Binding Activity of the Tubulin-3H-Colchicine Complex

Menjor Tino Unlap

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BINDING ACTIVITY OF THE TUBULIN-\(^3\)H-COLCHICINE COMPLEX

A Thesis

Presented to

the Faculty of the Department of Biology
Western Kentucky University
Bowling Green, Kentucky

In Partial Fulfillment
of the Requirements for the Degree
Master of Science

by

Menjor Tino Unlap

August, 1983
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BINDING ACTIVITY OF THE
TUBULIN-\(^3\)H-COLCHICINE COMPLEX

Approved December 13, 1953

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ACKNOWLEDGMENTS

I wish to express my sincere gratitude and deep appreciation to my advisor, Dr. Frank Toman, for his guidance and encouragement during the course of this study. His dedication, understanding, and professional inputs made my two years of graduate work enjoyable.

I am grateful to the members of my graduate committee, Dr. Larry Elliott, Dr. E.J. Hoffman, and Dr. Martin Houston, for their assistance throughout the duration of this study.

Appreciation is extended to Ms. Bobbie Dowden and Mr. Rod McCurry for their assistance in photographing the illustrations used in this thesis.
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BINDING ACTIVITY OF THE TUBULIN-$^3$H-COLCHICINE COMPLEX

Menjor T. Unlap 47 pages

Directed by: F.R. Toman, L.P. Elliott, E.J. Hoffman and M.R. Houston

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Microtubules, isolated from bovine brain, were used to study the effect of ultraviolet radiation on the tubulin-$^3$H-colchicine complex and the polymerizing ability of this complex to intact and disrupted microtubules. Dissociation of this complex was observed upon ultraviolet irradiation and continued to progress as exposure time increased. The binding of this complex to intact microtubules prior to irradiation did not seem to enhance its stability to ultraviolet radiation. There was a significant difference observed at the 0.05 level between the binding of the tubulin-$^3$H-colchicine complex to microtubules in mechanically disrupted and control samples. Statistically, there was no significant difference observed between the binding of tubulin-$^3$H-colchicine complex to microtubules in sonicated and control samples. A slight difference in the electrophoretic mobilities of the alpha and beta tubulins was observed upon electrophoresis of bovine brain microtubule preparations. Various microtubule preparations exhibited different binding levels to $^3$H-colchicine depending on their degrees of purity. The purer samples exhibited higher binding levels than the less pure samples.
INTRODUCTION

Microtubules are discrete filaments found primarily in eucaryotic cells. These organelles are an important part of the cell because they are involved in such vital cellular functions as cell shaping, receptor site distribution, cytokinesis, mitosis and axonal transport. Since these organelles are involved in such crucial cellular functions, they have become an area of high research interest.

Earlier research findings have shown that microtubules could be disrupted by various physical factors such as cold temperature (Barber and Callan, 1943; de Harven and Dustin, 1960) and hydrostatic pressure (Pease, 1941; Zimmerman and Marshall, 1964) and by chemical agents such as colchicine, podophyllotoxins, vinblastin, griseofulvin and many others (Bucher, 1939; Dustin, 1934; Oppenheim et al., 1973). These microtubule disruptors have come to be very useful in microtubule research and have contributed to the elucidation of the structure and functions of these organelles.

Colchicine, an aromatic compound isolated from *Colchicum autumnale*, has been used widely in microtubule research due to its specificity as a microtubule poison. It accomplished microtubule disruption by binding tightly and specifically to the dimer, a protein dimer of which the microtubule is composed (Ventilla et al., 1972; Weisenberg, 1972; Weisenberg et al., 1968; Wilson, 1970). This binding
results in the formation of the tubulin-$^3$H-colchicine complex and thus the inhibition of microtubule assembly.

This research was initiated with two objectives: (1) to determine the polymerizing ability of the tubulin-colchicine complex and (2) to determine the stability of the tubulin-colchicine complex to ultraviolet radiation (254 nm).
Microtubules

Microtubules are proteinaceous organelles found in eucaryotic cells. They are by no means restricted to eucaryotes. Roberts and Hyams (1979) indicated that microtubules are also found in parasitic spirochetes such as Diplocaulys, Pillocina and Hollandian. A study conducted a few years later substantiated these findings (Marquiliis et al., 1978). Gross studies of these organelles were done prior to the early sixties but the use of negative staining of sections have made the study of these organelles in fine detail possible.

Dimensions

The structure of microtubules has been elucidated by electron microscopy using various methods which include thin sectioning, negative staining, and freeze fracturing. The use of X-ray diffraction of purified microtubule preparations has also aided in studying the structure of these organelles.

Thin sectioning studies have shown that the outer diameter of microtubules range from 18-30 nm. The most frequently used value is 24 nm. It has been postulated that the variability in size may actually be due to differences in size of microtubules. This variability could
also be attributed to the effect of shrinkage during fixation (Roberts and Hyams, 1979).

Several x-ray diffraction studies on brain microtubules have revealed the outer diameter to be 30 nm and the inner diameter to be 14 nm (Cohen et al., 1971, 1975; Mandelkow et al., 1977). The outer diameter of 24 nm obtained from thin sectioning studies may therefore be attributed to shrinking and dehydration of the sections studied. This conclusion was supported by the results of a study which involved comparing the size of bacteriophage heads obtained by x-ray diffraction of hydrated specimens and electron microscopy of sectioned material. The results indicated that the bacteriophage heads studied in the latter case had shrunk by 20-30% (Earnshaw et al., 1978). The value of 30 nm for the outer diameter obtained from x-ray diffraction studies is in agreement with the range (27-32 nm) obtained for microtubules embedded in negative stain on a support film (Gall, 1966). The results of the two previous studies indicated therefore that the two procedures produced very little shrinking of the samples studied and that the outer diameter of microtubules may actually be 30 nm.

