

# Organization of Mammalian Neurofilament Polypeptides within the Neuronal Cytoskeleton

NOBUTAKA HIROKAWA,\* MARCIE A. GLICKSMAN,† and MARK B. WILLARD‡

*Departments of \*Physiology and Biophysics, and †Anatomy and Neurobiology and Biochemistry, Washington University School of Medicine, St. Louis, Missouri 63110. Dr. Hirokawa's present address is Department of Anatomy, University of Tokyo, School of Medicine, Tokyo 113, Japan*

**ABSTRACT** Neurofilaments in the axons of mammalian spinal cord neurons are extensively cross-linked; consequently, the filaments and their cross-bridges compose a three-dimensional lattice. We have used antibody decoration in situ combined with tissue preparation by the quick-freeze, deep-etch technique to locate three neurofilament polypeptides (195, 145, and 73 Kd) within this lattice. When antibodies against each polypeptide were incubated with detergent-extracted, formaldehyde-fixed samples of rabbit spinal cord, each antibody assumed a characteristic distribution: anti-73-Kd decorated the neurofilament core uniformly, but not the cross-bridges; anti-145-Kd also decorated the core, but less uniformly; sometimes the anti-145-Kd antibodies were located over the bases of cross-bridges. In contrast, anti-195-Kd primarily decorated the cross-bridges between the neurofilaments. These observations show that the 73-Kd polypeptide is a component of the central core of neurofilaments, and that the 195-Kd polypeptide is a component of the inter-neurofilamentous cross-bridges. It is consistent with this conclusion that we found few cross-bridges between neurofilaments in the optic nerves of neonatal rabbits during a developmental period when the ratio of 195 to 73 or 145-Kd polypeptides is much lower than in adults. The ratio of 195-Kd polypeptide to the other two neurofilament polypeptides also appeared much lower in the cell bodies and dendrites than in axons of adult spinal cord neurons, when the dispositions of the three polypeptides were studied by immunofluorescence experiments. The cell bodies apparently contain neurofilaments composed primarily of 145- and 73-Kd polypeptides, because we observed antibody decoration of individual neurofilaments in the cell bodies with anti-73- and -145-Kd, but not with anti-195-Kd. We conclude that the 195-Kd polypeptide participates in a cross-linking function, and that this function is, at least in certain neurons, most prevalent in the mature axon.

The axonal cytoskeleton is typically composed of longitudinally oriented neurofilaments and microtubules that are extensively cross-linked to themselves, to each other, and to the plasma membrane by thin fibrils (3, 10, 11, 22, 23, 28, 35, 40, 41). Embedded in the resulting three-dimensional lattice are membrane-bounded organelles (e.g., mitochondria, vesiculo-tubular structures, smooth endoplasmic reticulum) which are themselves extensively cross-linked to the neurofilaments, microtubules, or both. The system of cross-bridges appears to determine the spatial relationships between the various elements of the axoplasm; in addition, the cross-links between elements that may be moving past each other by the process of axonal transport raise the question of the dynamic

properties of these cross-bridges (i.e., how they are made and released) and whether they could affect the translocation of organelles down the axon. For these reasons, it is important to understand the morphology and chemistry of these cross-bridges.

Three populations of cross-bridges, each mediating different intra-axonal linkages, have been described in axons prepared for electron microscopy by the quick-freeze, deep-etch technique (10). Here we have attempted to identify the molecular nature of the cross-bridges (20–60 nm long, 4–6 nm wide) between neurofilaments.

Mammalian neurofilaments contain three major polypeptides. In the case of the rabbit, these have molecular weights

of ~195-, 145-, and 73-Kd. Several observations have nominated the 195-Kd polypeptide as a candidate for a component of the cross-bridges between neurofilaments. First, when partially purified neurofilaments were incubated with antibodies specific for 195-, 145- and 73-Kd polypeptides, anti-195-Kd decorated a structure that was peripheral to the core of the filament, and that occasionally cross-linked two filaments, whereas anti-73-Kd decorated the central core of the filament uniformly (38). A similar decoration pattern was observed when neurites of cultured dorsal root ganglion neurons were incubated with antineurofilament antibodies (29). Second, filaments could be assembled from their constituent polypeptides *in vitro* only when the 73-Kd polypeptide was included in the assembly mixture (4, 19, 24, 42); when the 195 Kd-polypeptide was also included, the resulting filaments had projections (4, 24). These observations showed that a portion of the 195-Kd polypeptide is peripheral to the filament core and in some cases links two filaments together *in vitro*. However, the cross-bridges between neurofilaments were not well preserved in these experiments, and therefore the role of the 195-Kd polypeptide in these cross-bridges was speculative. Here we provide evidence that the 195-Kd polypeptide is a component of the cross-bridges between neurofilaments in the axon. When we decorated nervous tissue with antineurofilament antibodies *in situ* and observed the resulting decoration in tissue prepared for electron microscopy by the quick-freeze, deep-etch technique, anti-195-Kd was localized over the interfilamentous cross-bridges. In certain immature axons, where the ratio of 195- to 145- or 73-Kd polypeptides is much lower than in adult axons (16, 18, 31, 39), we observed few interneurofilamentous cross-bridges. In adult neurons indirect immunofluorescence indicated that the ratio of 195- to 145- or 73-Kd polypeptides is much greater in the axons than in the cell bodies and dendrites. These observations support the conclusion that the 195-Kd polypeptide is a component of the cross-bridges between neurofilaments, and that the function of these cross-bridges is somewhat specific for mature axons.

## MATERIALS AND METHODS

**Preparation of Antisera:** Antibodies against SDS-denatured 195-, 145-, and 73-Kd rabbit neurofilament polypeptides were produced in goats as described previously (38). An IgG fraction of each antiserum was adsorbed by passing it sequentially through affinity columns containing each of the other two neurofilament polypeptides, and then purified by affinity chromatography on a column containing the polypeptide against which it was made. To assess the specificity of the antibodies, proteins from an SDS extract of rabbit spinal cord were transferred (Western blotted) onto nitrocellulose paper, after they had been separated by electrophoresis on SDS polyacrylamide gels (2). The specificity of the adsorbed, affinity-purified antibodies was assayed by incubating the polypeptides on the nitrocellulose paper with each antibody followed by biotinylated secondary antibody, and avidin-biotin-horseradish peroxidase (HRP), both from Vector Laboratories (Burlingame CA). The HRP was reacted with diaminobenzidine according to a previously described procedure (12). Fig. 1 shows that the anti-195-Kd and anti-73-Kd polypeptide reacted specifically with the 195- and 73-Kd polypeptide, respectively. Anti-145-Kd polypeptides reacted primarily with 145-Kd polypeptides, but also weakly stained a small number of additional polypeptides; our conclusion concerning the localization of 145-Kd polypeptides in the axon is subject to this reservation. The pattern of reactivity of each antibody was not altered when the proteins on the nitrocellulose sheets were incubated with alkaline phosphatase to remove phosphate by the method of Sternberger and Sternberger (34), prior to incubation with the antibodies.

Affinity-purified rabbit anti-goat IgG was purchased from either Gateway Immunologicals (St. Louis, MO) or Cappel Laboratories (West Chester, PA). Monovalent Fab fragments were prepared from rabbit anti-goat IgG using the method described by Mage (20).

**Quick-Freeze, Deep-Etch Technique and Immunocytochemistry:** Pieces (2–3 mm long) of rabbit spinal cord were cut in cross-section or in an oblique plane and were agitated gently with 1% Triton X-100 in K<sup>+</sup>-buffer (70 mM KCl, 5 mM MgCl<sub>2</sub>, 3 mM EGTA, 0.25 mM phenylmethylsulfonyl fluoride (PMSF), 30 mM HEPES, buffer pH 7.4) for 3 h at 4°C. After they were washed twice in K<sup>+</sup>-buffer, the spinal cord pieces were fixed with 1% paraformaldehyde in K<sup>+</sup>-buffer for 1 h at 4°C.

The Triton-treated tissue was trimmed and the anterior horn region including the bordering white matter was incubated overnight at 4°C with adsorbed, affinity-purified primary antibodies, or control nonimmune IgG (40–60 µg/ml) in K<sup>+</sup>-buffer containing bovine serum albumin (1 mg/ml). The tissue segments were washed in K<sup>+</sup>-buffer, and incubated overnight with a secondary rabbit anti-goat IgG (150 µg/ml) or monovalent Fab fragments (100 µg/ml). The tissue was washed in K<sup>+</sup>-buffer, and fixed in 2% paraformaldehyde for 3 h at 4°C.

To observe cytoskeletal structures in tissue that was not treated with antibodies, the tissue (either adult rabbit spinal cord or optic nerves from 1-, 5-, and 10-d-old and adult rabbits) was quick-frozen either directly after dissection, or after it was made permeable by incubation with saponin (0.1% saponin, 10<sup>-5</sup> M taxol) for 30 min at room temperature (10). Some of the saponin-treated tissues were fixed with 1% paraformaldehyde for 1 h at room temperature. The fixed tissue was first rinsed briefly in distilled H<sub>2</sub>O, followed by methanol (15%), and then rapidly frozen on a copper block cooled with liquid helium as described previously (9, 13). The frozen samples were freeze-fractured and deeply etched, rotary shadowed with platinum and carbon, and the replicas were viewed with a JEOL 100CX electron microscopy at 100 kV, with ±10° tilt.

