

Microtubules and Microtubule-associated Proteins from the Nematode *Caenorhabditis elegans*: Periodic Cross-links Connect Microtubules In Vitro

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Abstract. The nematode *Caenorhabditis elegans* should be an excellent model system in which to study the role of microtubules in mitosis, embryogenesis, morphogenesis, and nerve function. It may be studied by the use of biochemical, genetic, molecular biological, and cell biological approaches. We have purified microtubules and microtubule-associated proteins (MAPs) from *C. elegans* by the use of the anti-tumor drug taxol (Vallee, R. B., 1982, *J. Cell Biol.*, 92:435-44). Approximately 0.2 mg of microtubules and 0.03 mg of MAPs were isolated from each gram of *C. elegans*. The *C. elegans* microtubules were smaller in diameter than bovine microtubules assembled in vitro in the same buffer. They contained primarily 9-11 protofilaments, while the bovine microtubules contained 13 protofilaments. The principal MAP

had an apparent molecular weight of 32,000 and the minor MAPs were 30,000, 45,000, 47,000, 50,000, 57,000, and 100,000-110,000 mol wt as determined by SDS-gel electrophoresis. The microtubules were observed, by electron microscopy of negatively stained preparations, to be connected by stretches of highly periodic cross-links. The cross-links connected the adjacent protofilaments of aligned microtubules, and occurred at a frequency of one cross-link every 7.7 ± 0.9 nm, or one cross-link per tubulin dimer along the protofilament. The cross-links were removed when the MAPs were extracted from the microtubules with 0.4 M NaCl. The cross-links then re-formed when the microtubules and the MAPs were recombined in a low salt buffer. These results strongly suggest that the cross-links are composed of MAPs.

MICROTUBULES are hollow cylindrical structures present in most cells of eucaryotes. They play a part in many important cell functions including mitosis, meiosis, cell motility, particle transport, and morphogenesis. In neurons, microtubules are thought to be important for neurite outgrowth, shape maintenance, axonal transport, and sensory transduction. For a review, see Dustin (19).

Microtubules are formed from two similar proteins called α - and β -tubulin, which are $\sim 55,000$ mol wt, and are tightly associated to form a dimer. The dimers associate in a head-to-tail fashion to form linear protofilaments. The protofilaments associate through side-to-side interactions to form a microtubule. Microtubules usually contain 13 protofilaments, but other numbers of protofilaments are also possible (12). *C. elegans* contains microtubules with 11, 13, and 15 protofilaments (15, 16).

Microtubules often appear to be cross-connected to other structures and to each other in situ by thin filamentous bridges that probably mediate the association of microtubules with these structures (9, 19, 26, 34, 56). Proteins that bind to and co-isolate with microtubules are referred to as microtubule-associated proteins (MAPs)¹ (45). Several of

the MAPs decorate the walls of microtubules assembled in vitro with side arms similar to those seen in situ (24, 29, 43, 44, 53). Fluorescently labeled antibodies made against many of the MAPs stain the microtubule network in cultured cells (5, 6, 10, 11, 17, 28, 31, 41, 42), and MAPs mediate the association of microtubules in vitro with neurofilaments (1), pituitary secretory granules (40), actin filaments (20, 21, 38), and other microtubules (48).

Although microtubules are known to be part of many important biological structures, and involved in many biological processes, little is known about how they are organized, or how they perform many of their cellular functions. The nematode *C. elegans* should provide a system in which the role of microtubules in development and nerve function can be understood in the context of the living animal.

C. elegans is small, reproduces mainly as a self-fertilizing hermaphrodite, and has a mass doubling time of only 10.3 h (13). Hundred gram quantities of genetically identical animals can be easily grown in the laboratory. *C. elegans* has

teins; PEM buffer, 0.1 M Pipes, 1.0 mM EGTA, and 1.0 mM MgSO₄, pH 6.6; PEMMI buffer, 0.05 M Pipes, 1.0 mM EGTA, 1.0 mM MgSO₄, 0.5 M mannitol, 80 μ g/ml leupeptin, 80 μ g/ml pepstatin, 1.0 mg/ml TAME, and 2.0 mM dithioerythritol, pH 6.6; TAME, *N* α -*p*-tosyl-L-arginine methyl ester.

1. Abbreviations used in this paper: MAPs, microtubule-associated pro-

one of the simplest anatomies of any metazoan organism. This anatomical simplicity in conjunction with the animal's small size and transparency have made it possible to determine the fates and lineage histories of all 959 somatic cells in the adult hermaphrodite by light microscopic methods (47). The anatomical simplicity of *C. elegans* also facilitates immunofluorescence studies so cells expressing particular antigens can be readily identified. Furthermore, the small size of the adult has made it possible to reconstruct the entire wild-type nervous system from serial electron micrographs, so the positions, morphologies, and synaptic interactions of all of the neurons are known (55). Finally, because *C. elegans* has a very small genome, which is only about twenty times as complex as the genome of *E. coli* (46), molecular genetic methods can be effectively applied.

C. elegans contains several structurally distinct microtubule types that are located in different classes of cells. For example, most neurons in *C. elegans* contain 11 protofilament microtubules, but the six mechanosensory neurons contain 15 protofilament microtubules (15, 16). In addition, most receptor neurons are ciliated and the A-subfibers of the sensory cilia contain 13 protofilaments (15). Furthermore, several mutants of *C. elegans* have been identified that fail to form certain kinds of microtubules (14), or show abnormalities in the organization of the microtubules that are formed (3, 23).

