, pH 7.2, at 50°C for 2 min solubilized the B subfiber and a portion of the A subfiber as reported previously (Meza et al., 1972), but the solubilized tubulin had very little colchicine binding activity. However, considerable colchicine binding activity was obtained when the tubulin was solubilized by heating a suspension of outer doublet microtubules in phosphate-glutamate buffer. Heating a suspension of outer doublet microtubules at 50°C for 2.5 min in phosphate-glutamate buffer solubilized between 15 and 20% of the total outer doublet protein (Fig. 4). Maximum solubilization of protein was approximately 25%, and occurred after 7–8 min of heating. The decrease in soluble protein with longer periods of heating resulted from aggregation of previously solubilized protein. The degree of protein solubilization with various

heating time was similar, regardless of how long the outer doublet microtubule suspension was stored at 0°C, between 1 and 7 days of storage.

Decay of Colchicine Binding Activity during Solubilization by Heating at 50°C

Once it was ascertained that the solubilized outer doublet microtubule proteins possessed colchicine binding activity, the reason for our previous inability to detect significant colchicine binding activity was investigated. The reason lay in the very rapid rate of decay of colchicine binding activity during heating at 50°C. The half-time for this decay in 1 mM sodium phosphate pH 6.7 could not be measured accurately, but was no more than 30 s. In phosphate-glutamate buffer, the rate of decay was still quite rapid, but slow enough to allow one to obtain sufficient quantities of active protein by heating for a relatively short period of time (2.5 min) at 50°C. This half-time was between 5.2 and 5.6 min, and is at least 10 times longer than that obtained with low ionic strength buffers of the same pH.

Colchicine Binding Properties of Solubilized Outer Doublet Microtubules

The colchicine binding properties of solubilized outer doublet tubulin were qualitatively identical with those of tubulins from all other sources thus far investigated (e.g., Borisy and Taylor, 1967 *a, b*; Wilson and Friedkin, 1967; Wilson, 1970; L. Wilson, unpublished data). The molecular weight of the colchicine-outer doublet tubulin complex as determined by gel filtration was approximately 115,000 (Fig. 5). This was similar to the molecular weight of purified chick embryo brain tubulin-colchicine complex (Fig. 5) (Bryan and Wilson, 1971). Since the protein binds colchicine stoichiometrically (as will be described shortly) we can conclude that essentially all of the heat-solubilized protein was solubilized as tubulin (a protein complex) and not as dissociated α - and β -subunits.

As discussed above, the colchicine binding activity of the solubilized tubulin decayed according to first-order kinetics (data not shown). At 37°C the colchicine binding activity of the tubulin (120 μ g/ml) decayed with a half-life of 165 min. A similar concentration of purified tubulin from 13 day old chick embryo brain

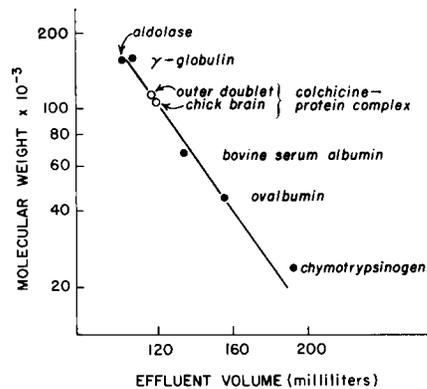


FIGURE 5 Molecular weight estimation of heat-solubilized outer doublet tubulin-colchicine complex. A solution of heat-solubilized (2.5 min, 50°C) outer doublet tubulin in phosphate-glutamate buffer (2 ml, 4.3 mg total protein) was incubated with labeled colchicine as described in the Methods section, cooled to 0°C, and passed through a calibrated Sephadex G-100 column, as described previously for chick embryo brain tubulin-colchicine complex (Bryan and Wilson, 1971). The labeled complex appeared as a single sharp peak at an effluent volume corresponding to a molecular weight of approximately 115,000. Purified chick embryo brain tubulin-colchicine complex run as a control appeared at an effluent volume corresponding to a mol wt of approximately 112,000.

