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RADIOIMMUNE ASSAY OF TUBULIN APPLIED TO OLIGOMERS, SYNAPTIC MEMBRANES, AND PLANTS

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SUMMARY. A radioimmune assay for microtubule protein, tubulin, is described, in which unknown amounts of native or denatured tubulin can be quantitated by the ability to compete with pure [^{125}I]tubulin for rabbit antibodies produced against purified bovine brain tubulin. The assay is used to demonstrate that crude extracts of mouse brain contain negligible amounts of 30-36S tubulin oligomers under conditions where purified tubulin forms substantial amounts of such structures. Also, the particulate fraction of osmotically shocked and sonicated brain synaptosomes contains negligible tubulin antigenic activity. By contrast, soluble extracts of soybean, especially rapidly dividing regions of the plant, were found to contain significant amounts of cross-reacting material, providing further evidence for the conservative evolutionary nature of this ubiquitous and important protein.

INTRODUCTION. Microtubule protein is an abundant component of all cells, participating in a variety of functions (1). In order to understand the regulation of these cellular functions, quantitative measurement of tubulin concentrations in various tissues, fractions, and stages of growth would be useful. Tubulin has been estimated by binding of radioactive colchicine, an anti-mitotic drug (2); the colchicine-binding assay is limited to the native protein, and colchicine binding capacity is rapidly lost by tubulin *in vitro* (2). Furthermore, colchicine does not bind well to oligomeric forms of tubulin nor to intact microtubules (3). Tubulin has also been measured by staining on polyacrylamide gel electrophorograms (4). This technique often involves some uncertainty with respect to identity of the protein bands. We describe here a sensitive radioimmune assay for tubulin and apply it to the quantitation of tubulin from several biologically interesting sources.

Abbreviations used: PBS: phosphate-buffered saline; IgG: immunoglobulin G; MES: 2(N-morpholino)ethanesulfonic acid; EGTA: ethylene glycol-bis (β-aminoethyl ether)N,N'-tetraacetic acid; MTAB: microtuble assembly buffer.

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EXPERIMENTAL PROCEDURES. Immunochemical Methods. Highly purified tubulin was obtained from bovine brain by 3-4 cycles of polymerization-depolymerization (5) in microtubule assembly buffer (MTAB: 0.1 M MES, 1 mM EGTA, 0.5 mM $MgCl_2$, pH 6.5), followed by phosphocellulose column chromatography to remove accessory proteins (3), and preparative polyacrylamide gel electrophoresis in sodium dodecyl sulfate (6). The unstained protein was freed of acrylamide, suspended in 1 mg aliquots in Freund's complete adjuvant, and injected into a rabbit near the lymph nodes. Booster injections of 1 mg of tubulin in incomplete adjuvant were made on three consecutive days during the seventh week. Blood was collected one week later and, following clotting, centrifuged at 5000 x g to yield anti-tubulin serum, which was stored at -20°. Dilutions were made in phosphate buffered saline (PBS: 0.15 M NaCl, 5 mM sodium phosphate, pH 7.4). Protein was measured by the Lowry method (7).

Tubulin purified by phosphocellulose column chromatography (3) was iodinated by the lactoperoxidase method (8) and purified by gel filtration to give [^{125}I]-tubulin of 1000 mCi/mmol. Tubulin was quantitatively determined by its ability to compete with [^{125}I]tubulin for antiserum raised against tubulin. The assay contained the following, added sequentially: 20-100 µl of tubulin standard or unknown, 50 µl of [^{125}I]tubulin (about 15,000 cpm representing <0.01 nmol), and 100 µl of antiserum, diluted 1:20 in a final volume of 250 µl of PBS. In the absence of nonradioactive tubulin, the antiserum was sufficient to precipitate about 50% of the labeled antigen, whereas increasing amounts of tubulin resulted in decreased binding of the labeled tubulin. After incubation at 23° for 30 min, 50 µl of goat anti-rabbit IgG, sufficient to precipitate all of the rabbit IgG, was added and incubation continued for 30 min at 23° and overnight at 4°. The precipitate was pelleted at 1000 x g for 30 min, washed once and repelleted, and counted for radioactivity (Beckman Biogamma II). Nonimmunized rabbit sera precipitated less than 10% of the labeled tubulin; this background was subtracted from triplicate experimental values.

Preparation of Tissue Samples of Unknown Tubulin Content. Plant extracts were a gift of John Howard of this Department. Two week old soybean seedlings were homogenized in four volumes of PBS, filtered through two layers of cheesecloth, and centrifuged at 10,000 x g for 10 min at 4°; the supernatant fraction was utilized.

