

# 270K Microtubule-associated Protein Cross-reacting with Anti-MAP2 IgG in the Crayfish Peripheral Nerve Axon

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**Abstract.** MAPs (microtubule-associated proteins) were isolated from crayfish walking leg nerves. A major MAP was identified as a high molecular weight protein (270K). This protein co-migrated with mammalian MAP2, stimulated the polymerization of rat brain tubulin into microtubules, and was heat resistant. Rotary shadowing revealed that the 270K MAP is a long thin flexible structure. It formed cross-bridges of fine strands, linking microtubules with each other in

vitro. These strands resemble the cross-bridges between microtubules observed in the crayfish axon permeabilized with saponin and quick-frozen, deep-etched. Antibodies against mammalian MAP2 cross-reacted with this crayfish MAP and stained the axoplasm of the walking leg nerves. Thus MAPs, especially the 270K MAP, appear to be a major component of the cross-linking strands between microtubules observed in the crayfish axon.

THE neuron is a highly polarized cell composed of dendrites, cell body, and axon along the direction of impulse propagation. Each part of the neuron takes characteristic shapes for which the cytoskeleton provides the framework. The cytoskeleton of the neuron is a static structure, but it does participate in the dynamic function of the cell, e.g., axonal transport (1, 7, 10) and neurite growth.

The crustacean axon is a unique system whose cytoskeleton is mainly composed of microtubules (25). This provides a simple model system for analysis of the microtubule cytoskeleton in axons which in other animals consist of complicated structures (8, 14, 15, 27).

Cross-bridges between microtubules have been observed in the crustacean axon (9, 18, 23, 26, 35). Concerning the chemical nature of the cross-bridge, it is reasonable to speculate that the cross-bridge, if present, may be composed of some kinds of MAPs.

In the mammalian nervous system several microtubule-associated proteins (MAPs)<sup>1</sup> have been identified. The MAPs include high molecular weight proteins MAP1A, 1B, 1C (4, 5) and MAP2A, 2B (11, 22, 32) as well as a group of low molecular weight proteins named Tau (34). The distribution of these MAPs is quite characteristic in a neuron. Usually, the MAP1 is present in dendrites, cell bodies, and axons (4, 5, 16), but the MAP2 is mainly present in dendrites and cell bodies (22). Recently it has been reported that Tau is restricted to the axon and that only a small amount of MAP2 exists in the axon (2, 24, 32).

The present study analyzes the cytoskeletal structure of axons that contain mainly microtubules and at the same time

tries to determine the chemical nature of the cross-bridges that connect the microtubule domain in an arthropod axon. By the quick-freeze, deep-etch approach, we have shown that there is a network of cross-bridges between microtubules in crayfish axons. Interestingly, we found a 270K MAP2-like protein as a main MAP in the peripheral axons of the crayfish, and this could very well be a main component of cross-bridges between microtubules found in the microtubule domain in the axon.

## Materials and Methods

### Quick-Freeze, Deep-Etch Electron Microscopy

Small bundles of axons including giant axons were dissected from walking leg nerves. Both ends of the nerves were tied with surgical threads and placed in Sylgard bottomed petri dishes containing internal medium (280 mM K<sup>+</sup> aspartate, 15 mM NaCl, 15 mM Hepes, 5 mM MgCl<sub>2</sub>, 3 mM EGTA) (21). Some of the axons were permeabilized by incubation for 20–30 min in internal medium plus 0.02% saponin. Saponin-permeabilized axons and some axons fixed with 2% glutaraldehyde in 0.1 M Pipes, 1 mM MgCl<sub>2</sub>, 1 mM EGTA, pH 6.8 and washed with distilled water were frozen by contact with a pure copper block cooled by liquid helium as previously described (12, 13). The surfaces of the frozen samples were fractured in Balzers 400 or 301 at –196°C at a vacuum of  $2 \times 10^{-6}$  torr and etched for 5 min at –95°C. Then they were replicated by rotary shadowing with platinum and carbon. Tissues were dissolved in chromic sulfuric acid, and replicas were cleaned with distilled water and picked up on formvar-coated grids. Stereo micrographs were taken by JEOL 100 CX or 1200 EX electron microscopy at 100 kV at  $\pm 10^\circ$  tilt.

### Preparation of MAPs from Walking Leg Nerves

For one experiment the walking leg nerves were dissected from 30 crayfish (12 experiments were carried out). MAPs were isolated using taxol according to the method developed by Vallee (32). About 0.1 g of nerves was taken

1. *Abbreviations used in this paper:* PEM buffer, 0.1 Pipes, pH 6.8, containing EGTA, 1.0 mM MgCl<sub>2</sub>; MAPs, microtubule-associated proteins.

from 30 animals. It was homogenized in 1.5 vol of 0.1 M Pipes, pH 6.8, containing 1.0 mM EGTA, 1.0 mM MgCl<sub>2</sub> (PEM buffer) with a Dounce glass homogenizer on ice. The homogenate was centrifuged at 36,000 g for 20 min at 4°C, and the pellet was discarded. The supernate was then centrifuged at 180,000 g for 90 min at 4°C, and the pellet again discarded. GTP was added to 1 mM, and the solution was warmed at 37°C for 10 min. Then taxol was added to 20 μM, and the solution was incubated for 5 min at 37°C. The solution was centrifuged at 35°C for 30 min through a cushion of 5% sucrose in PEM buffer containing 20 μM taxol and 1 mM GTP at 36,000 g. The resulting pellet contained microtubules and MAPs. The pellet was washed with PEM buffer containing 20 μM taxol and 1 mM GTP and then centrifuged for 30 min at 36,000 g.