Subunits: Tubulins and Protofilaments

Microtubules are polymers of proteins, the major one being tubulin. Tubulin occurs as a heterodimer consisting of two monomers called alpha and beta tubulins with each monomer having a molecular weight of 55,000 (Bryan and Wilson, 1971; Olmstead et al., 1971; Luduena and Woodward,
1973; Luduena et al., 1977). The proteinaceous nature of these tubulins was first detected by Behnke and Forer (1967) who took microtubules from various sources and tested their stability against papain. They found that papain destroyed these microtubules, some faster than others. Shelanski and Taylor (1968) showed that \underline{alpha} and \underline{beta} tubulins are acidic in nature.

Polyacrylamide gel electrophoresis of tubulin preparations showed two closely related bands with the \underline{beta} subunit having the greater electrophoretic mobility (Bryan and Wilson, 1971). The separation of these tubulin subunits resulted from a difference of charge. The amino acids in the two subunits have been sequenced and found to be very closely related (Luduena and Woodward, 1973, 1975). The sequenced tubulins were from two different sources: chicken brain and sea-urchin egg. Even though these two species are widely separated phylogenetically, there were no differences found in the first 24 N-terminal amino acids. The \underline{alpha} and \underline{beta} tubulins do differ in their location of colchicine, vinblastine, and guanine nucleotide binding sites and by the location of lateral and longitudinal sites that are essential for their assembly into tubules. The two subunits can also be differentiated by phosphorylation; beta tubulin is specifically phosphorylated and tyrosinated by ATP-dependent tubulin-tyrosine ligase which will attach a tyrosine molecule to the N-terminal of the \underline{beta} tubulin.

Tubulin dimers, 5 nm in diameter, polymerize to form
linear structures called protofilaments (Andre and Thiery, 1963; Gall, 1966; Grimstone and Gibbons, 1966). The number of protofilaments making up a microtubule was thought to vary from 10 to 15, but with the advent of negative staining the number most often found is 13. The protofilaments are arranged helically with a pitch of 10-25 degrees forming the wall of microtubules (Burton, 1966). Modern studies, employing the use of tannic acid, have shown that varying numbers of protofilaments making up microtubules are possible. For instance, microtubules with 15 protofilaments have been observed in the cockroach (Nagano and Suzuki, 1975), 16 protofilament microtubules have been found in the crickett (Kaye, 1970), and microtubules with less than 10 protofilaments have been observed in protozoan axopodia (Cachon and Cachon, 1974).

**Microtubule Disruptors**

The effects of several physical and chemical agents on microtubules have been studied. The results of these studies are essential since they give information about the thermodynamics of microtubule assembly and also useful information concerning the structure, function, and chemical properties of microtubules.

**Cold Temperature**

The effect of temperature on various species has been known since 1890 when Hertwig (1890) found that cold temperature arrested mitosis. In an article written by Buccianti
(1929) and quoted by Pierre Dustin (Dustin, 1978), it was stated that chicken fibroblasts appeared binucleated when cooled to 0°C during anaphase. This temperature effect was believed to be attributed to the destruction of spindle fibers. The effects of cold temperature (3°C) on the epithelial cells of Triturus vulgaris was found to be identical to those of colchicine. Olmstead and Borisy (1973) found that the assembly of tubulins into microtubules is inhibited by cold temperature. Behnke (1967) observed that blood platelet microtubules disappeared at 0°C and reappeared after the platelets were re-warmed. The same effect of cold temperature was found in Heliozoa (Tilney and Porter, 1967).

In this study, axopods of Actinosphaerium were studied under warm and cold temperatures. It was found that axopods lost their microtubules under cold temperature (4°C) and that microtubules appeared after the axopods were re-warmed.

**Hydrostatic Pressure**

Hydrostatic pressure of varying magnitudes have been shown to disrupt microtubules from different species. Kennedy and Zimmerman (197) found that hydrostatic pressure of 7,500 and 10,000 psi will disrupt the central ciliary microtubule of Tetrahymena. Later studies have shown that the effect of hydrostatic pressure on microtubules may be species related. In a study conducted by O'Connor et al., (1974), neurotubules from nerves of Rana pipiens were found to be resistant to pressures as high as 10,000 psi. Similar pressures did not affect microtubules polymerized from purified tubulin of cow
brain. The hydrostatic pressure effects have also been shown to be somewhat temperature related (Engelborghs et al., 1976). In this study rat brain microtubules were found to undergo complete dissociation only at 25°C.

Hydrostatic pressure as low as 2,000 psi has been shown to affect mitosis. Chromosome movements were retarded at 2,000 psi and completely arrested at 4,000 psi (Zimmerman and Marshall, 1964). Their study confirmed the results of an earlier study which showed that the spindle fibrils in the eggs of Urechis caupo were no longer observed at 3,000 psi. Chromosome movements were retarded at 3,000 psi and completely blocked at 6,000 psi.

