

SEROLOGICAL SIMILARITY OF FLAGELLAR AND MITOTIC MICROTUBULES

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ABSTRACT

An antiserum to flagellar axonemes from sperm of *Arbacia punctulata* contains antibodies which react both with intact flagellar outer fibers and with purified tubulin from the outer fibers. Immunodiffusion tests indicate the presence of similar antigenic determinants on outer-fiber tubulins from sperm flagella of five species of sea urchins and a sand dollar, but not a starfish. The antibodies also react with extracts containing tubulins from different classes of microtubules, including central-pair fibers and both A- and B-subfibers from outer fibers of sperm flagella, an extract from unfertilized eggs, mitotic apparatuses from first cleavage embryos, and cilia from later embryos. Though most tubulins tested share similar antigenic determinants, some clear differences have been detected, even, in *Pseudoboletia indiana*, between the outer-fiber tubulins of sperm flagella and blastular cilia. Though tubulins are "actin-like" proteins, antitubulin serum does not react with actin from sea urchin lantern muscle. On the basis of these observations, we suggest that various echinoid microtubules are built of similar, but not identical, tubulins.

INTRODUCTION

Microtubules are a component of all eucaryotic cells during at least part of their life histories (31, 43). Microtubules form the nine outer fiber doublets and the two central pair elements of cilia and flagella, the spindle fibers of the mitotic apparatus, and diverse cytoplasmic structures. More than one of these classes of microtubules often can be found in a single cell, or among the different cell types of a multicellular organism. The question considered in this paper is whether or not different classes of microtubules are built of the same structural protein.

All microtubules are relatively straight, unbranched tubular structures about 240 Å in diameter, with electron-opaque walls. Their substructures are also similar (see references 11, 21, 43). Microtubule structural proteins, tubulins

(25, 41), isolated from the outer fibers and central-pair fibers of cilia and flagella, from brain tissue, and from neuroblastoma cells all have virtually identical sedimentation coefficients, molecular weights, and amino acid composition (27, 33, 36, 38, 45, 51). Several different extracts containing tubulins have been found to bind colchicine (6, 35, 51) and also vinblastine (see references 27, 43). Outer fiber, central pair, and neurotubule tubulins have been found to contain bound guanine nucleotide (36, 46, 51). On the basis of these similarities, many authors have suggested that different classes of microtubules may be built of the same tubulin (some examples are reviewed in reference 4).

Microtubules differ from one another in their stability, both in and out of cells. In sea urchins,

as in other organisms, the microtubules of the mitotic apparatus are labile, easily solubilized by cold and by colchicine (14), easily lost from isolated mitotic apparatuses (19), and obliterated by fixatives that preserve flagellar microtubules (12). Cytoplasmic microtubules are also labile (49). The central-pair fibers of isolated sperm flagella are more readily solubilized than the outer fibers (10, 35). Under certain conditions the B-subfiber of the outer fiber doublets is more labile than the A-subfiber (41). On the basis of a detailed study of differences in stability and solubility of microtubules, Behnke and Forer (4) argued that four classes of tubules can be distinguished. These four classes are evident in sea urchins: (a) mitotic and cytoplasmic microtubules, which are the most labile; (b) central-pair tubules, more labile than outer fibers; (c) the B-subfibers of outer fibers; and (d) the A-subfibers. Behnke and Forer (4) postulated that the differences indicate "intrinsic physical and/or chemical differences between the tubules." In contrast, Tilney and Gibbins (49) suggested that all microtubules might be built of the same subunits, and their stability altered "either by the addition of material or through interactions between adjacent tubules or both."

On the basis of these differences in stability, one could argue for or against the hypothesis that different classes of microtubules are built of the same tubulin subunits. The first indication of a difference between tubulin subunits came from the studies of Stephens (41) on the tubulins from the A- and B-subfibers of the outer fibers of sea

urchin sperm flagella. These tubulins showed significant differences in pH solubility, electrophoretic mobility, and amino acid composition. Peptide mapping revealed that the tubulins share about 50 tryptic peptides, but the A-tubulin has five to seven unique peptides and the B-tubulin has two. These results indicate slight but unequivocal differences in the primary structure of the A- and B-tubulins.

Immunochemistry offers a means of detecting chemically undefined structural homologies among proteins. When an antiserum was found to contain antibodies against sea urchin tubulins, this offered a new opportunity to explore similarities and differences among microtubules. The results of our first explorations, in which various antigens were compared using Ouchterlony's (28) method of double diffusion in agar, are considered as further evidence for similarity but nonidentity of tubulins.

MATERIALS AND METHODS

A single antiserum was used to study antigens prepared from six species of echinoids (Table I).

Antiserum

The antiserum was prepared by immunizing a rabbit with *Arbacia* sperm flagellar axonemes. Purified flagella were extracted twice with digitonin and washed in buffer, as described by Stephens, Renaud, and Gibbons (46). This procedure removed most membrane and matrix material, and left mainly axonemes (10), consisting of outer-fiber doublets,

TABLE I
List of Echinoid Species Used

The classification is based on Mortensen (26).

Class Echinoidea				
Subclass	Order	Family	Genus and species	Source and season
Regularia				
Camarodonta				
		Toxopneustidae	<i>Pseudoboletia indiana</i>	Hawaii, perennial
			<i>Tripneustes gratilla</i>	Hawaii, perennial
		Echinometridae	<i>Colobocentrotus atratus</i>	Hawaii, winter
		Strongylocentrotidae	<i>Strongylocentrotus droebachiensis</i>	New England, winter
		Stirodonta	<i>Arbacia punctulata</i>	New England, summer
Irregularia				
		Clypeastroida	<i>Echinarachnius parma</i>	New England, summer

largely still arranged in cylindrical structures linked together by filamentous material (10, 43), with some arms (and associated dynein; reference 10) on the outer fibers and some central-pair microtubules. The axonemes were pelleted and frozen. Later the material, now amorphous and largely insoluble, was resuspended with the aid of mild sonication at 2 mg/ml in 2 mM Tris-HCl, pH 7.5, containing 0.14 M NaCl and 1 mM mercaptoethanol. Each injection consisted of 0.5 ml of antigen emulsified with an equal volume of complete Freund's adjuvant. After three toepad injections at weekly intervals, a bleeding, and a month's rest, the rabbit was given a booster injection and then bled a week later to obtain the antiserum used.

