

# Localization of Tektin Filaments in Microtubules of Sea Urchin Sperm Flagella by Immunoelectron Microscopy

R. W. LINCK,\* L. A. AMOS,<sup>‡</sup> and W. B. AMOS<sup>§</sup>

\*Department of Anatomy, Harvard Medical School, Boston, Massachusetts 02115; <sup>‡</sup>Laboratory of Molecular Biology, Medical Research Council, Hills Road, Cambridge CB2 2QH, U.K.; <sup>§</sup>Department of Zoology, Cambridge University, Downing Street, Cambridge, CB2 3EJ, U.K. Dr. Linck's present address is Department of Anatomy, University of Minnesota, Minneapolis, Minnesota 55455

**ABSTRACT** Extraction of doublet microtubules from the sperm flagella of the sea urchin *Strongylocentrotus purpuratus* with sarkosyl (0.5%)–urea (2.5 M) yields a highly pure preparation of “tektin” filaments that we have previously shown to resemble intermediate filament proteins. They form filaments 2–3 nm in diameter as seen by negative stain electron microscopy and are composed of approximately equal amounts of three polypeptide bands with apparent molecular weights of 47,000, 51,000, and 55,000, as determined by SDS PAGE. We prepared antibodies to this set of proteins to localize them in the doublet microtubules of *S. purpuratus* and other species. Tektins and tubulin were antigenically distinct when tested by immunoblotting with affinity-purified antitektin and antitubulin antibodies. Fixed sperm or axonemes from several different species of sea urchin showed immunofluorescent staining with antitektin antibodies. We also used antibodies coupled to gold spheres to localize the proteins by electron microscopy. Whereas a monoclonal antitubulin (Kilmartin, J. V., B. Wright, and C. Milstein, 1982, *J. Cell Biol.* 93:576–582) decorates intact microtubules along their lengths, antitektins labeled only the ends of intact microtubules and sarkosyl-insoluble ribbons. However, if microtubules and ribbons attached to electron microscope grids were first extracted with sarkosyl-urea, the tektin filaments that remain were decorated by antitektin antibodies throughout their length. These results suggest that tektins form integral filaments of flagellar microtubule walls, whose antigenic sites are normally masked, perhaps by the presence of tubulin around them.

It has been shown that sperm flagellar doublet microtubules can be dissociated in a number of ways to produce chemically resistant ribbons of two to four protofilaments (pf's)<sup>1</sup> (24, 31, 42, 43). More recently, the ribbons have been further fractionated with chemical agents to yield fibrous proteins that have been partially characterized (29). The proteins form highly insoluble filaments with physico-chemical properties similar to intermediate filaments. These insoluble filaments were referred to as “tektin filaments” and the constituent polypeptides as “tektins” (25, 26). The number and identity of the tektins was uncertain but they were tentatively identified as a group of three or more polypeptides with subunit molecular weights ranging from 46,000 to 68,000. Although the tektins were postulated to form longitudinal filaments or

pf's in the wall of the A-tubule of the doublet (25), proof for this model was lacking.

We have begun a new series of investigations to further define the properties and organization of the tektins in cilia and flagella. We have prepared antibodies against the tektins, and by immunoelectron microscopy (immuno-EM) have obtained evidence that these novel proteins exist as integral, filamentous components of the microtubule wall.

## MATERIALS AND METHODS

*Preparation and Purification of Proteins and Antibodies:* Sperm flagella axonemes and doublet microtubules were purified from the sea urchins *Strongylocentrotus purpuratus*, *Lytechinus pictus*, *Echinus esculentus*, and *Psammechinus miliaris* according to standard procedures (15, 28). Gill ciliary axonemes were isolated from the scallop *Aequipecten irradians*. In all preparations, the purity of the axonemes was judged by phase-contrast microscopy to be in excess of 100,000 to 1, sperm flagella (40  $\mu$ m per flagellum) to sperm heads. Axonemes were dialyzed extensively against 1 mM Tris-HCl, 0.1 mM EDTA (pH 8.0), to produce mixtures of doublet and singlet microtubules.

<sup>1</sup> Abbreviations used in this paper: anti-(*S.p.*) tektins, anti-(*Strongylocentrotus purpuratus*) tektins; EM, electron microscopy; FITC, fluorescein isothiocyanate; IgG, immunoglobulins; immuno-EM, immunoelectron microscopy; pf, protofilament.

and pf ribbons that result from depolymerization of A-tubules. To obtain the insoluble tektin filament fraction for immunization, we modified our previous procedure (29): doublet microtubules were twice extracted with a solution of sarkosyl-urea (0.5% sarkosyl [sodium dodecyl sarcosinate], 2.5 M urea, 10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) on ice for 2 h and centrifuged at 100,000 *g* for 2 h.

Mice were immunized with a sonicated mixture of this sarkosyl-urea-insoluble material with Freund's adjuvant. Antitekton immunoglobulins (IgG) were affinity-purified from the resulting antisera, using the antigen coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ). Bound IgGs were eluted from the column with 0.2 M glycine-HCl and the pH of the solution rapidly adjusted to neutral by adding drops of 3 M Tris. In subsequent immunolabeling experiments for fluorescence and electron microscopy, IgG with affinity for tubulin was absorbed out by passing the antitekton fraction through a Sepharose column to which was coupled trice-cycled, phosphocellulose-purified calf brain tubulin that had been prepared by the procedure described by Sloboda and Rosenbaum (34).

**SDS PAGE Immune-replica Procedures:** SDS PAGE was performed according to the method of Laemmli (23). SDS PAGE immune-replica procedures were modified slightly from those of Towbin et al. (39), in that only 15% methanol was used. Strips were cut from the slab gels and nitrocellulose transfers and stained appropriately with one of the following: (a) Coomassie Brilliant Blue, (b) amido black, (c) an appropriate dilution of affinity-purified mouse antitekton IgG followed by <sup>125</sup>I-labeled rabbit anti-mouse IgG, or (d) rat monoclonal YL1/2 antitubulin antibody (prepared and characterized by Kilmartin et al. [22]), followed by <sup>125</sup>I-labeled rabbit anti-rat IgG. Care was taken to ensure precise registration of the stained strips. Before radio-iodination, the rabbit anti-rat and rabbit anti-mouse IgGs (Miles Laboratories, Slough, England) were affinity-purified on a Sepharose 4B column to which purified rat or mouse IgG had been coupled. Radio-iodination was carried out using Iodo-gen (Pierce Chemical Co., Chester, England) by the method of Fraker and Speck (12).

**Immunofluorescence Microscopy:** Whole sperm and purified axonemes were fixed on polylysine-treated multiwell slides with 3.5% formaldehyde in sea water or phosphate-buffered saline for 15 min at room temperature, followed by treatment with 100% methanol (6 min) and acetone (30 s) on dry ice (22). Specimens were incubated at room temperature for 30–60 min with either affinity-purified mouse antitektons (absorbed free of antitubulin) or with a monoclonal mouse anti- $\beta$ -tubulin (W. B. Amos, unpublished), followed in each case by fluorescein isothiocyanate (FITC)-labeled rabbit anti-mouse IgG. All antibodies were diluted with phosphate-buffered saline containing 1% bovine serum albumin, that was included to inhibit nonspecific binding of antibodies. Also, the wells were washed with this saline after each incubation. The FITC-labeled rabbit anti-mouse IgG was obtained from Miles Laboratories and affinity-purified as described above. Specimens were viewed and photographed with a Zeiss epifluorescence microscope.

**Immuno-gold Labeling and Electron Microscope (EM) Analysis:** For immuno-EM localization, colloidal gold particles ( $5 \pm 1$ -nm diameter) were prepared using white phosphorus according to the procedures of Faulk and Taylor (10) and Frens (13), as modified by Slot and Geuze (35) and Kilmartin et al. (22). The gold particles were then complexed with saturating amounts of the relevant immunoglobulins at their respective isoelectric points and washed free of unbound IgG by centrifugation in 1% bovine serum albumin, 10 mM Tris-HCl, 0.15 M NaCl (pH 7).