To dissociate the MAPs from the microtubules, the microtubule pellet was resuspended to volume in assembly buffer plus taxol at 37°C and NaCl was added to 0.6 M. The solution was centrifuged again at 36,000 g for 30 min, leaving the MAPs in the supernate. The pellet was washed with PEM buffer containing 20 μM taxol and 1 mM GTP and then centrifuged for 30 min at 37°C. Pellets of MAPs plus microtubules and salt-extracted microtubules were quick-frozen and deep-etched. Other pellets were analyzed with SDS gel electrophoresis. For comparison MAPs plus microtubules were prepared from rat brain by the same procedure.

### ***Test for Heat Stability of MAPs and Stimulation of Polymerization of Rat Brain Tubulin***

Microtubules plus MAPs solutions from crayfish were made in PEM buffer containing 0.75 M NaCl, 10 mM 2-mercaptoethanol (protein concentration 8 mg/ml). They were rapidly pipetted into tubes that were kept in a boiling water bath for 4 min (11). Heavy precipitate formed. The tubes were rapidly cooled in ice water, and the solution was centrifuged at 4°C for 30 min at 10,000 g. The resulting pellets and supernatants were analyzed by SDS gel electrophoresis. The supernatant was dialyzed against PEM buffer and mixed with tubulin purified from rat brain by phosphocellulose column in

PEM buffer containing 1 mM GTP at 37°C for 10 min. In another case the boiled MAP and rat tubulin were mixed at 26°C, and OD<sub>350</sub> was scanned for 30 min. As a control a solution containing only tubulin was used. Finally solutions were warmed at 37°C for 5 min. Then some parts of the solutions were diluted 10–20-fold by PEM buffer and dropped on grids coated with formvar and carbon. They were stained with 2% uranyl acetate and examined by electron microscope. Other parts of the solutions were centrifuged at 10,000 g for 30 min. The resulting pellet was quick-frozen and deep-etched.

### ***Preparation of Rat Brain Tubulin***

Microtubule protein from rat brains was purified by three cycles of temperature-dependent assembly and disassembly in PEM buffer as described by Shelanski et al. (29). Tubulin was isolated from microtubule protein by phosphocellulose chromatography in the cold as described by Herzog and Weber (11). Pure tubulin was eluted with PEM buffer.

### ***Analytical Methods***

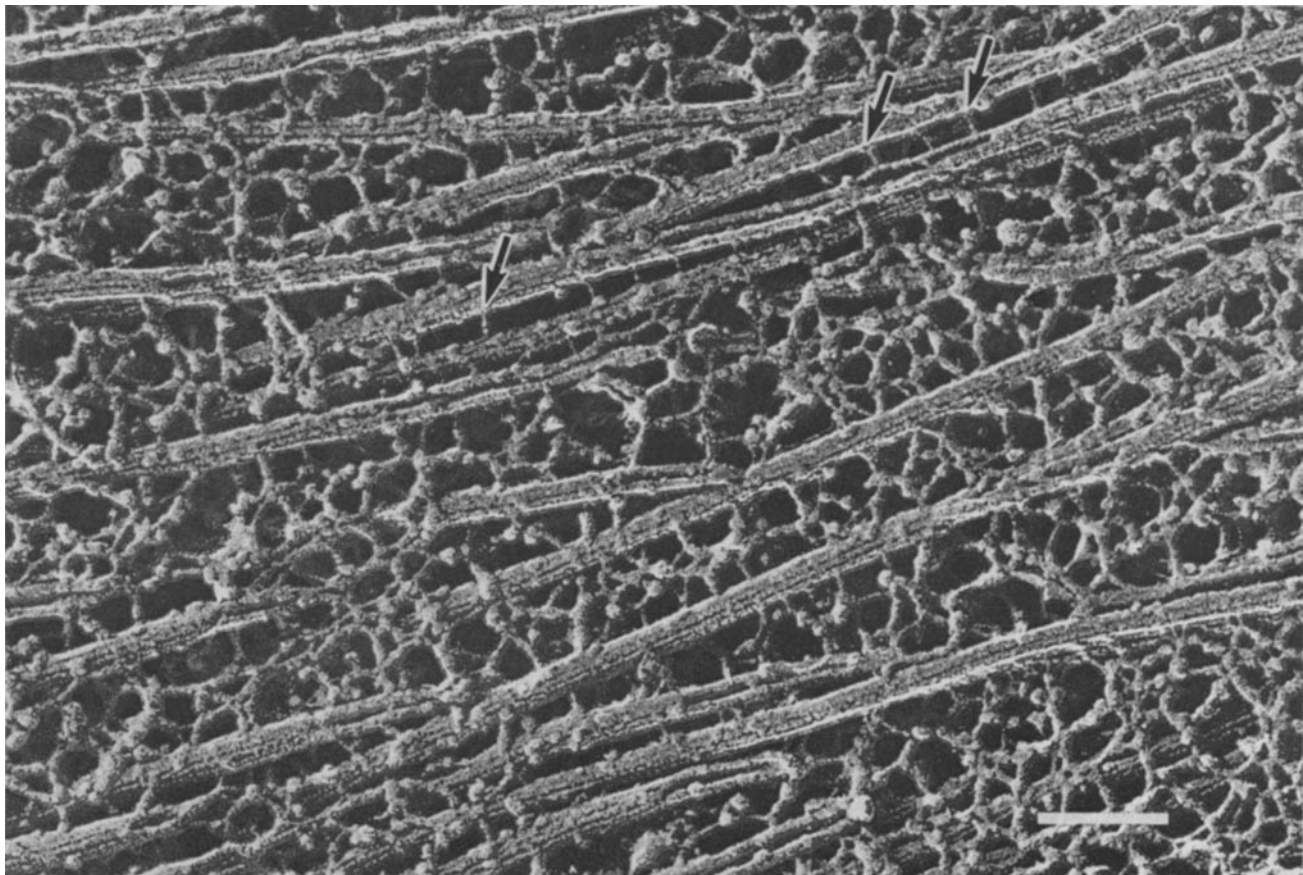
SDS gel electrophoresis was performed according to the method of Laemmli (20) using 7.5% acrylamide in the running gel and 3% acrylamide in the stacking gel. Gels were stained with Coomassie Brilliant Blue. Protein concentration was analyzed by a method described by Bradford (6).