**Immunofluorescence:** Rabbit spinal cord pieces, cut in cross-section or in an oblique plane were frozen in liquid freon, and 6–8-µm thick frozen sections were cut with a cryostat and collected on glass slides. The sections were incubated for 30 min with adsorbed, affinity-purified primary antibodies or with control nonimmune IgG (30 µg/ml), then washed three times in K<sup>+</sup>-buffer or PBS (150 mM NaCl, 10 mM NaPO<sub>4</sub>, pH 7.2) and incubated with a 50-fold dilution of fluorescein-conjugated rabbit anti-goat IgG (Cappel Laboratories). All sections were mounted in 90% glycerol (with 0.1% para-phenylenediamine added to reduce fading) and examined using a fluorescence microscope.

**Examination of Neurofilaments by the Quick-Freeze-Mica Technique (8):** Neurofilaments (100 µg/ml) in PBS, prepared from rabbit spinal cord as described previously [38]) were dropped onto fragmented mica flakes, and quick-frozen. The samples were freeze-fractured at –106°C, etched for 3 min in a Balzers 301 unit, and then rotary replicated with platinum at 24° or 11°. The mica and tissue were dissolved in hydrofluoric acid and chromesulfuric acid, and the platinum replicas were examined as described above.

## RESULTS

### *Antibody Decoration of Neurofilaments in Axons*

To determine the location of the three neurofilament polypeptides within the axonal cytoskeleton, we treated small pieces of the anterior horn region of rabbit spinal cord with Triton X-100, fixed them, and incubated them with antibodies that reacted specifically (see Fig. 1) with each of the three neurofilament polypeptides (or control nonimmune IgG) followed by a secondary antibody. The tissue was prepared for electron microscopy by the quick-freeze, deep-etch technique. Fig. 2 shows tissue incubated with nonimmune IgG. The neurofilaments and 20–60-nm cross-bridges were undecorated. When the tissue was incubated with anti-73-Kd, specific antibody complexes were attached to the central core of the neurofilaments, increasing their diameters by a factor of two; the cross-bridges appeared smooth and undecorated (Figs. 3 and 4A). Glial filaments, characterized by their lack of cross-bridges, were not decorated by the antibody (Fig. 4B). In contrast, anti-195-Kd-secondary-antibody complexes appeared as globular masses situated between the neurofilaments (Fig. 5, A and B); the cores of the neurofilaments appeared smooth. Fig. 5B shows, at higher magnification, that the anti-195-Kd polypeptide complexes were situated between the smooth neurofilament cores and appeared to overlie the cross-

bridges. When we used monovalent Fab fragments of rabbit anti-goat IgG as the secondary antibody, these complexes were smaller and more discrete (Fig. 5 C). Like anti-73-Kd, the anti-145-Kd antibody complexes decorated the central core, but less uniformly than anti-73-Kd complexes (Fig. 6).

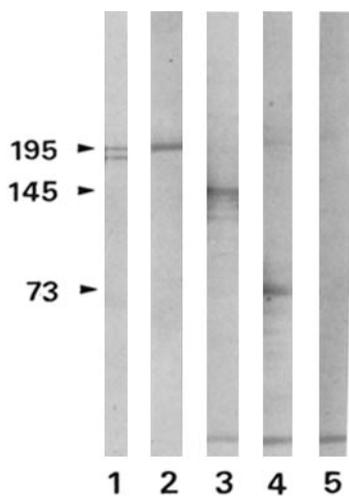


FIGURE 1 Polypeptides from SDS extracts of spinal cord electrophoresed and transferred onto nitrocellulose sheets (Western blotting) and incubated with affinity purified and adsorbed antibodies to the 195-Kd polypeptide, (lanes 1 and 2), the 145-Kd polypeptide (lane 3), the 73-Kd polypeptide (lane 4), or nonimmune IgG (lane 5); antibody reaction was detected by means of a peroxidase reaction as described in Materials and Methods. Lanes 3-5 are tissues samples from the

same rabbit. Lane 1 is from a different rabbit that was heterozygous for two different alleles ( $H_1$  and  $H_2$ ) of the gene for the 195-Kd polypeptide (36); this genetic polymorphism gives rise to the two bands on the blot.

Occasionally we observed anti-145-Kd complexes at the bases of cross-bridges. These results show that antigens associated with the 195-Kd polypeptides are located in the interfilamentous cross-bridges and that antigens associated with the 73- and 145-Kd polypeptides are located in the central core of neurofilaments.

### *Immunofluorescent Staining of Axons, Cell Bodies, and Dendrites with Antineurofilament Antibodies*

Using antibodies specific for each, we studied the distribution of the three neurofilament polypeptides in neurons of the anterior horn of the rabbit spinal cord by indirect immunofluorescence of cryostat sections. Fig. 7 shows oblique sections through the spinal cord, in which cell bodies, dendrites and axons in the grey matter, as well as axons cut in cross-section in the white matter, were available for staining. All three antineurofilament antibodies (but not nonimmune IgG) intensely stained the axons in both white matter and grey matter (Fig. 7, A-D). In contrast, cell bodies and dendrites were stained less intensely by anti-195- than by anti-145- and anti-73-Kd (Fig. 7, E-G). These observations suggest that the ratio of 195- to 145- or 73-Kd polypeptides is greater in the axons than in the cell bodies, and dendrites, and raises the question of how this deficiency of 195-Kd polypeptides may be expressed in the morphology of filaments in the cell bodies and dendrites.

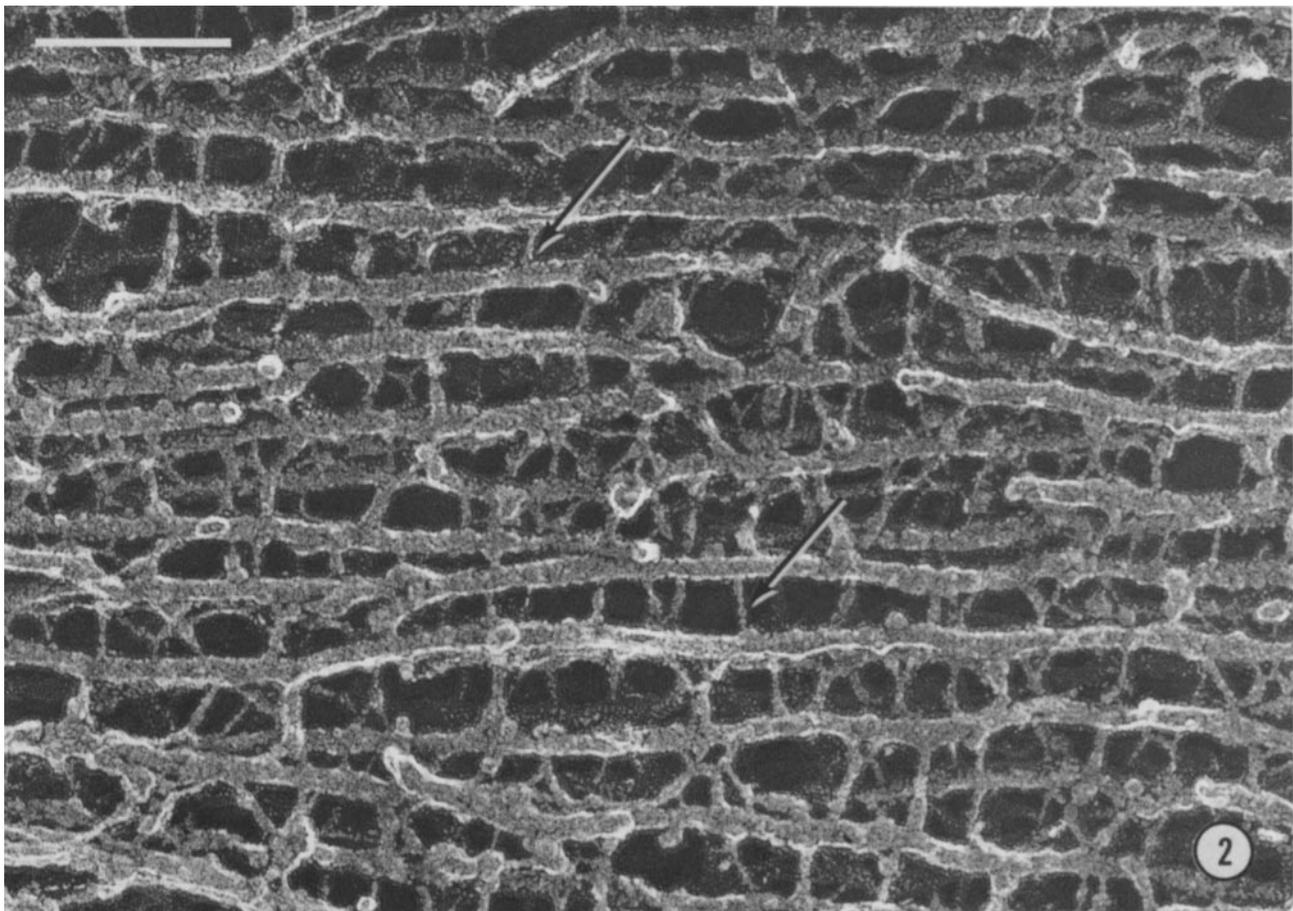


FIGURE 2 Axonal neurofilaments from Triton-extracted spinal cord incubated with nonimmune IgG and secondary IgG, and then prepared for electron microscopy by the quick-freeze, deep-etch method. No antibody complexes are apparent on the neurofilaments or their cross-bridges (arrows). Bar, 0.1  $\mu$ m.  $\times$  257,000.

