

PROPERTIES OF TUBULIN IN UNFERTILIZED SEA URCHIN EGGS

Quantitation and Characterization by the Colchicine-Binding Reaction

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ABSTRACT

The colchicine-binding assay was used to quantitate the tubulin concentration in unfertilized *Strongylocentrotus purpuratus* eggs and to characterize pharmacological properties of this tubulin. Specificity of colchicine binding to tubulin was demonstrated by apparent first-order decay colchicine-binding activity with stabilization by vinblastine sulfate, time and temperature dependence of the reaction, competitive inhibition by podophyllotoxin, and lack of effect of lumicolchicine. The results demonstrate that the minimum tubulin concentration in the unfertilized egg is 2.71 mg per milliliter or 5.0% of the total soluble cell protein. Binding constants and decay rates were determined at six different temperatures between 8°C and 37°C, and the thermodynamic parameters of the reaction were calculated. $\Delta H_0 = 6.6$ kcal/mol, $\Delta S_0 = 46.5$ eu, and, at 13°C, $\Delta G = -6.7$ kcal/mol. The association constants obtained were similar to those of isolated sea urchin egg vinblastine paracrystals (Bryan, J. 1972. *Biochemistry*. 11:2611-2616) but approximately 10 times lower than that obtained for purified chick embryo brain tubulin at 37°C (Wilson, L., J. R. Bamburg, S. B. Mizel, L. Grisham, and K. Creswell. 1974. *Fed. Proc.* 33:158-166). Therefore, the lower binding constants for colchicine in tubulin-vinblastine paracrystals are not due to the paracrystalline organization of the tubulin, but are properties of the sea urchin egg tubulin itself.

To understand the regulation of microtubule polymerization during cell division, it is important to determine the concentrations of soluble and polymerized tubulin during the cell cycle. As a first step in this investigation, we have characterized several parameters of the colchicine-binding reaction, and have used the reaction to define the lower

limit of the tubulin concentration in unfertilized eggs of the sea urchin *Strongylocentrotus purpuratus*.

The pharmacological properties of tubulin, the dimer subunit of microtubules, have been utilized to obtain information about the biochemistry and function of microtubules (see references 19 and 37

for reviews) as well as a means of quantitating tubulin levels in different systems. Based upon physical and pharmacological disruption, evidence has been obtained for different classes of microtubules (3, 31, 28, 17). Comparison of the drug-binding properties of tubulin from different sources reveals differences in binding affinities and protein stability. Therefore, in utilizing the colchicine-binding assay, one must define the characteristics of the binding reaction in order to accurately determine tubulin levels. It is with respect to these considerations that this investigation differs from others in which colchicine binding derived estimates of the tubulin level within the sea urchin egg have been reported.

There is one colchicine-binding site per mole of tubulin. The binding reaction is temperature and time dependent, and is characterized by a slow rate of complex formation and a slower dissociation rate (4, 5, 26, 39, 33, 7, 20). Consequently, long incubation times are required for the reaction to attain equilibrium, and the tubulin-colchicine complex can be isolated under nonequilibrium conditions. The reaction is complicated by the steady loss of colchicine-binding activity by free as well as colchicine-bound tubulin in an apparent first-order manner (33, 34). Although the initial binding capacity of tubulin is not affected by pH (between 5.5 and 8.5) or ionic strength, the decay rate of colchicine-binding activity is greatly affected by the incubation conditions (34).

The colchicine-tubulin interaction is influenced by other antimitotic drugs. Vinblastine sulfate decreases the decay rate of colchicine-binding activity without affecting the equilibrium of the reaction (34, 39). Therefore, the accuracy of binding constants determined when long incubations are required is improved when vinblastine is included in the reaction. Since colchicine is known to disrupt cell processes unrelated to microtubules (18), the specificity of binding to tubulin should be demonstrated. Wilson and Bryan (37) described five parameters which characterize formation of the tubulin-colchicine complex: temperature dependence, time dependence, first-order decay and stabilization by vinca alkaloids, competitive inhibition by podophyllotoxin (34, 39), and lack of effect of lumicolchicine on colchicine-binding activity (34, 39). On the basis of these parameters, it will be demonstrated that all the colchicine binding observed in sea urchin egg supernatant fractions is due to association with tubulin. Therefore, the colchicine-binding assay is an accurate method for

quantitating tubulin in the unfertilized sea urchin egg.