### ***Low Angle Rotary Shadowing of 270K MAP***

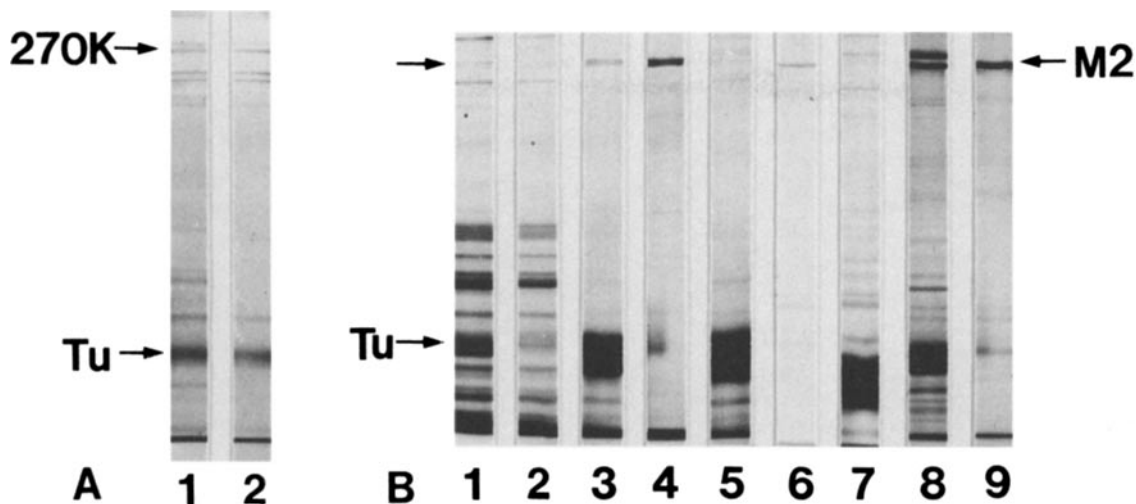
Rotary shadowing was accomplished as described by Shotton et al. (28) and Tyler and Branton (31).

The supernatant of the boiled MAPs, which contained the 270K protein as a main band according to SDS gel analysis, was dialyzed against PEM buffer.

The solution was mixed with glycerol (50 μg/ml protein in 30% glycerol-



**Figure 1.** Axoplasm in an axon permeabilized with saponin. The axoplasm is composed of longitudinally oriented microtubules and a network of fine strands between them. The strands are either a straight and short type (arrows) or anastomosing type. Bar, 0.1 μm.