We have isolated microtubules and MAPs from *C. elegans* by the taxol-dependent method of Vallee (51). These microtubules were smaller than microtubules isolated from bovine brain. They contained predominantly 9–11 protofilaments, while the bovine microtubules contained predominantly 13 protofilaments. The microtubules isolated from *C. elegans* were cross-connected by stretches of periodic cross-links that occurred with a frequency of one cross-link per dimer along the protofilaments. These cross-links appeared to be formed from one or more of the MAPs. The predominant MAP had a molecular weight of 32,000, and the minor MAPs had molecular weights of 30,000, 45,000, 47,000, 50,000, 54,000, 57,000, and 100,000–110,000.

Materials and Methods

Materials

Taxol was generously provided by the Natural Products Branch, Division of Cancer Treatment, National Cancer Institute. Other materials were from the following sources: Sigma Chemical Co., St. Louis, MO (GTP [type I], MgSO₄, EGTA, Pipes, mannitol, sucrose, leupeptin, pepstatin, *N*-p-tosyl-L-arginine methyl ester [TAME], phenylmethylsulfonyl fluoride, aprotinin, soybean trypsin inhibitor, and dithioerythritol); Ladd Research Industries, Inc., Burlington, VT (formvar); and Ted Pella, Inc., Irvine, CA (uranyl acetate).

PEMMI buffer contained 0.05 M Pipes, 1.0 mM EGTA, 1.0 mM MgSO₄, 0.5 M mannitol, 80 μg/ml leupeptin, 80 μg/ml pepstatin, 1.0 mg/ml TAME, and 2.0 mM dithioerythritol at pH 6.6. PEM buffer contained 0.1 M Pipes, 1.0 mM EGTA, and 1.0 mM MgSO₄ at pH 6.6. M 9 buffer has been described (46).

Growing and Harvesting *C. elegans*

Bristol wild-type *C. elegans* strain N₂ was used. Stocks were cultured as described by Brenner (8) on petri plates containing nutrient agar and a lawn of *E. coli* strain OP50 (NGM plates). Large quantities of *C. elegans* were grown on NGM plates containing cooked egg, a method suggested to us by D. L. Ballie and R. Rosenbluth. Six chicken eggs were homogenized, poured into stirring boiling water (350 ml), boiled (2 min), and homogenized. This slurry (7 ml) was added to NGM plates. These plates were inoculated with 0.05 g of *C. elegans*, and the animals were grown for 4 d.

To harvest *C. elegans*, each plate was dipped three times into two beakers containing 800 ml of M 9 buffer at room temperature. *C. elegans* were sedimented by centrifugation at 676 g for 5 min at 0°C, resuspended in cold 14% (wt/vol) sucrose in distilled water, and centrifuged again. *C. elegans* sedimented to the bottom of the tube, while some visible debris remained in the supernatant which was discarded. The animals were then resuspended in cold 35% sucrose, and centrifuged at 676 g for 10 min at 0°C. *C. elegans*, which float in 35% sucrose (46), were aspirated into a side-arm flask, washed three times with water, and stored in small aliquots at –80°C. Typically 0.6–1.0 g of *C. elegans* were isolated from each plate. All of the developmental stages were present, and this preparation contained no visible contamination.

While *C. elegans* can be grown in a liquid culture, we found that the use of egg containing NGM plates was easier, cheaper, and less work. The plates took up less room than a fermenter, and in our hands, the animals grew faster and appeared healthier when grown on plates.

Isolation of Microtubules and MAPs from *C. elegans*

C. elegans were thawed, mixed with two parts of ice cold PEMMI buffer, and homogenized in a French press at 12,000 psi. The homogenate was centrifuged at 40,000 g for 30 min at 0°C. The pellet was discarded, and the supernatant was centrifuged at 140,000 g for 90 min at 0°C. The second pellet was also discarded, and 0.4 M Pipes at pH 6.6 was added to the supernatant to bring the final Pipes concentration to 0.1 M. GTP and taxol were added for a final concentration of 1.0 mM GTP and 20 μM taxol, and this mixture was left on ice for 20 min. The solution was then layered onto a cushion of PEMMI buffer containing 10% sucrose, and centrifuged at 22,500 g for 30 min at 0°C to sediment the microtubules. The microtubules were rinsed once by resuspension and sedimentation, and the MAPs were eluted from the resuspended microtubules by the addition of 0.4 M NaCl as described (51, 52).

Isolation of Bovine Brain Microtubule Proteins

Microtubule proteins were isolated from bovine brain by the method of Shelanski et al. (39), with three cycles of assembly and disassembly. The tubulin and MAPs were separated by phosphocellulose chromatography (18, 54), as described in Aamodt and Williams (1).

Electron Microscopy

To prepare negatively stained samples for electron microscopy, 20 μl of an appropriately diluted sample was applied to a formvar- and carbon-coated electron microscope grid for 15 s. The grid was rinsed with three drops of 0.1 M ammonium acetate and three drops of 1% uranyl acetate. The last drop of uranyl acetate was left on the grid for 15 s, and then all but a thin coat of the stain was removed by aspiration.

Thin section micrographs were prepared by the method of Tilney (49), as modified by Kim et al. (29), except the samples were dehydrated in ethanol rather than acetone. Samples were photographed in a JEOL 100CX electron microscope at 80 kV and at magnifications ranging from 10,000 to 58,000. The microscope was calibrated at each magnification by the use of a 54864 lines/in. carbon replica of a diffraction grating.

Other Methods

Protein concentrations were determined by the method of Bradford (7). Bovine serum albumin (BSA) was used as a standard. SDS-gel electrophoresis was performed with 10% polyacrylamide gels (32). Proteins used for molecular weight standards were myosin, β-galactosidase, phosphorylase B, BSA, and ovalbumin (Bio-Rad Laboratories, Richmond, CA).