Microtubule specimens were applied to carbon-coated gold grids. Some samples were made 20  $\mu$ M in taxol (obtained from the Drug Development Branch, National Cancer Institute, Bethesda, MD), which was also included in the solutions subsequently applied to these grids. The grids were rinsed in wash buffer, consisting of Tris-buffered saline containing a soluble protein; similar results were obtained with each of the three proteins tried, namely 0.1–0.5% bovine serum albumin, 0.02–0.2% cytochrome *c* and 0.5% beef brain calmodulin. After the grids had been drained on filter paper, they were placed on drops of antibody in wash buffer for 30–60 min in a moist chamber at room temperature. At the end of the incubation, the grids were washed with seven drops of wash buffer and then left for 15 min on an eighth drop. After similar incubation in gold-labeled anti-mouse IgG and subsequent washing, the grids were negatively stained with 1% uranyl acetate (19). In addition, some grids were labeled with the monoclonal antitubulin, YL1/2. In this case, since the antitubulin itself had been labeled with colloidal gold, no incubation in a second antibody was required.

For in situ sarkosyl-urea extraction, *S. purpuratus* specimens were applied to the carbon grid and the excess material washed off. The microtubules and ribbons that remained sparsely attached to the carbon were rigorously extracted by washing rapidly with five drops of sarkosyl-urea solution, incubated on a sixth drop for 1 h at 2°C, followed by two more drops of sarkosyl-urea and seven drops of Tris-EDTA buffer. The grid sample was then processed by primary and secondary antibody treatments and negatively stained.

## RESULTS

### Fractionation of Flagellar Microtubules into Tektin Filaments

Flagellar microtubules from *S. purpuratus* axonemes were fractionated into singlet microtubules following previously published procedures (37), into sarkosyl-insoluble ribbons of two to four pf's (24, 31, 42, 43) similar to those in Fig. 7, *e* and *f*, and into a sarkosyl-urea insoluble filamentous material (29). In negative stain, the latter appears as thin filaments (and groups thereof), each measuring 2–3 nm in diameter (Fig. 4, *b* and *c* [see also Fig. 9 in reference 29]). Fig. 1 shows

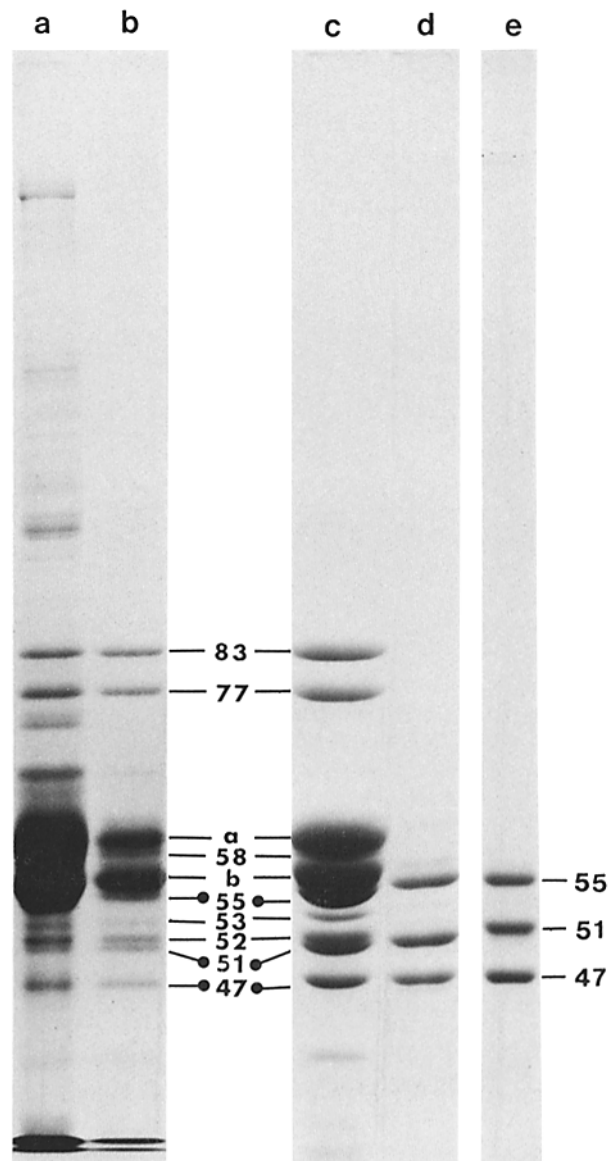


FIGURE 1 SDS PAGE of *S. purpuratus* flagellar microtubule fractions stained with Coomassie Blue. Calibrated molecular weights are given  $\times 10^{-3}$ ;  $\alpha$ -tubulin (a) and  $\beta$ -tubulin (b) are indicated. (Lane a) Singlet A-tubules (100,000 *g*-h pellet derived from 37.5  $\mu$ g doublet microtubules after heating [see references 28, 37]); (lane b) sarkosyl-insoluble pf ribbons (pellet derived from 0.75% sarkosyl extraction of 37.5  $\mu$ g doublet tubules); (lane c) sarkosyl-insoluble pf ribbons (pellet derived from 0.5% sarkosyl extraction of 130  $\mu$ g doublet tubules); (lane d) sarkosyl-urea insoluble fraction (pellet derived from extraction of 130  $\mu$ g doublet tubules with 0.5% sarkosyl and 2.5 M urea); (lane e) insoluble material obtained after two extractions of doublet microtubules with sarkosyl-urea.

the polypeptide composition of each of these three fractions. The tektins, a set of polypeptides with molecular weights of 47,000, 51,000, and 55,000, are present in each case. After two extractions with 0.5% sarkosyl and 2.5 M urea (Fig. 1, lane *e*), these three bands, present in approximately equal amounts, constitute >95% of the Coomassie-stained protein. As reported briefly by Linck and Langevin (29), the sarkosyl-urea extraction gives a significantly better purification of the tektins than urea alone or sodium thiocyanate. Apart from tubulin and the three tektin bands in the final filament fraction, the ribbons (Fig. 1, *b* and *c*) contain other proteins (notably those with apparent molecular weights of 77,000 and 83,000), but little is known about their properties (29).

### Characterization of Antitekton Antibodies

Mouse antibodies were raised against the complete sarkosyl-urea-extracted tektin filament complex from *S. purpuratus* sperm flagella, affinity-purified using the original antigen coupled to Sepharose 4B, and characterized as to their specificity using SDS PAGE immune-replica staining techniques (39). These results are shown in Fig. 2. The affinity-purified anti-(*S. purpuratus*) tektin antibodies (referred to here as anti-(*S.p.*) tektins) react disproportionately with the three principal tektins (lane *c*: anti-47,000 > anti-51,000 > anti-55,000). The monoclonal YL1/2 antitubulin (22), which is specific for tyrosylated  $\alpha$ -tubulin (41), does not bind to the tektins with