**Figure 2.** SDS gel electrophoresis of proteins from crayfish peripheral nerves and MAPs of rat brain. Running gel 7.5%; stacking gel 3%. (A) Lane 1, isolated whole giant axons. Lane 2, isolated giant axons after permeabilization with saponin. *Tu*, tubulin. Arrow, 270K proteins remain after permeabilization. (B) Lane 1, homogenate of crayfish peripheral nerves. Lane 2, supernatant after centrifugation of the first supernatant of the homogenate warmed at 37°C and incubated with taxol. Tubulin and 270K protein are decreased in amount. Lane 3, pellet after centrifugation (at 36,000 *g* for 30 min) of the first supernatant of the homogenate incubated with taxol at 37°C for 10 min. This contains tubulin and MAPs from crayfish peripheral nerves. In the pellet, tubulin and 270K protein are the main bands. Lane 4, supernatant after centrifugation of crayfish MAPs and tubulin boiled for 4 min. 270K proteins clearly stay in the supernatant after heat treatment. Lane 5, pellet after centrifugation of crayfish MAPs and tubulin boiled for 4 min. Tubulin and other proteins are found in the pellet. Lane 6, supernatant after centrifugation of crayfish MAPs plus tubulin extracted with 0.6 M NaCl. 270K protein is extracted by high-salt treatment. Lane 7, pellet after centrifugation of crayfish MAPs plus tubulin treated with 0.6 M NaCl. 270K protein is extracted from the pellet. Lane 8, rat brain MAPs and tubulin isolated by a method using taxol. Lane 9, supernatant after centrifugation of MAPs and tubulin boiled for 4 min. MAP2 clearly stays in the supernatant. Arrow points to 270K protein. *Tu*, tubulin.

PEM buffer) and sprayed onto mica with nitrogen gas. The mica was dried under vacuum and rotary shadowed with platinum at an angle of 7° by an electron beam evaporator. Specimens were then coated with a thin carbon film. The replicas were detached from mica with hydrofluoric acid, washed with distilled water, and observed with an electron microscope.

### Immunocytochemistry and Immunoblotting Using Anti-MAP2 Antibodies

Heat stable MAP was subjected to SDS PAGE on a 7.5% polyacrylamide gel. It was then transferred to nitrocellulose paper. Nitrocellulose replicas of SDS gels were stained with either preimmune serum or anti-MAP2 serum (3) (courtesy of R. Vallee and G. Bloom, the Worcester Foundation). The second antibody was horseradish peroxidase-conjugated IgG fraction of goat anti-rabbit IgG (Cappel Laboratories, Cochranville, PA), which was reacted with diaminobenzidine to reveal the immunoreactive proteins.

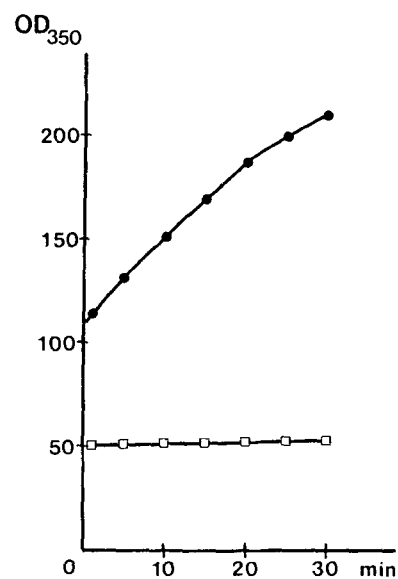
Crayfish walking leg nerves were dissected out and fixed with 2% paraformaldehyde and 0.2% glutaraldehyde in internal medium for 10 h. They were cut into small pieces and incubated with NaBH<sub>4</sub> (1 mg/ml) for 30 min and washed with phosphate-buffered saline (PBS). They were immersed in 1.5 M sucrose in PBS for 3 h and frozen with liquid Freon. 8- $\mu$ m frozen sections were cut with a Damon cryostat (Needham, MA). The sections were incubated with 20% goat serum in PBS followed by rabbit anti-MAP2 serum (3) in PBS plus 1% bovine serum albumin (BSA), or preimmune serum in PBS + BSA for 1 h. After washing with PBS plus 0.1% BSA, they were incubated with horseradish peroxidase-labeled goat anti-rabbit IgG which was reacted with 4-Cl-1-naphthol.