decays with a half-life of 230 min at 37°C (Bamburg et al., 1973 *b*). Addition of the vinca alkaloids vinblastine sulfate and vincristine sulfate markedly reduced the rate of decay. For example, in the presence of 5.2×10^{-4} M vinblastine sulfate, the half-time for loss of colchicine binding activity of outer doublet tubulin was decreased by a factor of 3 to 485 min at 37°C. Negligible binding of colchicine occurred during incubation for 3 h at 0°C. As expected, the addition of a mixture of β - and γ -lumicolchicine, photoinactivated isomers of colchicine which do not disrupt cytoplasmic microtubules nor bind to tubulin (Wilson and Friedkin, 1967, Wilson, 1970) did not affect the binding of colchicine to solubilized outer doublet tubulin. The binding of colchicine was time dependent as shown in Fig. 6. In the absence of vinblastine, binding of 2.6×10^{-6} M colchicine at 37°C increased to a maximum level at about 60 min, then decreased as incubation was continued. Similar results have been obtained with tubulins from other sources (Borisy and Taylor, 1967 *a*; Wilson and Friedkin, 1967; Wilson, 1970). The

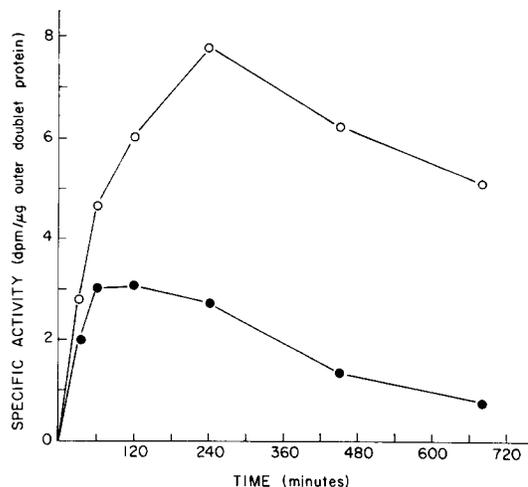


FIGURE 6 Binding of colchicine to heat-solubilized outer doublet tubulin with time at 37°C in the presence and absence of vinblastine sulfate. Solutions of heat-solubilized (50°C) outer doublet tubulin in phosphate-glutamate buffer (3 ml, 145 μg/ml) were incubated with 2.6×10^{-6} M [*acetyl*-³H]colchicine at 37°C in the absence (closed circles) and in the presence (open circles) of 5.2×10^{-4} M vinblastine sulfate. This concentration of vinblastine sulfate did not produce any precipitation of the solubilized tubulin. Aliquots (400 μl) were removed at the times indicated and the concentration of colchicine complex determined by gel filtration (see Methods section). Half-times for loss of colchicine binding activity in the presence and absence of vinblastine were determined as described previously (Wilson, 1970). In the presence and absence of vinblastine, the half-times were 486 min and 165 min, respectively, at 37°C.

decrease in binding activity with increased time of incubation and the reaching of an apparent plateau after 1 h of incubation were due to the decay of colchicine binding activity ($t_{1/2} = 166$ min). In the presence of 5.2×10^{-4} M vinblastine which was added to prevent decay (half-time = 485 min), maximal binding was not reached until 4 h (Fig. 6). Colchicine binding activity was greatly increased in the vinblastine-stabilized sample, demonstrating the marked effects that the decay rate can have on apparent values of colchicine binding activity. The decay rate varied markedly with the pH in a manner similar to that reported for chick embryo brain tubulin (Wilson, 1970). The pH optimum was 6.8–6.9. The initial colchicine binding capacity was unaffected between pH 5.8 and 7.9 (data not shown).

Similar to the colchicine binding properties of tubulins from other sources (Wilson and Friedman, 1967; Wilson, 1970; Bryan, 1972) the binding of colchicine was prevented in a competitive manner by the addition of podophyllotoxin (Fig. 7). The inhibition constant (K_i) for podophyllotoxin in this experiment was 1.3×10^{-6} M. The inhibition constant for podophyllotoxin is analogous to the dissociation constant which describes the affinity of podophyllotoxin for the colchicine binding site. The binding constant for colchicine, and the number of colchicine binding sites on tubulin, were determined with the use of a Scatchard plot as shown in Fig. 8

