anterior end possesses stout prezygapophyses that extend far beyond the recessed procoelous centrum and the weakly developed prehypapophyses. In the Big Bend specimens the anterior end of the cervicals is composed of two large prezygapophyses forming a yoke that would surround, and a dorsoanteriorly extended procoelous centrum that would rest upon, the bulbous end of the preceding vertebra. This articular arrangement apparently allowed great lateral movement and significant upward movement but restricted downward movement.

The mandibles are long and slender, probably a meter in length. Although the anteriormost part of the dentary is missing, what is preserved indicates that the jaw expanded anteriorly, became triangular toward the tip, and bore no teeth. The strangely elongated jaw would seem to indicate an animal similar to *Pterodaustro guinazui* of Argentina (*18*) or *Pterodactylus antiquus* of Germany (*20*), but unlike *Dsungaripterus weii* of China (*19*) and *Ornithocheirus* of England (*17*), both of which possess extensively toothed, deep, short jaws.

The most distinctive feature of this Big Bend pterosaur is its immense size, for it is without doubt the largest flying creature presently known. The largest specimen from Big Bend has a humerus 52 cm long (Fig. 1, b and c), over twice that of *Pteranodon*. The deltopectoral crest is about half the length of the humerus, making it proportionally longer than in other pterosaurs. The expanded distal end of the humerus is equaled in proportion only by that of the Oregon pterosaur (*5*). Since this large specimen consists of a humerus and partial radius, proximal carpal, distal carpal, metacarpal, first phalanx, and second phalanx, an estimate of the wingspan can be made. Plotting the estimated wingspan against length of the humerus of *Pterodactylus antiquus, P. longicollum* (*20*), *Dsungaripterus* (*19*), *Nyctosaurus*, and *Pteranodon* (*21*) shows that for the pterodactyloids the wingspan increased more rapidly than the length of the humerus (Fig. 2), but within any one taxon the ratio of wingspan to length of humerus is constant. Therefore, if one uses the humerus as a standard and increases *Pterodactylus antiquus*—one of the most closely related forms with completely preserved wings—to an appropriate size, a wingspan of only 11 m is obtained. However, if one takes the dimensions of the wing bones preserved in the smaller Texas specimens and applies an allometric increase to these bones and to complementary wing bones in *Dsungaripterus* and *Pteranodon*, a wingspan estimate of 15.5 m results. Following the general trend for pterodactyloids from small to large taxa and extending it to the appropriate humerus length gives a wingspan of 21 m. The intermediate wingspan is accepted at this time.

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22. I thank Dr. Wann Langston, Jr., for suggesting the project that brought these specimens to light and allowing me to undertake a detailed description now in preparation, at which time the specimens will be diagnosed. Cooperation of the authorities at the Big Bend National Park is gratefully acknowledged. The specimens were collected under Department of the Interior Antiquities Act permits issued to the Texas Memorial Museum, University of Texas at Austin. All material collected under these permits is preserved at the Vertebrate Paleontology Laboratory (Texas Memorial Museum). Supported by the American Oil Company through the Geology Foundation of the Department of Geological Sciences, University of Texas at Austin, and by the Society of the Sigma Xi.

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## Immunofluorescence of Mitotic Spindles by Using Monospecific Antibody against Bovine Brain Tubulin

Abstract. *Monospecific antibody directed against bovine brain tubulin has been purified by affinity chromatography and tested against soluble tubulin and intact microtubules of brain and mitotic apparatus. Binding of the tubulin antibody to the mitotic spindle of rat kangaroo cells was demonstrated in all stages of mitosis by indirect immunofluorescence.*

Within the past 5 years, considerable interest has been directed toward elucidating the molecular structure, mode of assembly, and drug-binding capability of the protein tubulin (*1–3*). Revised methods of purification (*4, 5*) have permitted the isolation of a relatively large amount of tubulin which is nearly electrophoretically homogeneous and retains the capacity to assemble into microtubules. Production of antibody directed against tubulin has been reported (*6, 7*); however, the monospecificity of the antiserum (homogeneity of the antigen tubulin) has not been confirmed. Previous methods of tubulin purification for antibody production involved the formation of paracrystals of tubulin by using high concentrations of vinblastine (*7*). Although a large amount of the crystalline material was undoubtedly tubulin, the presence of other proteins was not ruled out in the preparation, since it was demonstrated that the resolubilized paracrystals possessed an appreciable amount of adenosine triphosphatase activity. To date, there has been no confirmation of such activity in purified 6*S* brain tubulin. A radioimmunoassay for outer doublet tubulin from *Naeglaria gruberi* has been reported (*8*).