Antigens

Most of the fractionation procedures for preparing the antigens have been described in detail in the cited references. Unless otherwise stated, all operations were performed at 0°–4°C. A frequently used buffer, Tris-DTT, contained 10 mM Tris-HCl, pH 8 (sometimes pH 7.5), and 0.1 mM dithiothreitol (1 mM 2-mercaptoethanol was also used).

Outer-fiber doublet microtubules were prepared from purified sperm flagella (41, 46) in several different ways. Prolonged dialysis against low ionic-strength buffer containing ethylenediaminetetraacetate (EDTA) solubilized central fibers, dynein, and matrix proteins (10, 41). Usually dialysis was preceded or followed by a detergent extraction to remove the flagellar membranes, using either 1% Triton X-100 (41), 0.05% Sarkosyl (40), or 0.5% digitonin (10, 46). Membrane protein could also be extracted by storing the preparations, after dialysis, at –20°C in 50% glycerol (40). Outer fibers were recovered from glycerol by dilution (at least 1:4 in Tris-DTT) and centrifugation. Outer fibers also could be purified by extraction of axonemes with 0.6 M KCl (10, 41). These procedures were combined in several ways. One of the simplest was to dialyze the flagella, and then to extract with glycerol (see Fig. 1). In a more elaborate, frequently used procedure, the flagella were extracted twice with 1% Triton X-100 in Tris-DTT, dialyzed against two 18-hr changes of 0.1 mM EDTA and 0.1 mM DTT in 1 mM Tris-HCl, pH 7.5, stored and extracted at –20°C in 50% glycerol in Tris-DTT, and finally washed in Tris-DTT by repeated centrifugation for 15 min at 35,000 g. Purified outer fibers were always contaminated with at least a little of the material that links them together in groups of nine, but they could often be induced to separate into doublets by exposing them briefly to 0.05% Sarkosyl at room temperature. Purified outer fibers were washed by two or more centrifugations in Tris-DTT before use. Usually they were solubilized

in 0.5% Sarkosyl in Tris-DTT at room temperature (39), after which they were stored at 0°C.

The A- and B-microtubule components were fractionated from outer fibers by thermal depolymerization of the B-subfibers as described by Stephens (41). The A-subfibers were recovered in the form of singlet microtubules by centrifugation, leaving essentially pure B-tubulin in the supernatant. The A-subfibers were then solubilized in Sarkosyl or in low ionic-strength buffer at pH 2.5 (41).

Central-pair protein was extracted from sperm flagella by dialysis against low ionic-strength buffer (10, 35, 41).

An acetone powder was prepared from sperm flagella (33, 40), and extracted to obtain the portion (35–40%) soluble in Tris-DTT.

Cilia were isolated from embryonic sea urchins using hypertonic seawater (3, 15). Blastulae and gastrulae were washed in seawater by hand centrifugation, and resuspended in seawater containing NaCl in concentration dependent on the species, ranging from 3.0 g/100 ml for *Pseudoboletia* to 4.4 g/100 ml for *Arbacia*. The deciliated embryos were removed, and the cilia were sedimented for 10 min at 12,000 g, and washed in seawater. If the embryos were returned to seawater, they regenerated cilia within a few hours (3, 15), so it was possible to prepare several (three or four) batches of cilia from the same batch of embryos. The first batch prepared was contaminated usually with debris and was discarded; subsequent batches were much cleaner. Outer fibers from cilia were prepared by the same methods used with sperm flagella.

Acetone powders of unfertilized eggs (37) were extracted with Tris-DTT and sedimented for 4 hr at 100,000 g. The supernatant contained 4-6S protein, presumably tubulin, which was further purified by gel filtration on Sephadex G-200.

Mitotic apparatuses were isolated from zygotes at first cleavage metaphase by the hexylene glycol method (16, 17). The mitotic apparatuses were stored in the isolation medium at 0°C for from 4 to 10 hr, until the gross structure became stable (19). Then the mitotic apparatuses were centrifuged, suspended in Tris-DTT, and dialyzed for 18 hr against 100 volumes of Tris-DTT containing 0.1 mM EDTA. The insoluble material was removed by centrifugation, and the supernatant used as crude mitotic tubulin (6, 19).

Actin was extracted from the muscles surrounding the lantern of adult *Arbacia* (32) by standard procedures (7, 48). The muscles were stripped of their membranes and plunged into cold 50% glycerol. A day later the insoluble material was washed with buffer and then thrice precipitated with acetone. The dried acetone powder was immediately suspended, at 20 mg/ml, in CO₂-free water containing 0.1 mM adenosine triphosphate (ATP). The soluble fraction,

containing about 10% of the acetone-powder protein, gave a single band on gel electrophoresis.

Protein concentration was measured by the Lowry method (24) using a serum albumin standard. Negative staining with uranyl acetate was performed by the method of Huxley (13). Mitotic apparatuses for electron microscopy (Fig. 5) were fixed in 2% glutaraldehyde in iced isolation medium, washed in isolation medium, and postfixed in 1% OsO₄. Sperm (Fig. 1) were fixed directly in 1% OsO₄.