MATERIALS and METHODS

Preparation of Sea Urchin Egg Supernatant Fractions

S. purpuratus gametes were obtained by injection of 0.6M KCl into the body cavity. Eggs were collected in cold Millipore-filtered (Millipore Corp., Bedford, Mass.) seawater and gently washed three times with 20 mM sodium phosphate and 100 mM sodium glutamate, pH 6.75 (phosphate-glutamate buffer). Eggs were homogenized at low speed with a motor-driven Teflon-glass tissue homogenizer in an equal volume of phosphate-glutamate buffer and centrifuged at 150,000 g for 1 h at 4°C.

Colchicine-Binding Assay

Aliquots of egg supernatant fractions containing 6.6–7.0 mg total soluble protein per milliliter, except as otherwise indicated, were incubated with [*acetyl*-³H]colchicine with a sp act of 145 mCi/mmol or [*methyl*-³H]colchicine with a sp act of 2.41 Ci/mmol obtained from New England Nuclear Corp., Boston, Mass. Binding reactions were carried out in phosphate-glutamate buffer, and unless stated otherwise, all samples contained 1.0×10^{-4} M vinblastine sulfate to decrease decay of colchicine-binding activity (34), and 0.02% sodium azide to prevent bacterial growth during long incubations. This concentration of sodium azide affects neither the initial binding capacity of the tubulin nor the decay rate of colchicine-binding activity (data not shown).

The tubulin-colchicine complex was separated from free colchicine by gel filtration (4, 5, 34) on 1×18 cm columns of Bio-Gel P10 (Bio-Rad Laboratories, Richmond, Calif.). Radioactivity was determined by adding 1 ml of the bound colchicine fractions to 10 ml of 3 gm Omnifluor (New England Nuclear Corp., Boston, Mass.) per liter, 25% Triton X-100 in xylene (1) or Bray's solution and counted in a Packard Tri-Carb Liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). Efficiency was determined with tritiated water as an internal standard.

Miscellaneous Procedures and Chemicals

The method for induction and isolation of vinblastine crystals from unfertilized sea urchin eggs has been described by Bryan (6). Tubulin was solubilized by incubating 2.56 mg of crystals in 1.6 ml of 1 mM sodium phosphate, pH 6.8, for 5 min at 5°C. The solution was diluted to a vol of 10 ml with phosphate-glutamate buffer, and contained final concentrations of 1.0×10^{-4} M vinblastine sulfate and 0.02% sodium azide. The preparation was clarified by centrifugation at 100,000 g for 45 min.

Protein concentrations were determined by the method of Lowry et al. (15). For determination of average soluble protein content per cell, the number of eggs in an aliquot of a homogeneous suspension was determined with a hemacytometer. The suspension was homogenized in phosphate-glutamate buffer, centrifuged at 100,000 g for 2 h, and the protein concentration of the supernatant fraction was measured.

Podophyllotoxin was obtained from the Aldrich Chemical Co., Milwaukee, Wisc. Vinblastine sulfate was a gift from Dr. Koert Gerzon of Eli Lilly & Co., Indianapolis, Ind. Lumicolchicine was prepared as described by Mizel and Wilson (18).

RESULTS

Decay of Colchicine-Binding Activity

The colchicine-binding activity of tubulin is unstable and decays according to apparent first-order kinetics; the half time for loss of binding activity varies with incubation conditions (33, 34). To obtain accurate values for bound colchicine, it was necessary to correct for the loss of binding activity which occurred during incubation at each temperature.