In this report we present evidence for the production of monospecific antibody directed against pure bovine brain 6*S* tubulin. The antibody is relatively simple to produce and purify, and it has the ability to bind with microtubules as well as soluble tubulin. Studies using the indirect immunofluorescence technique demonstrate specific fluorescence of mitotic spindles during

mitosis. The antibody can become a useful molecular probe for understanding more fully the architecture of this ubiquitous and important protein, in addition to providing a means of following the attachment and fate of spindle fibers during cell division.

Bovine brain tubulin was prepared by a modification of the procedure of Shelanski *et al.* (*5*). Fresh bovine brain tissue was washed and homogenized at 4°C in reassembly buffer: 0.1*M* 2-(*N*-morpholino)ethanesulfonic acid, 1 m*M* ethylenebis(oxyethylenenitrilo)-tetraacetic acid, 0.1 m*M* guanosine triphosphate, and 0.5 m*M* $MgCl_2$ (*p*H 6.85). After centrifugation at 100,000*g* for 1 hour at 4°C, an equal volume of reassembly buffer containing 8*M* glycerol was added to the supernatant, which was then divided into portions and frozen at −70°C until needed. Pure tubulin was obtained from the supernatants by allowing polymerization to proceed for 30 minutes at 37°C, followed by centrifugation at 100,000*g* for 30 minutes at 25°C. The pellet, containing polymerized tubulin, was resuspended and resolubilized in reassembly buffer at 0°C for 1 hour. Insoluble aggregated tubulin was removed from solution by a 30-minute centrifugation at 4°C and 100,000*g*. To the soluble tubulin was added an equal volume of 8*M* glycerol–reassembly buffer, and the tubules were again allowed to form at 37°C. After another 100,000*g* centrifugation at 25°C for 30 minutes, the tubules were solubilized and made 4*M* with respect to glycerol, as previously described. The twice repolymerized tubulin was then chromatographed on a Sepharose 4B (Pharmacia) column (2.5 by 60 cm) at 4°C in order to separate a small amount of higher molecular weight material from the 6*S* tubulin. The pure tubulin was stored in small portions at −20°C. The purity of the tubulin protein was determined by polyacrylamide gel electrophoresis on 6 percent acrylamide gels made up in 8*M* urea and 0.1 percent sodium dodecyl sulfate, as described by Weisenberg *et al.* (*2*). The gels were stained with 0.05 percent Coomassie brilliant blue in 25 percent isopropanol and 10 percent acetic acid, then destained in 25 percent isopropanol and 10 percent acetic acid.

Sepharose 4B was activated with cyanogen bromide by the procedure of Cuatrecasas *et al.* (*9*). Cyanogen bromide (3.5 g) was added to 20 ml of Sepharose 4B at 0°C. The *p*H of

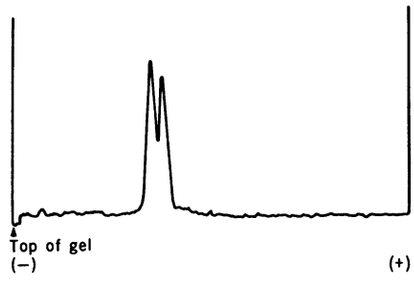


Fig. 1. Densitometric scan of a polyacrylamide gel containing 0.1 percent sodium dodecyl sulfate and 8*M* urea. Approximately 30 μg of purified tubulin was applied to the gel. Following electrophoresis the gel was stained with Coomassie brilliant blue, destained, and scanned at 550 nm.

the solution was maintained at 11.0 by the addition of 4*N* NaOH; the reaction was allowed to proceed for 15 minutes at 0°C with constant stirring. Following activation, the Sepharose was immediately washed in a sintered glass funnel at 4°C, first with 50 ml of distilled water and then twice with 50 ml of a solution containing 100 m*M* boric acid, 25 m*M* sodium borate, and 75 m*M* NaCl (*p*H 8.4). The Sepharose was then allowed to react with 40 to 50 mg of tubulin in 30 ml of the borate-saline buffer, while being stirred gently at 4°C for 24 hours. The coupled Sepharose-tubulin material was washed extensively in a column (2.5 by 5 cm) with 100 m*M* HCl to remove unbound protein, and was then equilibrated with borate-saline buffer (*p*H 8.4).