Immunodiffusion

The immunodiffusion tests were performed in 60 mm Petri dishes (47). The agar medium contained 1% Difco Bacto-agar (Difco Laboratories, Inc., Detroit, Mich.) (washed after weighing with cold distilled water on a Buchner funnel), 0.85% NaCl, 0.1 mg/ml merthiolate, and 2 mM Tris-HCl, pH 7.5. The agar was washed to avoid precipitation of Sarkosyl by divalent ions present in unwashed agar, but otherwise washing the agar did not change the results. The dishes contained a bottom layer of 1.2 ml agar, and a 3.5 ml top layer containing wells 8 mm in diameter. The outer wells were 5–8 mm, edge to edge, from the center well. From 0.05 to 0.1 ml of antigen or antiserum was placed in each well. Antigens usually were diluted for assay in Tris-DTT, sometimes with 0.5% Sarkosyl added. When it was necessary to test larger concentrations of antigen than could be contained in 0.1 ml, the wells were refilled the next day; otherwise the wells were filled only once. Usually 0.05 ml of antiserum was used directly, but $\frac{1}{10}$ of that amount was sufficient to give a visible precipitin band. The dishes were incubated in a humidified atmosphere at 22°C, and observed at intervals from 1 day to 2 wk.

Absorptions were performed by incubating the antiserum with an equal volume of antigen at high concentration for several hours in the cold. If the antigen was outer-fiber or central-pair protein, large quantities of visible precipitate formed on this first absorption. The precipitate was removed by centrifugation, another volume of antigen was added, and incubation and centrifugation repeated. This procedure diluted the antiserum 1:2, so absorbed sera were compared with untreated serum at a 1:2 dilution.

Quantitative estimates of the minimum concentration of antigen forming a visible precipitin band were made by testing serial twofold dilutions of antigen against adjusted concentrations of antiserum. The tests were most sensitive if performed with antigen against 0.05 ml of a $\frac{1}{4}$ dilution of antiserum, but some of the tests were performed with 0.05 ml of undiluted antiserum. The end-point titrations were reproducible, between tests, to within about two dilutions. In many titrations of *Arbacia* outer fiber tubulins in Sarkosyl, where usually about 1 μ g of antigen

protein was required to give a visible precipitin band, the amount required ranged from 0.6 to 3 μ g protein. The values given in the text and in Table II are based on repeated titrations. Differences of less than threefold are not considered significant.

At least two independent preparations of each antigen, except actin, were tested.

RESULTS

The Antiserum Contains Antitubulin Antibodies

In Ouchterlony double-diffusion tests, the anti-*Arbacia*-axoneme serum reacted against solubilized *Arbacia* axonemes to give three precipitin bands. The main band regularly was somewhat broad. The minor bands, one on either side of the main band, were visible only at high-antigen concentrations. Fractionation of axonemes suggested that the main precipitate was formed in a reaction of antiserum with tubulin. Outer-fiber tubulin was chosen for detailed study.

Outer-fiber doublet microtubules, isolated from *Arbacia* sperm flagella, purified, and solubilized in Sarkosyl, reacted against the antiserum to give a single, diffuse precipitin band, equivalent to the main band from axonemes. (When outer-fiber preparations were tested at high concentrations, in excess of 100 μ g protein per well, most preparations also showed one or two minor bands. Only the main band is considered here.) The outer fibers were purified to electron microscopically pure doublets, as individuals or loosely associated in sheets of up to nine, with only traces of wispy material linking them together (Fig. 1). Such preparations have been estimated to consist of at least 95% tubulin (38).

A recurrent problem in immunochemistry is that minor contaminants can induce the production of antibodies, if they happen to be good antigens. Even though most of the outer-fiber protein is tubulin, it is important to determine whether the main precipitin band is due to tubulin or to a contaminant. Several observations strongly support the conclusion that the band is formed by a tubulin-antibody reaction.

PURITY: Pure outer-fiber doublets from sperm flagella, prepared by several different procedures, always have given the main band when solubilized. A quite different procedure for making flagellar microtubule protein, extracting the tubulin from an acetone powder of whole flagella (33, 40), also gave the main band. These diverse purification

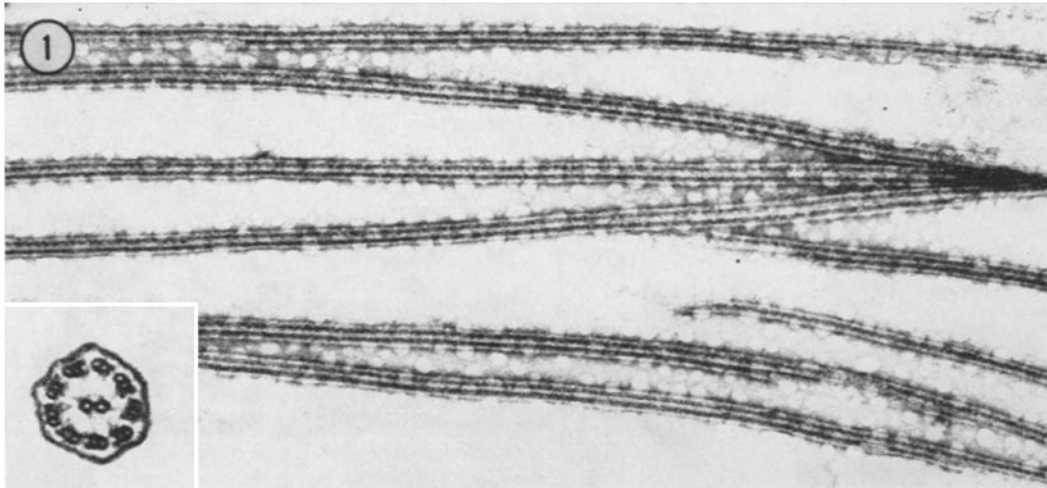


FIGURE 1 Outer-fiber doublet microtubules from *Arbacia* sperm flagella, negatively stained with uranyl acetate. These microtubules were prepared by dialysis against low ionic-strength buffer, then storage at -20°C in 50% glycerol, and finally washing in buffer. When solubilized in Sarkosyl they gave a reaction with antiserum at dilutions as low as $1\ \mu\text{g}$ antigen protein per Ouchterlony well. The insert shows a transverse section through an intact *Arbacia* sperm flagellum. Both $\times 70,000$.

procedures might be expected to eliminate different minor contaminants, but tubulin and the antigenic activity have always purified together.