The first-order decay rates of binding activity were determined for six different temperatures. Decay rates were determined by preincubating aliquots of an egg supernatant fraction at the various temperatures followed by incubation with labeled colchicine at the preincubation temperatures. Half times for loss of binding activity were calculated and are shown as a function of temperature in Fig. 1. Decay rates were markedly decreased by addition of 1.0×10^{-4} M vinblastine sulfate (Fig. 1). Unlike colchicine, vinblastine binds rapidly to tubulin, with maximal binding attained after 15 min of incubation at 0°C (20, 35). Comparison of the slopes obtained in Fig. 1 demonstrates an approximately constant percentage of stabilization by vinblastine at each temperature. Extrapolation of the first-order decay lines to zero incubation time (data not shown) indicated that the initial binding capacity of the tubulin when vinblastine was present was identical to that obtained when vinblastine was not included in the incubation samples. Therefore, vinblastine is affecting only the decay rate of binding activity and not the affinity of tubulin for colchicine.

Colchicine Binding as a Function of Time

The rate of colchicine-tubulin complex formation, particularly at low temperatures and low colchicine concentrations, is slow (4, 5, 7). In order

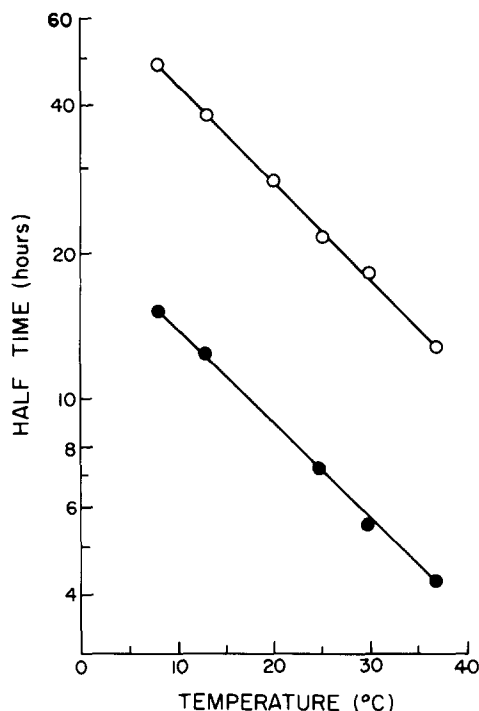


FIGURE 1 Decay rate of colchicine-binding activity as a function of temperature. Sea urchin egg supernatant fractions containing 6.6 mg total soluble protein per milliliter were preincubated at different temperatures in the presence (open circles) or absence (closed circles) of 1.0×10^{-4} M vinblastine sulfate. Subsequent incubations with labeled colchicine were carried out for 3 h at 37°C, 5 h at 30°C, 6 h at 25°C, 6 h at 20°C, 9 h at 13°C, and 10 h at 8°C. Bound colchicine was determined as described in the Materials and Methods section. Half times were calculated from the slope of the first-order decay curve obtained for each temperature.

to determine incubation times required for equilibrium measurements, it was necessary to measure the kinetics of the binding reaction. Values for bound colchicine were corrected for decay to obtain an accurate determination for maximal binding; the plateau region was attained after approx. 16 h of incubation at 13°C (Fig. 2). For determination of binding constants at low temperatures, the reactions were allowed to proceed for longer periods of time to ensure that equilibrium had been reached.

Equilibrium Constants for Colchicine Binding to Sea Urchin Egg Tubulin

Equilibrium constants for the colchicine-binding reaction were determined at six different temperatures between 8°C and 37°C by incubating ali-

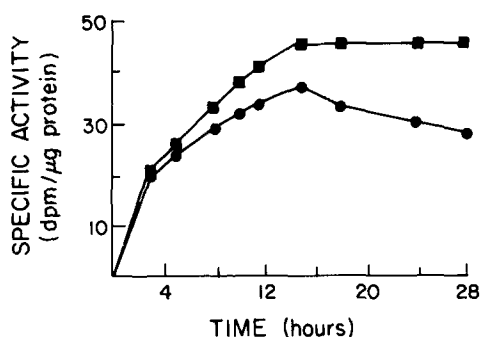


FIGURE 2 Colchicine binding with time at 13°C. A 150,000 g supernatant fraction containing 4.0 mg protein per milliliter and 1.0×10^{-4} M vinblastine sulfate was incubated with 1.18×10^{-5} M labeled colchicine at 13°C. Aliquots (0.5 ml) were removed at the times indicated for determination of bound colchicine (circles). The values obtained were corrected for decay of colchicine-binding activity (squares) using a half time of 38.9 h.

quots of egg supernatant fractions with different concentrations of labeled colchicine. In each case, values obtained for bound colchicine were corrected for decay of binding activity which occurred during the incubation. The data for four temperatures are shown in the form of Scatchard plots (25) in Fig. 3. The slope of each line, from which the binding constants (K_A) are obtained, was calculated by a least squares fit.