**Ultraviolet Radiation**

The effects of ultraviolet (UV) radiation on microtubules have been studied in general. Most of the studies were done on mitotic spindles using an ultraviolet microbeam. Experimental results indicated that irradiation of the mitotic spindle with the UV microbeam at a controlled dose produced a localized area of reduced birefringence in the spindle fibers. Indications are that at least 30-50% of spindle birefringence is of microtubule origin which means that UV radiation likely has somewhat of a destructive effect on microtubules (Forer, 1965; Forer and Zimmerman, 1976). The same study was carried out on the spermatocytes of the crane-fly Nephatoma naturalis. The results indicated that irradiation of a localized area of the mitotic spindle lead to a temporary loss of birefringence and mitosis (Forer,
Two more direct studies on the effect of UV radiation on spindle microtubules have shown that rapid disassembly of spindle microtubules occurred within a few seconds of irradiation (Bajer, 1968; Zirkle, 1970).

Irradiation of the colchicine-tubulin complex with UV radiation has been shown to cause the conversion of colchicine to lumicolchicine, and the colchicine binding site was partially destroyed (Amerhein and Filner, 1973). A lipid complex, perhaps a part of the tubulin molecule, has been observed to be liberated upon irradiation of the colchicine-tubulin complex with UV radiation (366 nm). This lipid complex is believed to combine with an intermediary product to form lumicolchicine which is inactive in inhibition of microtubule assembly (Wilson et al., 1974; Bryan, 1972).

Colchicine

The tubulin dimer is known to interact with such antimitotic drugs as daunomycin (Na and Timasheff, 1977), vinblastin, podophylotoxin, and colchicine (Wilson et al., 1974). These chemical agents have similar effects on mitotic spindles but colchicine appears to be the most specific microtubule poison acting on all types of cells in both animals and plants.

Colchicine is an alkaloid found in Colchicum autumnale and has also been found in some plants of the Liliaceae family. This substance has been used as a medicine to treat gout since the 18th century (Rodan and Benedek, 1970). Prior to the end of the 19th century, many cases of colchicine
poisoning were observed. The diagnostic symptoms appeared to be diarrhea and intestinal ulcerations. In a study conducted in 1889 by Pernice and quoted by Dustin (Dustin, 1978), the effect of colchicine on dogs appeared to be limited as to the germinative zones of the small intestine, as evidenced by the prominence of arrested mitosis in the cells of the intestine. That effect on mitosis was attributed to the fact that colchicine will bind to the microtubules of the mitotic spindle (Borisy and Taylor, 1967), and that the binding of colchicine to tubulin has an inhibitory effect on microtubule assembly (Borisy and Olmstead, 1972).

Colchicine is an aromatic compound containing three rings (Figure 1). In order for it to be active, colchicine must have the following two characteristics: (1) there has to be at least one methoxy group on ring A and (2) ring C must be a seven membered ring possessing a methoxy group which can be replaced by a thiomethyl group (Dustin, 1963). The use of colchicine as a research tool was enhanced by Taylor who introduced the use of colchicine labeled with tritium on the methoxy group of ring C (Taylor, 1965). The use of tritiated colchicine as a research tool has enabled researchers to study microtubules in finer detail than before.

Colchicine, even at low concentrations (less than $10^{-6}$M), will inhibit microtubule formation (Oppenheim et al., 1973; Bucher, 1939; Sluder, 1976; Borisy et al., 1972). The inhibition is possible since the tubulin dimer has one binding site for colchicine (Weisenberg et al., 1968; Wilson and Friedkin,
Figure 1. Structure of colchicine.
1967) which is freely reversible, stereospecific, and temperature sensitive (Bryan, 1972). The binding of colchicine prevents assembly and appears to be non-covalent since unaltered colchicine could be re-isolated from the tubulin dimer upon extraction with organic solvents (Wilson and Friedkin, 1967).

The question of whether or not colchicine will act on assembled microtubules still remains a matter of controversy. Past studies have shown that assembled microtubules have very low affinity for colchicine (Wilson et al., 1974; Wilson and Meza, 1973). Their results indicated that preformed microtubules were resistant to colchicine since the binding sites on the tubulin dimers were probably not exposed. A contradiction, however, is apparent, since not all assembled microtubules are resistant to colchicine. The only ones resistant to colchicine are those found in cilia and flagella (Wilson et al., 1974) and those found in the marginal bundle of nucleated erythrocytes and thrombocytes (Behnke, 1970; Behnke and Forer, 1967). The resistance of these microtubules to colchicine could be explained by their slow turn-over rates as opposed to the rapid turn-over rate of the mitotic spindle microtubules which are very sensitive to colchicine.

Another possible answer to the aforementioned question could be that the tubulin comprising microtubules exists in two forms, one form binds colchicine and the other is resistant to colchicine (Kirschner et al., 1974). Another answer could be that colchicine binds to the growing end of
a microtubule thus preventing the further addition of tubulin dimers to the growing end (Wilson, 1975). This reasoning is substantiated by the results of a study which showed that Chinese hamster fibroblasts, after being treated with colcemid, grew an increased number of cilia which were shorter than normal in length (Stubblefield and Brinckley, 1966).

The most probable answer to the previous question considers the arrangement of microtubules in cilia and flagella. These microtubules are arranged in doublets. This arrangement involves different orientation of the tubulin dimers from those in single microtubules and this orientation probably confers protection upon the colchicine site (Behnke and Forer, 1967; Behnke, 1970; Amos and Klug, 1974). The destruction of microtubules upon the addition of colchicine could therefore be the consequence of a block in their assembly (Dustin, 1978).
MATERIALS AND METHODS

Chemicals

$^{3}$H-colchicine was obtained from New England Nuclear Co. Morpholino ethane sulfonic acid (MES), ethyleneglycol-bis-(B-aminoethyl ether) N,N'-tetraacetic acid (EGTA), guanosine triphosphate (GTP), dimethyl sulfoxide (DMSO), and 2,5-diphenyloxazole (PPO) were obtained from Sigma Chemical Co.