## Results

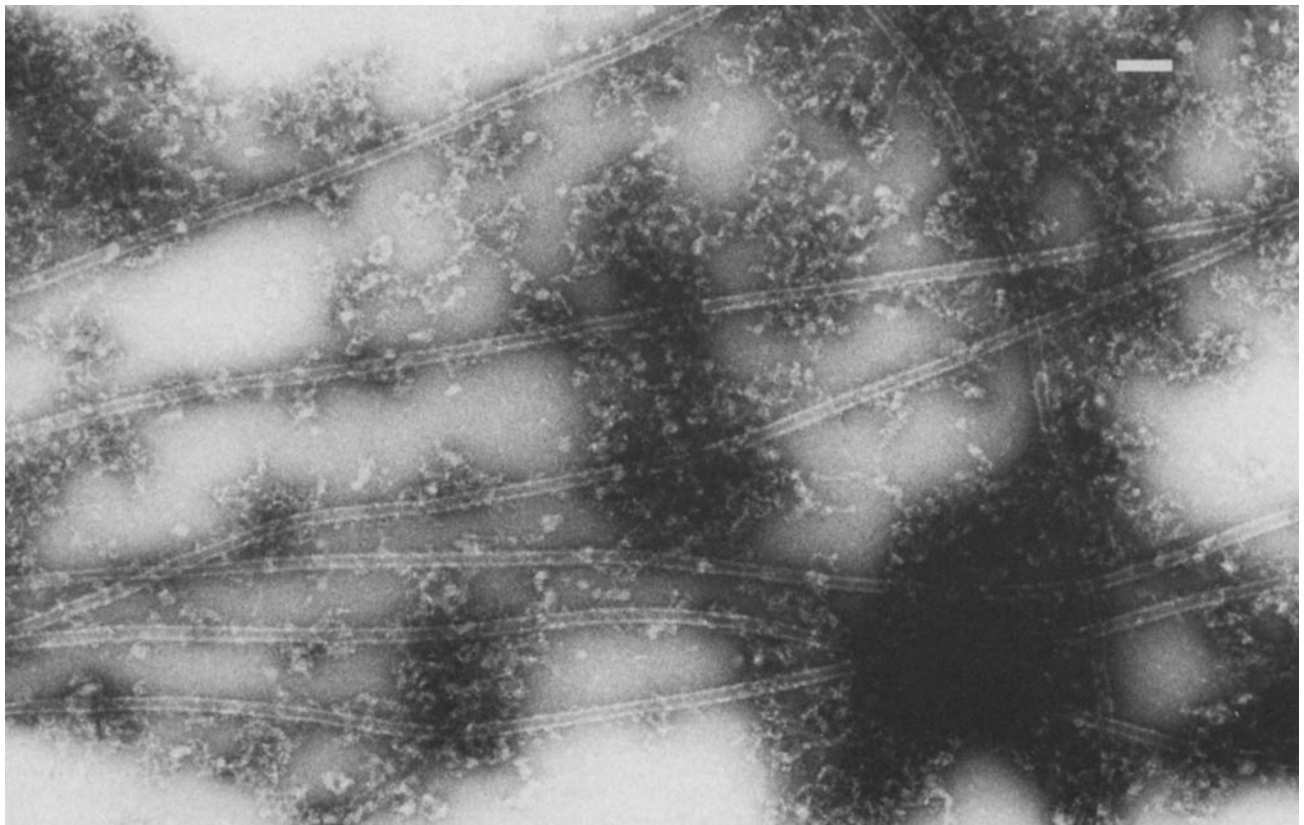
### Visualization of the Cytoskeletal Organization in the Saponin-permeabilized Crayfish Axon with Quick-Freeze, Deep-Etch Electron Microscopy

The axoplasm in saponin-treated nerves was composed of longitudinally oriented microtubules and an extensive net-

work of cross-bridges 6–9 nm in diameter ( $6.6 \pm 1.4$ -nm wide) (Fig. 1). When the microtubules are close to each other, the cross-bridges tend to be straight and short (~30-nm long). However, frequently microtubules tended to be separated far more than 30 nm and the cross-bridges tended



**Figure 3.** OD<sub>350</sub> of solution A containing 224  $\mu$ l of rat tubulin (1.5 mg/ml), 150  $\mu$ l of crayfish-boiled MAP (0.56 mg/ml), and 1 mM GTP in PEM buffer (●) and solution B containing 224  $\mu$ l of rat tubulin (1.5 mg/ml), 150  $\mu$ l of PEM buffer, and 1 mM GTP in PEM buffer (□).



**Figure 4.** Negative-stained solution A containing rat brain tubulin and crayfish boiled MAP in PEM buffer plus 1 mM GTP after incubation for 35 min.

to form anastomosing networks (Fig. 1). Basically similar results were obtained from fixed samples washed with distilled water before freezing.

#### ***Identification of a MAP (270K) in the Crayfish Peripheral Nerve and Its Molecular Structure***

MAPs were isolated from crayfish leg nerves using taxol. A prominent band of protein co-migrating (270K) with rat brain MAP2 was identified by SDS gel electrophoresis in the MAPs polymerized with tubulin and by using taxol (Fig. 2). This protein was also identified in an SDS gel of isolated giant axons (two to three axons, Fig. 2 A). After saponin treatment this MAP clearly remained in the isolated giant axons (Fig. 2 A). This protein stayed in the supernatant after boiling for 5 min, so it somewhat resembled mammalian MAP2 (Fig. 2 B). 270K MAP purified by boiling enhanced polymerization of tubulin purified from rat brain by a phosphocellulose column (Figs. 3 and 5). OD<sub>350</sub> was scanned for 30 min after incubating rat brain tubulin with 270K MAP at 26°C in PEM buffer containing 1 mM GTP (Fig. 3). OD<sub>350</sub> of the control solution containing only tubulin was also scanned (Fig. 3). After 30 min both solutions were warmed to 37°C and were then incubated for another 5 min. After that the solutions were examined by the negative-stain technique. Microtubules were formed in the samples of 270K MAP plus rat tubulin (Fig. 4). Microtubules were not found in the sample containing only tubulin. Polymerized tubulin plus 270K MAP were centrifuged and the pellets were quick-frozen, deep-etched. Microtubules were cross-linked with

each other mostly by simple straight cross-bridges (25–50-nm long), but sometimes by anastomosing cross-bridges (Fig. 5). These cross-bridges looked just like the fine strands between microtubules observed in *in vivo* axons. However, the cross-bridges tended to be much more anastomosing *in vivo* than those in the replica of 270K plus tubulin *in vitro*. Low angle rotary shadowing of 270K MAP on mica revealed that the 270K MAP is a long flexible thin molecule (Fig. 6). The length of the 270K MAP on mica varied somewhat but its average was  $104 \pm 22$  nm.