The temperature profile of the equilibrium constants (Fig. 4) is characteristic of a temperature-dependent reaction with increased binding affinity at higher temperatures. The same data are shown in the form of a van't Hoff plot (Fig. 4, inset) from which the thermodynamic parameters were calculated (16). The reaction is characterized by positive enthalpy and entropy changes, and a large, negative-free energy change: $\Delta H_0 = 6.6$ kcal/mol, $\Delta S_0 = 46.5$ eu(entropy units), and, at 13°C, the ambient temperature of the organism, $\Delta G = -6.7$ kcal/mol.

Specificity of Colchicine-Binding Activity and Inhibition by Podophyllotoxin

Lumicolchicine, a compound which is structurally similar to colchicine but lacks antimetabolic activity and which apparently does not bind to tubulin (34), can be used in ascertaining the specificity of colchicine binding. When present in a 10-fold excess, lumicolchicine had no effect upon colchicine-binding activity at 8°C in the egg supernatant fractions (data not shown).

Podophyllotoxin competitively inhibits colchicine binding to tubulin (39, 7, 40). In addition to providing evidence supporting specificity of colchicine binding, inhibition by podophyllotoxin can also be used to compare the relative affinities of tubulin from different sources for the two drugs. The competition experiment was done in the egg supernatant fractions at 37°C (Fig. 5) and at 13°C, and competitive inhibition was demonstrated in each case (14). The inhibition constants were 6.6×10^{-7} M at 37°C and 7.6×10^{-7} M at 13°C.

Tubulin Content per Egg

The amount of tubulin present in the unfertilized egg can be calculated from the intercept on the abscissa (infinite colchicine concentration) of the Scatchard plots shown in Fig. 3. Based upon one colchicine-binding site per tubulin molecule and a mol wt of 110,000, the minimum tubulin content is 5.0% of the total soluble protein. This value

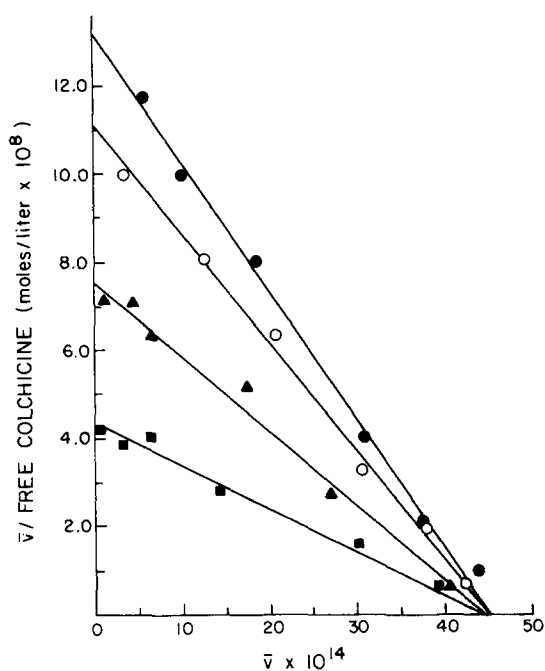


FIGURE 3 Scatchard plots for four temperatures. Aliquots (0.5 ml) of a 150,000 g supernatant fraction were incubated with six different concentrations of labeled colchicine. The reaction was allowed to proceed for 8 h at 37°C (closed circles), 13 h at 30°C (open circles), 26 h at 13°C (triangles), and 38 h at 8°C (squares). The data were corrected for decay of colchicine-binding activity which occurred during incubation periods. \bar{V} = moles of bound colchicine per microgram total soluble protein.