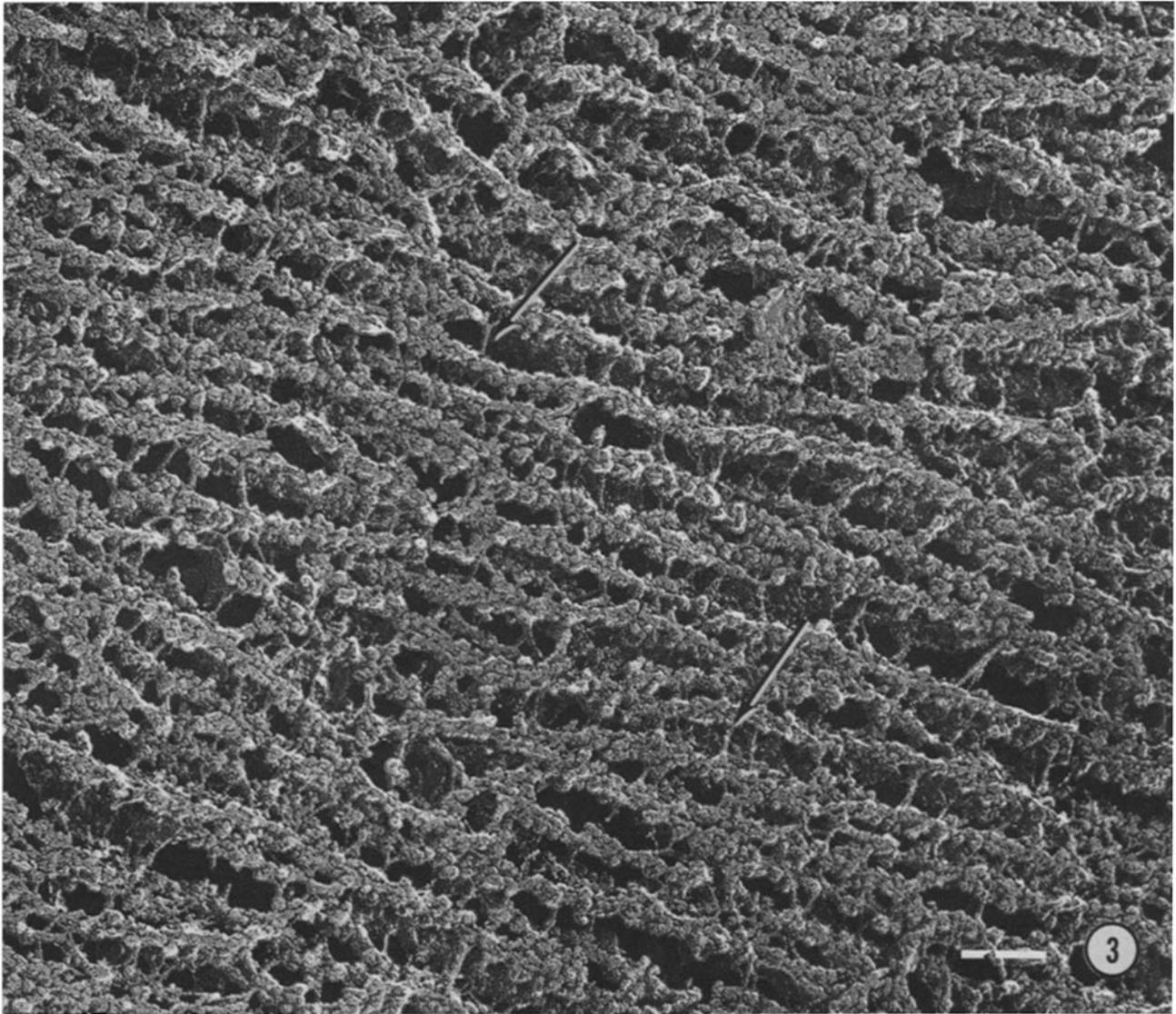


FIGURE 3 Low magnification view of axonal neurofilaments decorated with anti-73-Kd and secondary antibody (IgG). Antibodies decorate the neurofilament core uniformly, but cross-bridges (arrows) are not decorated. Bar, 0.1  $\mu\text{m}$ .  $\times 125,000$ .

### Organization of Neurofilaments in Axons, Cell Bodies, and Dendrites

We examined the filamentous cytoskeleton in neurons from the anterior horn of the rabbit spinal cord, prepared by the quick-freeze, deep-etch technique. Prior to freezing, the samples were either (a) untreated; (b) made permeable with saponin; or (c) made permeable with saponin or demembrated with Triton X-100, and then fixed and washed with distilled water. In axons, neurofilaments 20–60 nm apart ran parallel to the longitudinal axis of the axon cylinder. Neighboring neurofilaments were cross-linked to each other at frequent intervals (25 per  $\mu\text{m} \pm 7.5$  SD  $N = 109$ ), and the resulting lattice occupied much of the axoplasm (Fig. 8A and reference 10). In contrast, intermediate filaments in the cell body were randomly oriented, and the frequency of cross-bridges (an average of 10.5 per  $\mu\text{m} \pm 14.5$  SD  $N = 41$ ) was lower than in axons (Fig. 9). Some of the filaments formed small bundles, and within these bundles the frequency of cross-bridges was the same as in axons (Fig. 9). These organ-

izational features were apparent whether the tissue was prepared by method 1, 2, or 3 above.

The arrangement of the microtubular-neurofilamentous cytoskeleton in dendrites was also different than in axons. The ratio of microtubules to neurofilaments was much higher in dendrites and the neurofilaments coursed through the parallel arrays of microtubules as single filaments, pairs, or as small fascicles (Fig. 10). Although the average frequency of cross-bridges between neurofilaments was lower in dendrites (15 per  $\mu\text{m} \pm 13$  SD  $N = 161$ ), the frequency within a dendritic fascicle was similar to the axonal frequency. However, the filaments within a dendritic fascicle were often closer together, and the cross-bridges appeared to be shorter than in axons, as illustrated in Fig. 8.

### Antibody Decoration of Neurofilaments in the Cell Body

The apparent scarcity of 195-Kd polypeptides relative to 145- or 73-Kd polypeptides in the cell body compared with