Purified tubulin containing only the 6*S* molecule was cross-linked with glutaraldehyde to form an insoluble complex. To 10 mg of tubulin in 2.0 ml of buffer was added an equal volume of 2 percent glutaraldehyde at 0°C. The precipitated tubulin was collected by centrifugation and washed twice with 0.15*M* NaCl. Freund's complete adjuvant (5 ml) was added and the mixture was homogenized. The rabbits were given an injection of 3.5 mg of protein, followed by two booster injections of 1.5 to 2.0 mg at 10-day intervals. One week after the last injection the rabbits were bled via cardiac puncture. The serum was precipitated by adding an equal volume of saturated ammonium sulfate. The precipitate was collected by centrifugation and dialyzed against borate-saline buffer.

Monospecific antibody against tubulin was isolated by passing the rabbit gamma-globulin fraction previously dialyzed against the borate-saline buffer, through the immunosorbent column. After all the unbound gamma globulin was eluted, the elution buffer

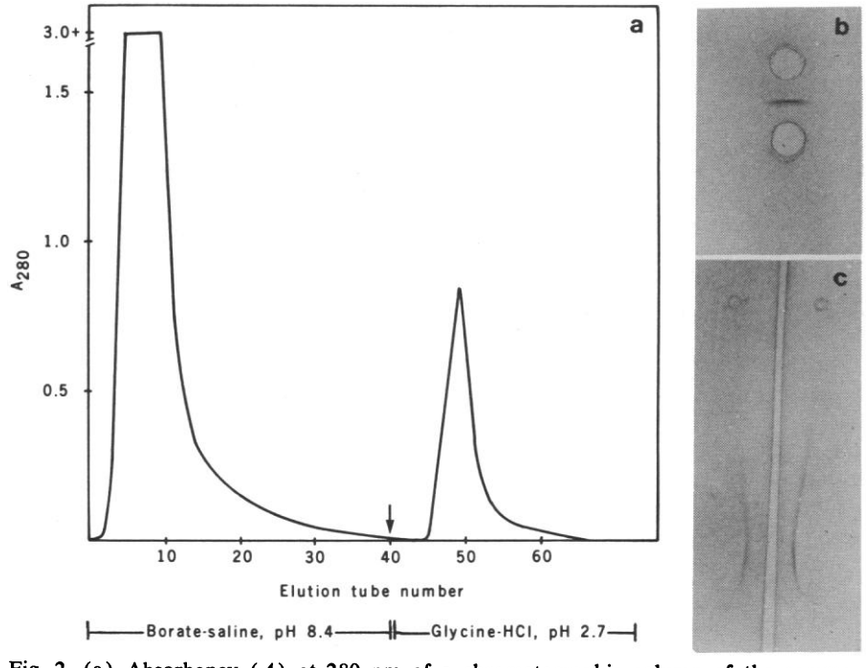


Fig. 2. (a) Absorbancy (*A*) at 280 nm of a chromatographic column of the gamma-globulin fraction from the immunosorbent column to which tubulin was covalently bound. The arrow indicates the point at which 200 m*M* glycine-HCl buffer (*p*H 2.7) is added to disrupt the antigen-antibody complex. (b) Double immunodiffusion of pure tubulin (6*S*) against antibody eluted from the immunosorbent column. (c) Immunoelectrophoresis in barbital buffer (*p*H 8.2).

was changed to 200 m*M* glycine-HCl (*p*H 2.7), at which point the bound tubulin antibody was released. This monospecific antibody was concentrated by ammonium sulfate precipitation and dialyzed extensively against 0.15*M* NaCl before being stored at −20°C.

Double immunodiffusion was carried out in 1 percent Bacto agar (Difco) in 0.15*M* NaCl with 0.1 percent $NaN_3$ (*10*). Immunoelectrophoresis was performed with 2 percent Bacto agar in 0.05*M* barbital buffer (*p*H 8.2), according to the method of Scheidegger and Roulet (*11*). Electrophoresis was carried out for 75 minutes at 4°C with a constant voltage of 30 volts across the agar.

Tubulin prepared from bovine brain by the in vitro reassembly procedure was contaminated with a small amount of higher molecular weight material. The additional step using gel filtration on Sepharose 4B separated the higher molecular weight proteins from 6*S* tubulin as judged by polyacrylamide gel electrophoresis (Fig. 1).

All six rabbits injected with tubulin produced an antibody against the antigen, although the titers, as determined by hemagglutination and hemagglutination inhibition assays, were low. Antibody directed against tubulin was isolated and concentrated by passing the gamma-globulin fraction over an affinity column to which tubulin had been covalently bound (Fig. 2a). The specificity of the tubulin antibody was determined by double immunodiffusion and immunoelectrophoresis (Fig. 2, b and c).