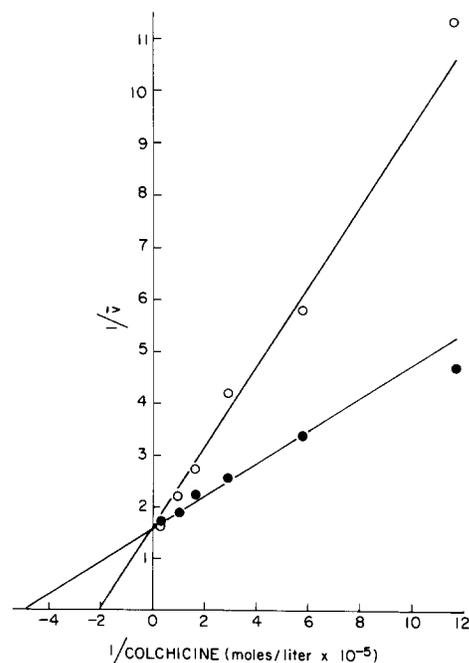


FIGURE 7 Inhibition of colchicine binding activity by podophyllotoxin. Solutions of heat-solubilized (50°C) outer doublet tubulin in phosphate-glutamate buffer (0.5 ml, 183 μg/ml) containing 5.2×10^{-4} M vinblastine sulfate (to stabilize colchicine binding activity) were incubated with various concentrations of [*acetyl*-³H]colchicine in the presence (closed circles) or absence (open circles) of 2.0×10^{-6} M podophyllotoxin for 350 min at 37°C. Bound colchicine complex was determined by the filter paper assay procedure (see Methods section). Binding values were corrected for loss of colchicine binding activity during heat solubilization (2.5 min; half-time = 5.2 min) and during incubation with colchicine at 37°C (350 min; half-time = 480 min). \bar{V} , the total number of moles of colchicine bound per mole of tubulin (mol wt, 115,000).

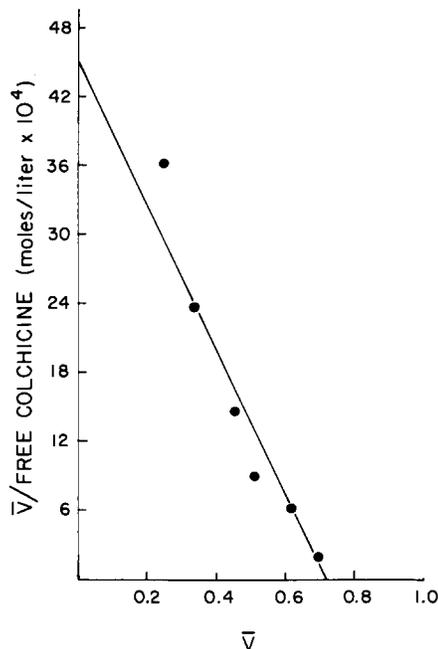


FIGURE 8 Binding constant (K_A) for colchicine and the number of colchicine binding sites on solubilized outer doublet tubulin. Data from the experiment described in Fig. 7 (closed circles) were recalculated to determine the moles of free and bound colchicine at each colchicine concentration, and are shown in the form of a Scatchard plot (Scatchard, 1949). The value of n (the extrapolated number of binding sites per mole of tubulin (mol wt, 115,000) at infinite colchicine concentration) was 0.73. The binding constant is derived from the slope of the straight line (slope = $-K_A$).

(Scatchard, 1949). The association constant (K_A) was 6.3×10^5 liters/mol at 37°C . This value is not very different than that obtained with purified tubulin from 13 day old chick embryo brain. An identical experiment carried out with chick embryo brain tubulin yielded a value for the binding constant at 37°C of 2.0×10^6 liters/mol. A similar value of 2.3×10^6 liters/mol was obtained by Borisy and Taylor (1967 *b*) in cell-free extracts of *S. purpuratus* eggs with the use of a kinetic analysis.

The number of moles of colchicine bound per mole of tubulin (n) in this experiment at infinite colchicine concentration was 0.73. This value includes corrections for decay of colchicine binding activity during heating at 50°C , and during incubation with colchicine for 6 h at 37°C . In six determinations by several procedures, the mean value of n was 0.9 with variation at the

95% confidence level equal to ± 0.2 . Therefore, there appears to be one colchicine binding site per mole of solubilized outer doublet tubulin.

Colchicine Binding Activity of Intact Outer Doublet Microtubules

Once it was established that tubulin solubilized from outer doublet microtubules possessed a colchicine binding site, it became meaningful to determine whether the site was free or blocked in the assembled microtubule. In one type of experiment, a suspension of outer doublet microtubules in phosphate-glutamate buffer (330 μl ; 495 μg of total protein) was incubated for 1.5 h at 37°C with 5×10^{-5} M [*acetyl*- ^3H]colchicine. The suspension was cooled to 0°C , diluted to 10 ml with additional phosphate-glutamate buffer, and centrifuged at 27,000 g for 15 min. After a second identical wash step, the amount of labeled colchicine associated with the outer doublet microtubules was determined. This value was less than 0.001 mol of colchicine per mol of tubulin (mol wt 115,000).