Results

Control of Proteolysis

To isolate microtubules and MAPs from *C. elegans* we wished to establish conditions that minimized the proteolysis of MAPs by proteases present in *C. elegans* homogenates. When bovine MAPs (4.3 mg/ml) were mixed with a 40-fold dilution of *C. elegans* homogenate in PEM buffer and incubated at 37°C, nearly all of the MAPs were proteolyzed within 16 min as assayed by SDS PAGE. Neither phenyl-

methylsulfonyl fluoride, TAME, nor soybean trypsin inhibitor detectably reduced proteolysis, but leupeptin (at 20 $\mu\text{g/ml}$) and pepstatin (at 20 $\mu\text{g/ml}$) did. Phenylmethylsulfonyl fluoride, TAME, aprotinin, and soybean trypsin inhibitor were tested to see if they would further reduce proteolysis in the presence of 20 $\mu\text{g/ml}$ leupeptin and 20 $\mu\text{g/ml}$ pepstatin. Under these conditions only TAME further controlled the rate and extent of proteolysis of the MAPs.

The concentrations of TAME, leupeptin, and pepstatin that gave a maximal yield of microtubules and MAPs from *C. elegans* were determined (Fig. 1). All three protease inhibitors significantly improved the yield of microtubules; however, TAME, at concentrations >1.0 mg/ml, apparently inhibited microtubule assembly. Based on these results, we decided to use 80 $\mu\text{g/ml}$ of pepstatin and leupeptin and 1.0 mg/ml TAME in the experiments described below.

Protein Composition of the Isolated Microtubules and MAPs

The yield (Table I) and the polypeptide composition (Fig. 2) of a typical microtubule preparation are shown. The major protein in the MAP fraction (Fig. 2, lane H) had an apparent molecular weight of 32,000. The minor proteins in this fraction had apparent molecular weights of 30,000, 45,000, 47,000, 50,000, 54,000, 57,000, and several proteins in the range of 100,000–110,000. A small amount of a 180,000-mol-wt protein discussed below is present as well as a small amount of a 210,000-mol-wt protein, which co-migrates with *C. elegans* myosin.

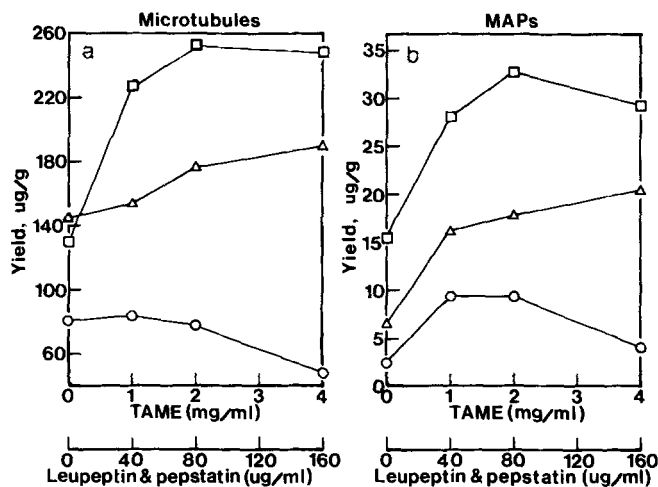


Figure 1. The yield of microtubules and MAPs isolated from *C. elegans* at various protease inhibitor concentrations. Microtubules were isolated as described in Materials and Methods except that the Pipes concentration was kept at 0.1 M throughout the procedure. First, the TAME concentration was varied while leupeptin and pepstatin were held at 40 $\mu\text{g/ml}$ (\circ). TAME at 1.0 mg/ml caused an increase in the yield of both microtubules and MAPs, but at higher concentrations TAME caused the yield of microtubules to decrease, presumably by inhibiting microtubule assembly. Next, the leupeptin concentration was varied, while TAME and pepstatin were held at 1.0 mg/ml and 40 $\mu\text{g/ml}$, respectively (Δ). The yield of microtubules and MAPs increased with increasing leupeptin concentration up to 160 $\mu\text{g/ml}$, although most of this increase occurred between 0 and 80 $\mu\text{g/ml}$. Finally, the pepstatin concentration was varied, while TAME and leupeptin were held at 1.0 mg/ml and 80 $\mu\text{g/ml}$, respectively (\square). The yield of microtubules and MAPs increased with increasing pepstatin concentration up to 80 $\mu\text{g/ml}$.

Table I. Purification of *C. elegans* Microtubule Protein

Step	Yield from 3 g of <i>C. elegans</i>	
	Volume	Total protein
	ml	mg
<i>C. elegans</i> homogenate	9.0	108.8*
Low speed supernatant	5.5*	52.3*
High speed supernatant	4.7	42.5
Microtubules	1.0	0.761
Salt-extracted microtubules	1.0	0.517
MAPs	1.1	0.098

* Estimation based on previous preparations.

A Second Self-Assembling Protein Structure

The 180,000-mol-wt protein, present in the MAP fraction, was also isolated when, as a control, the *C. elegans* homogenate was taken through the procedure to isolate microtubules except that no taxol or GTP was added to cause microtubule assembly. The protein composition and an electron micrograph of this material, negatively stained with uranyl acetate, are shown (Fig. 3). This material contained filaments with diameters of 2–20 nm. The larger filaments appear to be formed by the association of the smaller filaments. To be present in the MAP fraction, the 180,000-mol-wt protein