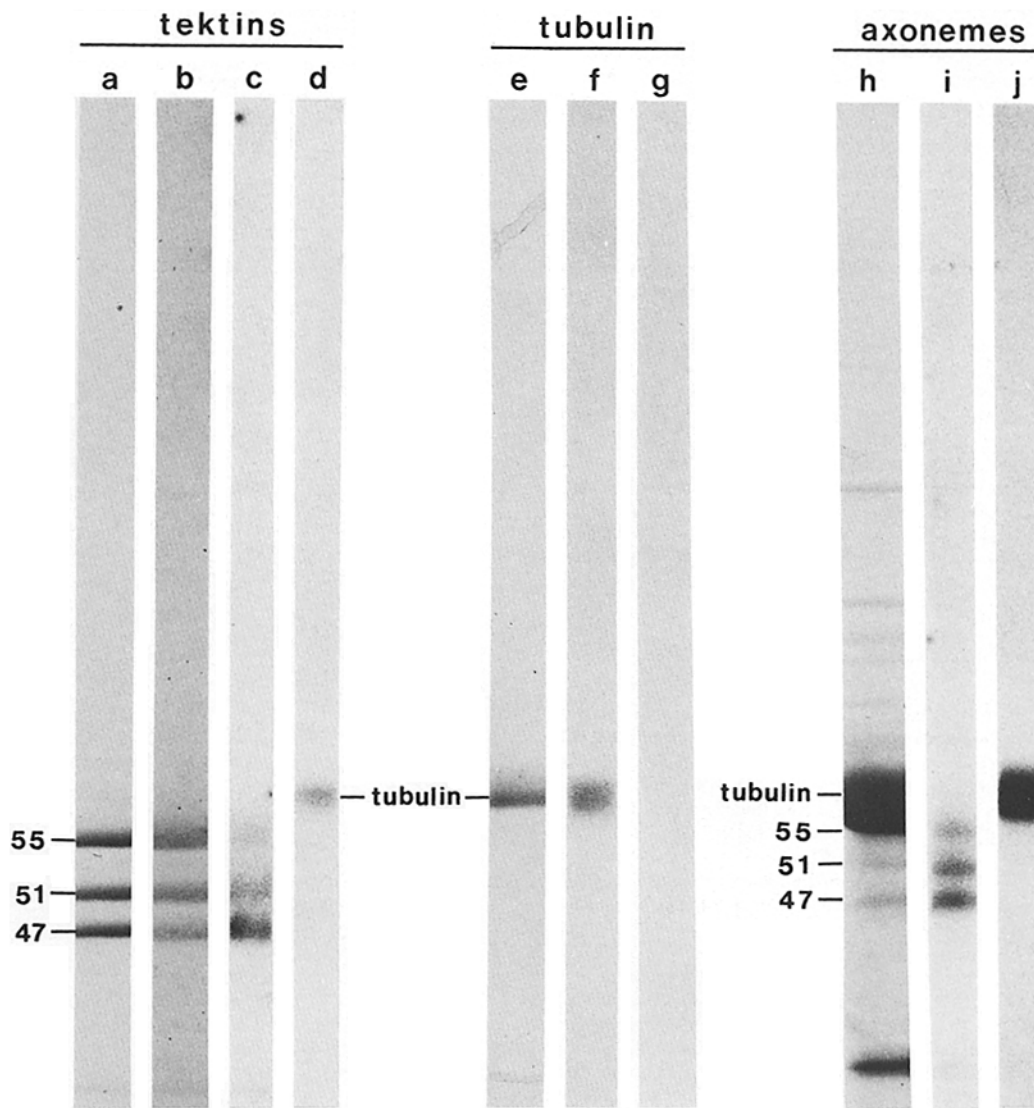


FIGURE 2 Characterization of affinity-purified antitekton antibodies by SDS PAGE immune-replica staining. SDS PAGE was carried out on samples of sarkosyl-urea-extracted tektin filaments from *S. purpuratus* (~12  $\mu$ g per lanes a-d), calf brain tubulin, phosphocellulose-purified (4  $\mu$ g per lanes e-g), and whole (25  $\mu$ g per lanes h-j) flagellar axonemes. Relevant molecular weights are indicated  $\times 10^{-3}$ . The slab gel lanes were cut and treated as follows: *Tektins*: (Lane a) Gel stained with Coomassie Blue; (lane b) transferred to nitrocellulose paper and stained with amido black; (lane c) autoradiograph of replica incubated in affinity-purified mouse antitekton antibody, followed by  $^{125}$ I-rabbit anti-mouse IgG; (lane d) autoradiograph of replica incubated in YL1/2 rat monoclonal antitubulin, followed by  $^{125}$ I-rabbit anti-rat IgG. *Tubulin*: (Lane e) Gel stained with Coomassie Blue; (lane f) autoradiograph of replica incubated in YL1/2 rat antitubulin, followed by  $^{125}$ I-rabbit anti-rat IgG; (lane g) autoradiograph of replica incubated in mouse antitekton antibodies, followed by  $^{125}$ I-rabbit anti-mouse IgG. *Axonemes*: (Lane h) Stained with Coomassie Blue; (lane i) autoradiograph of replica incubated with mouse antitekton antibodies followed by  $^{125}$ I-rabbit anti-mouse IgG; (lane j) autoradiograph of replica incubated with YL1/2 rat antitubulin, followed by  $^{125}$ I-rabbit anti-rat IgG.

apparent molecular weights of 47,000, 51,000, or 55,000<sup>2</sup> (lanes *d* and *j*), but does reveal a residual amount of tubulin present in the original tektin filament preparation (lane *d*). This amount is below the level of detection with Coomassie Blue or amido black staining. In spite of this residual trace of tubulin in the injected antigen, antitektons do not recognize sea urchin flagellar or calf brain tubulin (lanes *c*, *g*, and *i*). Thus, under the conditions of the SDS PAGE immune-replica technique, the antibodies to the 47,000-, 51,000-, and 55,000-mol-wt tektins are specific to these three protein bands and do not cross-react strongly, if at all, with other polypeptides in *S. purpuratus* flagellar axonemes. However, to ensure that the IgG fraction used for the structural work described below contained no trace of antitubulin affinity, it was passed through a tubulin-Sepharose column first.

The anti-(*S.p.*) tektins also cross-react with similar but not identical sets of proteins in SDS PAGE replicas of axonemes from cilia and flagella of other sea urchin species, but their affinity is somewhat reduced (data not shown); proof of cross-reaction is also seen in the structural studies that follow.

### Immunofluorescence Staining

Demembrated, purified axonemes from *S. purpuratus* and three other species of sea urchin sperm were fixed on multiwell slides as described under Materials and Methods, and incubated with purified mouse anti-(*S.p.*) tektins followed by FITC anti-mouse IgG. Provided the initial formaldehyde fixation was done in a simple saline, all showed uniform immunofluorescent staining (e.g., Fig. 3 *c*), although the antitekton concentration required for a positive result was 100 times higher for *L. pictus*, *E. esculentus*, or *P. miliaris* than for *S. purpuratus*. If millimolar concentrations of EDTA and MgCl<sub>2</sub> were added to the fixation medium, however, no staining was observed. Preimmune mouse serum, used as a control during the initial screening of the immune sera, also gave no fluorescent staining, no matter how the axonemes were fixed. Mouse monoclonal antitubulin gave staining after either type of fixation.

To check that the Triton extractions carried out during axoneme purification were not causing a redistribution of the antigens, we also examined whole fixed sperm from *S. miliaris* by indirect immunofluorescence staining, both with the affinity-purified mouse anti-(*S.p.*) tektins and with the mouse monoclonal antitubulin. Both treatments stained the flagella (i.e., axonemes) and basal body regions (Fig. 3). The antitektons also stained sperm heads reproducibly, whereas antitubulin sometimes stained the heads and sometimes did not, in an unpredictable manner.

### Immuno-EM

To study the possible molecular organization of the tektins in microtubules, we conducted several sets of EM investigations, using immunolabeling and negative staining techniques. 5-nm gold particles were not difficult to detect in thin uranyl acetate stain, especially since they often appear with a light halo due to the layer of antibody coating the gold surface and excluding the negative stain (for clearest examples, see Figs. 4 *b*, 5, *i* and *j*, and 7 *c*).

<sup>2</sup> Whole rabbit antisera against vinblastine-tubulin crystals from *S. purpuratus* eggs also does not cross-react with these proteins on SDS PAGE immune-replicas (R. W. Linck and K. Fujiwara, unpublished observations).