The binding of antibody against tubulin to spindle microtubules was tested by using an indirect immunofluorescence procedure (*12*). Rat kangaroo cells (strain Pt K1) were grown to confluence on glass cover slips. After being rinsed in phosphate-buffered saline (PBS), the cells were fixed for 20 minutes in PBS containing 3.5 percent formaldehyde at 22°C. The cover slips were rinsed in PBS and subsequently fixed in absolute acetone at −10°C for 7 minutes. The cover slips were air-dried and then incubated with the antibody (0.20 mg/ml in borate-saline buffer) at 37°C for 1 hour. The preparations were again washed in PBS and incubated in a 1 : 1.5 dilution of fluorescein-tagged goat antiserum against rabbit immunoglobulin G (Meloy Laboratories, Springfield, Virginia) for 1 hour at 37°C. The cover slips were rinsed in PBS followed by distilled $H_2O$ and mounted on glass slides in a drop of PBS-glycerol (1 : 1) for viewing in a Leitz microscope adapted for dark-field ultraviolet microscopy. Photographs were recorded on Tri-X Pan film (Kodak).

The mitotic spindle was clearly delineated by fluorescent stain and both chromosomal (kinetochore to pole) and interpolar (pole to pole) fibers were apparent (Fig. 3). In addition, a weak cytoplasmic fluorescence was apparent throughout mitosis. At prophase (Fig. 3a), a faint background fluorescence was apparent in the vicinity of the nucleus and around the condensing chromosomes. During various stages of prometaphase, the fluorescence pattern progressed from a central spot surrounded by chromosomes (Fig. 3b) to a more spindle-shaped pattern with chromosomes distributed throughout (Fig. 3, c and d). At metaphase, the spindle was fully formed and the chromosomes were positioned at the cell equator (Fig. 3e). On separation of the daughter chromosomes at anaphase, both chromosomal and interpolar fibers were apparent (Fig. 3f). At telophase, interpolar fibers were faintly fluorescent, but cytoplasmic fluorescence increased sharply (Fig. 3g), possibly due to increased free tubulin derived from spindle microtubule disassembly. Except for a brightly fluorescent midbody between daughter cells (Fig. 3h), fluorescence was greatly diminished at late telophase or the early $G_1$ phase. Electron microscopic studies of rat kangaroo cells (*13*) showed that microtubules were present in all the brightly fluorescent regions indicated in Fig. 3.

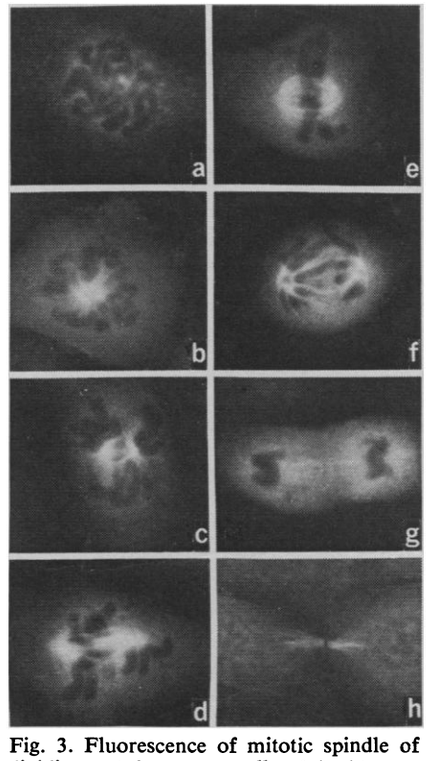


Fig. 3. Fluorescence of mitotic spindle of dividing rat kangaroo cells. (a) At prophase, a weak background fluorescence is seen around condensing chromosomes. (b) At a later stage a single bright fluorescent spot is surrounded by chromosomes. (c and d) During subsequent states of prometaphase the fluorescence becomes more spindle-shaped. (e) Metaphase. (f) Anaphase, showing both chromosomal and interpolar fibers. (g) Telophase; note the weak interpolar fibers and increased cytoplasmic fluorescence. (h) Midbody between two daughter cells.

The procedure for preparing antibody to purified brain tubulin is relatively simple. The antibody is able to bind not only to soluble 6*S* tubulin but also to intact microtubules of another cell type in a different species. This observation supports and extends the concept of the ubiquitous nature of microtubule protein (*7, 14*). Moreover, it supports the idea that the gene for tubulin is highly conserved. Antibody to purified 6*S* tubulin should be a useful molecular probe for studies of the function of this important molecule.

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