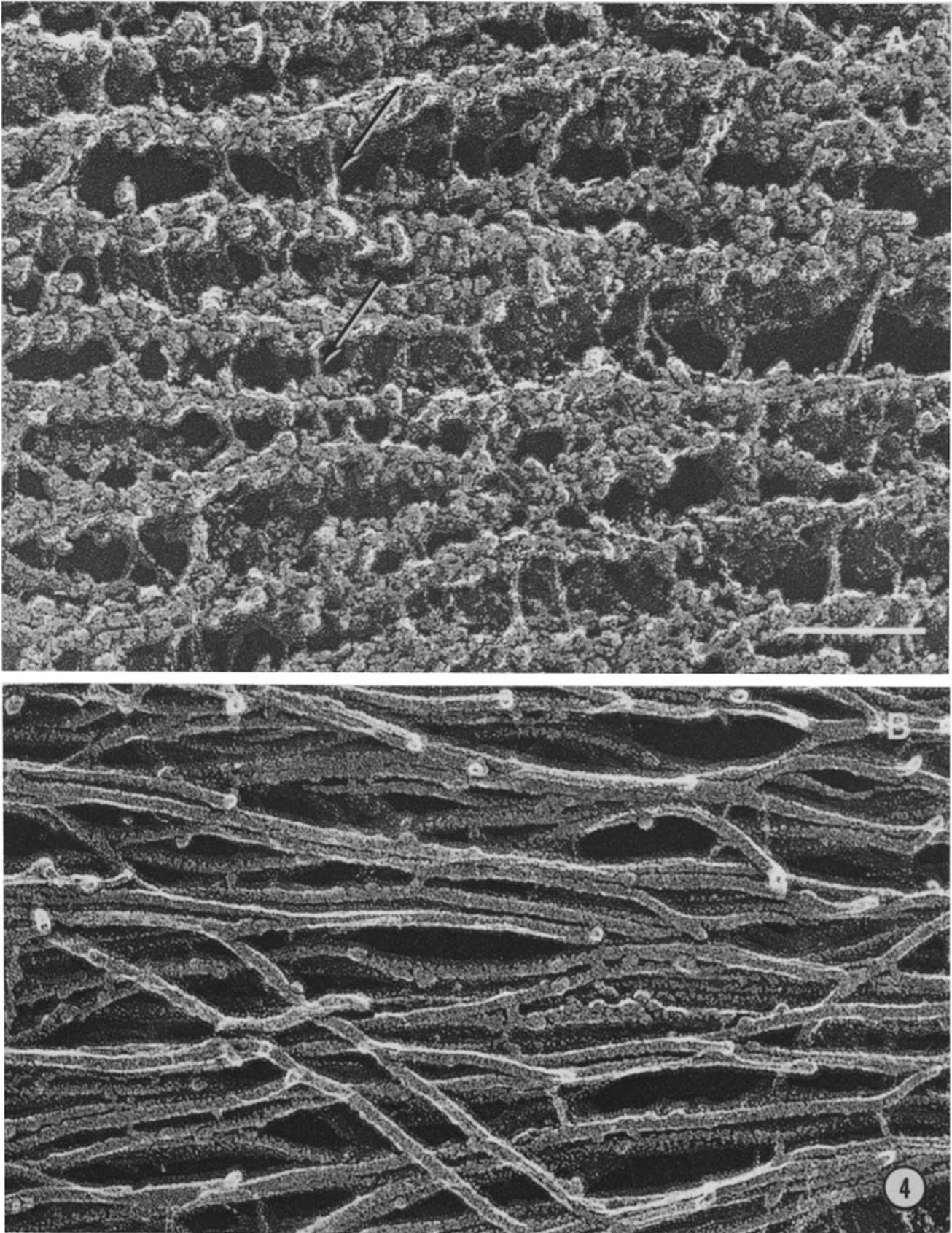
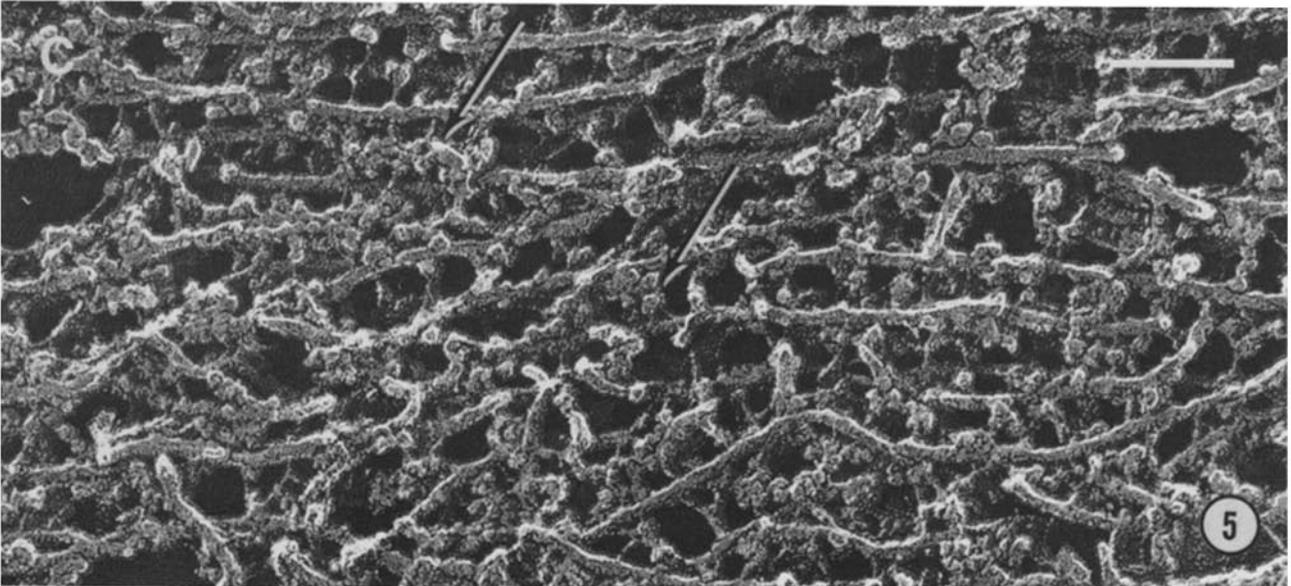
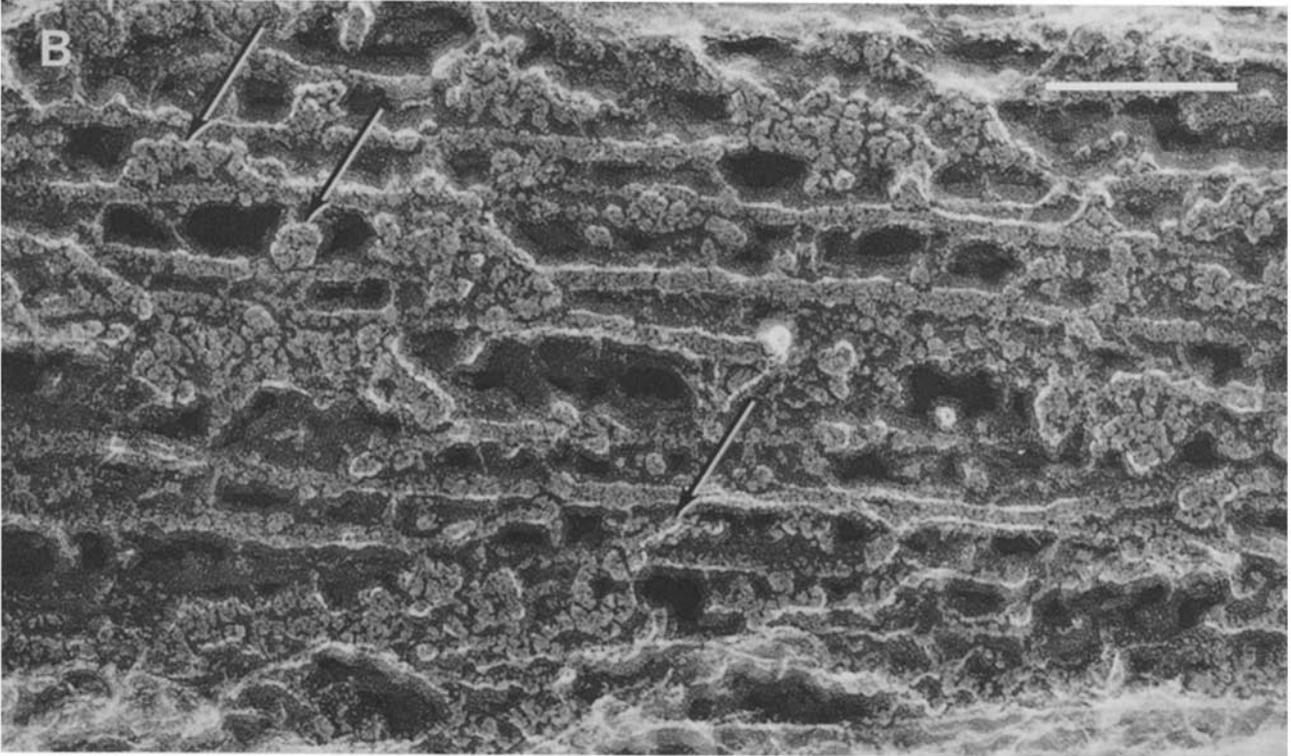
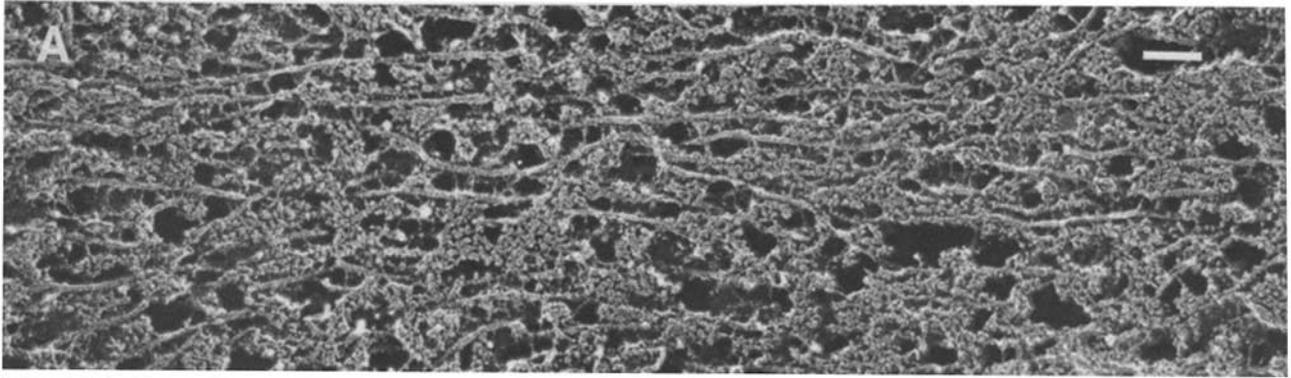


FIGURE 4 (A) Higher magnification view of axonal neurofilaments decorated with anti-73-Kd polypeptides and secondary antibody (IgG). The neurofilament cores are increased in diameter twofold by the antibodies, whereas the cross-bridges (arrows) are not decorated. (B) Glial filaments in an astrocyte in the sample incubated with anti-73-Kd antibody. The glial filaments look smooth and undecorated. Bar, 0.1  $\mu\text{m}$ .  $\times 257,000$ .