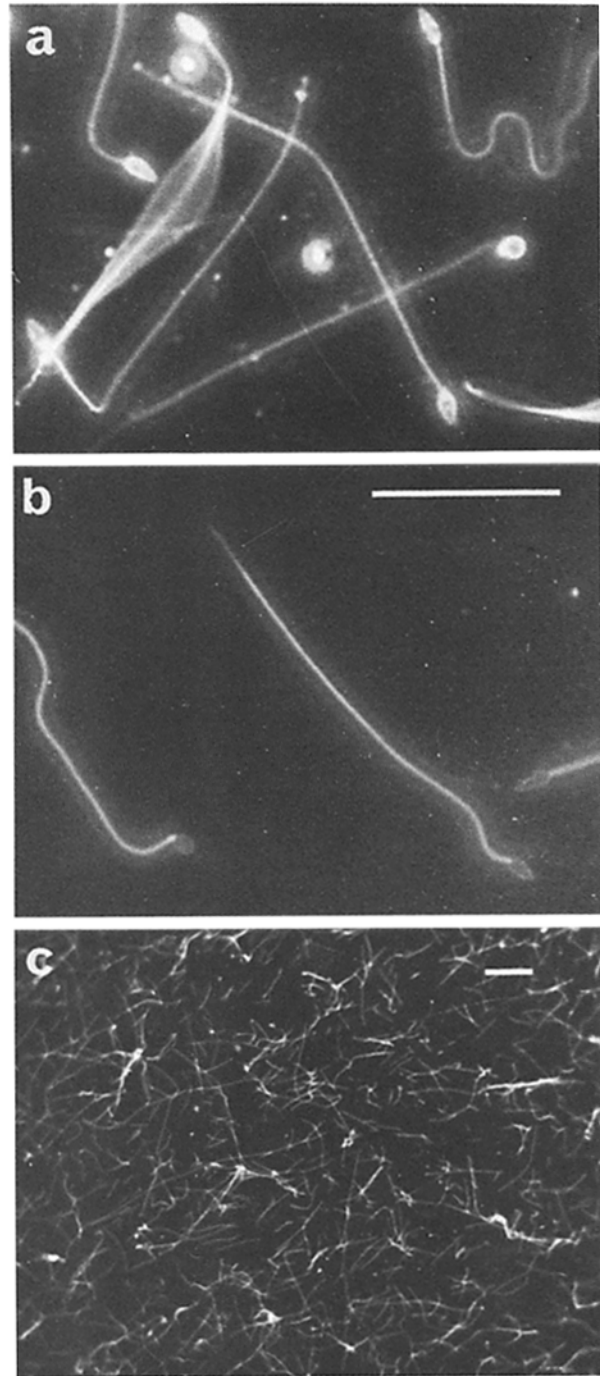
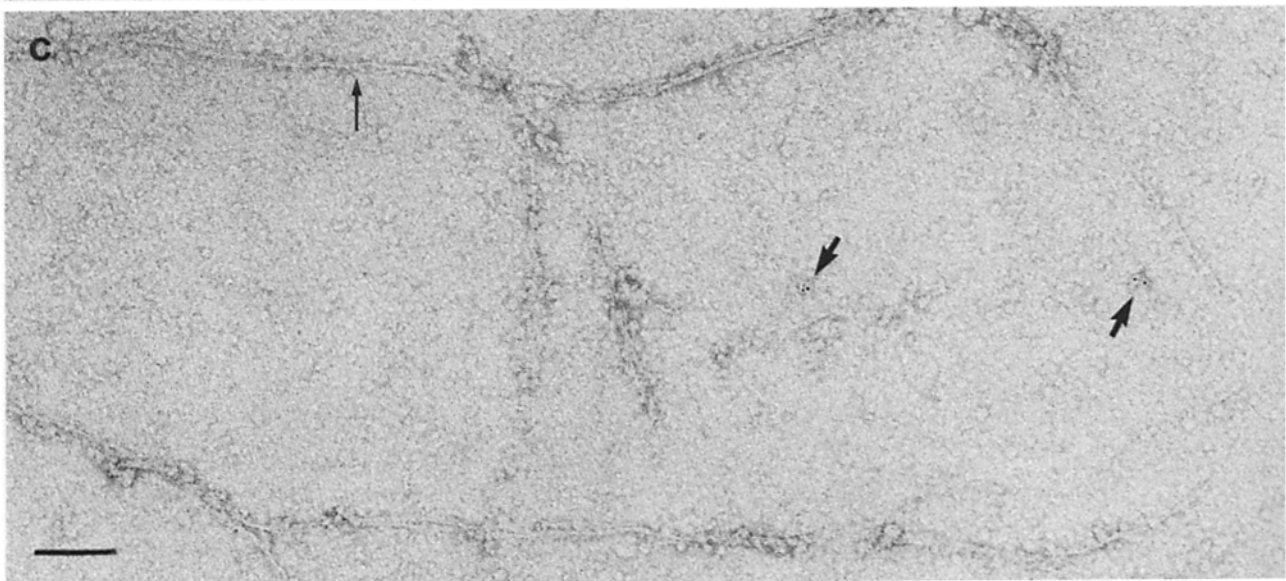
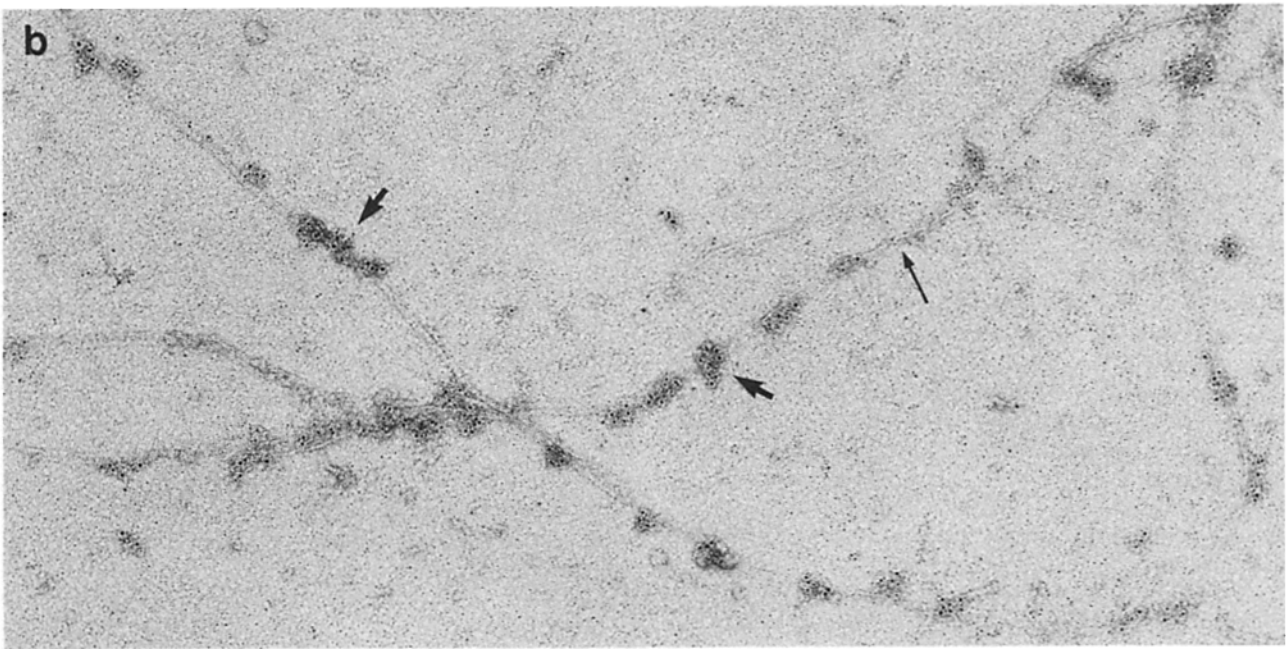
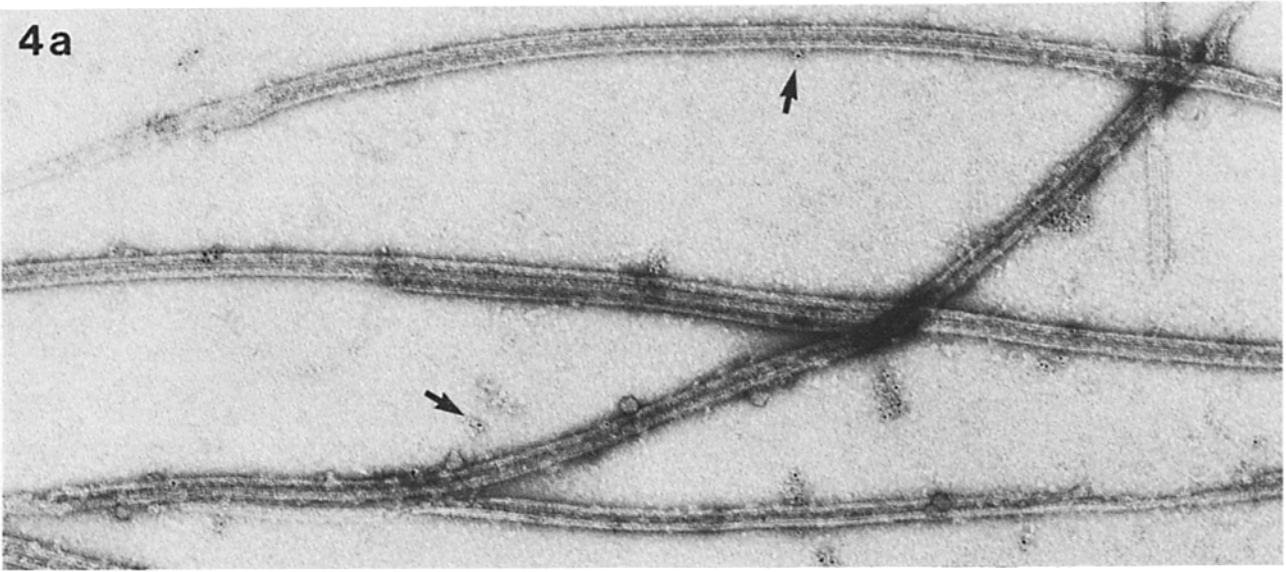