Crude extracts of mammalian brain were obtained as the supernatant fraction of a 100,000 x g, 60 min centrifugation at 0° of a solution of whole brain from adult Swiss mice, homogenized and briefly sonicated in 8 volumes of MTAB. Synaptosomes were prepared by sucrose gradient centrifugation of the crude mitochondrial pellet of whole brain from adult mice (9). This fraction, recovered from the 0.8-1.2 M sucrose interface, was diluted with four volumes of H_2O and pelleted at 50,000 x g for 20 min, followed by osmotic shock in 10 volumes of H_2O, brief sonication, and pelleting as before. The pellet was washed once in H_2O, then resuspended in PBS.

Colchicine Binding Assay. The quantitative measure of colchicine-binding activity (tubulin) was performed by the centrifugation/charcoal method (2), with variation between replicates of less than 10%, as previously described (10).

RESULTS. Radioimmune Assay for Tubulin. Rabbit antiserum was prepared against highly purified bovine brain tubulin and shown to precipitate the pure antigen by an Ouchterlony double diffusion test (11). Tubulin was assayed by its ability to compete with [^{125}I]tubulin for reaction with the anti-tubulin. The antigen-antibody complex was separated from free antigen by precipitation with goat

antibody against rabbit anti-immunoglobulin G. Figure 1 shows that purified tubulin (0.3-10 μg) inhibited the reaction of [^{125}I]tubulin with antiserum according to a linear-logarithmic standard curve. Equivalent responses were observed with native tubulin (freshly prepared by polymerization and able to bind 0.7 ± 0.05 moles of [^{3}H]colchicine/110,000 daltons) or with denatured tubulin. Crude extracts of cow or mouse brain (Fig. 1 inset) were also able to compete for the anti-tubulin with the same logarithmic dependence on the amount of antigen. Quantitative colchicine binding estimates of tubulin from either source agreed with measurements of tubulin by the radioimmune assay, and the antigen from mouse was exactly equivalent to that of cow. Up to 100 μg of albumin (egg or bovine) were unable to compete for the specific anti-tubulin-[^{125}I]tubulin reaction.

Oligomeric Tubulin. Polymerization-purified tubulin exists in a concentration-dependent equilibrium between monomeric 6S subunits and 30-36S oligomeric rings or discs, when depolymerized *in vitro* (3). These oligomers contain several minor proteins in addition to tubulin and have been correlated with the ability of tubulin to polymerize *in vitro* (3, 4). Agarose colume chromatography can be employed to separate the subunit and oligomeric tubulin (3, 12); samples of polymerization-purified bovine brain tubulin (>90% polymerization-competent) and crude extracts of mouse brain (neonatal or adult) were fractionated on a column of Biogel A15-m (Figure 2). The void volume fractions 16-21 were pooled and assayed for tubulin by radioimmune assay. Table 1 shows that under the conditions used (1.22 mg/ml = 11 μM tubulin), almost 13% of the purified tubulin eluted in the void volume, with the residue eluting in the position of roughly 100,000 molecular weight proteins (yield 90%), in agreement with the results of others (3). By contrast, crude extracts containing similar concentrations of tubulin eluted from the column with less than 2% of the tubulin in the void volume, indicating an absence of oligomeric tubulin under these conditions. This experiment also indicates that both oligomeric and subunit tubulin reacted in the radioimmune assay (as shown with the polymerization-purified protein).

Synaptosome Membranes. Tubulin antigenicity was measured in the particulate

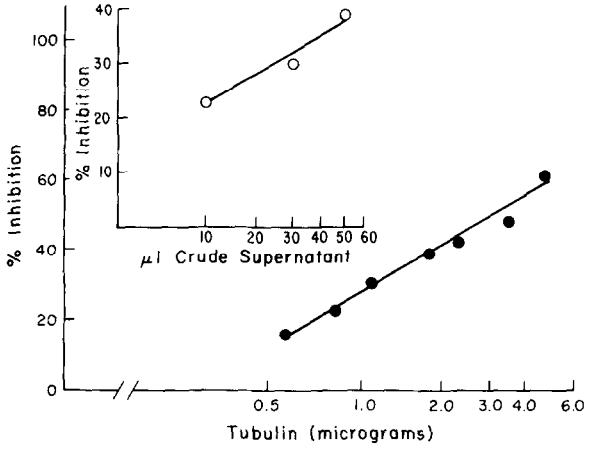

Figure 1. Radioimmune standard curve for tubulin. Bovine brain tubulin, purified by polymerization and ion-exchange chromatography, was added in varying amounts to give an increasing percent inhibition of the binding of [^{125}I]tubulin to antiserum as described under Methods. The inset shows the ability of crude soluble extracts of mouse brain to compete for the anti-tubulin.