Microtubule Isolation

A bovine skull was obtained from a local slaughter house within an hour after slaughter. A V-shaped incision was made above the foramen magnum and a 100 g portion of brain was removed. Membraneous tissues and blood vessels were removed from the brain sample, and it was placed in a Waring blender containing modified microtuble medium (MTM). MTM was composed of 50% glycerol, 10% DMSO, 30% distilled water, 5% of 100 mM MgCl$_2$, and 5% of 100 mM MES and represents a modification of the medium described by Filner and Behnke (1973). The brain sample was homogenized, at low speed, for 15 sec. The homogenate was placed in a pre-cooled dissecting bowl and cooled to 5°C within a minute.

The cooled homogenate was transferred to a Sorvall Super Speed RC2-B centrifuge and centrifuged at 16,300 x g at 4°C for 15 min. The pellets were discarded, and the supernatants were combined and filtered through four layers of cheese
cloth. The filtrate was centrifuged for an hour at 39,000 x g at 4°C. Separate pellets were suspended in 5 ml of MTM and syringed through an 18 G hypodermic needle to ensure homogeneity. The supernatants were combined and centrifuged in an International Preparative Ultracentrifuge (Model B-35) at 110,000 x g for 2.5 hr. The pellets were again re-suspended in MTM and syringed for homogeneity. The supernatants were combined and stored in the refrigerator.

**Microtubule Purification**

One milliliter of 100 mM EGTA and one milliliter of 100 mM GTP were added to 100 ml of supernatant (110,000 x g). This mixture was incubated at 37°C for 1.5 hr in a water bath. After incubation, the mixture was centrifuged at 110,000 x g for 2.5 hr. The supernatants were discarded, and the pellets were combined and homogenized in MTM as previously described. A 25-ml quantity of the homogenate was removed and placed in a depolymerizing medium containing 1.25 ml of 100 mM MgCl₂, 22.75 ml of 100 mM MES, 25 ml glycerol, and 76 ml distilled water. This mixture was incubated in a water bath for 1.5 hr at 37°C and then centrifuged at 110,000 x g for 2.5 hr. The supernatants were discarded and the pellets were re-suspended in MTM as before. This entire procedure was repeated three times to ensure purity.

**Coating of Electron Microscope Grids**

A 0.75% formvar solution was prepared in a 250 ml film caster by dissolving 0.75 g of formvar in 100 ml of chloro-
form. After standing for 24 hr, this solution was used to coat grids in the following manner: Formvar was pumped onto a clean slide and allowed to air dry for three minutes. Both sides of the slide were cut along the edges with a sharp razor blade and breathed upon three times. The slide was held perpendicularly to the surface of distilled water in a dissecting bowl and lowered gently until the formvar film on each side separated from the slide and floated on the water surface. Grids were placed, with their shiny side up, on the formvar films. The coated grids were removed from the water surface by placing a clean slide on top of each film and lifting it gently out of the water. The films were allowed to dry over night.

Preparation of Electron Microscope Grids

A small drop of microtubule preparation was placed on the rough surface of the grid and allowed to stand for 10 sec. A paper towel was used to blot off the excess sample leaving a thin layer of microtubule preparation on the grid. The thin layer was stained with a drop of 1.0% uranyl acetate for 29 sec. Excess stain was removed and air-dried for a few minutes before it was viewed under the electron microscope. Viewing of microtubule samples under the electron microscope was done in order to ascertain the presence of microtubules.

Protein Determination

The protein content of each microtubule preparation was
determined by the method of Waddell as modified by Murphy and Kies (1960). Twenty μl of each preparation was diluted 300-7000 fold using 5 mM phosphate buffer (pH 6.5). The UV absorbance was determined at 215 and 225 nm using a Bausch and Lomb Precision Spectrophotometer. Protein concentration in each preparation was calculated using the following formula: 

\[ C = \frac{A}{K} \]

where C is the extinction coefficient (0.00486) for the particular spectrophotometer used; A is the change in absorbance; and K is the protein concentration in μg/ml.

**Binding Activity Assay**

The amount of time required for maximum binding of \(^3\)H-colchicine to tubulin was determined by taking 0.8 ml of each microtubule preparation and placing it in a test tube containing 1.9 ml of 5 mM MESMg, 0.15 ml \(^3\)H-colchicine (30 μCi), and 0.15 ml of 20 mM GTP in 5 mM MESMg. At 15-min intervals, a 0.2 ml aliquot was removed and placed in a test tube containing 0.01 ml colchicine (10 mM) and 0.2 ml of a DEAE cellulose slurry. The slurry was prepared by adding 10 g of DEAE cellulose to 100 ml of 5 mM MESMg. The binding activity for each incubation mixture was determined by removing 0.1 ml of each mixture and filtering it through a 20 mm millipore prefilt (AP2002000). The filter was rinsed five times with 5 ml of MES buffer which was prepared by combining 5.85 g MES, 12.0 ml MgCl\(_2\) (100 mM), 300 ml glycerol, and 300 ml deionized water. The filter was removed and placed in a scintillation vial with 10 ml scintillation fluid, and the radioactivity was determined.
Liquid Scintillation Counting

The $^3$H-activity in counts per minute (cpm) was determined by placing 0.1 ml of each sample in a 20 ml scintillation vial containing 10 ml of scintillation fluid. The scintillation fluid was prepared by combining 4.0 g of 2,5-diphenyloxazole (PPO), 0.1 g of 2,2'-p-phenylene bis-(5-phenyl) oxazole (POPOP), 330 ml of trition x-100, and 670 ml of toluene in a reagent bottle. The activity was determined using a Beckman Liquid Scintillation Counter (Model LS-100C).