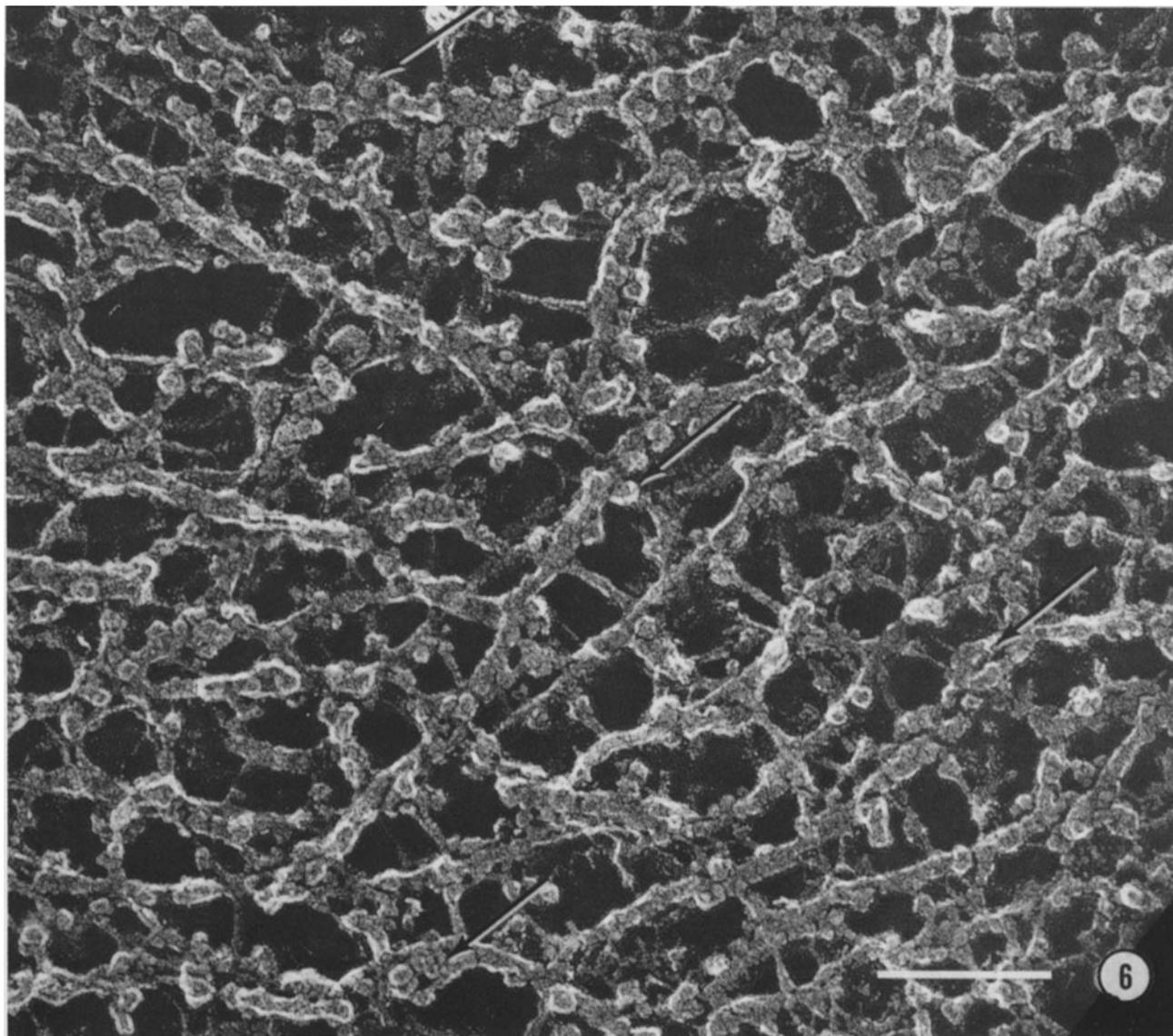


FIGURE 6 Axonal neurofilaments decorated with anti-145-Kd polypeptides and secondary IgG. Globular masses are attached sporadically on the surface of neurofilaments (arrows), sometimes at the base of cross-bridges. Bar, 0.1  $\mu\text{m}$ .  $\times 257,000$ .

the axons indicated by immunofluorescence raised the question of whether the 145- and 73-Kd proteins in the cell body are in the form of filaments lacking 195-Kd proteins, or in a nonfilamentous form. To determine whether the intermediate filaments in the cell body are neurofilaments, we examined cell bodies from preparations that had been incubated with the antineurofilament antibodies (or nonimmune IgG) followed by a secondary antibody (Fig. 11), as described in Materials and Methods. After they were incubated with either anti-145 Kd (Fig. 11B) or anti-73-Kd (Fig. 11C), the cell body filaments appeared knobby, indicating that the globular antibody complexes had attached to the neurofilament core.

We did not observe antibody decoration of the cell body filaments when the tissue was incubated with either anti-195-Kd polypeptides (Fig. 11A) or nonimmune IgG (Fig. 11D). These observations indicate that the cell body contains neurofilaments, and suggests that these are composed primarily of 145- and 73-Kd polypeptides, with less 195-Kd polypeptides than axonal neurofilaments.

#### *Organization of Filaments in Immature Axons*

During early postnatal development of the rabbit optic nerve, the ratio of 195- to 145- or 73-Kd polypeptides in

FIGURE 5 (A) Low magnification view of axonal neurofilaments decorated with anti-195-Kd polypeptide and secondary antibody (IgG). Antibodies form globular masses localized between the neurofilaments, but the neurofilament core is smooth. (B) Axonal neurofilaments decorated with anti-195-Kd polypeptides and secondary antibody (IgG). Globular masses of antibody complex decorate cross-bridges (arrows). The core of the neurofilaments tend to be smooth. (C) Axonal neurofilaments decorated with anti-195-Kd polypeptides and secondary Fab fragments. The antibody complexes attached to the cross-bridges (arrows) are smaller and more discrete. Bars, 0.1  $\mu\text{m}$ .  $\times 76,000$  (a);  $\times 257,000$  (b);  $\times 167,000$  (c).