FIGURE 3 (a and b) Immunofluorescence staining of whole fixed sperm from *P. miliaris*. (a) Sperm stained with anti-(*S.p.*) tektins (primary antibody) and FITC-rabbit anti-mouse (secondary antibody). (b) Monoclonal mouse anti- $\beta$ -tubulin, followed by FITC-rabbit anti-mouse. (c) Immunofluorescence staining of isolated axonemes from *E. esculentus*, stained with anti-(*S.p.*) tektins and FITC-rabbit anti-mouse. No staining of axonemes was observed if preimmune mouse serum was used in place of primary antibody. Bars, 50  $\mu$ m. (a and b)  $\times$  100. (c)  $\times$  25.

When *S. purpuratus* preparations of doublet and singlet microtubules and pf ribbons were applied to carbon-coated grids, incubated with affinity-purified mouse antitektons and gold-labeled rabbit anti-mouse IgG, as described in Materials and Methods, none of the microtubules and pf ribbons appeared to decorate with antibody markers (Fig. 4 *a*). Reason-



ing that in such mildly treated specimens, the tektins may be inaccessible to the antibody, we performed an in situ extraction with sarkosyl-urea (see Materials and Methods) of microtubules on the grid, followed by the antibody treatments. As seen in Fig. 4*b*, the extraction of individual microtubules in situ left behind multiple longitudinal strands of filaments 2–3 nm in diameter. The filaments became heavily decorated with gold particles when treated with mouse antitektins and gold-labeled rabbit anti-mouse IgG (Fig. 4*b*). In control experiments, where the primary antibody was omitted, no secondary gold-labeling was seen (Fig. 4*c*).

Preparations from *P. miliaris* behaved somewhat differently; the conditions described for incubating with primary and secondary antibodies on the EM grids (see Materials and Methods) were sufficient to break down the microtubules and pf ribbons into filaments and amorphous aggregates (Fig. 5, *i* and *j*). The filaments became labeled with gold in a similar fashion to the *S. purpuratus* sarkosyl-urea filaments. When 20  $\mu$ M taxol was included as a microtubule-stabilizing agent (32) in the incubation buffers, the doublet microtubules broke down only to pf ribbons (Fig. 5, *a–h*, *k*), similar to those produced by sarkosyl extraction (Fig. 7, *e* and *f*). Gold-labeling was then found mostly at the broken ends of pieces of ribbon (as analyzed in Fig. 6), from which thin filaments 2–3 nm in diameter were often seen protruding (Fig. 5, *c–g*).

As an additional control and as a check on the immunolabeling procedure, dialyzed flagellar microtubules from *P. miliaris* were decorated with monoclonal YL1/2 antitubulin that had been labeled directly with gold (Fig. 7*a–d*). In this case, although the doublet microtubules tended to break down to singlet A-tubules, most of the latter remained intact without taxol, either because the incubation was shorter (in only one antibody), or because antitubulin stabilized the polymerized state. The gold label appeared preferentially on singlet A-tubules and on pf ribbons; the tubulin in doublet microtubules may perhaps be masked by accessory proteins.

## DISCUSSION

### Protein Characterization and Identification

The goals of this work have been to purify and characterize further the tektins from sea urchin sperm flagellar microtubules and, using antibodies to these proteins, to localize them in whole sperm and particularly doublet microtubules. We have purified a tektin filament fraction from *S. purpuratus* that is composed almost exclusively of individual thin filaments and bundles of filaments 2–3 nm in diameter, as seen by negative stain EM (Fig. 4, *b* and *c*; see also Fig. 9 in reference 29), that are composed of three polypeptide bands with apparent molecular weights of 47,000, 51,000, and 55,000, present in roughly equimolar amounts, as judged by SDS PAGE. Similar results have been obtained from several

other species of sea urchin sperm and also the gill cilia of *A. irradians* (unpublished results).

The present work shows that the tektins are antigenically similar in different sea urchin species and quite distinct from tubulin. This conclusion is strengthened by the polyclonal nature of the antitektin preparations. The general properties of the tektins have suggested to us that they are related to the proteins of intermediate filaments (14, 25). They are certainly more similar to intermediate filament proteins than to tubulin, by several criteria. (*a*) Although the three tektins from *S. purpuratus* differ slightly in molecular weight and are heterogeneous in their isoelectric points (see reference 29), they cofractionate and are highly insoluble in solutions of sarkosyl and urea. (*b*) Their sensitivities to limited proteolytic cleavage by *S. aureus* protease are different from that of tubulin and similar for two of the tektins, the 47,000- and 51,000-mol-wt polypeptides (29). (*c*) As a group they appear as fine fibrils with high  $\alpha$ -helical content, as shown by circular dichroism (29). (*d*) Finally, x-ray fiber diffraction of this 3-polypeptide set yields strong  $\alpha$ -type patterns (5).

### Tektin Filament Localization and Organization

In an effort to determine to a first approximation the origin and organization of the tektins in sperm cells, we have employed immunolabeling techniques. Although we have been limited by the fact that our antibodies come from mouse antisera directed against the whole 3-polypeptide set of tektins, the antibodies are highly pure and specific for their antigens. The affinity-purified anti-(*S.p.*) tektins cross-react on SDS PAGE replicas only with the original three bands in gels of whole *S. purpuratus* flagellar axonemes; antitektins do not recognize tubulin or other axoneme proteins by this technique (Fig. 2).

Immunofluorescence staining with anti-(*S.p.*) tektins demonstrates that the antibodies cross-react with other species and that the antitektins stain (as does antitubulin) the entire length of sperm tails and isolated axonemes after a fixation procedure designed to expose a large proportion of antigenic sites (Fig. 3); thus the tektins are continuously arranged along the axoneme. The antitektins reproducibly stain the sperm heads approximately as intensely as they do the tails and basal body regions. The reason for this phenomenon requires further investigation. Since antitubulin also stains the heads sometimes, the antibody binding here may be nonspecific. Alternatively, tektinlike proteins may be present in the heads, perhaps in a form similar to intermediate filaments. In mammalian cells, intermediate filaments have been shown to be associated with centrioles (11, 20, 36, 40) and with the nucleus (6).

Though the tektins may originate solely from the heads, this is unlikely since the tails of whole sperm are stained under

---

FIGURE 4 Immuno-EM labeling of tektin filaments in microtubules and pf ribbons. The samples, after being spread on carbon-coated EM grids, were treated as indicated, followed by negative staining with uranyl acetate. (*a*) Intact microtubule sample, treated with purified mouse antitektins, followed by gold-labeled rabbit anti-mouse IgG (arrows). Neither microtubules nor pf ribbons are labeled with gold. (*b*) Adhered microtubules and pf ribbons extracted on the grid by rapid drop-wise addition of sarkosyl-urea to remove tubulin, then treated with mouse antitektins, followed by gold anti-mouse IgG. Strands of filaments 2–3 nm in diameter are left behind and are heavily labeled in stretches with antibody (large arrows). The unsaturated nature of the labeling is probably a result of using dilute antibody solutions to keep the nonspecific background low. (*c*) (Control). Adhered microtubules and pf ribbons extracted with sarkosyl-urea, followed by gold-anti-mouse IgG (no antitektins). The filamentous strands (large arrows) are not labeled with gold-particles. Bar, 100 nm (for *a–c*).  $\times 110,000$ .