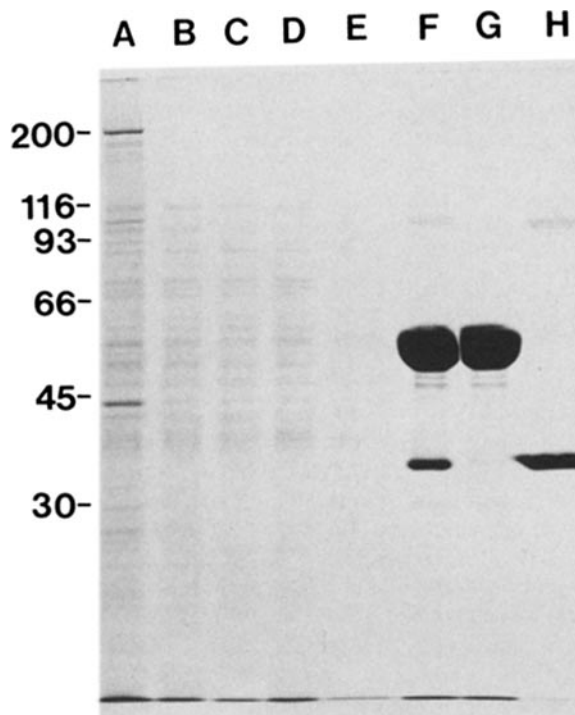


Figure 2. Fractions from a *C. elegans* microtubule preparation electrophoresed on an SDS-polyacrylamide gel and stained with Coomassie Blue. Lane A, 20 μg of *C. elegans* homogenate. Lane B, 20 μg of the proteins in the supernatant after the 40,000 g centrifugation. Lane C, 20 μg of the proteins in the supernatant after the 140,000 g centrifugation. Lane D, 20 μg of the proteins in the supernatant after removal of the microtubules by sedimentation. Lane E, 10 μg of the proteins in the supernatant from the rinse step. Lane F, 20 μg of isolated microtubules. Lane G, 20 μg of the microtubules after extraction of the MAPs with 0.4 M NaCl. Lane H, 4 μg of isolated MAPs.

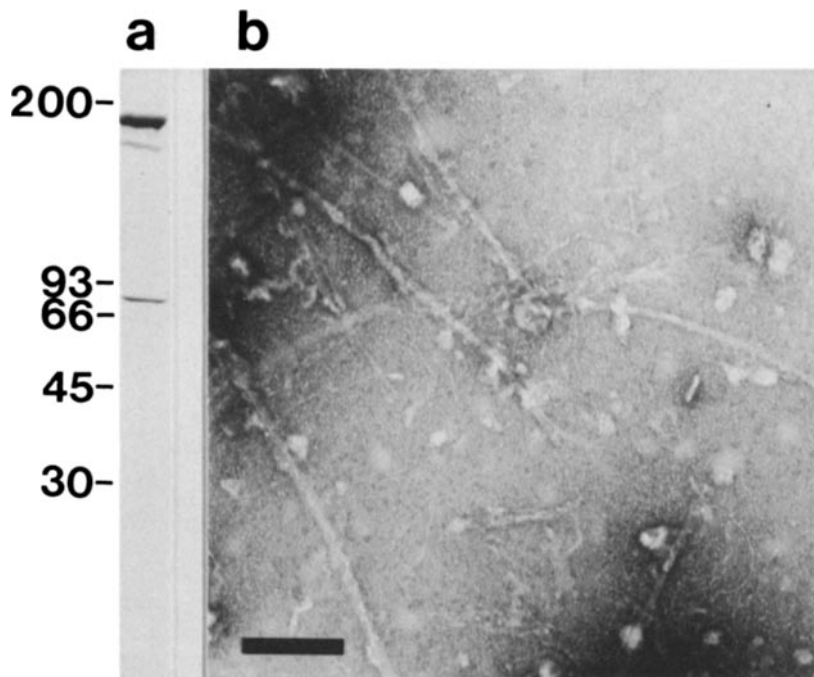


Figure 3. Material isolated in a control preparation where the procedure for isolating the microtubules was followed except that the homogenization buffer contained 0.1 M Pipes, and no GTP or taxol was added. (a) Coomassie Blue-stained SDS-polyacrylamide gel of this material (8 μ g). (b) A micrograph of this material negatively stained with uranyl acetate. The principal protein in this preparation had a molecular weight of 180,000, and the only structures visible were filaments of 2–20-nm diam. Bar, 200 nm.

must be part of a large structure (presumably these filaments), and this structure must dissociate in high salt. To reduce the amount of the 180,000-mol-wt protein in the microtubule preparation, we homogenized and carried out the first two centrifugations in PEMMI buffer containing 0.05 M Pipes, and then before assembling the microtubules we brought the Pipes concentration to 0.1 M by the addition of 0.4 M Pipes.

Reconstitution of Microtubules and MAPs

To determine whether the MAPs would rebind to the microtubules, microtubules and MAPs were prepared as described above. The resuspended microtubules were stored overnight at 4°C in PEM buffer containing 1.0 mM GTP and 20 μ M taxol, while the MAPs were dialyzed against PEM buffer. The MAPs were centrifuged at 22,500 g for 30 min to remove aggregated proteins, and then mixed with the microtubules to a concentration of 0.03 mg/ml MAPs and 1.5 mg/ml microtubules. Controls containing 0.03 mg/ml MAPs but no microtubules and 1.5 mg/ml microtubules but no MAPs were also prepared. The samples were warmed to 37°C for 5 min, left on ice for 20 min, and then centrifuged at 22,500 g for 30 min at 0°C. In the sample where microtubules were present all of the MAPs sedimented, while in the sample where microtubules were absent none of the MAPs sedimented. Fig. 4 shows the fractions run on an SDS-polyacrylamide gel.