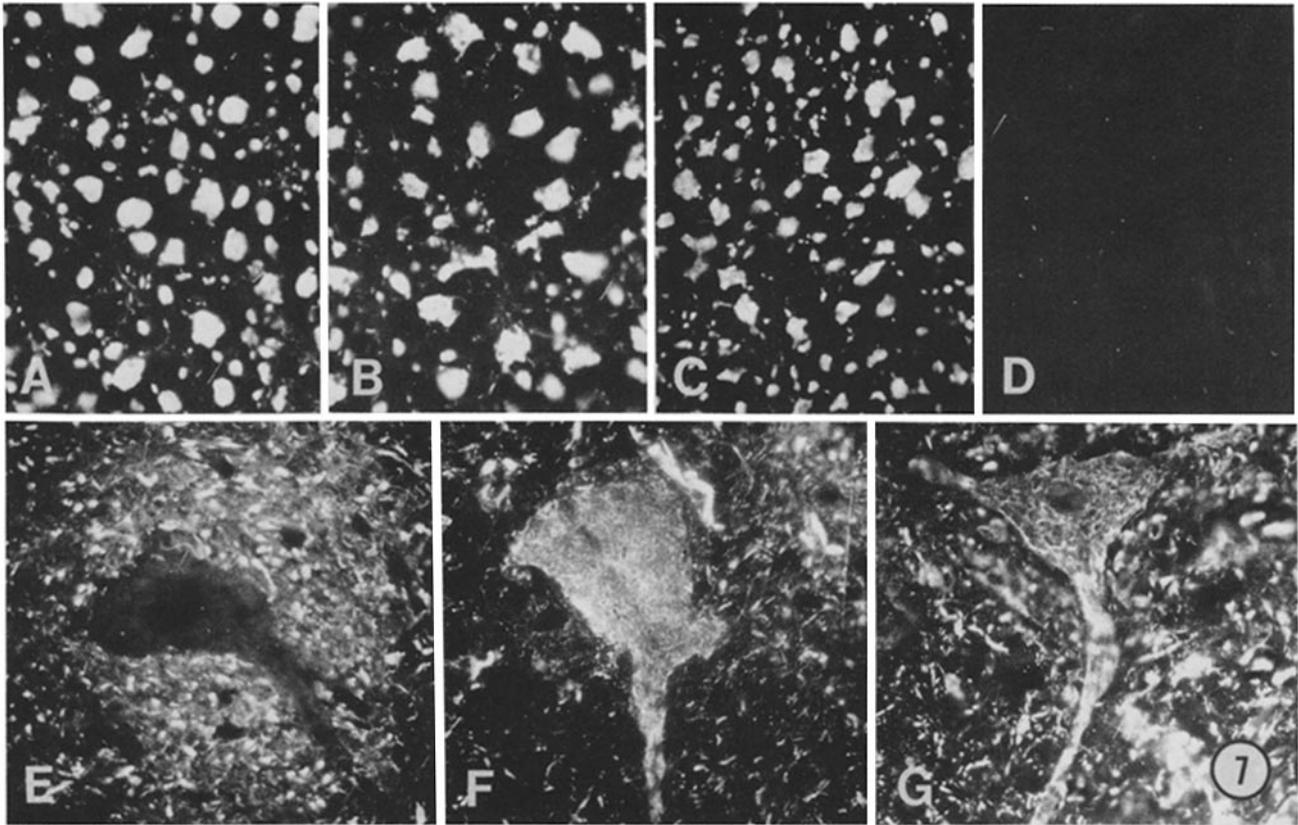


FIGURE 7 Immunofluorescent microscopy of the spinal cord using anti-195-, -145, and -73-Kd antibody and fluorescein-labeled second antibody. (A-D) Cross-section of white matter of spinal cord. Anti-195- (A), anti-145- (B), and anti-73-Kd antibody (C) stain axons intensely, but nonimmune IgG (D) did not. (E-G) Neurons in anterior horn region. (E) Anti-195-Kd antibody stains axons intensely, but cell bodies much less intensely. (F) Anti-145-Kd antibody stains cell bodies and dendrites, although somewhat less intensely than axons. (G) Anti-73-Kd antibody stains cell bodies and dendrites, although somewhat less intensely than axons. Anti-195-Kd antibody (E) stained cell bodies less intensely relative to surrounding axons than did anti-145- (F) or 73-Kd antibody (G).  $\times 900$ .

retinal ganglion cells is much smaller than in the adult (16, 39). Fig. 12A shows a saponin-treated optic nerve from a 1-d-old rabbit. Solitary neurofilaments run between the microtubules, which are the major filamentous organelle at this developmental stage. The intermediate filaments are connected to the microtubules by infrequent cross-bridges. At later stages of development, (Fig. 12B) when the 195-Kd polypeptide becomes a major protein of the optic nerve, the neurofilaments are extensively cross-linked to each other, forming bundles.

#### *Appearance of Neurofilaments That Have Been Quick-Frozen, Deep-Etched on Mica*

A technique for observing macromolecules adsorbed to mica by quick-freezing and deep-etching has been developed (8). Isolated neurofilaments prepared in this way appeared to be composed of two, and sometimes three parallel strands that were often helically entwined; the sense of the helix was right-handed, as judged from comparison with hemocyanin, which contains a right-handed helix, and which was frozen in the same sample. The strands had occasional spine-like projections from their surfaces (Fig. 13).

#### DISCUSSION

The observations reported here show that the 195-Kd neurofilament polypeptide is a component of the cross-bridges

between neurofilaments within the axonal cytoskeleton, and that the 73- and 145-Kd polypeptides are components of the central core of these filaments. The 195-Kd polypeptide is much more abundant in the axons than in the cell bodies and dendrites, suggesting that filaments lacking 195-Kd polypeptides can be elaborated *in vivo*, and that the function of 195-Kd polypeptides reflects special requirements of the axon.

#### *Evidence that the 195-Kd Polypeptide Is a Component of Cross-bridges Between Neurofilaments*

The most direct evidence for the arrangement of the neurofilament polypeptides within the neurofilamentous cytoskeleton was that, in axons, antibodies specific for the 195-Kd polypeptide decorated cross-bridges between neurofilaments, whereas antibodies against the 145- and 73-Kd polypeptide decorated the central core of the filaments. Because the tissue was fixed, and the cross-bridges were well preserved by the quick-freeze, deep-etch technique, this observation establishes the 195-Kd polypeptide as a component of the cross-bridge between neurofilaments in axons. Because we do not know the disposition within each polypeptide of the antigenic determinants recognized by our adsorbed antibodies (38), nor the accessibility of these determinants within the tissue preparation, the lack of core decoration by anti-195-Kd polypeptides does not rule out the possibility that a portion

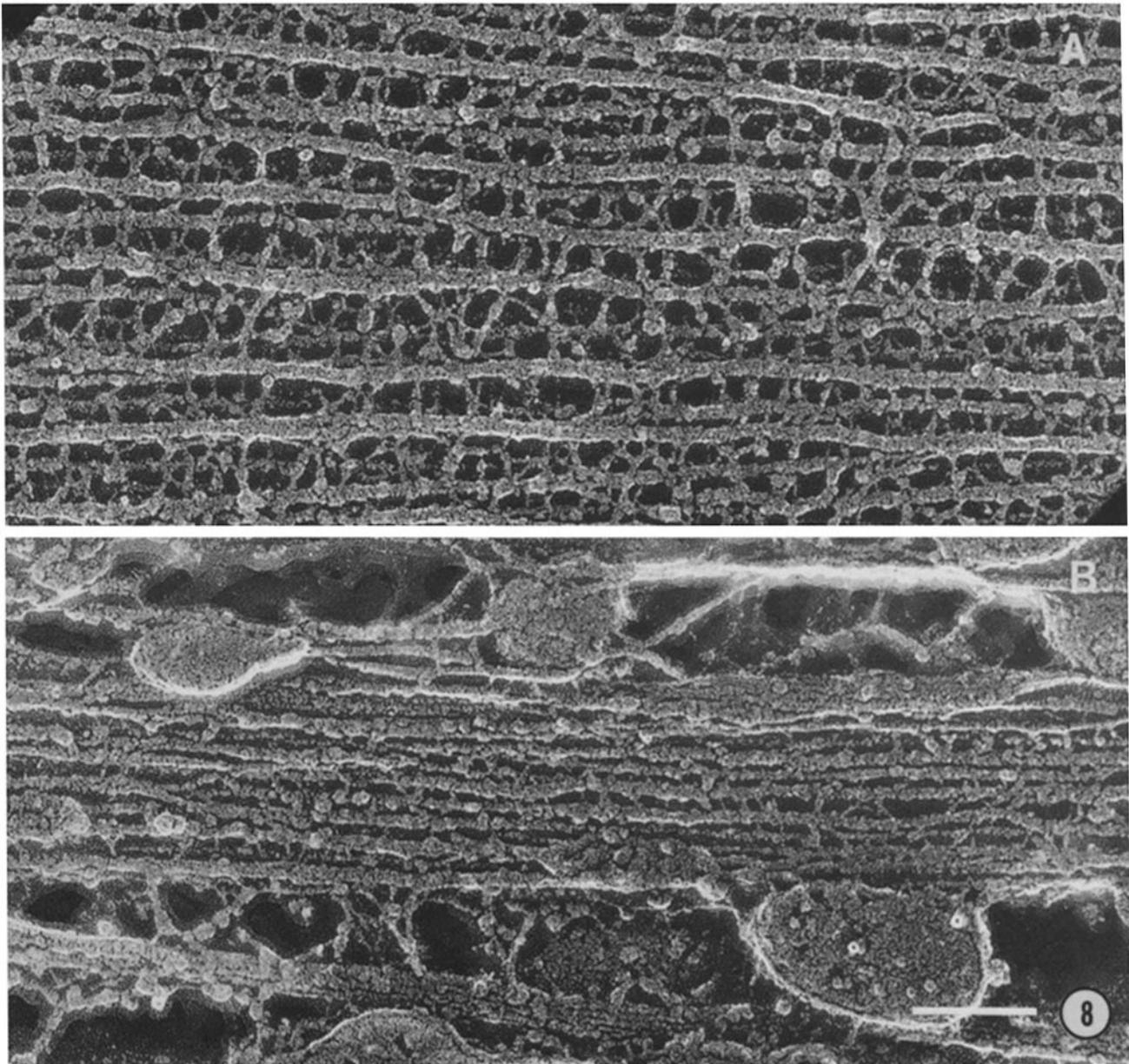


FIGURE 8 Comparison between axonal neurofilaments (A) and dendritic neurofilaments (B). (A) Neurofilaments fill the axoplasm. They are more evenly spaced and the cross-bridges between neurofilaments tend to be longer and more numerous. (B) Neurofilaments in fascicles in dendrites tend to be tightly packed. Although there are cross-bridges between neurofilaments, they appear to be shorter and less frequent than those in the axons. Bar, 0.1  $\mu\text{m}$ .  $\times 190,000$ .

of the anti-195-Kd polypeptide resides in the core. For the same reason, these results do not preclude the possibility that a portion of the 73- and 145-Kd polypeptides are components of cross-bridges; this possibility is particularly worth considering in the case of the 145-Kd polypeptide in view of our observation that anti-145-Kd polypeptides decorated the central core non-uniformly, and sometimes over the bases of cross-bridges.