Isolation of Tubulin-$^3$H-colchicine Complex

Four grams of Sephadex G 100-120 were swollen overnight and under vacuum in 25% glycerol MESMg buffer at pH 7.0. This slurry was used to prepare a 3 x 46 cm column which was equilibrated with 50% glycerol/MESMg buffer. A 3.0-ml microtubule incubation mixture, consisting of 0.3 ml purified microtubule preparation, 0.15 ml of $^3$H-colchicine (30 µCi), 0.6 ml of 20 mM MESMgGTP, 0.6 ml of glycerol, and 1.35 ml of MESMg, was placed on the column and eluted with 50% glycerol/MESMg buffer. Fractions of 1 ml were collected using an Instrumentation Specialties Company Fraction Collector (Model-272). $^3$H-colchicine activity in each fraction was determined by liquid scintillation counting as previously described. Fractions found to contain tubulin-$^3$H-colchicine complex were combined and stored in 50% glycerol in the refrigerator at 8°C.
The Effect of UV Radiation on Tubulin-$^3$H-colchicine Complex

Three-ml samples of the tubulin-$^3$H-colchicine complex in 10-ml beakers were irradiated for different lengths of time with UV radiation (254 nm). The UV radiation source was located 40.6 cm above the samples. After irradiation, each sample was placed on the Sephadex column and eluted with 50% glycerol MESMg buffer. The effect of UV radiation on the complex was determined by the amount of dissociation to free $^3$H-colchicine.

Effect of UV Radiation on the Binding of Tubulin-$^3$H-colchicine Complex to Microtubules

Incubation mixtures consisting of 0.8 ml of purified microtubules, 0.8 ml of tubulin-$^3$H-colchicine complex, 0.71 ml of glycerol, 0.02 ml of EGTA (20 mM), 0.06 ml of GTP (5 mM), and 0.34 ml of MESMg buffer were incubated at 37°C in a water bath for 1 hr. After incubation, the incubation mixtures were irradiated with UV radiation (254 nm) at a distance of 40.6 cm for 90 min. Following irradiation, the binding activity for each incubation mixture was determined in the same manner previously described.

Microtubule Disruption

Disruption of microtubules was done either by sonication or by mechanical disruption using a 27 G hypodermic needle. Sonication of purified microtubules was accomplished using a Branson Sonifier Cell Disruptor, Model W120D (Heat System Ultrasonics, Inc.) at a maximum output of 42 W for 15 sec,
30 sec, and 15 min. The sonicated microtubules were incubated with tubulin-\(^3\)H-colchicine complex in the same manner as previously described. After incubation, 0.1 ml of the mixture was filtered through a 20 mm millipore pre-filter as described previously.

Microtubules were mechanically disrupted by syringing for 2 min through a 27 G hypodermic needle. Binding of the microtubule to tubulin-\(^3\)H-colchicine complex was determined as previously described.

**SDS-Gel Electrophoresis**

Electrophoresis was carried out according to the method of Laemli (1970). Gel plates (14 x 17.5 cm) were assembled using plastic spacer strips, one on each side and one on the bottom, with vaseline as sealant. The plates were held together by large paper clamps, one on each side and one on the bottom. Separating gel (pH 8.8) was prepared in a 120 ml Erlenmeyer side-arm flask by combining 10.61 ml of distilled water, 4.33 ml of 30% acrylamide/0.8% bis stock solution, 5.0 ml 4X-concentrated separating buffer, and 15 \(\mu\)l of tetramethylethylenediamine (TEMED). This mixture was deaerated for 30 sec and polymerized in the assembled gel plates using 60 \(\mu\)l of 10% persulfate as a catalyst. The 30% acrylamide/0.8% bis stock solution was composed of 30.0 g acrylamide, 0.8 g methylene bisacrylamide, a pinch of activated charcoal, diluted to 100 ml with distilled water and filtered through no. 1 Watman paper. The 4X-concentrated
separating buffer was made up of 18.7 g of Trishydroxy-methylaminoethane (Tris), 4.0 ml of 10% SDS stock solution, 2-4 ml of concentrated HCl and diluted to 100 ml with distilled water.

Stacking gel (pH 6.8) was prepared in a 120 ml Erlenmeyer side-arm flask by mixing 6.5 ml of distilled water, 2.5 ml of 4X-concentrated stacking buffer, 1.0 ml of 30% acrylamide/0.8% bis stock solution, 10 μl TEMED, and 30 μl of 10% persulfate. The 4X-concentrated stacking buffer was made up of 6.06 g of Tris, 4.0 ml of 10% SDS stock solution, 2.4 ml of concentrated HCl and diluted to 100 ml with distilled water. After a comb was positioned above the separating gel, the stacking gel was poured and left to polymerize.