#### ***270K MAP Reacted with Anti-MAP2 IgG Which Stained Crayfish Axons***

As shown in Fig. 7, 270K MAP is composed of two closely associated bands, both of which reacted with anti-MAP2 IgG. Anti-MAP2 stained axoplasm of both giant axons and smaller axons in the walking leg nerves (Fig. 8). The cytoplasm of the satellite cells were not stained (Fig. 8). These data mean that 270K MAP cross-reacts with anti-MAP2 IgG and is localized in the axoplasm of crayfish peripheral nerves.

#### ***Discussion***

##### ***A Network of Cross-bridges Exists between Microtubules in Crayfish Axons***

The present study clearly revealed a network of fine cross-bridges between microtubules. These cross-bridges were

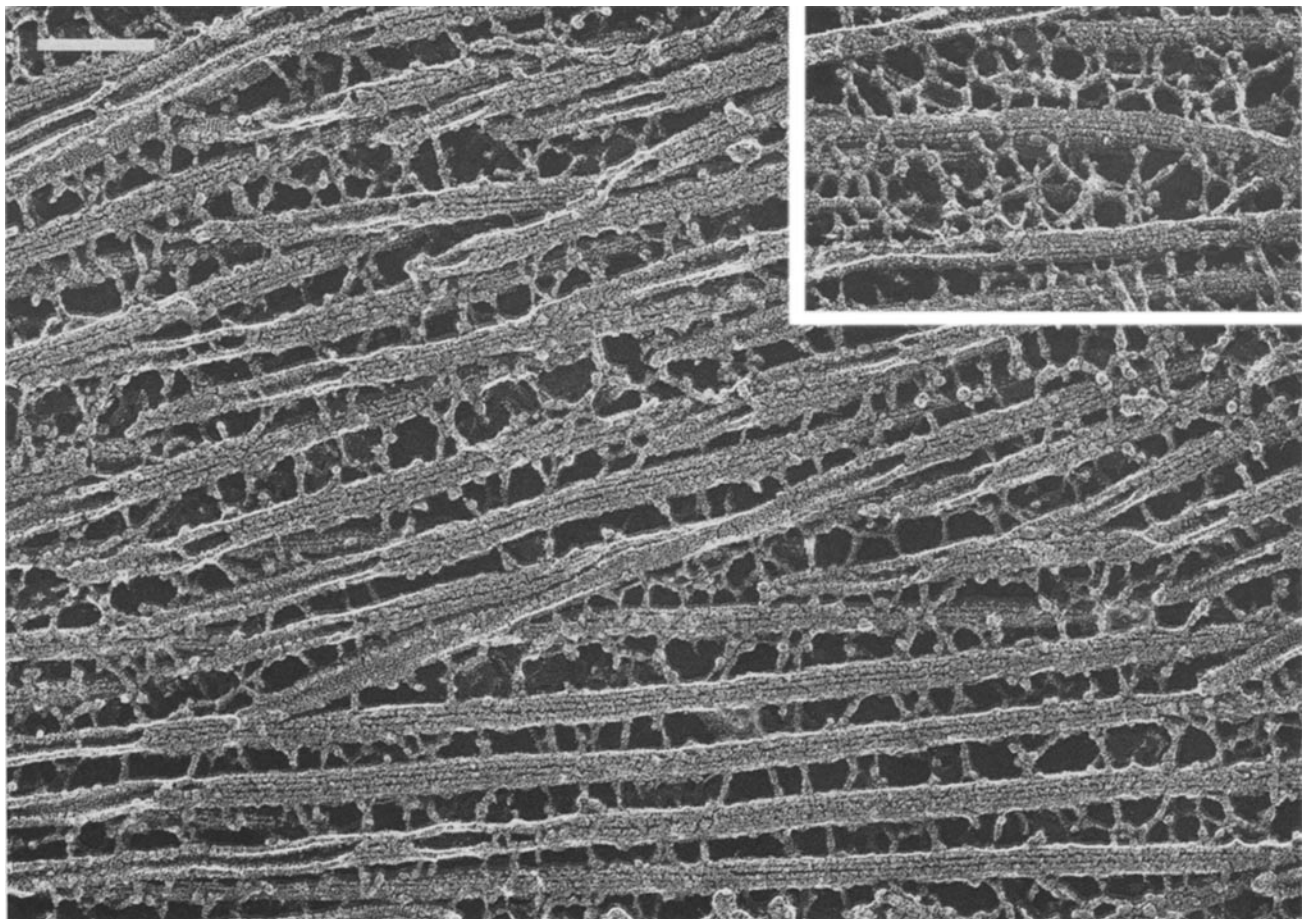


Figure 5. Quick-frozen, deep-etched pellet of crayfish 270K MAP and rat tubulin polymerized in vitro. Microtubules are cross-linked with each other via fine strands of a mainly straight and simple type but sometimes anastomosing type. Bar, 0.1  $\mu\text{m}$ . (Inset) Anastomosing cross-bridges.

most clearly demonstrated in saponin-treated axons. Because we have found similar cross-bridges in axons permeabilized with saponin and reactivated with ATP in which organelles were actively transported, it is reasonable to conclude that the cross-bridges are real functional structures in the axon (17).