An observation consistent with a cross-linking role for 195-Kd polypeptides is that the optic axons of neonatal rabbits that have reduced amounts of 195-Kd polypeptides relative to 145- or 73-Kd polypeptides, have infrequent cross-bridges between neurofilaments. Electrophoretic analysis of both total proteins and radiolabeled axonally transported proteins of the rabbit optic nerve has shown that the 195-Kd polypeptide

accumulates to detectable levels only after the first postnatal week, whereas 145- and 73-Kd polypeptides can be detected at birth (16, 39). (A similar delayed appearance of the 195-Kd polypeptide has been reported in rat cerebral cortex [31] and rat optic nerve [18].) When we examined the optic axons of a 1-d-old rabbit by the quick-freeze, deep-etch technique, the intermediate filaments were usually single, and lacked cross-bridges to other neurofilaments; on the other hand, these filaments were cross-linked to microtubules, which were the major filamentous organelle at this stage of development.

The correlation between the relative abundance of 195-Kd polypeptides and the frequency of cross-bridges appeared incomplete when we compared the axons of nerve cells in anterior horn regions with their cell bodies and dendrites. Immunofluorescent staining of the spinal cord indicated that

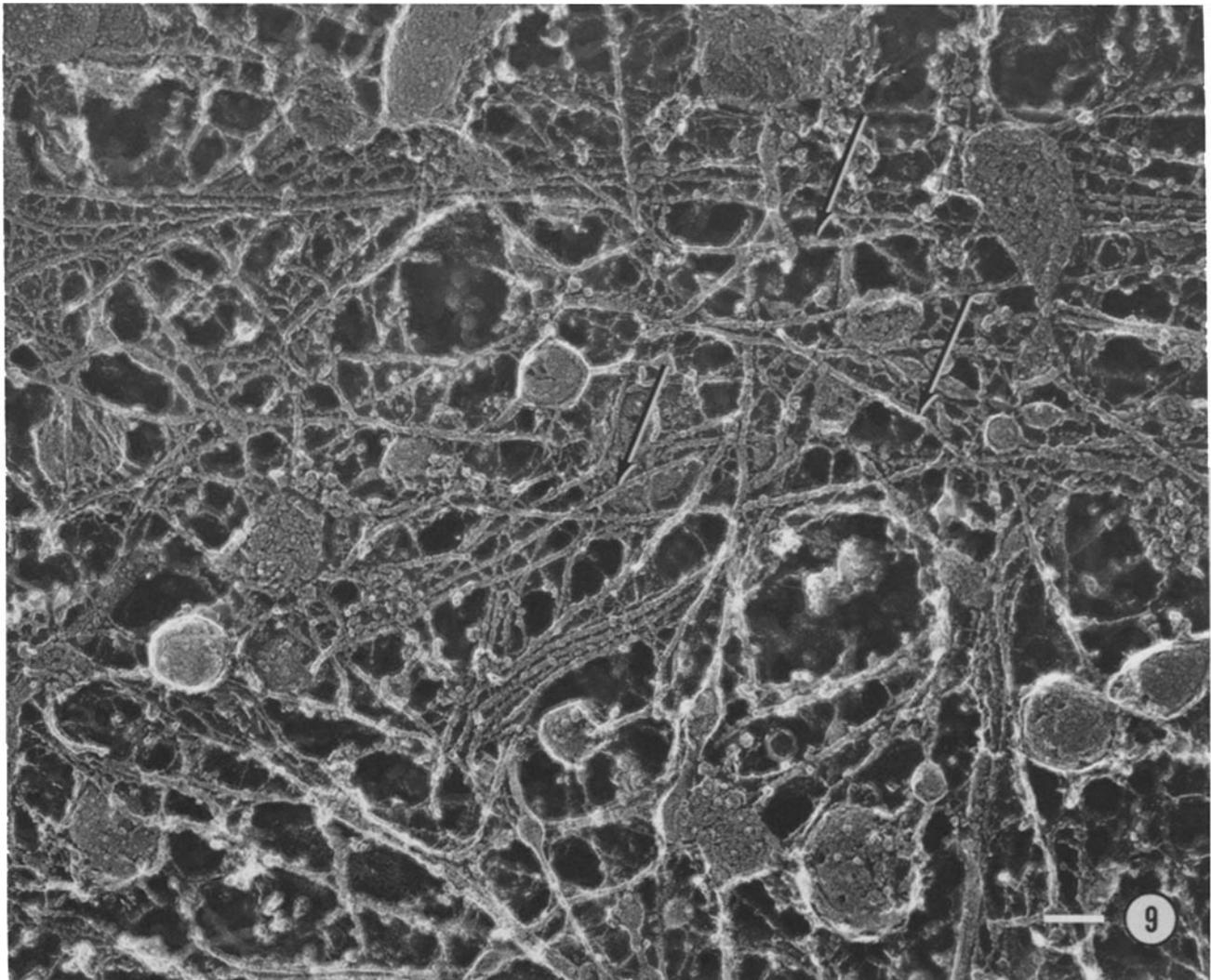


FIGURE 9 Organization of neurofilaments in a neuronal cell body that has been quick-frozen and deep-etched without chemical fixation. Individual neurofilaments (11-nm diam) are randomly oriented (arrows) or contained in small fascicles. The frequency of cross-bridges is lower than in the axon, and these cross-bridges tend to be shorter. Bar, 0.1  $\mu\text{m}$ .  $\times 88,000$ .

the ratio of 195- to 145- or 73-Kd polypeptides was much greater in axons than in cell bodies or dendrites. A similar segregation of neurofilament polypeptides has been reported previously in other neurons (30). Although a preliminary estimate indicates that the average frequency of cross-bridges is lower in both cell bodies and dendrites than in axons, it is not clear whether the difference in frequency (about twofold) would be sufficient to account for the differences in immunofluorescent staining, or alternatively, whether this observation suggests that polypeptides other than 195-Kd can cross-link neurofilaments.

The conclusion that 195-Kd polypeptides is a component of the interneurofilament cross-bridges is consistent with several previous observations. When purified neurofilaments or detergent-extracted cytoskeletons of cultured neurons were decorated with antineurofilament antibodies, anti-195 Kd reacted with a structure peripheral to the central core (29, 38). This 195-Kd polypeptide structure often appeared helically wrapped around the central core, and occasionally extended from the core to cross-link adjacent filaments, suggesting a cross-linking function *in vivo*. In view of the current results,

the helical conformation of 195-Kd polypeptides may represent cross-bridges that collapsed onto the central core when they were disrupted during the neurofilament preparation, as considered previously (38). Such disengaged cross-bridges might account for certain long strands visible in the partially unraveled filament that has been adsorbed to mica and viewed by the quick-freeze, deep-etch technique (Fig. 13). The short projections from these filaments might also be composed of the 195 or 145-Kd polypeptides.

Studies on the *in vitro* reconstitution of neurofilaments from the isolated polypeptides have also suggested that the 195-Kd polypeptide composes a structure that can extend from the filament core, and that this structure is not required for filament formation (4, 19, 24, 42). Filaments have been assembled from the purified 73-Kd polypeptide alone, but not from the 195-Kd polypeptide alone. A mixture of 73- and 195-Kd polypeptides produced filaments with thin projections from the surface. The experiments reported here show that this peripheral 195-Kd containing structure indeed composes a cross-bridge *in situ*.