In addition, outer-fiber doublets were solubilized in Sarkosyl and diluted under conditions which allowed some of the tubulin to reassociate into singlet tubules (39, 40). These tubules were washed in buffer and solubilized again in Sarkosyl. The material still gave the main precipitin band. This "recrystallization" step might be expected to eliminate some contaminants, but the antigenic activity remained with the tubulin.

SENSITIVITY: Purified outer-fiber preparations, in 0.5% Sarkosyl, regularly have given the precipitin band, visible without staining, at antigen concentrations as low as $1\ \mu\text{g}$ of protein in the Ouchterlony well. On the basis of the experience of other workers with double diffusion in agar in macro-Ouchterlony tests, a reaction with $1\ \mu\text{g}$ of protein is good sensitivity for the method (9, 56).

FRACTIONATION OF TUBULIN. Solubilized outer-fiber preparations were fractionated by gel filtration on a Sephadex G-200 column by elution with buffer containing Sarkosyl. The protein eluted as a single peak. This peak corresponded precisely, in two trials, with the distribution of antigenic activity. Samples taken from the two ends and the middle of the protein peak were indistinguishable. They all gave Ouchterlony (28) "reactions of

identity" with each other, and they were equally active, each one giving a visible precipitin band with as little as 5–7 μg protein per well. Thus, in an outer-fiber extract, where the majority of protein is known to be tubulin, the protein and the antigenic activity fractionated together.

These three observations indicate that the main precipitating antigen fractionates with outer-fiber tubulin.

ABSORPTION: Intact, purified outer-fiber doublets absorbed, from the antiserum, all antibodies able to form the precipitin band against solubilized outer-fiber protein. This indicates that similar antigenic determinants are exposed on intact outer fibers and in solubilized tubulin preparations.

COATING OF TUBULES WITH ANTIBODY: When intact outer fibers were incubated with the antiserum, all parts of the doublets became coated with protein (Fig. 2). Control serum (two different sera were tested) did not coat the tubules. The sample treated with control serum and washed (Fig. 2 *c*) is indistinguishable from untreated tubules. The sample treated with control serum on the grid (Fig. 2 *a*) shows indications of residual serum proteins, but not specific precipitation on the tubules. In contrast, the walls of microtubules treated with antiserum (Fig. 2 *b* and *d*) appear thicker and less regular than those treated with control serum. The antibody protein