The Structure of Microtubules Isolated from *C. elegans*

When viewed in cross-section, the *C. elegans* microtubules, which had been fixed in the presence of tannic acid (Fig. 5 a), had a smaller diameter and fewer protofilaments than bovine microtubules (Fig. 5 b). They also lacked the conspicuous long filamentous side-arms present on the bovine microtubules. The distribution of protofilaments per microtubule for *C. elegans* and for bovine microtubules are shown in

Fig. 6. The most prevalent nematode microtubules had 11 protofilaments, whereas the most prevalent bovine microtubules had 13 protofilaments. The *C. elegans* microtubules also had a broader distribution of protofilaments per microtubule than the bovine microtubules.

Periodic Cross-links

Microtubules were the only structures visible in negatively stained samples of the microtubule preparation from *C. elegans*. These microtubules were smaller than microtubules from bovine brain, and they were connected by stretches of periodic cross-links. High magnification micrographs of the cross-links are shown (Fig. 7). The large arrows point to the cross-links, while the small arrows point to tubulin monomers along one protofilament. There were two monomers, or one dimer, for each cross-link. The center-to-center distance between cross-links was 7.7 ± 0.9 nm ($n = 17$), while the center-to-center distance between dimers was 7.6 ± 1.3 nm ($n = 17$). The cross-links were ~ 5.7 -nm long and 3.0-nm wide.

To determine whether the cross-links are formed from MAPs, we dissociated the MAPs from the microtubules, shown in Fig. 8 a, by adding NaCl to a concentration of 0.4 M. After the addition of NaCl the cross-links were no longer present (Fig. 8 b). When the microtubules were centrifuged away from the MAPs, and then resuspended back into NaCl-free PEM buffer, the cross-links did not re-form (Fig. 8 c). However, when the MAP fraction was desalted into PEM buffer, and then added to the MAP-free microtubules, the periodic cross-links were re-formed (Fig. 8 d). These results strongly suggest that the cross-links are formed from MAPs.

Very occasionally, microtubules were found in negatively stained samples of the desalted MAP fraction. In this situation, where there were very few microtubules and a large excess of MAPs, the microtubules became covered with short

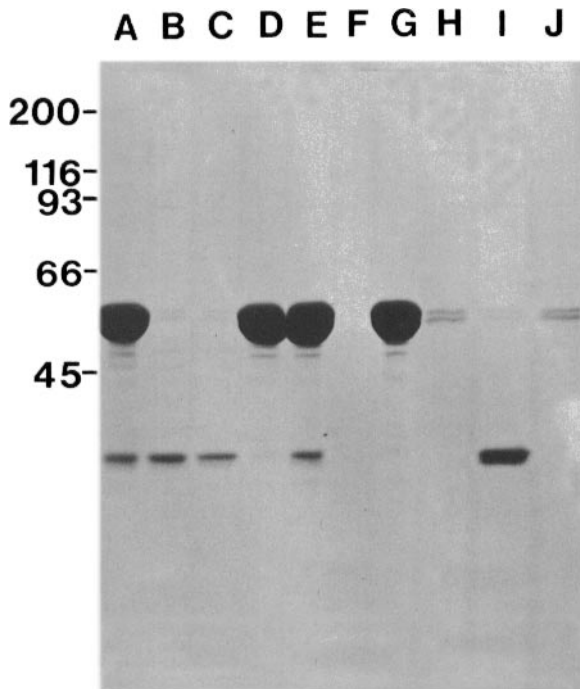


Figure 4. Fractions from the microtubule and MAP reconstitution experiment electrophoresed on an SDS-polyacrylamide gel and stained with Coomassie Blue. Lane A, 20 μ g of isolated microtubules. Lane B, 8 μ g of MAPs which was eluted from the microtubules with 0.4 M NaCl. Lane C, 5 μ g of MAPs after centrifugation at 22,500 g for 30 min. Lane D, 20 μ g of microtubules after removal of the MAPs. Lanes E-G, pellets from the reconstitution experiment. Lane E, 26 μ l (20 μ g) of the resuspended pellet from the microtubule and MAP mixture. Lane F, 26 μ l of the resuspended pellet from the sample containing only MAPs. Lane G, 26 μ l of the resuspended pellet from the sample containing only microtubules. Lanes H-J are supernatants from the reconstitution experiment. Lane H, 100 μ l of the supernatant from the microtubule and MAP mixture. Lane I, 100 μ l of the supernatant from the sample containing only MAPs. Lane J, 100 μ l of the supernatant from the sample containing only microtubules.

arms that appeared to be the same as the cross-links (Fig. 9). Each dimer of the microtubules apparently bound a side-arm, and this decoration enhanced the surface lattice of the microtubule. The side-arms appeared to follow a single helix. This would correspond to a two-start helix of tubulin subunits, since there is one cross-link per dimer. The inset to Fig. 9 shows a drawing of a 10 protofilament microtubule with a two-start helix. The pitch of this helix is nearly identical to the pitch of the decorations along the surface of the microtubule in the micrographs.

Discussion

C. elegans seems to be an excellent system in which to study the role of microtubules in mitosis, embryogenesis, morphogenesis, and nerve function, since this system is amenable to most of the methods for studying microtubules in vivo, in situ, and in vitro. We have shown that milligram quantities of pure microtubules and MAPs could be easily and rapidly isolated from *C. elegans* by the taxol-dependent method of Vallee (51). The predominant MAP in this preparation had an apparent molecular weight of 32,000. There were also