After both gels were polymerized, the comb was removed carefully. The bottom spacer strip was removed and the gel plates were inserted into the electrophoretic apparatus and clamped to backing which was coated with vaseline. The bottom and top reservoirs were filled with 10X-concentrated reservoir buffer (pH 8.3) which was prepared by combining 121.2 g of Tris, 576.4 g of glycine, and 40 g of SDS in a reagent bottle and diluted to 4 l with distilled water. Air bubbles were removed from the bottom of the gel slab with a 10 cc syringe with a bent, blunted 20 G hypodermic needle.

Microtubule samples were prepared by mixing equal amounts of sample and 2X-concentrated buffer which was
composed of 5.0 ml of 4X-concentrated stacking buffer, 8.0 ml of 10% SDS, 6.0 ml of glycerol, 0.4 ml of 0.1% bromophenol blue, and 1.0 ml of 2-mercaptoethanol. These were prepared in 1.5 ml self-capping plastic tubes and stored in the freezer at -18°C.

Proteins were denatured by immersing the self-capping tubes in boiling water for 2-3 min. A Helena 5-50 μl Hand Pipettor with a Leur-end Catheter cut off to about 8 cm was used to place the samples into the wells. After the samples were in the wells, the electrophoretic apparatus was connected to a power supply and run at 25 mA until the tracking dye reached the bottom of the separating gel. The plates were taken from the apparatus and the gels removed. The stacking gel was discarded but the separating gel was placed in 15 ml of fixing solution (25% isopropanol and 10% acetic acid) and left overnight. The gel was removed and stained, 2-10 hrs, with coomassie blue composed of 10% isopropanol, 10% acetic acid, and 0.025 - 0.05% coomassie blue R-250. The gel was destained in 5% acetic acid solution which was changed 2-3 times or until the protein bands could be seen clearly.

**Statistical Analysis**

To determine whether or not there was any significant difference between the observations, statistical analyses were carried out. One-way analysis of variance with the use of a mean separation technique, LSD, and Student t test were used to determine the effects of each treatment (Steel and Torrie, 1980).
RESULTS

Pellets Obtained from Centrifugation

Pellets were saved after each step of centrifugation and designated with the proper number of thousand rpm. The pellet from the 16,300 x g (10,000 rpm) was designated 10p, the one from the 39,000 x g (18,000 rpm) was designated 18p, and that from the 110,000 x g (35,000 rpm) was designated 35p. The pellets obtained after repolymerization-depolymerization-repolymerization were abbreviated RDR followed by a number designating the number of cycles. The RDR pellets were obtained by centrifugation at 110,000 x g.

Protein Determination

The protein content of the 18p was found to be 54.45 μg/ml. The content of the 35p was found to be 49.01 μg/ml and that of the RDR-3 was found to be 38.89 μg/ml. Each number is an average of two values taken a few minutes apart.

Electrophoresis

Figure 2 shows the SDS-gel electrophoretic mobilities of the proteins in the pellets obtained from the isolation process. S3 represents a standard containing alpha and beta tubulins. The standard was used to ascertain the location of the alpha and beta tubulin bands. The extremely dark band in each of the first five columns represents the alpha and beta tubulins. All of the other bands represent non-
Figure 2. SDS-gel electrophoresis of bovine brain proteins contained in the pellets of various centrifugations. Each pellet was re-suspended in modified microtubule medium. 10p is the 16,300 x g pellet. 18p is the 39,000 x g pellet and 35p is the 110,000 x g pellet. RDR-1,2,3, are pellets from the 110,000 x g spin after 1,2, and 3 re-polymerization-depolymerization-re-polymerization cycles. The bands represent high molecular weight proteins with the thickest band representing the alpha and beta tubulins. S is a standard containing Heliozoan alpha and beta tubulins.
tubulin proteins. In pellet RDR-3 the alpha and beta tubulins are somewhat separated. Repolymerization-depolymerization-repolymerization was limited to three cycles on the supernatant from the 110,000 x g spin since most of the non-tubulin proteins had been eliminated (Figure 2) and enough alpha and beta tubulins still remained for experimental work.

**Binding Activity Assay**

The binding activity of each pellet is represented in Figure 3. Each point on the graph is an average of four different values taken within a one-week period.

The graph shows that the RDR-3 pellet (hexagons) exhibited the highest binding activity after 75 min of incubation, followed by the 35p (squares) and finally the 18p (circles). Based on these results, the RDR-3 pellet or the most purified microtubule preparation was chosen to be used throughout the course of this study. Since the three different microtubule preparations exhibited maximum binding to radio-labeled colchicine at 75 min, this incubation period was chosen.

**Isolation of Tubulin-{\textsuperscript{3}}H-Colchicine Complex**

The tubulin-{\textsuperscript{3}}H-colchicine complex was separated from free {\textsuperscript{3}}H-colchicine on a Sephadex G 100-120 column. The elution profile is shown in Figure 4. The graph shows two major peaks. The first peak, fraction 12 through 23, represents the tubulin-{\textsuperscript{3}}H-colchicine complex. It was eluted
Figure 3. $^3$H-colchicine binding activity to tubulin from 18p (circles), 35p (squares), and RDR-3 (hexagons) at 15-min intervals from 0 to 105 min. Incubation mixtures contained 0.15 ml $^3$H-colchicine (30 μCi) plus 0.8 ml of re-suspended pellets.
Figure 4. Elution profile of an incubation mixture consisting of 0.15 ml $^3$H-colchicine (30 µCi) and 0.3 ml purified microtubules (RDR-3) from a Sephadex G 100-120 column after incubation for 75 min at 37°C. Fractions of 1 ml were collected in each tube.
from the column faster due to its larger size. The second peak on the right, beginning at fraction 26 and ending at fraction 75, represents free or unbound $^3$H-colchicine which was eluted slower from the column due to its smaller size.