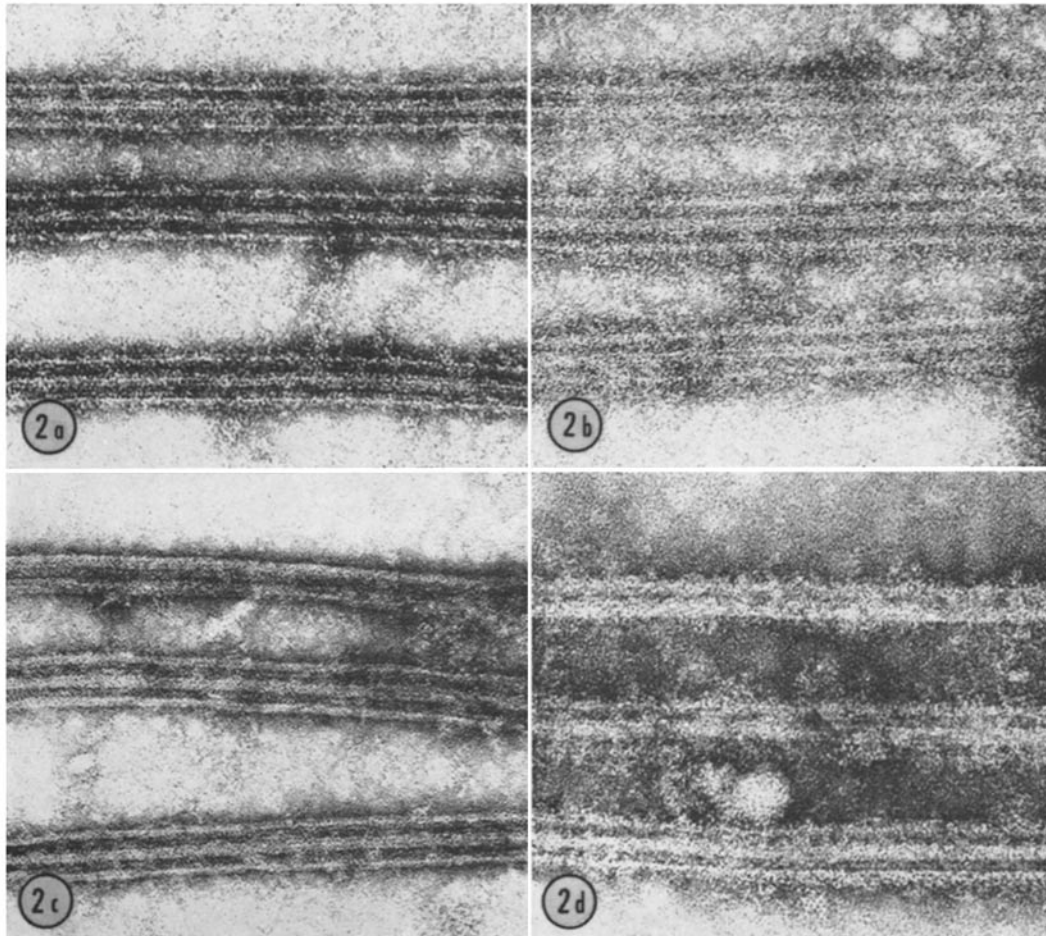


FIGURE 2 Outer-fiber doublet microtubules of *Strongylocentrotus*, negatively stained with uranyl acetate. All microtubules were treated with serum before staining. The microtubules on the left (*a* and *c*) were treated with control serum from an unimmunized rabbit, whereas those on the right (*b* and *d*) were treated with anti-*Arbacia*-axoneme serum. The upper microtubules (*a* and *b*) were treated with serum and rinsed on the grid, whereas the lower ones (*c* and *d*) were treated in a centrifuge tube and then washed twice with saline before putting them on the grid and staining. The singlet tubules seen in *c* and *d* are commonly found in doublet preparations (cf. Fig. 1), and result from solution of one of the two tubules, presumably the B tubule (41). $\times 150,000$.

seems to precipitate all over the tubulin, rather than in any selective place. The characteristic camouflaging of the microtubules treated with antiserum on the grid (Fig. 2 *b*) was observed regularly.

The tubulin subunits are too close-spaced about 40 Å (43)—for the antibodies to “decorate” the tubules in a specific pattern (cf. reference 13). The antitubulin antibodies appear to simply coat the tubules, as antibodies against bacterial flagellin coat bacterial flagella (2).

The uniform deposition of antibody on outer

fibers appears to exclude any possibility that the reaction is with a “contaminating” protein. Since absorption with intact outer fibers removes the ability to react with solubilized tubulin, it appears that the same antibodies react with similar antigenic determinants on both intact tubules and solubilized tubulin. Thus, the main precipitin band is formed by a reaction of tubulin with anti-tubulin antibodies.

The antigenic activity of outer-fiber tubulin is quite stable. Normally the antigen was assayed after dissolving the outer fibers in 0.5% Sarkosyl

(39), under which conditions the antigenic activity remained after several months of storage at 0°C. The precipitin band also formed after the outer fibers were solubilized in the mercurial salyrgan (40), and even after reduction and alkylation of the protein in 8 M urea (33). Though these preparations form the main precipitin band, in some cases the antigenic activity was reduced.

Diverse Echinoids Have Similar Antigenic Determinants in Their Outer-Fiber Tubulins

Outer-fiber tubulins from sperm flagella or diverse echinoid species (all those listed in Table I) all gave the main precipitin band with the anti-*Arbacia* serum. Fig. 3 shows the marked similarity of the outer-fiber antigens from sea urchins of two orders, *Arbacia* and *Strongylocentrotus*, and a sand dollar, *Echinarachnius*, in a separate subclass. The

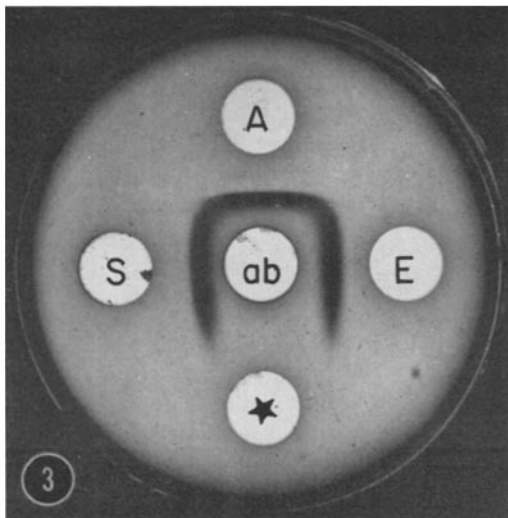


FIGURE 3 Similarity of outer fiber tubulins from various echinoid species. An antiserum (*ab*) prepared using *Arbacia* axonemes forms a continuous precipitin band against outer fiber preparations from *Arbacia* (*A*), *Strongylocentrotus* (*S*), *Echinarachnius* (*E*), and other sea urchins not shown. Small spurs from *A* toward both the *S* and *E* antigens indicate the presence of antigenic determinants in *A* that are lacking in *S* and *E*. The antiserum does not form this precipitin band against outer fibers from the starfish *Asterias* (*). The outer fiber preparations were tested in 0.5% Sarkosyl, with *E* at 65 μ g protein in the well and the others at 105–115 μ g.

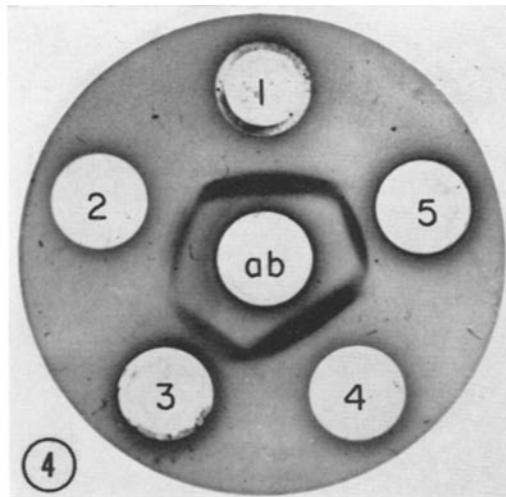


FIGURE 4 Similarity of several *Arbacia* microtubule extracts, tested against anti-axoneme serum (*ab*). The extracts compared are (protein per well is given in parentheses): (1) Outer fibers from sperm flagella (44 μ g, in Sarkosyl); (2) central-pair extract of sperm flagella (23 μ g); (3) crude outer fibers from cilia of blastulae and gastrulae (60 μ g, in Sarkosyl); (4) acetone powder protein of sperm flagella (23 μ g); and (5) mitotic apparatus extract (62 μ g). The main precipitin band is continuous, and bends at each junction, except between flagellar central-pair protein and ciliary outer-fiber tubulins.