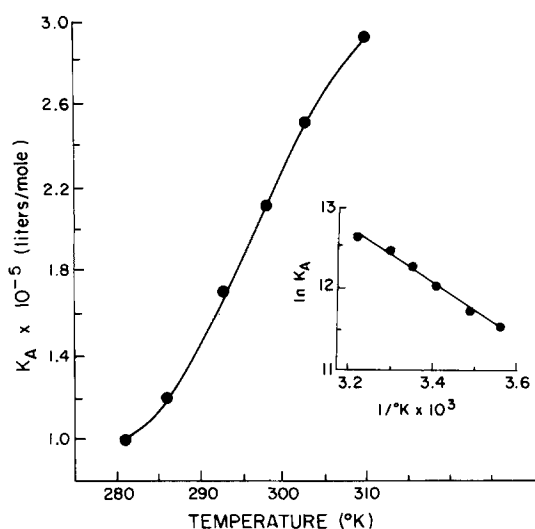


FIGURE 4 Equilibrium constants as a function of temperature. Binding constants were calculated from the slopes of Scatchard plots (see Fig. 3). Inset: The same data shown as a van't Hoff plot from which the thermodynamic parameters of the colchicine-binding reaction can be calculated (16).

represents the lower limit for the amount of tubulin present, as the data were not corrected for decay of colchicine-binding activity which occurred during the preparation of supernatant fractions. A value of $1.45 \times 10^{-2} \mu\text{g}$ total soluble protein per cell was obtained by determining the number of eggs in a volume of a homogeneous suspension and the protein concentration of a supernatant prepared from this suspension. Therefore, based upon an average cell diam of $80 \mu\text{m}$, the concentration of tubulin in the unfertilized sea urchin egg is 2.71 mg/ml .

DISCUSSION

Quantitation of Tubulin

From direct counts of mitotic spindle microtubules in eggs of *Arbacia punctulata*, Cohen and Rebhun (9) estimated that the microtubule protein present in the spindle and asters constitutes 0.11% of the total cell protein. Treatment of marine eggs with hexylene glycol, ethyleneglycol, ethoxyethanol, dimethylsulfoxide, and D_2O increases the size and birefringence of the mitotic spindle in vivo, suggesting that not all of the tubulin present in the egg is normally incorporated into the mitotic apparatus (24, 13). Drug binding and amino acid incorporation experiments have

demonstrated that unfertilized sea urchin eggs contain a store of microtubule protein (5, 22, 7) and that this pool is replenished by *de novo* synthesis of tubulin throughout early development (21, 22, 12, 29, 30).

Raff et al. (22) found that vinblastine sulfate precipitated 2–5% of the soluble protein in *A. punctulata* eggs and 50–77% of the colchicine-binding activity which had occurred after incubation of 150,000 g supernatant fractions with $2.3 \times 10^{-6} \text{ M}$ colchicine for 1 h at 37°C . Quantitation of vinblastine precipitates in *Arbacia* egg supernates by SDS/urea gel electrophoresis suggested that tubulin constitutes 1.1% of the soluble cell protein (23). Using an isotope dilution-vinblastine precipitation method, Burnside et al. (8) determined that approx. 3.3% of the total *Spisula solidissima* egg protein is tubulin. The extent of tubulin precipitation by vinblastine is dependent upon vinblastine concentration, temperature, and the pH of the buffer (37). Burnside et al. (8) found that vinblastine may not have precipitated all the