Irradiation of the Tubulin-$^3$H-Colchicine Complex

Figure 5 represents the elution profile of a sample of tubulin-$^3$H-colchicine complex unexposed to UV radiation. The first peak represents the tubulin-$^3$H-colchicine complex and the one on the right represents free $^3$H-colchicine.

Exposure of the tubulin-$^3$H-colchicine complex to UV radiation (254 nm) resulted in the dissociation of the complex. The degree of dissociation increased when exposure time was increased from 30 to 60 min and then to 90 min. The amount of colchicine dissociation was seen when the graph of each of the exposed samples was superimposed onto the graph of the control or unexposed sample (Figures 6-8).

Effect of UV Radiation on the Binding of the Tubulin-$^3$H-Colchicine Complex to Microtubules

Table 1 shows the effect of UV radiation on the binding of tubulin-$^3$H-colchicine complex to microtubules. The left column represents the amount of tubulin-$^3$H-colchicine binding to microtubules in incubation mixtures which were exposed to UV radiation for 90 min after 75 min of incubation. The right column represents the amount of binding in incubation mixtures which were not exposed to UV radiation after the incubation period.
Figure 4. Elution profile of a 3-ml sample of tubulin-\(^3\)H colchicine complex unexposed to UV radiation from a Sephadex G 100-120 column. Fractions of 1 ml were collected.
Figure 6. Elution profile of a 3-ml sample of tubulin-$^3$H-colchicine complex after 30 min exposure to UV radiation (hexagons) superimposed onto that of the control (squares). They were eluted from a Sephadex G 100-120 column and collected in 1-ml fractions.
Figure 7. *Elution profile of a 3-ml sample of tubulin-\(^{3}H\)-colchicine complex after 60 min exposure to UV radiation (circles) superimposed onto that of the control (squares). They were eluted from a Sephadex G 100-120 column; 1-ml fractions were collected.*
Figure 8. Elution profile of a 3-ml sample of tubulin-$^3$H-colchicine complex after 90 min exposure to UV radiation (triangles) superimposed onto that of the control (squares); 1-ml fractions were collected.
The data were collected within a two-week period. Statistical analysis of the data was carried out using Student t test to determine whether or not there was any significant difference between the mean for each data column.

**Microtubule Disruption**

The results of microtubule disruption on the binding of the tubulin-$^3$H-colchicine complex to microtubules are shown in Table 2. Each treatment was repeated three times for the sonicated samples and four times for the syringed samples. Statistical analysis, using one-way analysis of variance, was carried out to determine whether there were significant differences between the treated samples and the controls in terms of the levels of binding of tubulin-$^3$H-colchicine complex to microtubules.
Table 1. Binding activities of incubation mixtures consisting of 0.8 ml purified microtubules, 0.8 ml tubulin-\( ^{3}H\)-colchicine complex, 0.71 ml glycerol, 0.2 ml EGTA, 0.6 ml GTP, and 0.34 ml MES. After a 75-min incubation period, some of the mixtures were exposed to UV radiation for 90 min (*) and some were not (right column) prior to filtration.

<table>
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<th>(^{3}H)-CLC-TB+MT</th>
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<td>600</td>
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Abbreviations: \(^{3}H\)-CLC - radio-labeled colchicine; TB - tubulin; MT - microtubules; * - UV irradiated samples
Table 2. Binding activities of incubation mixtures consisting of 0.8 ml of purified microtubules (disrupted either by sonication for 15 sec, 30 sec, and 15 min or by syringing for 2 min), 0.8 ml tubulin-H-colchicine complex, 0.71 ml glycerol, 0.02 ml EGTA, 0.06 ml GTP, and 0.34 ml MES. Control (CON) equals non-disrupted microtubules.

<table>
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<th></th>
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Abbreviations: CON - control samples; SYR - syringed samples
DISCUSSION

The results of the SDS-gel electrophoresis (Figure 2) show that the beta and alpha tubulins differ in their electrophoretic mobilities. The difference was observed clearly in RDR-3 preparation where the two tubulin bands were separated. The separation of the two tubulins was the result of the action of mercaptoethanol and SDS. Mercaptoethanol was used to reduce all disulfide bonds present in the proteins, and SDS was used to unravel all intermolecular protein associations by binding to all regions of the proteins. This lead to the dissociation of the tubulin dimers and each tubulin, as well as each of the other proteins, was in the form of a denatured polypeptide chain. The relative mobility of each protein, therefore, was due to its molecular weight. Since earlier findings have shown that beta tubulin has greater electrophoretic mobility than alpha tubulin (Bryan and Wilson, 1971), it can be deduced that the lower band (fourth from the electrophoretic front) in RDR-3 was the beta tubulin band and the top band (fifth from the front) was the alpha tubulin band.

The binding activity experiment showed that the level of binding activity exhibited by each pellet, when incubated with $^3$H-colchicine, was determined by the degree of purity of the pellet. The most purified preparation, RDR-3 (hexagons), exhibited the highest level of binding, and the least
purified preparation, 18p (circles), exhibited the lowest level of binding (Figure 3). The binding difference was attributed to the fact that the less purified pellets were contaminated with non-tubulin proteins which could have interfered with the binding interaction between the tubulin dimer and $^3$H-colchicine. The degree of purity of the 34p (squares) was greater than that of the 18p and less than that of the RDR-3. As expected, its level of binding was between those of the 18p and RDR-3.