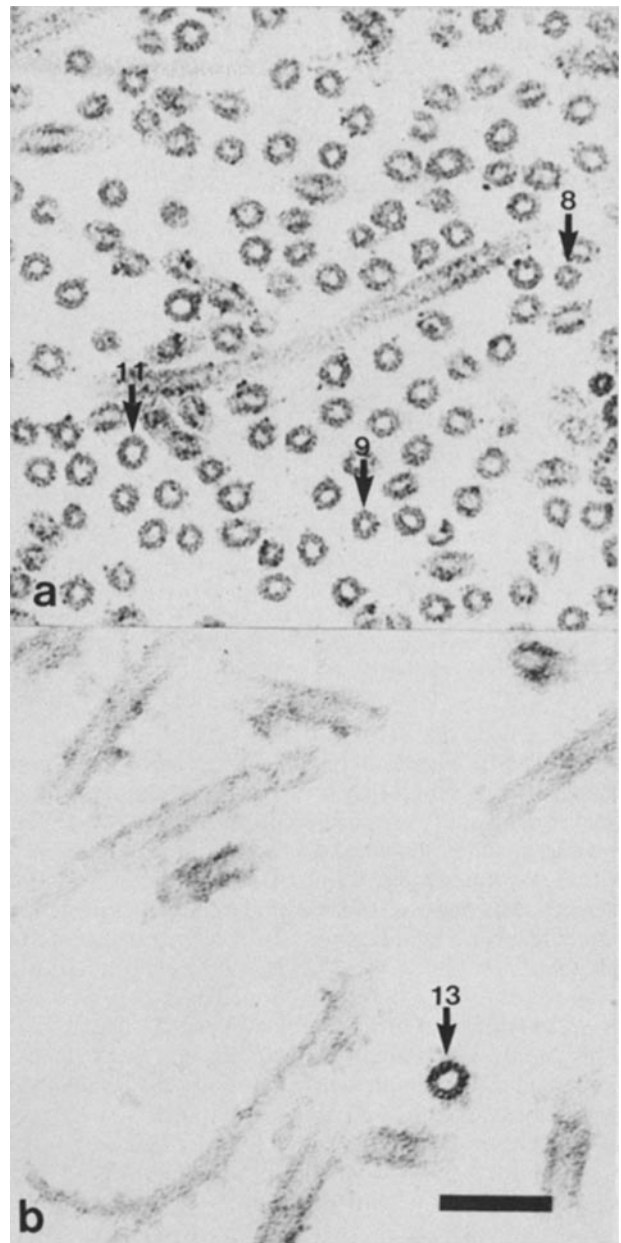


Figure 5. Micrograph of thin-sectioned microtubules isolated from *C. elegans* (a) and bovine brain (b). The microtubules were fixed in the presence of tannic acid to enhance the resolution of the protofilaments. The *C. elegans* microtubules had a smaller diameter than the bovine microtubules and contained fewer protofilaments. The numbers indicate the number of protofilaments contained in the microtubules indicated by the arrows. Bar, 100 nm.

several minor proteins that co-isolated with the microtubules and were eluted from the microtubules by high salt. We refer to all of these proteins as MAPs based on their association with microtubules in vitro. Further research will be required to determine whether each of these proteins associates with microtubules in vivo.

The Structure of Microtubules from *C. elegans*

The *C. elegans* microtubules were smaller in diameter than microtubules isolated from bovine brain. This smaller size was reflected in the protofilament numbers of the micro-

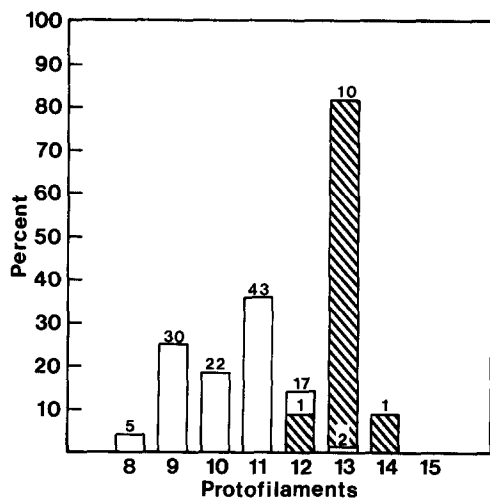


Figure 6. Protofilament number of *C. elegans* microtubules (□), and bovine microtubules (▨) determined from micrographs of tannic acid fixed microtubules as shown in Fig. 5. The number above each bar indicates the number of microtubules counted. The microtubules from *C. elegans* had fewer protofilaments per microtubule and a broader distribution of protofilaments per microtubule than the microtubules from bovine brain.

tubules. The *C. elegans* microtubules contained fewer protofilaments and had a broader distribution of protofilament numbers compared to the bovine microtubules. The *C. elegans* microtubules contained primarily 9–11 protofilaments, while the bovine microtubules contained primarily 13 protofilaments. Both the smaller number and broader distribution of protofilament numbers appear to be related to the size of microtubules in situ. In situ, *C. elegans* have primarily 11 protofilament microtubules, but in ciliated dendrites the A-subfibers of the doublet microtubules contain 13 protofilaments, and the touch sensitive microtubule neurons contain 15 protofilament microtubules. In cow brain almost all of the microtubules contain 13 protofilaments. These results suggest that whatever determines protofilament number in vivo has been preserved in our in vitro preparation. Thus it may now be possible to identify the factors that determine the protofilament number of microtubules assembled in vitro.

Two problems were encountered during the adaptation of the taxol-dependent method to *C. elegans*. First, homogenates of *C. elegans* contain a large amount of proteases that rapidly proteolyzed exogenous MAPs. These proteases were controlled by the addition of leupeptin, pepstatin, and TAME. Second, a 180,000-mol-wt protein was present in the MAP fraction that was also present in control preparations where microtubules were not induced to assemble. This protein, and some minor proteins associated with it, were nearly eliminated from the microtubule preparation by reducing the ionic strength of the homogenization buffer to 0.05 M Pipes during the first two centrifugation steps (before microtubules were formed) and then raising the ionic strength to 0.1 M Pipes while the microtubules were assembled and isolated. Presumably this worked because the structures that contain the 180,000-mol-wt protein are dissociated by high salt. By keeping the ionic strength low during the first two centrifugation steps most of the 180,000-mol-wt protein remained assembled and was eliminated. By keeping the ionic strength high during microtubule assembly and isolation, most re-



Figure 7. Periodic cross-links connecting microtubules isolated from *C. elegans*. The large arrows point to cross-links, and the small arrows point to monomers along a single protofilament. The cross-links occurred at a frequency of one cross-link per tubulin dimer along a protofilament. Bar, 100 nm.

maining 180,000-mol-wt protein was prevented from assembling, and was, therefore, not isolated with the microtubules.