The arrangement of microtubules in the crayfish axon was different from axons in vertebrates. In crayfish the distance between adjacent microtubules was wider than in the vertebrate. So the network of cross-bridges was more extensive in the crayfish axons. In this regard the cytoskeletal architecture in the crayfish axon resembles that in the dendrites of

mammalian neurons where the microtubules are dominant cytoskeletal components and the network of cross-bridges is more extensive than in axons. It is interesting that both in the dendrites of mammalian neurons and in the crayfish axon, MAP2 (22) or MAP2-like protein is the main component of microtubule-associated cross-bridges.

#### *MAPs in Crayfish Peripheral Nerves*

A MAP (270K) co-migrating with mammalian MAP2 was identified as a major MAP in the crayfish peripheral nerve in the present study. It was found in isolated fresh giant axons and remained in the axon after saponin extraction. Of course

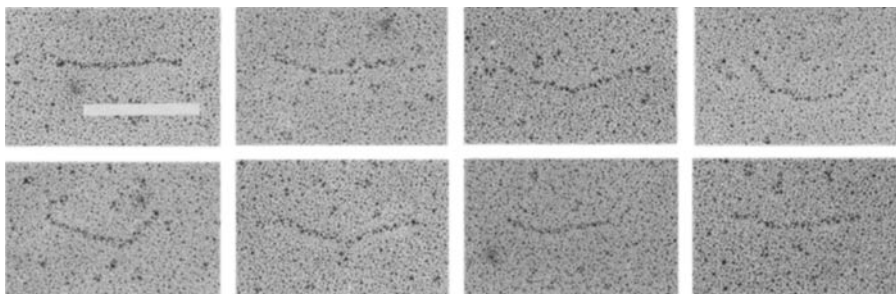
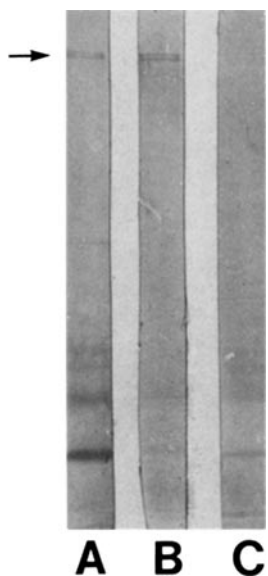


Figure 6. Rotary-shadowed 270K MAP molecules. It looks like a long flexible thin rod. Bar, 0.1  $\mu\text{m}$ .



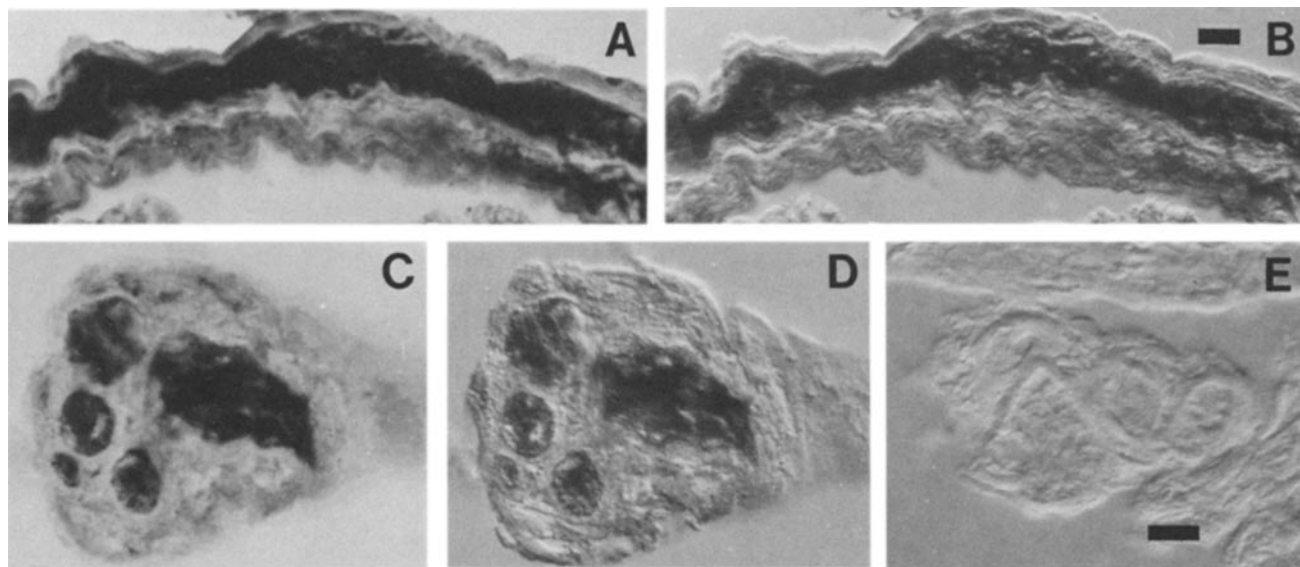
**Figure 7.** Nitrocellulose replicas of SDS gels of crayfish heat stable MAP. (A) A micrograph of an original gel stained with Coomassie Brilliant Blue. (B) A nitrocellulose replica stained with anti-MAP2 serum. (C) A replica stained with preimmune serum. Anti-MAP2 antibodies react with 270K MAP (arrow).