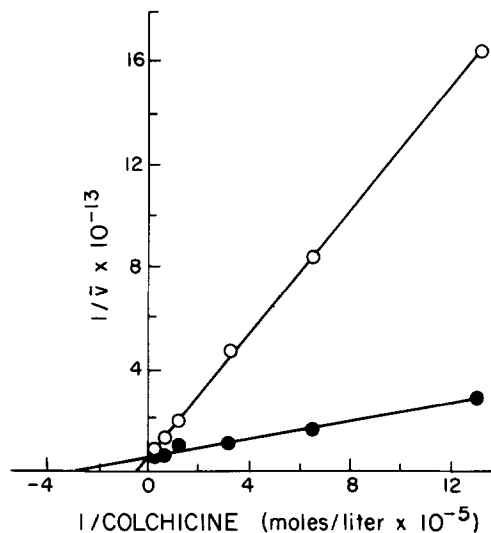


FIGURE 5 Competitive inhibition of colchicine binding by podophyllotoxin at 37°C . Aliquots (0.5 ml) of sea urchin egg supernate containing 7.0 mg protein per milliliter were incubated for 8 h with different concentrations of labeled colchicine in the presence (open circles) or absence (closed circles) of $4.0 \times 10^{-6} \text{ M}$ podophyllotoxin. Bound colchicine was determined as described in the Materials and Methods section. The data were corrected for loss of colchicine-binding activity which occurred during the incubation period (half time at $37^\circ\text{C} = 12.8 \text{ h}$). \bar{V} = moles of bound colchicine per microgram total soluble protein.

tubulin present in egg homogenates, and Wilson et al. (38) found that vinblastine can precipitate a number of acidic proteins. Consequently, vinblastine precipitation is not an accurate method for quantitating low concentrations of tubulin. Gel electrophoresis cannot be used to quantitate low levels of tubulin either. The lower the relative tubulin concentration in a cell extract, the more difficult it is to accurately resolve the tubulin band. When specificity of colchicine binding can be demonstrated on the basis of the previously mentioned characteristics of the reaction (37), the colchicine-binding assay provides the most accurate quantitation of low tubulin levels. With the use of values of colchicine binding which have been corrected for decay, the present results demonstrate that tubulin comprises a minimum of 5.0% of the total soluble protein in the unfertilized egg of *S. purpuratus*.

Differences in Colchicine-Binding Properties of Tubulins

Three properties of the colchicine-binding reaction can be used to characterize tubulin from different sources: the thermodynamic parameters of the reaction, stability of binding based upon decay rates, and the relative affinities for colchicine and podophyllotoxin. In vinblastine crystals isolated from *S. purpuratus* eggs, Bryan (7) determined that the maximum equilibrium constant obtained for the colchicine-binding reaction was about 10 times lower than that obtained for chick embryo brain tubulin. The similarity of binding constants obtained for vinblastine crystals and soluble tubulin at low temperatures (Table I) and tubulin solubilized from vinblastine crystals (data not shown) demonstrates that the lower binding constants are due to a lower affinity of sea urchin egg tubulin for colchicine, and not a result of the paracrystalline conformation.

In the vinblastine crystal, the equilibrium constants attain an apparent maximum at approx. 20°C (7), whereas the values for soluble tubulin continue to increase above 20°C. It is possible that the decrease observed at higher temperatures is due to solubilization of the crystals and subsequent decay of colchicine-binding activity.

Unlike tubulin from grasshopper embryos, chick brain, HeLa cells, and KB cells, vinblastine crystals demonstrate considerable binding at 0°C (7). Other colchicine-binding constants obtained for the reaction at 37°C are 2.0×10^6

TABLE I
Equilibrium Constants for Colchicine Binding to Tubulin in Sea Urchin Egg Supernatant Fractions and Vinblastine-Induced Crystals

Temperature	K_A (liters/mol)	
	150,000 g supernatant fraction	Vinblastine-induced crystals*
°C		
37	2.9×10^6	0.3×10^6
30	2.5	0.8
25	2.1	2.0
20	1.7	2.6
13	1.2	1.2
8	1.0	0.9

* From Bryan, reference 7.

liters/mol for purified chick embryo brain tubulin (36), 6.3×10^5 liters/mol for sea urchin sperm tail outer doublet tubulin (40), 1.8×10^6 liters/mol for purified porcine brain tubulin (20), 7×10^5 liters/mol for bovine thyroid tubulin (41), and 4.0×10^2 liters/mol for what has tentatively been identified as yeast tubulin (11).