The elution profile shown in Figure 5 contains two major peaks. The smaller peak (on the left) represents the tubulin-$^3$H-colchicine complex and the larger one (on the right) represents unbound $^3$H-colchicine. The separation of these two compounds was made possible by their different sizes. The tubulin-$^3$H-colchicine complex was larger than the free colchicine; therefore, the larger molecule migrated freely down the Sephadex column. The smaller molecule, on the other hand, was small enough to penetrate the gel granules in the column which slowed down its migration, thereby causing a longer elution time from the column than the tubulin-$^3$H-colchicine complex.

The results of the tubulin-$^3$H-colchicine complex irradiation experiment showed that this complex was not stable to UV radiation (Figures 5-8). This experiment showed that irradiation of the complex for 30 min caused dissociation of 25% of the complex in the sample (Figure 6). When irradiation time was increased to 60 min the amount of
complex dissociated was increased to 44% (Figure 7). A dissociation of 71% of the tubulin-$^3$H-colchicine complex was observed when exposure time to UV radiation was increased to 90 min (Figure 8). The total decrease in the area of the tubulin-$^3$H-colchicine complex peak after 90 min was approximately equal to the total increase in the area of the $^3$H-colchicine peak after 90 min. This was an indication that the complex underwent dissociation into $^3$H-colchicine and tubulin dimer.

These findings agree with the results of earlier studies that showed UV radiation affects the tubulin site on the tubulin dimer. For instance, Forer (1966) showed that irradiation of the mitotic spindle with UV radiation lead to the cessation of mitosis. His experimental findings were supported by Bajer (1968) and Zirkle (1970) who showed that spindle microtubules disappeared within a few seconds after UV irradiation. These findings suggest that UV radiation affects the tubulin binding site on the tubulin dimer. It can be deduced, therefore, that not only does UV radiation affect the tubulin binding site on the tubulin dimer but also the colchicine binding site. These results were substantiated by Amerhein and Filner (1973), who showed that irradiation of the tubulin-$^3$H-colchicine complex with UV radiation (366 nm) caused the conversion of colchicine to lumicolchicine, an inactive derivative, which is incapable of inhibiting microtubule assembly, and also caused the destruction of part of the colchicine binding site. Figures 5-8 showed that dissociation
of the complex occurred upon UV irradiation indicating that part of the colchicine binding site was probably affected.

The results in Table 1 showed that the tubulin-$^3$H-colchicine complex could polymerize with intact microtubules. Any binding activity less than 100 cpm would have indicated that no binding occurred, but since the binding activity in each of the incubation mixtures exceeded 100 cpm, one can deduce that binding of the complex to intact microtubules had occurred. These results agree with earlier research, which showed that the tubulin-$^3$H-colchicine complex could polymerize with intact microtubules (Toman and Filner, 1975). The polymerizing ability of this complex was also demonstrated by two studies which showed that tubulin-$^3$H-colchicine complexes could polymerize, under the same condition that promotes the assembly of microtubules from tubulins, to form polymers that are different from microtubules (Andreu and Timasheff, 1982; Saltarelli and Pantaloni, 1982).

It is evident in Table 1 that UV radiation still caused dissociation of the tubulin-$^3$H-colchicine complex even though it was bound to microtubules prior to UV irradiation. The Student t test showed that there was a significant difference between the means of the two columns at the 0.05 level. This indicated that the amount of tubulin-$^3$H-colchicine complex dissociation, as a result of UV irradiation, was high. The value of 2,985 in the right column is unusually larger than the other values indicating possible experimental error. To avoid any error in the statistical analysis, this value was not included.
The results of the microtubule disruption experiment (Table 2) showed that the amount of binding of tubulin-$^3$H-colchicine complex to microtubules was greater in the syringed samples (SYR) than in the control samples (CON). One-way analysis of variance with the use of a mean separation technique (LSF) showed a significant difference between the means of the SYR and CON samples at the 0.05 level, which indicated that syringing of microtubules had a significant influence on the binding of tubulin-$^3$H-colchicine complex to microtubules. Syringing broke the intact microtubules into shorter segments thereby providing more binding sites for the tubulin-$^3$H-colchicine complex, which indicated that the binding of the complex occurred at the free ends of the microtubules.

The sonicated samples, on the other hand, exhibited a binding level which was not significantly different from that of the control samples. It can be reasoned that the sonicated samples did not exhibit higher binding activity than the controls because the microtubules in these samples were not disrupted by the frequency of sound waves used in the experiment. The number of binding sites in the sonicated samples remained the same, and the binding activity exhibited in these samples was comparable to that of the control samples.
SUMMARY

1. Alpha and beta tubulins showed different electrophoretic mobilities due to a slight difference in their molecular weights.

2. Various microtubule preparations exhibited different binding levels with $^3$H-colchicine. The level of binding exhibited by each microtubule preparation depended upon its degree of purity. The purer samples exhibited higher binding than the less pure samples.

3. The tubulin-$^3$H-colchicine complex was not stable to UV radiation (254 nm). UV radiation caused its dissociation which continued to progress as irradiation time increased. The stability of this complex was not enhanced upon its binding to intact microtubules prior to UV irradiation.

4. The tubulin-$^3$H-colchicine complex could polymerize with intact and disrupted microtubules. Disruption of microtubules by syringing through a 27 G needle had a significant influence on complex binding to microtubules. Sonication of microtubules on the other hand did not enhance the binding of tubulin-$^3$H-colchicine to microtubules.
LITERATURE CITED