In order to eliminate the possibility that the colchicine was bound to the outer doublet microtubules, but removed by the washing step in the previous experiment, a suspension of outer doublet microtubules was incubated at 37°C with 1.69×10^{-5} M [^3H]colchicine for increasing periods of

TABLE I
Lack of Colchicine Binding to Assembled Outer Doublet Microtubules

Time of Incubation	Colchicine remaining in soluble fraction
min	nmol/0.5 ml
0 (control)	8.45 (± 0.19)*
15	8.45
30	8.45
60	8.40
120	8.40
180	8.56

Suspensions of outer doublet microtubules (0.5 ml, 4.0 nmol of tubulin, mol wt 115,000) were incubated with 8.45 nmol of ^3H -colchicine at 37°C . At the times indicated, the microtubules were pelleted by centrifugation (11,000 g , 20 min) and the amount of colchicine remaining in the supernatant fraction was determined.

* Variation at the 95% confidence level (Student's t test).

time. The outer doublet microtubules were then pelleted by centrifugation at 16,000 *g* for 20 min, and the amount of [³H]colchicine associated with the microtubules ascertained by measuring the amount of radioactivity remaining in the supernatant fraction (Table I). The protein concentration in this experiment was 8×10^{-6} mol/liter, therefore there was a 2 to 1 excess of colchicine over potential colchicine binding sites. No detectable colchicine was removed from the supernatant fraction (pelleted along with the outer doublet microtubules). The binding of 0.1 mol of colchicine per mol of tubulin (mol wt, 115,000) to the microtubules would have decreased the amount of colchicine remaining in the soluble fraction by 0.42 nmol/0.5 ml. Since it was also conceivable that upon the binding of colchicine, the tubulin-colchicine complex dissociated from the microtubules, the rate of dissociation (dissolution) of the microtubules was measured in the presence and absence of colchicine (Fig. 9). The outer doublet microtubules dissolved slowly at 37°C, but in the presence of colchicine, there was no detectable alteration in the rate of solubilization. Thus, colchicine does not bind to the outer doublet microtubules nor

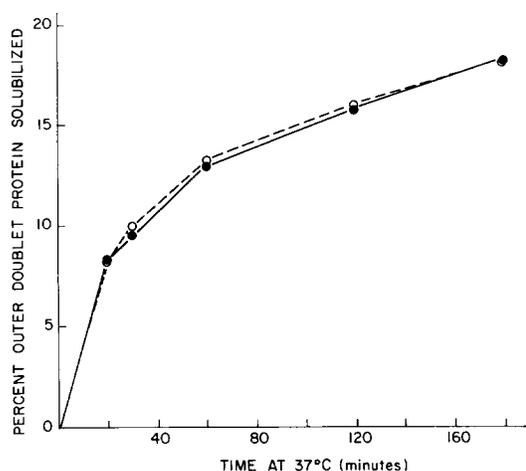


FIGURE 9 Solubilization of outer doublet microtubules in the presence and absence of colchicine. Suspensions of washed outer doublet microtubules (3.0 ml, 900 μ g protein/ml) in phosphate-glutamate buffer were incubated in the presence (closed circles) and absence (open circles) of 1.7×10^{-5} M colchicine at 37°C. At the times indicated, aliquots (0.5 ml) were removed, centrifuged at 16,000 *g* for 20 min (4°C), and the quantity of protein in the supernatant and particulate fraction was determined.

does it solubilize the microtubules. Therefore, since the solubilized tubulin does possess a colchicine binding site, that site must be blocked (or the affinity substantially decreased) in the assembled outer doublet microtubules.

DISCUSSION

Solubilized tubulin from *S. purpuratus* sperm tail outer doublet microtubules can be obtained in a form which binds colchicine by heating a suspension of the microtubules in 20 mM sodium phosphate, 100 mM sodium glutamate, pH 6.8, for short periods of time at 50°C. The colchicine binding properties of the solubilized tubulin are qualitatively identical with those of tubulins from other sources (Borisy and Taylor, 1967 *a, b*; Shelanski and Taylor, 1967; Wilson and Friedkin, 1967; Wilson, 1970; Bryan, 1972; Bamburg et al., 1973 *a, b*). The quantity of colchicine binding activity which can be recovered in solubilized outer doublet tubulin depends upon the rate of loss of colchicine binding activity under the conditions utilized for solubilization. Heating outer doublet suspensions in low ionic strength solutions or at high or low pH, solubilizes the tubulin efficiently, but the rate of decay during solubilization is so rapid under these conditions, that little or no colchicine binding activity can be recovered.