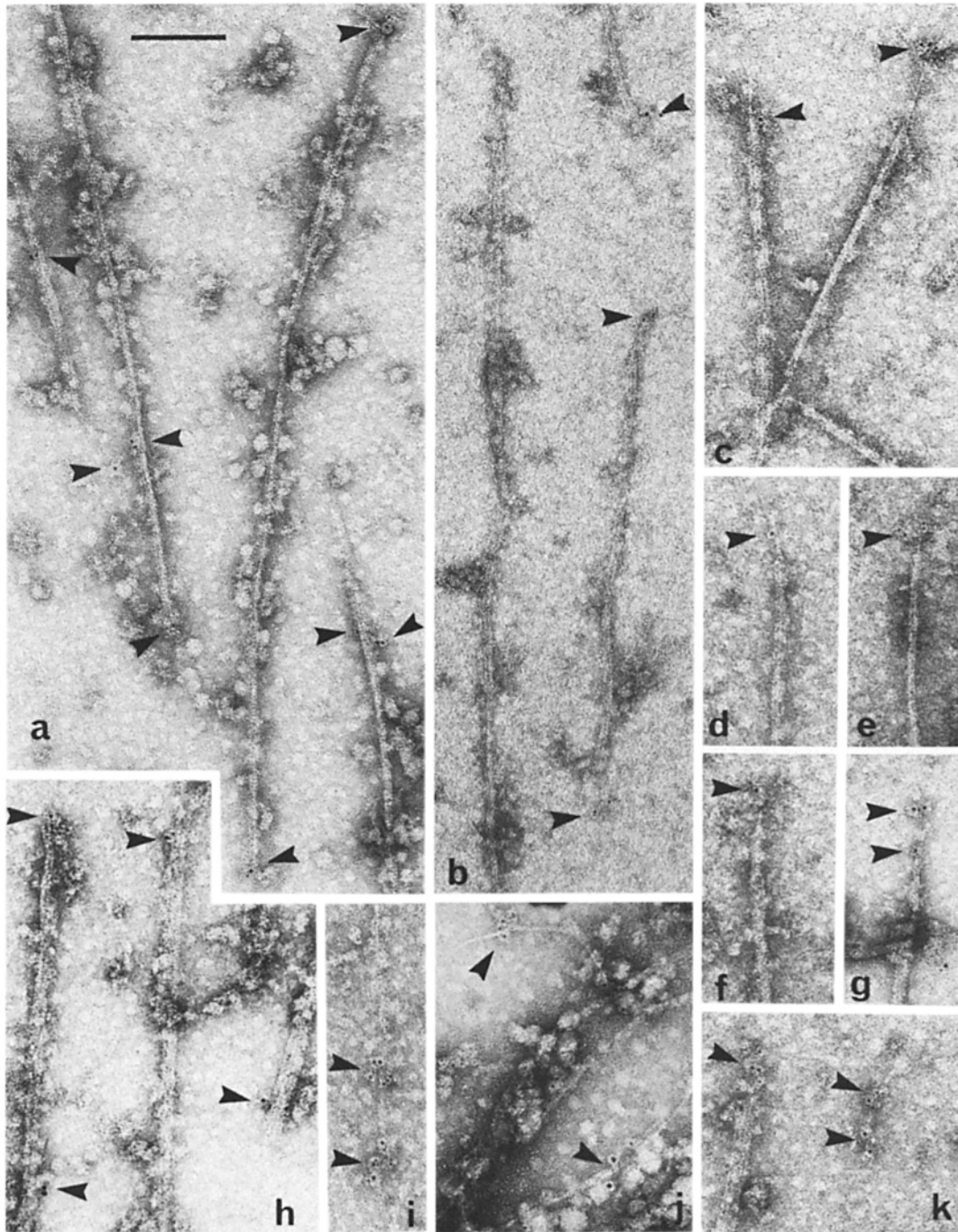


FIGURE 5 Immuno-EM labeling of tektin filaments in pf ribbons from *P. miliaris*. Doublet microtubules spread on carbon-coated EM grids were incubated with mouse antitektons followed by gold-labeled rabbit anti-mouse IgG, before being negatively stained with uranyl acetate. 20  $\mu$ M taxol was present in the incubation buffers, except for the examples in *i* and *j*. Apart from the latter, gold label (arrows) is seen almost exclusively at the ends of the pf ribbons that survive the incubations (see Fig. 6). Bar, 100 nm (for *a-k*).  $\times 140,000$ .

conditions in which the heads obviously remain intact (Fig. 3). Furthermore, during the purification of tektins, sperm head contamination of isolated axonemes is orders of magnitude below concern. Similarly, if the tektin filaments that we have isolated originated from sperm membranes or a submembrane skeleton, we would expect to see structural or biochemical evidence of this in the axoneme preparations following our customary two or three Triton washes. Stephens (38) has shown that the major sperm flagellar membrane protein has an approximate molecular weight of 250,000 and

is soluble in Triton. We find no trace of this on SDS PAGE. Nor do we see any evidence of membranes in the light or electron microscopes and, since filaments are only observed after the microtubules are chemically dissociated, we conclude that the filaments we have isolated arise from the axoneme, not from the flagellar membrane or a membrane-associated skeleton.

At the molecular level, we were particularly concerned with whether the tektins pre-exist as extended filamentous polymers in the microtubules or whether they are arranged along

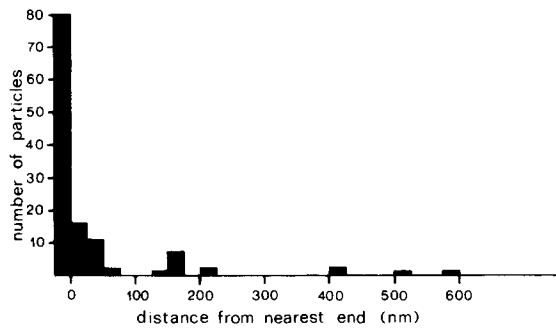


FIGURE 6 Histogram showing the distribution of gold particles on *P. miliaris* pf ribbons, such as those illustrated in Fig. 5, after incubation with mouse antitektons and gold-labeled anti-mouse IgG. Twelve micrographs (at  $\times 45,000$ ) were selected for showing well-spread ribbons and a low background of gold particles. The position of any particle closer than 20 nm to a ribbon was measured relative to the end of that ribbon. Any particle attached to a filament protruding from the end of a ribbon was counted as being in the zero position. Forty-nine particles were found to be  $>20$  nm from any ribbon on the same micrographs, as compared with 123 particles which were close enough to be attached to ribbons.

them as periodic structures (e.g., radial spoke components or microtubule-membrane linkers [see reference 9]) which spontaneously associate to form filaments upon extraction with chaotropic solvents. Though antitubulin labels intact microtubules all along their lengths (Fig. 7), antitektons do not appreciably label intact doublet or single microtubules or the fractionated pf ribbons (Figs. 4a and 5). These results, together with the fact that antitektons do stain the entire length of denatured axonemes (Fig. 3) but not those fixed in buffers designed to preserve their structure intact, strongly suggest that tektins are in some way integrated into the microtubule wall rather than associated with the external surface as are radial spokes or other axoneme matrix components, which would be readily accessible to antibodies. A direct demonstration that the tektins form longitudinally arranged filaments in the microtubules comes from our experiments in which microtubules were dissociated directly on EM grids and labeled with antibodies; the 2–3-nm filaments left by extraction of *S. purpuratus* microtubules and pf ribbons specifically label with the antitektons. The rapid application of drops of sarkosyl-urea to microtubules attached to EM grids would be expected to solubilize and remove tubulin and microtubule-associated proteins from the grid. Under these conditions, it does not seem possible that periodically arranged tektins (whether spokes or membrane linkers) would be present at sufficient concentrations to assemble within seconds into uniform 2-nm filaments that are nearly as long and numerous as the original microtubules (see Fig. 4, b and c).