Cross-links and Side-Arms

Perhaps the most interesting feature of these microtubules was that they were cross-connected by stretches of highly periodic cross-links. These links formed between the adjacent protofilaments of aligned microtubules with the same periodicity as the tubulin dimers along the protofilaments. Cross-links connecting microtubules to each other and to other structures have been described in many organisms (19, 34). These links are probably important for maintaining the organization of microtubule arrays, and they are also interesting because some of them may be directly involved with cell motility. Like dynein, they may hydrolyze high-energy phosphates and do mechanical work.

The cross-links described here are morphologically similar to the short cross-links that connect microtubules in the mitotic apparatus of several organisms (9, 26, 34, 56), but are shorter than those previously described. They are also similar to arms that cross-link microtubules isolated from sea urchin eggs by the taxol-dependent method (52), but the sea urchin cross-links have a periodicity of ~ 16 nm rather than 8 nm. These cross-links appear similar, in thin section micrographs, to the cross-links that connect the microtubules in the mechanosensory neurons of *C. elegans* (reference 16, Fig. 2). Both the cross-links described here, and those in the mechanosensory neurons, hold the microtubules ~ 6 nm apart.

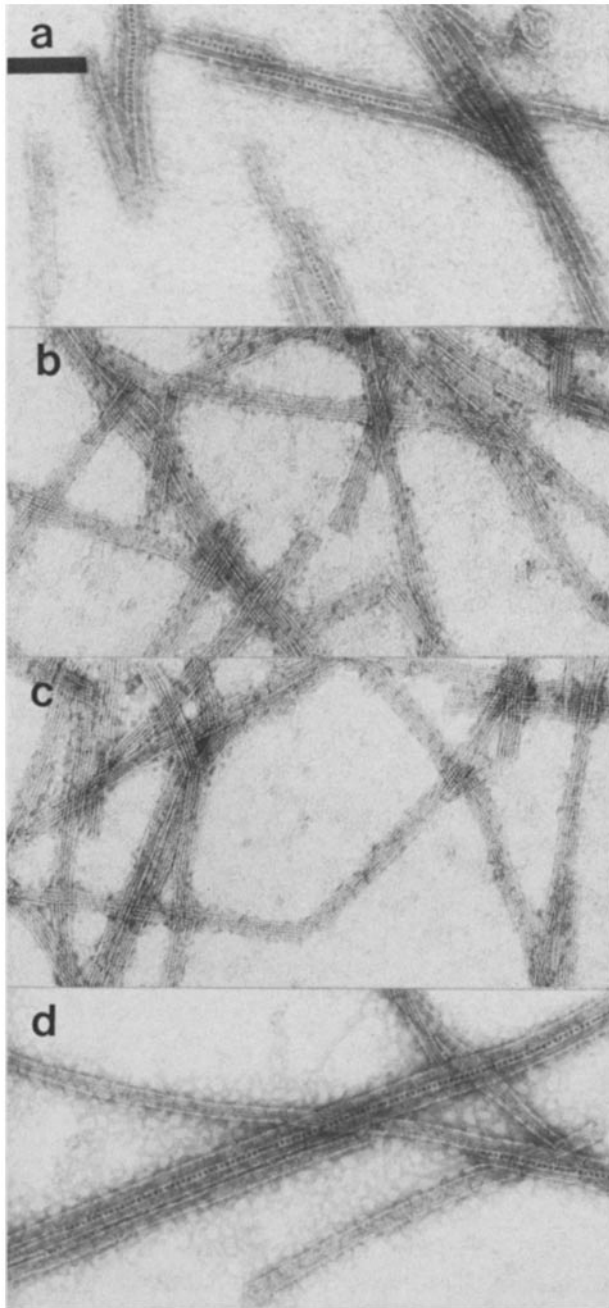


Figure 8. Dissociation and re-formation of the cross-links. (a) Cross-linked microtubules; (b) the same sample after the addition of 0.4 M NaCl. At this salt concentration most of the MAPs are dissociated from the microtubules. The cross-links present in a were also dissociated at this salt concentration. When the microtubules were centrifuged out of the high salt- and MAP-containing buffer at 22,500 g for 30 min at 0°C, and resuspended in fresh PEM buffer containing GTP and taxol, the cross-links did not re-form (c). When the MAP-containing supernatant was desalted by centrifugation through Sephadex G50-80 equilibrated with PEM buffer, and then 60 μ l of MAPs at 0.03 mg/ml were recombined with 60 μ l of the MAP-free microtubules at 1.36 mg/ml, the crosslinks were reformed (d). Bar, 60 nm.