antigens of different species often were so similar as to be indistinguishable in double-diffusion tests, giving an Ouchterlony “reaction of identity”, though slight spurs, observed especially with *Echinarachnius* but also with some other species, indicate that the antigenic determinants probably are not identical. The outer fibers of *Strongylocentrotus*, like those of *Arbacia*, have been extensively studied. Both gave a visible band with as little as 1 μ g protein per well. *Echinarachnius* outer fibers gave a visible band to 2 μ g per well (which is not a significant difference; see Methods). Absorption of the antiserum with *Echinarachnius* outer fibers removed all detectable precipitating activity against *Arbacia* outer-fiber tubulins.

The retention of structural homology during evolution is not unlimited. Outer fibers isolated from sperm of the starfish *Asterias forbesi*, a member of the class Asteroidea, did not form the main precipitin band with the *Arbacia* antiserum (Fig. 3), even when the *Asterias* outer fibers were tested with 500 μ g protein in the Ouchterlony well.

Thus, the tubulin antigenic determinants measured by the antiserum are not shared between the two classes, and do not extend throughout the echinoderms.

Tubulins from Different Classes of Microtubules Share Similar Antigenic Determinants but They Probably Are Not Identical Molecules

Tubulins from several different classes of microtubules, in addition to flagellar outer fibers, formed the main precipitin band with the antiserum. A sample of the reactions with extracts from *Arbacia* is shown in Fig. 4. In most cases where two tubulin antigens have been compared, there was continuity at the junction of the two precipitin bands, with fusion and bending of the band. Often spurs were observed at the junction, indicating that some but not all the antigenic determinants are shared. The "fusion" reactions observed indicate the presence of similar antigenic determinants, but cannot be considered to demonstrate identity or nonidentity of the antigen molecules (see 9, 28, 29, 55, 56).

Although no echinoid tubulins have failed to form the main precipitin band, there are clear indications that not all the tubulin molecules are

identical, even within a single species. Most of the evidence for differences is based on semiquantitative comparisons of the amount of antigen required to give a visible precipitin band. The antigens tested with two species, *Arbacia* and *Pseudoboletia*, are listed in Table II.

Several tubulin-containing extracts were studied.

OUTER FIBERS OF SPERM FLAGELLA: As already described, flagellar outer-fiber tubulins gave a visible precipitin band to 1-3 μg protein per well. Both the A- and B-tubulins, isolated from *Strongylocentrotus*, formed the main precipitin band. The A-tubulin formed a visible band to about 3 μg protein per well, whereas the B-tubulin band only remained visible to about 8 μg per well, a reproducible, but barely significant, difference. The A-tubulin formed a spur toward the B-tubulin. Thus, at least after the fractionation procedure, which could itself alter the antigenic determinants (see reference 56), the A-tubulin has antigenic determinants not present in the B-tubulin.

CENTRAL PAIR PROTEIN FROM SPERM FLAGELLA, AND ACETONE POWDER EXTRACTS FROM SPERM FLAGELLA: Either of these extracts, prepared from flagella of *Arbacia*, *Pseudoboletia*, or *Strongylocentrotus*, gave precipitin bands with as little as 1-2 μg protein per well. Both extracts are mainly tubulin; the central-pair extract

TABLE II
Extracts of Arbacia and Pseudoboletia Tested for the Presence of Microtubular Antigen
The amount of antigen required or tested by Ouchterlony immunodiffusion is given as microgram protein per well.

Preparation		<i>Arbacia</i>	<i>Pseudoboletia</i>
A. Extracts giving a positive reaction. Amounts given are those required to give a visible precipitin band (see Methods).			
Sperm flagella	Outer fibers*	1	3
	Central pair extract	1	2
	Acetone powder	1	2
Blastular cilia	Outer fibers*	30	40
	Extract	2‡	≥300
Unfertilized egg	Extract	8	30
	6S proteins	1	—
B. Extracts giving no reaction under any conditions tested. Amounts given are the maximum tested.			
22S protein from unfertilized eggs		600	—
Hyalin from unfertilized eggs		330	—
Actin from adult lantern muscle		200	—

* Tested in 0.5% Sarkosyl.

‡ Best preparation; the least active required 40 μg protein per well.

may contain some outer fiber protein and the acetone powder extract may contain some central pair protein.

All pairwise combinations of these sperm flagellar tubulins—outer fibers in Sarkosyl, central-pair protein, and acetone powder extracts—gave clear fusion of the precipitin bands with bending at the junctions, indicating the presence of shared antigenic determinants. Absorption of the antiserum with central-pair protein (from *Strongylocentrotus*) removed all activity against outer fibers, and vice versa, absorption with outer fibers removed all activity against central-pair protein.

OUTER FIBERS OF CILIA FROM BLASTULAE AND GASTRULAE: Sarkosyl-solubilized ciliary outer fibers from embryos of *Arbacia*, *Colobocentrotus*, and *Strongylocentrotus* all gave the main precipitin band with the antiserum, and this band fused with the band formed against sperm flagellar outer fibers of the same species. It has not been possible, however, to get clear fusion between the precipitin bands of ciliary outer fibers and of sperm-flagellar central-pair protein from the same species (e.g., Fig. 4). The ciliary outer-fiber preparations were relatively unreactive. The best preparations from *Arbacia*, for example, required 30 μg protein per well to give a visible band, which is roughly 30 times the amount required with sperm flagellar outer fibers. These ciliary outer fibers were contaminated with membrane material, but it seemed unlikely that the great difference in antigen titer was due to greater impurity of the ciliary outer fibers.