Furthermore, experiments with *P. miliaris* support the conclusion that the tektin filaments pre-exist as longitudinal polymers in the microtubule wall. In these experiments, antitektons mainly label thin filaments that protrude from the ends of taxol-stabilized pf ribbons and tubules that have not been exposed to the possibly denaturing sarkosyl-urea solvent (Figs. 5 and 6). In these cases, it cannot easily be argued that the extracting media promoted the artifactual polymerization of tektin filaments.

We have not yet determined whether the tektin filaments are laterally associated with the outside or inside surfaces of the microtubule (e.g., as filaments running along the grooves

between pf's, or whether they themselves form certain pf's, possibly in association with other nontubulin proteins, such as the 77,000- and 83,000-mol-wt polypeptides of *S. purpuratus*. We have shown that neither intact microtubules nor pf ribbons bind antitektons along their lengths (Figs. 4a and 5 [and data not shown]), indicating that the tektin filaments (except at their ends) are inaccessible to antibody from either the outside or inside surfaces. If the tektin filaments lie between tubulin pf's, they could simply be masked by tubulin. If they form pf's themselves, it may be that they are masked by associated nontubulin proteins. However, at present we do not know whether there is more than one class of sarkosyl-insoluble ribbon (29); if so, the other nontubulin proteins could be associated with a different class from those containing the 47,000-, 51,000-, and 55,000-mol-wt tektins. Perhaps a lack of antibody-binding to pf's consisting only of tektins suggests that the antigenic sites are hidden within the filaments themselves, but treatments that dissociate the tubulin and other proteins from them may also denature the tektin filaments to some extent or otherwise open up their antigenic sites. However, the relatively mild conditions required to expose the antigenic sites in *P. miliaris* microtubules suggests that denaturation is not necessarily required.

Whichever of these explanations is correct, the tektin filaments are almost certainly structural components of the A-microtubule wall, integrated in some manner with the microtubule surface lattice (see references 2, 3, 16, 28). In this respect, it is interesting that McLachlan and Stewart (30) have found evidence for a regular pattern of charged residues in the primary sequences of the intermediate filament proteins, desmin and vimentin, with a period of 28 residues. In a coiled-coil, this periodicity would correspond to an axial distance of 4.2 nm, which is close to the tubulin monomer repeat in microtubules (3, 16).

### Implications of This Work

Our results from this and previous studies indicate that the proteins we refer to as tektins are integral components of the chemically resistant pf ribbons in flagellar doublet microtubules (24). We would also expect tektins or related proteins to be present in the triplet microtubules of basal bodies and centrioles; this suggestion is supported by a recent report (7). We have suggested earlier that the arrangement of tubulin and tektins within the microtubule wall could specify the three-dimensional organization of the accessory components of ciliary and flagellar axonemes, such as the dynein arms and radial spokes. Thus, genetic mutations affecting tektins might be the cause of abnormal arrangements of axoneme components, such as those occurring in ciliary dyskinesia syndromes (1, 4, 8, 18, 33).

The existence of relatively stable filaments associated with the walls of microtubules may also be of general significance. We have noted that the presence of chemically stable ribbons of pf's is not simply a property of doublet microtubules (24, 31, 42, 43), since flagellar singlet microtubules (29) and mitotic spindle microtubules (21) also exhibit such stable moieties. Moreover, recent studies have shown mitotic spindles to contain structural proteins with properties remarkably similar to flagellar tektins (7, 17). Our current results and these related observations are thus in agreement with our earlier proposal that tektin filaments may be fundamentally important in the structure, assembly, and function of cytoplasmic microtubules and filaments (27).



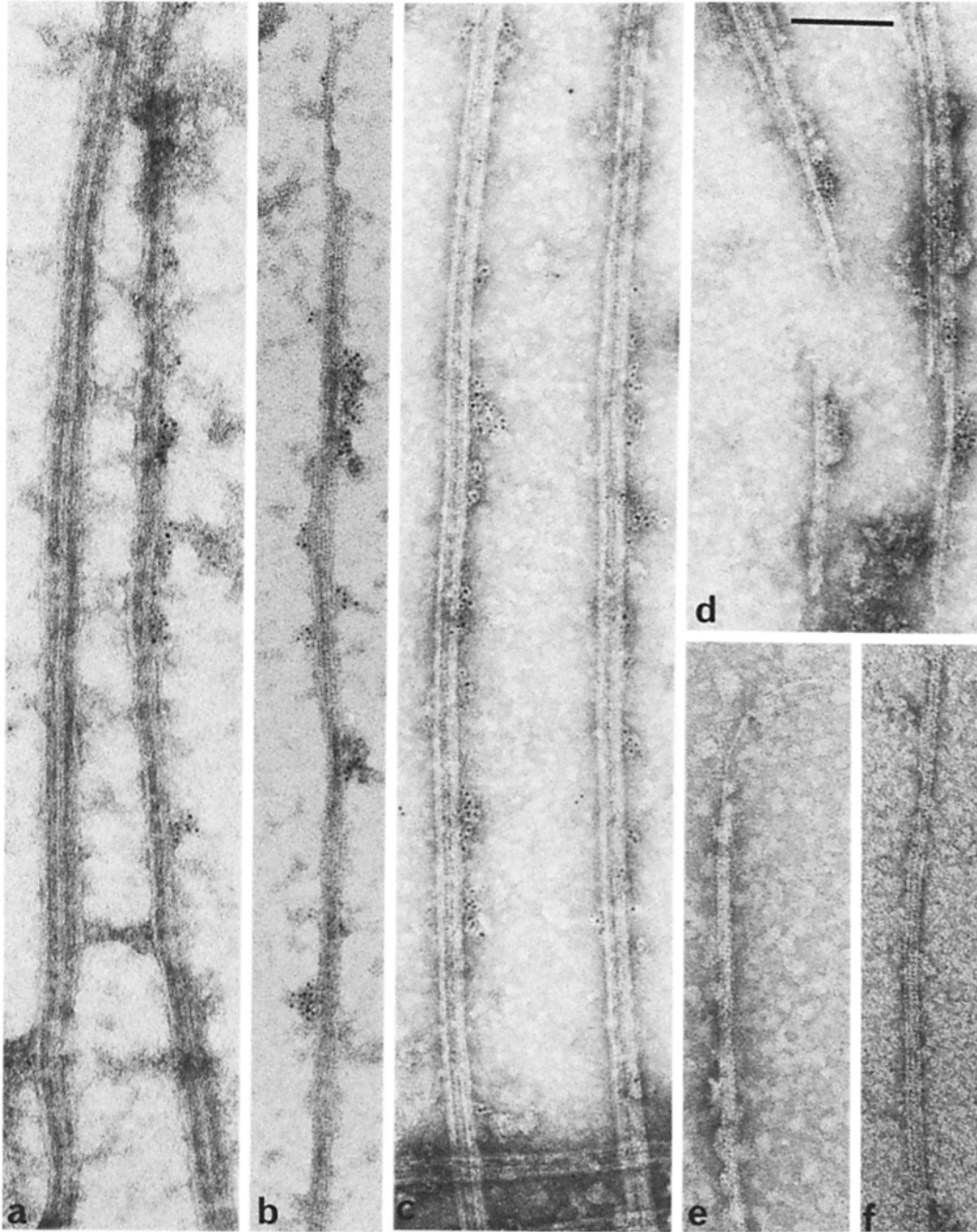


FIGURE 7 Immuno-EM labeling of tubulin in microtubules and pf ribbons from *P. malariae* that have been negatively stained with uranyl acetate. (a-d) Samples that were incubated on EM grids in YL1/2 monoclonal antitubulin, coupled directly to gold. Singlet microtubules (a-c) and pf ribbons (d) are labeled but doublet microtubules (a) are not. The labeling occurs along the whole length of singlet microtubules and ribbons; there is no preferential labeling of broken ends as with the antitektons. (e and f) *P. malariae* pf ribbons prepared using 0.5% sarkosyl; the substructure is more visible than in the antibody-treated samples. Bar, 100 nm (for a-f).  $\times 145,000$ .

We thank J. V. Kilmartin for help and advice, and for gifts of gold-labeled and unlabeled YL1/2 antitubulin.