Equilibrium constants obtained by kinetic analysis (5, 10, 27) are approx. 5–20 times larger than those obtained by direct measurements. Kinetic determinations are based upon the assumption that the binding of colchicine to tubulin has the characteristics of a simple bimolecular reaction. However, evidence has been obtained which suggests that the reaction is more complicated than previously thought (10). The long incubation times required to attain equilibrium imply that the reaction is not described by a simple bimolecular mechanism. Bryan (7) has suggested that colchicine may bind to an energetically unfavorable conformation of the tubulin dimer. On the basis of circular dichroism studies, Ventilla et al. (32) have suggested that a slow conformational change of the tubulin molecule is required for colchicine binding. The principal reason for using a kinetic analysis is to avoid the decay of colchicine-binding activity. In vinblastine crystals, Bryan (7) found that no decay occurred at low incubation temperatures during a 5-day period. The similarity of binding constants obtained at low temperatures for vinblastine crystals, and tubulin from supernatant fractions, demonstrates that, when correction is made for loss of binding activity, direct equilibrium measurements yield accurate binding constants.

TABLE II
Comparison of Dissociation Constants (K_D) for Colchicine and Podophyllotoxin Binding to Tubulin

Source of tubulin	Temperature	K_D colchicine	K_D podophyllotoxin	$\frac{K_D \text{ colchicine}}{K_D \text{ podophyllotoxin}}$
	$^{\circ}\text{C}$	<i>mol/liter</i>	<i>mol/liter</i>	
Chick embryo brain* (purified)	37	9.1×10^{-7}	3.9×10^{-7}	2.3
Chick embryo brain* (supernate)	37	8.5	5.4	1.6
<i>S. purpuratus</i> sperm tail outer-doublet microtubules*	37	21	13	1.6
<i>S. purpuratus</i> eggs (supernate)	37	34	6.6	5.2
<i>S. purpuratus</i> eggs (supernate)	13	83	7.6	11.0
Vinblastine crystals from <i>S. purpuratus</i> eggs*	13	85	300	0.28

* From Wilson and Bryan, reference 37.

Decay of colchicine-binding activity can also be used to compare tubulins from different sources. At 37°C, binding activity decayed with a half time of 4.2 h in sea urchin egg supernatant fractions, 4.3 h for chick embryo brain tubulin (34), and 2.8 h for sea urchin outer doublet tubulin (40). Inclusion of 1.0×10^{-4} M vinblastine sulfate increased the half time for decay at 37°C to 12.8 h for the supernatant fractions, and 16.1 h for chick embryo brain tubulin (34). The half time for decay of binding activity at 37°C for sea urchin outer doublet tubulin in the presence of 5.2×10^{-4} M vinblastine sulfate was 8.1 h (40). However, direct comparison of colchicine-binding stability requires more standardized experiments since decay rate is dependent upon protein concentration (2) and possibly other cellular components present in the incubation solutions.

Comparison of the dissociation constants for colchicine and podophyllotoxin (Table II) reveals differences in relative affinities of tubulin from different sources for the two drugs. While the affinity of vinblastine crystals for colchicine is approx. 10 times lower than that of brain tubulin, the affinity for podophyllotoxin is decreased approx. 75 times (37). However, the affinity of sea urchin egg supernatant tubulin for podophyllotoxin is approx. five times that for colchicine at 37°C and 11 times that for colchicine at 13°C. Therefore, the decrease in podophyllotoxin binding in the vinblastine crystal appears to be due to either a conformational change of the binding site when tubulin is in the paracrystalline form which does not affect colchicine binding, or to steric hindrance. The increased relative affinity for podophyllotoxin at 13°C observed for tubulin in super-

natant fractions was expected since the binding of podophyllotoxin is not as temperature-dependent as that of colchicine (35).

Comparison of drug-binding properties of tubulin is useful for understanding the chemical and functional differences of tubulin from different species and also different populations of microtubules within the same cell. The exact mechanism of the colchicine-binding reaction still remains to be determined. Whether or not differences in the pharmacological properties of tubulin are due to differences in the primary structure of the protein or the presence of cytoplasmic factors which alter protein conformation and hence which may be physiologically important in the regulation of microtubule assembly is an area warranting further research.

This work was supported by American Cancer Society grant CH-4A and National Institute of Health grant NS09335. Dr. Clara F. Asnes is a recipient of a Postdoctoral Fellowship from the Pharmaceutical Manufacturers Association Foundation, Inc.

Part of this investigation was done at the University of Washington, Friday Harbor Laboratories, Friday Harbor, Washington.

Received for publication 28 October 1975, and in revised form 17 February 1976.

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