Although the cross-links from *C. elegans* do connect multiple microtubules together, they do not act like any of the previously described microtubule bundling proteins (2, 22, 25, 27, 30, 35, 36, 50). The bundling proteins connect many

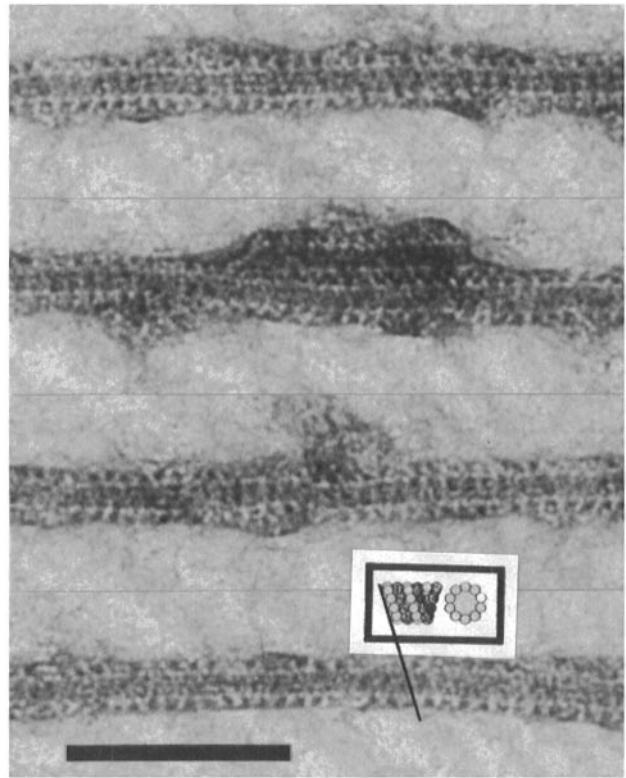


Figure 9. Micrographs of a negatively stained microtubule present in the desalted MAP fraction. Under these conditions the rare microtubules present became decorated with many short side-arms. Every tubulin dimer appears to have bound a side-arm, and this decoration seems to follow a single helix. The inset shows a drawing of a 10 protofilament microtubule with a two-start helix of subunits demonstrating that the pitch of the two-start helix is nearly identical to the pitch of the decorations on the microtubule in the micrographs. Bar, 100 nm.

microtubules together with a small number of cross-links; the cross-linking protein from *C. elegans* connected a few microtubules (2–4) together with many cross-links. Many of the microtubule bundling proteins are dissociated by ATP (25, 30, 50); however, 1.0 mM ATP had no discernable effect on the cross-links described here, as assayed by electron microscopy of negatively stained preparations (unpublished results).

The cross-links from *C. elegans* are almost certainly formed from one or more of the MAPs since they were eluted from the microtubules under the same ionic conditions as the MAPs, and re-formed when the MAPs and microtubules were recombined (Fig. 8). Because the cross-links are seen frequently, the proteins that compose the cross-links probably represent a substantial portion of the protein in the MAP fraction. The 32,000-mol-wt MAP was by far the most predominant protein in the MAP fraction, which suggests that this protein forms all or part of the cross-link. Furthermore, the cross-links appear to be 5.7-nm long and 3.0-nm wide in negatively stained samples. If we assume that they are also 3.0-nm deep and that they have a partial specific volume of 0.72 cm³/g, they would have a molecular weight of 43,000. Thus, given the uncertainty of this size estimate, the cross-links are in the size range of one or two 32,000-mol-wt MAPs.

In the microtubule preparation the cross-links only bound between microtubules (Fig. 7), but when the MAPs were in excess, structures that appeared to be the same as the cross-links appeared to bind to every dimer on the microtubule (Fig. 9). This suggests that in the microtubule preparation there were far more cross-link binding sites than cross-link proteins, and that the sites where the cross-links bound to two microtubules were favored. This result is expected since the change in free energy when two bonds are formed is greater than the change in free energy when one bond, of the same type, is formed. We expect, however, that the cross-linking of two free microtubules is rare since the cross-links appear to bind weakly and thermal motion is likely to dissociate microtubules connected by a single cross-link. However, once cross-links have formed between two microtubules, adjacent binding sites on aligned tubules are brought closer together, so the formation of additional cross-links between these microtubules should be favored. Therefore, cross-link formation is likely to be a cooperative reaction. This cooperativity could explain why there are few gaps in the stretches of periodic cross-linking. It is not yet known whether the cross-links bind to α - or β -tubulin, or between the subunits, but once this is determined they may serve as a useful tool for labeling α - or β -tubulins in microtubules.

The Surface-Lattice of Microtubules from *C. elegans*

In the standard model of a microtubule, the protofilaments are positioned so that the tubulin subunits form three parallel right-handed helices. Each helix contains 13 subunits and rises 12 nm in a turn (4, 33, 37). The pitch can be described as the ratio of the rise/turn to the number of subunits/turn or $P = 12 \text{ nm}/13 \text{ subunits} = 0.923 \text{ nm/subunit}$. Because microtubules of different sizes all contain protofilaments that are nearly parallel to their long axis rather than spiraling around the microtubule, the surface lattice must adjust to different numbers of protofilaments by adjusting the relative position of the protofilaments to each other laterally. A nine protofilament microtubule with approximately the same surface lattice as a 13 protofilament microtubule will be formed from two parallel right-handed helices each of which contain nine subunits in a turn of 8-nm rise, so $P = 8/9 = 0.889 \text{ nm/subunit}$. Similarly, for 8, 10, and 11 protofilament microtubules, P would be 1.0, 0.8, and 0.727 nm/subunit, respectively. The seam, which is present in 13 protofilament microtubules (37), is not predicted for microtubules that are formed with a two-start rather than a three-start helix. The microtubules shown in Fig. 9 apparently had a pitch of 0.8 nm/subunit, suggesting that these microtubules were formed with 9 or 10 protofilaments and contained a two-start helix.

Electron microscopy provides a rapid and sensitive assay for the cross-links, and the cross-links can be dissociated and re-formed *in vitro*. They should, therefore, be easy to isolate and further characterize. Antibodies made against the cross-links should show where and at what developmental stages these structures are present in *C. elegans*.

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