The clearest evidence for a difference between echinoid tubulins came from careful comparison of the outer fibers from sperm flagella and blastular cilia of *Pseudoboletia*. In this species it proved straightforward to isolate pure outer fibers from both sperm and embryos. This was done by a procedure which allowed the two preparations to be done simultaneously as "replicates". Sperm flagella and blastular cilia were isolated, extracted with Triton X-100, dialyzed against low ionic-strength buffer, extracted with glycerol at -20°C , and extracted with dilute Sarkosyl. The result, in both preparations, was electron microscopically pure outer-fiber doublets, of similar morphology. These were dissolved in Sarkosyl for comparison. The two preparations gave immunodiffusion reactions indicating the presence of shared antigenic determinants. However, though the sperm flagellar outer fiber preparations reacted to give a visible band with as little as 3 μg protein per well, the

ciliary outer fibers of embryos required at least 40 μg per well (Table II), a difference of more than 10-fold. This comparison was repeated several times, with different preparations but similar results. Since these preparations were subjected to the same chemical treatments, and were of comparable purity, this result indicates that in *Pseudoboletia* the outer-fiber tubulins of cilia from embryos contained fewer antigenic determinants recognized by the antiserum than the tubulins of sperm flagella.

MITOTIC APPARATUS EXTRACTS: A relatively small proportion of the isolated mitotic apparatus consists of microtubules—less than 15% of the protein (8, 18)—and these tubules are quite labile. Spindle microtubules have never been isolated directly, so we used a method to selectively extract them (Fig. 5). Under our conditions of extraction the supernatant contained roughly 15% of the protein. The amounts of material were small, and no detailed chemical characterization of these extracts has been attempted.

Extracts from mitotic apparatuses of *Arbacia* gave the main precipitin band (Fig. 4), and this band coalesced with the bands produced by sperm outer fibers, central-pair protein, acetone-powder extracts, but showed poor homology with ciliary outer fibers. Two mitotic apparatus extracts were tested quantitatively; these varied tremendously in activity. One gave a visible band at dilutions as low as 2 μg , and the other 40 μg , protein per well. This difference is presumably due to variability in the proportion of tubulin and of other material solubilized in the procedure. The degree to which the mitotic apparatus ghosts retained structural integrity varied considerably.

Similar extracts of mitotic apparatuses from *Pseudoboletia* were nearly inactive. Of several tested, none gave a reaction with less than 300 μg of protein. This is 100-fold more than the amount required with outer fibers, but since these mitotic apparatus extracts have not been characterized this result is less definite evidence for a difference between tubulins than the difference between flagellar and ciliary outer fibers.

UNFERTILIZED EGG EXTRACTS of *Arbacia* and of *Strongylocentrotus* were fractionated to give the material sedimenting at 4–6S (18, 37), and this material gave the precipitin band with as little as 1 μg protein per well (Table II).

Two other proteins from echinoid eggs, both known not to be tubulins, and chemically quite different, gave no reactions. The 22S protein, a

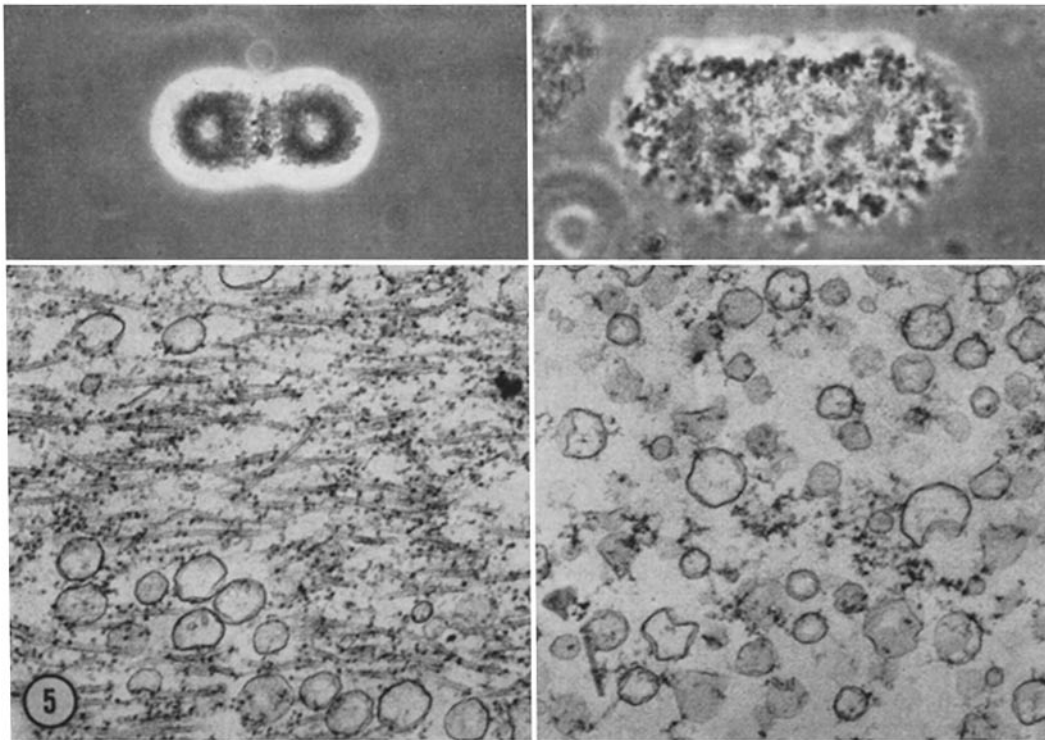


FIGURE 5 Mitotic apparatuses of *Arbacia*, before (left) and after (right) extraction of microtubules. The phase-contrast micrographs show a typical metaphase mitotic apparatus, and the swollen ghost that remains after extraction. Microtubules are abundant in the mitotic apparatuses before extraction, but are absent after extraction. $\times 600$ and $\times 36,000$.