In phosphate-glutamate buffer, the quantity of protein which dissolves during heating at either 37°C or 50°C is less than that obtained with low ionic strength buffers, but the decay rate is slow enough at both temperatures to allow recovery of considerable colchicine binding activity. The half-life at 37°C in phosphate glutamate buffer is 165 min. Increasing the temperature to 50°C decreases the half-life by a factor of 30 to approximately 5.4 min. However, heating at 50°C in phosphate-glutamate buffer for short periods of time solubilizes considerably more tubulin than does heating for long periods of time at 37°C. Thus, the largest quantity of active (colchicine-binding) tubulin has been obtained utilizing a temperature of 50°C and a heating time of 2.5 min. It is clear that the colchicine binding activity of outer doublet microtubules is completely stable (does not decay) when the tubulin is contained within the assembled microtubule. Decay occurs only after dissociation of the tubulin complex from the microtubules.

Although outer doublet tubulin can bind colchicine once it is in a soluble form, tubulin contained within the assembled outer doublet microtubules cannot bind the drug. Two possible mechanisms could explain these results. First, the colchicine binding site may be *one* of the protein-protein interaction sites between tubulin molecules, and the binding of colchicine at that site prevents normal assembly. Second, the colchicine binding site, which might not be one of the protein-protein interaction sites, could be modified during assembly in a manner such that it can no longer bind the drug, or has a markedly decreased affinity. Either mechanism is consonant with the known physiological response of stable outer doublet microtubules to colchicine. For example, outer doublet microtubules in intact cilia or flagella are not disrupted by colchicine (Behnke and Forer, 1967; Tilney and Gibbons, 1968; see also Stephens, 1971). In addition, regeneration of flagella is prevented by colchicine (Rosenbaum and Carlson, 1969), presumably due to the prevention of microtubule assembly by colchicine. This same view regarding the mechanism of action of colchicine can be logically extended to labile (cytoplasmic) microtubules as well. The most widely held hypothesis has been that colchicine disrupts cytoplasmic microtubules in just this manner (cf. Borisy and Taylor, 1967 *a, b*; Wilson and Friedkin, 1967; Tilney, 1971; Stephens, 1971) preventing microtubule assembly by binding to soluble tubulin. Thus, the ability of colchicine to disrupt an assembled microtubule would depend upon the dynamic equilibrium (see Inoué and Sato, 1967) which existed at the time between tubulin in soluble pools and tubulin in assembled microtubules. The more stable the microtubule, the more resistant it will be to the action of colchicine.

The origin (i.e. from the A or B subfiber) of the heat-solubilized tubulin has been investigated. Examination of electron micrographs of the outer doublet material remaining after heat solubilization revealed considerable and in many cases complete absence of B subfibers. Less of the A subfiber tubulin was dissolved under the conditions employed in this study. However, stoichiometric binding of colchicine was obtained in all of the preparations analyzed, thus we can argue that tubulin solubilized from both subfibers binds colchicine in an identical manner. However, a firm conclusion regarding the colchicine binding

properties of A subfiber tubulin must await results of further experiments.

Urea polyacrylamide gel electrophoresis of reduced and carboxymethylated proteins obtained by heat solubilization of outer doublet microtubules in phosphate-glutamate buffer always showed equal quantities of the α - and β -subunits regardless of the time of heating, or the length of time the outer doublet microtubules were stored prior to heating. These results are in support of the model proposed for the subunit composition of outer doublet microtubules by Meza et al. (1972) and the heterodimer model of microtubule composition (Bryan and Wilson, 1971) in which both the A and the B subfibers are composed of dimers containing equal quantities of the α and β subunits.

In addition to possessing very similar colchicine binding properties, the chemical properties of the stable outer doublet tubulin subunits from *S. purpuratus* sperm tails have also been shown to be very similar to those of tubulins from presumed labile microtubules (cf. Bryan and Wilson, 1971; Feit et al.; 1971; Eipper, 1972; Meza et al., 1972). In a recent study it was found that the amino acid sequences from the N-terminal end of the α - and β -chains of *S. purpuratus* outer doublet microtubules were almost identical to the respective α - and β -chains of purified chick embryo brain tubulin (Luduena, 1973). Thus, the chemical basis for the differences in stability (and perhaps function) of microtubules from various sources remains an exciting and important area for future research.

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