TABLE 1. Radioimmune Assay of Tubulin in Various Tissue Fractions

Sample	Quantity of Tubulin
Brain tubulin	Oligomeric Tubulin/Total Tubulin (μg)
a. Polymerization-purified	153 ± 23/1219 ± 150 (12.6%)
b. Crude extract (adult)	28 ± 4/1514 ± 186 (1.8%)
c. Crude extract (neonatal)	7 ± 1/1215 ± 87 (0.6%)
	Tubulin/Total protein (μg)
Synaptosome membranes	< 0.3/700 (<0.1%)
Plant extracts	
a. Shoot tip and primary leaves	25.5 ± 3.4/3300 (0.77%)
b. Hypocotyl	3.2 ± 0.4/1100 (0.29%)

Samples were assayed for tubulin antigenic activity in the competitive radioimmune assay as described in Methods and in Figure 1. Oligomeric brain tubulin was obtained by pooling fractions 16-21 of the agarose column chromatogram shown in Figure 2.

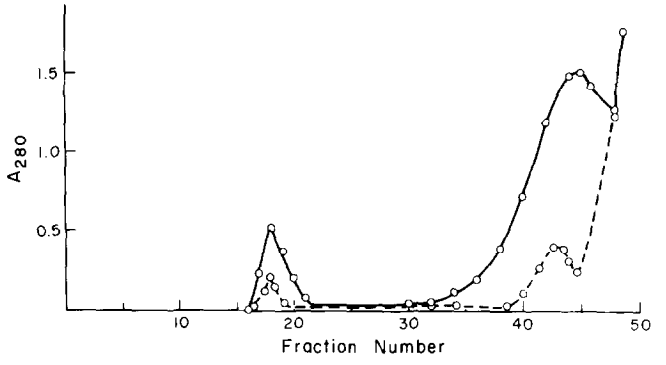

Figure 2. Agarose column chromatography of tubulin. Soluble crude extract of adult mouse brain, 9.0 mg protein including 1.5 mg tubulin in 1 ml of MTAB (——), or 3 x polymerized bovine brain microtubules, 1.4 mg protein including 1.2 mg tubulin in 1 ml of MTAB (- - -) were chromatographed on a 2.5 x 40 cm column of Biogel A15M at 0° in MTAB. Fractions of 1.0 ml were monitored for protein by aborbance at 280 nm (flow rate 10 ml/hr). Neonatal brain extracts were similarly chromatographed (not shown).

fraction of osmotically shocked and mildly sonicated crude mouse brain synaptosomes prepared by sucrose gradient centrifugation. No tubulin was detected in the radioimmune assay, indicating a maximal content of antigenically active tubulin of <0.1% of the protein (Table 1).

Plant Extracts. Soluble fractions of soybean seedlings had detectable levels of tubulin antigenic activity, with shoot tip/primary leaves containing over twice the specific activity of hypocotyl regions (also shown in Table 1).

DISCUSSION. The assay described here provides a specific and quantitative measurement of tubulin from a variety of sources and species with a sensitivity of 300 ng (2.5 pmol). Similar assays have recently appeared (13-15). Either native (oligomeric or subunit) or denatured protein is equivalent by this radioimmune assay, and this method can be applied to tubulin problems which were previously difficult to analyze.

For example, we wished to determine whether quantitative differences in tubulin oligomerization state might explain our previously reported observations that tubulin in crude extracts of neonatal mouse brain was more competent to polymerize (on a molar basis) than that of adult (10). Oligomers of tubulin do

not bind [^{3}H]colchicine well, and quantitation of tubulin on gel electrophorograms of crude extracts is difficult. The radioimmune assay showed that crude extracts of neonatal or adult mouse brain contained no significant oligomeric tubulin under conditions where polymerization-purified tubulin had considerable amounts of higher molecular weight species. Polymerization competency in cold-depolymerized crude extracts therefore cannot be correlated with the presence of oligomeric tubulin, nor are age differences in assembly explained by oligomerization.

Tubulin has been proposed to interact with cellular membranes (16, 17) and perhaps to somehow control the mobility of membrane proteins during differentiation of specific cell surface structure and function. Some tubulin has been identified with particulate fractions of tissue homogenates, including purified synaptic membranes (18) and postsynaptic densities (19) of mammalian nervous system. It is not clear whether tubulin is an integral or peripheral membrane protein, or indeed, if trapped aggregates of cytoplasmic microtubules account for the particulate tubulin. Our radioimmune assay detected no tubulin antigenic activity in mouse brain synaptosome particulate fractions. Variable reports on this subject are likely to reflect the method of membrane preparation. Furthermore, if tubulin were an intimate part of synaptic membranes or postsynaptic densities, it might be inaccessible to antibody and not detectable in the assay employed here.

Despite the ability of colchicine to affect microtubules in the mitotic apparatus of plants, no tubulin-like protein having the same colchicine-binding properties as tubulin from animals has been isolated. We report the presence of significant cross-reacting material in plant extracts using antiserum against bovine tubulin. This response cannot be considered a quantitative measure of plant tubulin since the antigen may differ slightly between species. Interestingly, rapidly dividing portions of the plant showed a greater proportion of tubulin-like protein than more quiescent regions.

In conclusion, the ability to quantitate tubulin by radioimmune assay

procedures can be useful in studying various biological questions.

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