major component of the isolated mitotic apparatus but not a component of microtubules (18, 37, 38), was inactive (Table II). Bibring and Baxandall (5) have studied an antiserum against 22S protein, and found that it does not react with microtubule preparations. In addition the protein hyalin (44), a component of the cortical granules, was isolated from *Arbacia* and *Colobocentrotus* eggs, and was inactive at all tested concentrations.

ACTIN: Since actin has been found similar to tubulin by superficial chemical criteria, a serological relatedness was also sought. Actin isolated from the lantern muscles of adult *Arbacia* gave no trace of a reaction with the antiserum, even when it was tested at $200 \mu\text{g}$ protein per well. Thus, no serological similarity could be found between the actin and the tubulins of *Arbacia*.

DISCUSSION

This study has demonstrated that it is possible to obtain antibodies to tubulins of echinoids, and

that similar antigenic determinants are widespread among diverse echinoid tubulins from different classes of microtubules and different species of sea urchins. All echinoid tubulins tested have given a reaction with the antitubulin antibodies, though the reactions obtained indicate that the tubulins are not identical. Thus, this serological study provides evidence of a new kind for the conclusion that, in the Orwellian sense, all microtubules are equal, but some microtubules are more equal than others.

There have been several previous serological studies of sea urchin sperm and mitotic apparatus, but none of these have been concerned specifically with microtubules. Went (52, 53), in his study of the mitotic apparatus, found an antigen, "precursor-2," common to the mitotic apparatus, unfertilized eggs, extracts of ovaries and testes, but not of gut, and common to three echinoid species (reference 52: Figs. 7 and 8). In a prophetic study, Ruby (34) found that antibodies to an "actin-like"

extract of sperm flagella reacted with a mitotic apparatus extract. Others have found antigens common to sperm and eggs (20, 30).

Comparisons of the outer-fiber tubulins from sperm flagella of several species revealed that some common antigenic determinants have been retained during evolution of six members of the class Echinoidea, but not one representative of the Asteroidea. Other authors have seen the conservation of antigenic similarities in echinoderms. Studies of sperm agglutination by antiserum have shown that various echinoids possess similar antigens, but that this similarity does not extend to starfish (22, 50). However, other serological studies have found not only some reactions between echinoids and asteroids, but even a little between echinoids and chordates (1, 54).

Though tubulin is an actin-like protein, it is increasingly clear that there are many chemical differences between tubulins and actin (41, 42, 45). Our experiments revealed no serological similarity.

Interpretation of our results is limited to the qualitative conclusion that tubulins are serologically similar but not identical. A more quantitative evaluation of the degree of similarity among tubulins from different classes of microtubules, theoretically possible by the use of a method like micro-complement fixation, has not been attempted because of three technical limitations. First, the immunizing antigen, axonemes, contained the tubulins of three different classes of microtubules: the A- and B-subfibers of outer fibers and the central pair fibers. It is possible that the antiserum contains a mixture of antibodies to antigenic determinants common to and unique to the specific tubulins. If this is so, different combinations of these antibodies may have reacted with each tubulin tested, thus suggesting more similarity among the tubulins than is present. Second, of the tubulin antigens studied, only the outer-fiber doublets were purified. Other extracts may contain mixtures of tubulins. Central-pair extracts, for example, may contain outer-fiber tubulins. Again, some of the reactions suggesting similarity could be due to mixtures of tubulins in the extracts. Third, the different classes of microtubules, and their tubulins, differ in solubility and in the chemical treatments required to isolate and solubilize them. The different treatments could result in chemical or physical alteration of the tubulins and their antigenic determinants during

isolation. Because of these limitations, the qualitative Ouchterlony procedure for comparing antigens seems as direct and simple as any that could be employed at present. It should be emphasized that none of these limitations undermine the qualitative evaluation of similarities and differences presented here.

The clearest evidence for a serological difference between tubulins is the marked difference in the amount of protein required to give a visible precipitin band in the comparison of outer-fiber proteins of sperm flagella and blastular cilia from *Pseudoboletia*. Since these tubulins were exposed to the same treatments during isolation and solubilization, the difference cannot be due to alteration of the antigenic determinants during preparation. It is unexpected to find this difference between the tubulins of outer fibers of very similar organelles, of similar structure and presumably similar function. Yet, these two outer-fiber tubulins of a single species share fewer antigenic determinants than the outer fiber tubulins from sperm flagella of different species. This is explainable if it is assumed that the ciliary and flagellar tubulins are made by different structural genes. Presumably, the common ancestor of all echinoids already had flagellated sperm and ciliated embryos, so the evolutionary divergence of these ciliary and flagellar tubulins started long before the evolution of different echinoid species.

Stephens (41) clearly demonstrated small differences in the primary structure of the A- and B-tubulins of *Strongylocentrotus* sperm flagella. These differences probably indicate that the A- and B-tubulins are the product of different structural genes, but they could result from alteration of the tubulins after synthesis (41). Linck (23) has found differences in the solubility of outer fibers from sperm flagella and gill cilia in the scallop *Aequipecten irradians*. In the case of the serological differences, we do not know what causes them. Since the echinoid flagellar and ciliary outer fibers can be purified, comparison of the primary structure of these proteins can be made to determine the nature of the differences.

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