This work was supported by grants from the U. S. National Institutes of Health (GM 21527 and S07-RR-05381-20), the U. S. National Science Foundation (PCM 78-16158) to R. W. Linck and from the Medical Research Council of Great Britain to W. B. Amos.

Received for publication 12 June 1984, and in revised form 27 August 1984.

#### REFERENCES

1. Afzelius, B. A. 1976. A human syndrome caused by immotile cilia. *Science (Wash. DC)*. 193:317-319.
2. Amos, L. A., and A. Klug. 1974. Arrangement of subunits in flagellar microtubules. *J. Cell Sci.* 14:523-549.
3. Amos, L. A., R. W. Linck, and A. Klug. 1976. Molecular structure of flagellar microtubules. *Cold Spring Harbor Conf. Cell Proliferation*. 3(Book A):847-867.
4. Baccetti, B., A. G. Burrini, V. Pallini, and T. Renieri. 1981. Human dynein and sperm pathology. *J. Cell Biol.* 88:102-197.
5. Beese, L. 1984. Microtubule structure: an analysis by x-ray diffraction. Ph.D. thesis. Brandeis University, Waltham, MA.
6. Capco, D. G., K. M. Wan, and S. Penman. 1982. The nuclear matrix: three-dimensional architecture and protein composition. *Cell*. 29:847-858.

7. Chang, X. J., and G. Piperno. 1983. Antigens associated with complex microtubules of different origins. *J. Cell Biol.* 97(5, Pt. 2):195a. (Abstr.)
8. Chao, J., J. A. P. Turner, and J. M. Sturgess. 1982. Genetic heterogeneity of dynein deficiency in cilia from patients with respiratory disease. *Am. Rev. Respir. Dis.* 126:302-305.
9. Dentler, W. L., M. M. Pratt, and R. E. Stephens. 1980. Microtubule-membrane interactions in cilia. II. Photochemical cross-linking of bridge structures and the identification of a membrane-associated dynein-like ATPase. *J. Cell Biol.* 84:381-403.
10. Faulk, W. P., and G. M. Taylor. 1971. An immunocolloid method for the electron microscope. *Immunochimistry.* 8:1081-1083.
11. Fawcett, D. W. 1981. *The Cell.* W. B. Saunders Co., Philadelphia. 558 pp.
12. Fraker, P. J., and J. C. Speck. 1978. Protein and cell membrane iodinations with a sparingly soluble chloramide 1,3,4,6-tetrachloro-3 $\alpha$ ,6 $\alpha$ -diphenyl-glycouril. *Biochem. Biophys. Res. Commun.* 80:849-857.
13. Frens, G. 1973. Controlled nucleation for the regulation of the particle size in monodisperse gold suspension. *Nature (Lond.)* 241:20-22.
14. Fuchs, E., and I. Hanukoglu. 1983. Unraveling the structure of the intermediate filaments. *Cell.* 34:332-334.
15. Gibbons, I. R. 1965. Chemical dissection of cilia. *Arch. Biol.* 76:317-352.
16. Grimstone, A. V., and A. Klug. 1966. Observations on the substructure of flagellar fibres. *J. Cell Sci.* 1:351-362.
17. Hayes, T. S., and E. D. Salmon. 1983. A non-microtubular component of sea urchin isolated spindles. *J. Cell Biol.* 97(5, Pt. 2):44a. (Abstr.)
18. Huang, B., G. Piperno, and D. J. L. Luck. 1979. Paralyzed flagella mutants of *Chlamydomonas reinhardtii*. *J. Biol. Chem.* 254:3091-3099.
19. Huxley, H. E. 1963. Electron microscope studies on the structure of natural and synthetic protein filaments from striated muscle. *J. Mol. Biol.* 7:281-308.
20. Ishikawa, H., R. Bischoff, and H. Holtzer. 1968. Mitosis and intermediate-sized filaments in developing skeletal muscle. *J. Cell Biol.* 38:538-555.
21. Jensen, C., and A. Bajer. 1973. Spindle dynamics and arrangement of microtubules. *Chromosoma (Berl.)* 44:73-89.
22. Kilmartin, J. V., B. Wright, and C. Milstein. 1982. Rat monoclonal antitubulin antibodies derived by using a new nonsecreting rat cell line. *J. Cell Biol.* 93:576-582.
23. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680-685.
24. Linck, R. W. 1976. Flagellar doublet microtubules: fractionation of minor components and  $\alpha$ -tubulin from specific regions of the A-tubule. *J. Cell Sci.* 20:405-439.
25. Linck, R. W. 1982. The structure of microtubules. *Ann. NY Acad. Sci.* 383:98-121.
26. Linck, R. W., D. F. Albertini, D. M. Kenny, and G. L. Langevin. 1982. Tektin filaments: chemically unique filaments of sperm flagellar microtubules. *Cell Motil.* 1(Suppl):127-132.
27. Linck, R., D. Albertini, G. Langevin, G. Olson, and D. Woodrum. 1981. The structure and function of microtubules. *Biophys. J.* 33(2, Pt. 2):215a. (Abstr.)
28. Linck, R. W., and G. L. Langevin. 1981. Reassembly of flagellar B( $\alpha\beta$ ) tubulin into singlet microtubules: consequences for cytoplasmic microtubule structure and assembly. *J. Cell Biol.* 89:323-337.
29. Linck, R. W., and G. L. Langevin. 1982. Structure and chemical composition of insoluble filamentous components of sperm flagellar microtubules. *J. Cell Biol.* 58:1-22.
30. McLachlan, A., and M. J. Stewart. 1982. Periodic charge distribution in the intermediate filament proteins desmin and vimentin. *J. Mol. Biol.* 162:693-698.
31. Meza, I., B. Huang, and J. Bryan. 1972. Chemical heterogeneity of protofilaments forming the outer doublets from sea urchin flagella. *Exp. Cell Res.* 74:535-540.
32. Parnass, J., C. F. Asnes, and S. B. Horwitz. 1983. Taxol binds differentially to flagellar outer doublets and their reassembled microtubules. *Cell Motil.* 3:123-130.
33. Pedersen, H., and N. Mygind. 1976. Absence of axonemal arms in nasal mucosa cilia in Kartagener's syndrome. *Nature (Lond.)* 262:494-495.
34. Sloboda, R. D., and J. L. Rosenbaum. 1982. Purification and assay of microtubule-associated proteins (MAPs). *Methods Enzymol.* 85:409-416.
35. Slot, J. W., and H. J. Geuze. 1981. Sizing of protein A-colloidal gold probes for immunoelectron microscopy. *J. Cell Biol.* 90:533-536.
36. Starger, J. M., W. E. Brown, A. E. Goldman, and R. D. Goldman. 1978. Biochemical and immunological analysis of rapidly purified 10-nm filaments from baby hamster kidney (BHK-21) cells. *J. Cell Biol.* 78:93-109.
37. Stephens, R. E. 1970. Thermal fractionation of outer fiber doublet microtubules into A- and B-subfiber components: A- and B-tubulin. *J. Mol. Biol.* 47:353-363.
38. Stephens, R. E. 1977. Major membrane protein differences in cilia and flagella: evidence for a membrane-associated tubulin. *Biochemistry.* 16:2047-2058.
39. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350-4354.
40. Vogl, A. W., R. W. Linck, and M. Dym. 1983. Colchicine induced changes in the cytoskeleton of the golden-mantled ground squirrel (*Citellus lateralis*) Sertoli cells. *Am. J. Anat.* 168:99-108.
41. Wehland, J., M. C. Willingham, and I. V. Sandoval. 1983. A rat monoclonal antibody reacting specifically with the tyrosylated form of  $\alpha$ -tubulin. *J. Cell Biol.* 97:1467-1475.
42. Witman, G. B., K. Carlson, J. Berliner, and J. L. Rosenbaum. 1972. *Chlamydomonas* flagella. I. Isolation and electrophoretic analysis of microtubules, matrix, membranes, and mastigonemes. *J. Cell Biol.* 54:507-539.
43. Witman, G. B., K. Carlson, and J. L. Rosenbaum. 1972b. *Chlamydomonas* flagella. II. The distribution of tubulins 1 and 2 in the outer doublet microtubules. *J. Cell Biol.* 54:540-555.