the peripheral nerves also contain Schwann cells (satellite cells) so that the data from biochemistry alone are not enough to verify the idea that the 270K MAP is a component in the crayfish peripheral axon. However, in the present study immunocytochemistry using anti-MAP2 antibodies that cross-react with 270K MAP demonstrated clearly that anti-MAP2 antibodies stained axoplasm. Therefore, we conclude that 270K MAP, a MAP2-like protein, exists in the axoplasm of the peripheral nerves in crayfish. When the 270K MAP was polymerized with rat brain tubulin *in vitro*, it formed cross-bridges between microtubules. It took a long rodlike form on mica.

Mammalian MAP2 was shown by a thin section study to form armlike structures on microtubules (19). The platinum-replication technique also demonstrated that MAP2 is a long rodlike structure (33). By the quick-freeze method MAP2 was revealed as cross-bridges between microtubules (unpublished data). Furthermore, anti-MAP2 IgG cross-reacted with the 270K MAP.

The fact that this MAP in many ways resembles MAP2, but in fact exists in the axons, is extremely interesting, because MAP2 has been shown to exist mainly in dendrites in vertebrate neurons (22). One of the main functions of this MAP could be, of course, to build a framework in the crayfish axon. However, at the same time this structure ought to be elastic or dynamic, because rapidly transported vesicles must pass through it (1, 10, 17). It has also been shown that MAPs cross-link secretory granules with microtubules in the absence of ATP, but release them in their presence (30). The fact that the 270K MAP resembles mammalian MAP2 may be related to the structural similarity between dendrites of mammalian neurons and crayfish axons. In both cases microtubules are predominant cytoskeletal elements and are distributed throughout the cytoplasm. There are similar types of networks of cross-bridges between microtubules in both crayfish axons and dendrites of mammalian neuron (Shiomura, Y., and N. Hirokawa, manuscript in preparation).

However, although MAP1 and MAP2 are two main MAPs in the dendrites of mammalian neurons, crayfish axons contain only 270K protein as a main high molecular weight MAP. We found straight cross-bridges as well as anastomosing cross-bridges in the crayfish axons. In the preparation containing 270K MAP and rat tubulin we recognized mainly straight and short cross-bridges, but sometimes anastomosing ones between microtubules. Because the straight and



**Figure 8.** Immunocytochemical staining of crayfish peripheral nerves with anti-mammalian MAP2 antibodies. (A) A longitudinal section of a medium-sized nerve stained with anti-MAP2 antibody. The axoplasm is prominently stained. The cytoplasm of satellite cells surrounding the axon is not stained. (B) A Nomarski photomicrograph of the same axon as shown in A. The reaction products are clearly observed. Bar (for A and B), 10  $\mu$ m. (C) A cross-section of several nerves stained with anti-MAP2 antibody. The axoplasm is stained positively. (D) A Nomarski photomicrograph of the same section as shown in C. It is evidently shown that the reaction products localize in the axoplasm and not in the cytoplasm of the surrounding satellite cells. (E) A Nomarski photomicrograph of a cross-section of several nerves stained with preimmune serum. The axoplasm is not stained. Bar (for C, D, and E), 10  $\mu$ m.

short cross-bridge ( $\sim 30$  nm) was shorter than a single 270K molecule ( $104 \pm 22$  nm), it could be composed of one 270K molecule, and a 270K molecule could have binding sites to tubulin at both ends.

There are probably two possibilities to explain the structure of the anastomosing cross-bridges at the molecular level: (a) 270K MAP can bind tubulin as well as 270K protein at both ends. Thus a single molecule can cross-link microtubules with each other, and several 270K molecules can form networks. (b) A 270K molecule has binding sites to tubulin at both ends. In this case the oligomer of the tubulin may serve as molecules which locate at the anastomosing points of cross-bridges. In addition, because in the *in vivo* axon the cross-bridges are so extensively anastomosed, the possibility remains that there may be some unknown proteins that connect 270K MAP with each other. Further studies are necessary to understand the structure of anastomosing cross-bridges at the molecular level. So far the crayfish axoplasm provides a simple, clear system for understanding how the MAP interacts with microtubules to build the *in vivo* structure further at the molecular level.

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