The intimate relationship of 195, 145, and 73-Kd polypep-

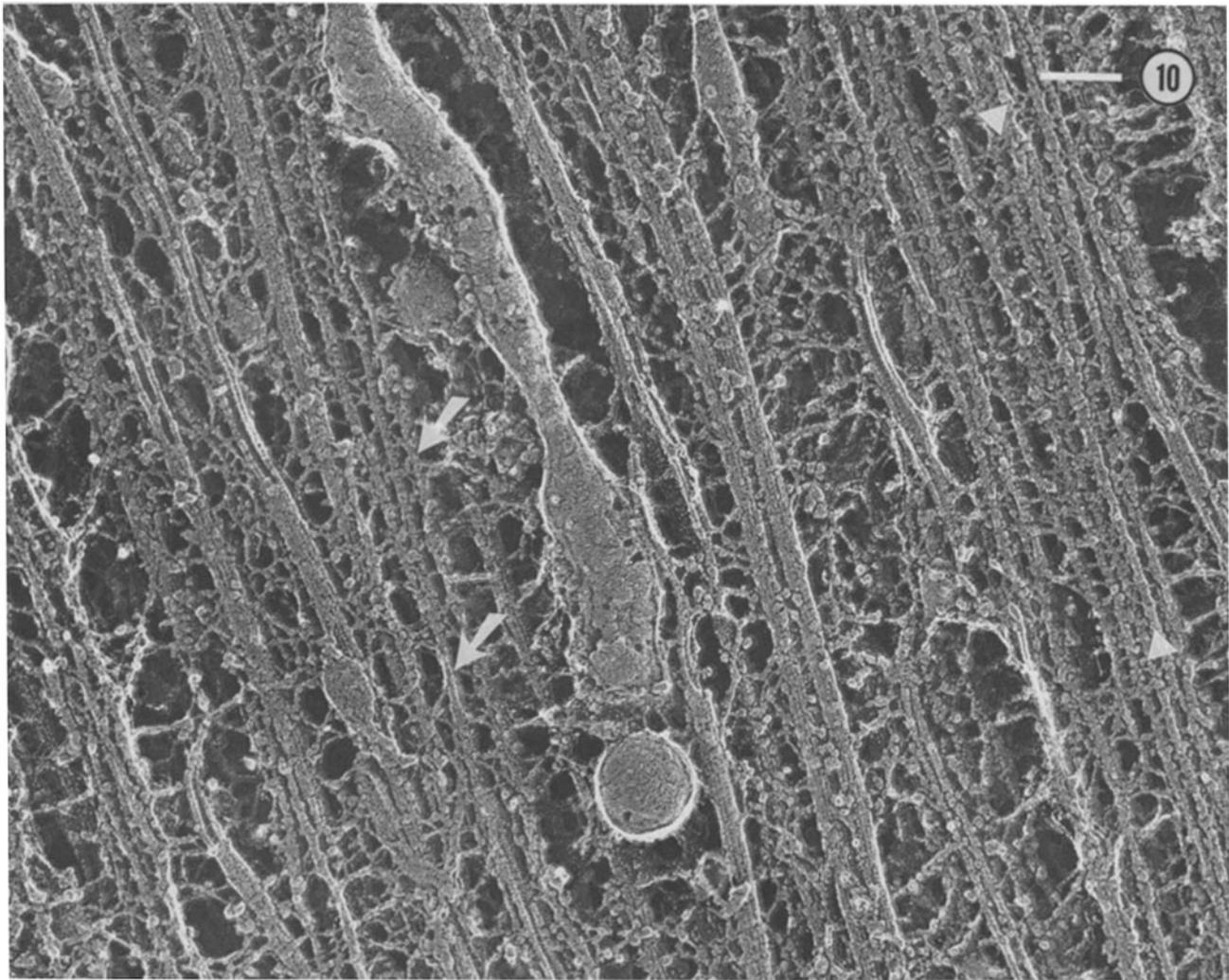


FIGURE 10 Architecture of a dendrite that has been quick-frozen and deep-etched without chemical fixation. Neurofilaments (arrows) run singly or in small fascicles (small fat arrows) between microtubules, which are the major filamentous organelle in the dendrite. The microtubules are extensively cross-linked to other microtubules, to membrane organelles, and to neurofilaments. The cross-bridges between neurofilaments are less frequent, and usually shorter than those in axons. Bar, 0.1  $\mu\text{m}$ .  $\times 113,000$ .

tides within the neurofilaments is not known. By analogy to models for the structure of other intermediate filaments (5, 33), it has been considered that the core of neurofilaments could be constructed from units composed of a common length ( $\sim 50$  Kd) of each of the three neurofilament polypeptides (37). If so, the remainder would be available to project from the filament core and participate in cross-bridges at intervals ( $\sim 500$  Å) corresponding to the length of the unit. A shorter segment of the 145-Kd polypeptide would also be available for such projections, and could conceivably produce shorter cross-bridges, such as those between neurofilaments in cell bodies and dendrites. If the portion of each polypeptide that contributed to the central core were sufficiently similar among the three polypeptides, several different combinations of subunits might be able to form filaments (37).

#### *Unequal Distribution of 195-, 145-, and 73-Kd Polypeptides in Dendrites, Cell Bodies, and Axons*

If the relative intensities of immunofluorescence staining with anti-195-, anti-145-, and anti-73-Kd polypeptides in

axons and cell bodies reflect the relative concentrations of these polypeptides, then the ratio of 195- to 73- or 145-Kd polypeptides is much greater in the axon than in the cell body or the dendrites of neurons of the anterior horn. A similar relative paucity of anti-195-Kd polypeptide immunofluorescence in cell bodies has been noted previously in pyramidal cells of the rat cerebral cortex and hippocampus, as well as in large cells of the brain stem (30). These observations suggest that, at least in these neurons, the 195-Kd polypeptide performs a cross-linking function specialized for axons. This axonal specialization might be reflected in the greater spacing between filaments within bundles in the axon, compared with filaments within some fascicles in the cell bodies and dendrites. In addition, the reduced levels of 195-Kd polypeptides relative to 145- or 73-Kd polypeptides in the cell body raises the possibility that neurofilaments in the cell body are composed primarily of 145- and 73-Kd polypeptides. (An alternative would be that somal and dendritic neurofilaments contain the same ratio of the three polypeptides as do axonal filaments, but that there are additional pools of 145- and 73-Kd polypeptides in a nonfilamentous form.) We observed that intermediate filaments in the cell body could be decorated

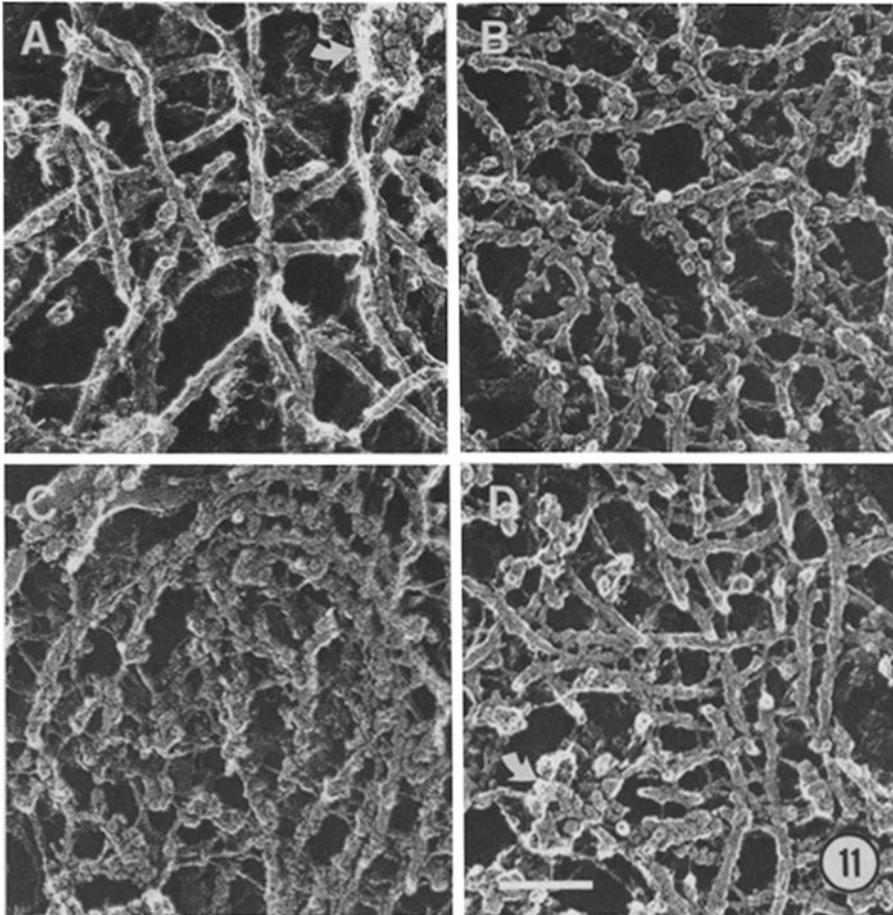


FIGURE 11 Antibody decoration of neurofilaments in cell bodies. (125,000X) Bar: 0.1  $\mu$ m. (A) neurofilaments are not decorated with anti-195-Kd and secondary antibody (IgG). (B) Globular masses are attached on the neurofilaments incubated with anti-145-Kd and a secondary antibody (IgG). (C) Neurofilament cores are decorated with anti-73-Kd and secondary IgG. (D) Neurofilaments incubated with nonimmune IgG and secondary antibody (IgG). No decoration is apparent. Polysomes (arrows) are frequently associated with neurofilaments.

with anti-145- and anti-73-Kd polypeptides, but decoration of these filaments with anti-195-Kd polypeptides was too infrequent to be observed. Although this negative evidence does not conclusively demonstrate that neurofilaments lacking 195-Kd polypeptides can be elaborated *in vivo*, it supports this possibility and is consistent with the observation that filaments lacking 195-Kd polypeptides can be reconstituted from purified subunits *in vitro* (4, 19, 24, 42).

#### *Relationship Between the 195-Kd Polypeptide and Other Cross-bridges*

In a previous quick-freeze, deep-etch analysis of axons of the frog sciatic nerve, three classes of cross-bridges were recognized (10). One class (4–6-nm diam and 20–60 nm long) served to link neurofilaments to other neurofilaments, to microtubules, and to membrane-bounded organelles. The experiments reported here show that the 195-Kd polypeptide is a component of this class, but does not rule out the possibility that other polypeptides may participate; for example, we do not know whether 195-Kd polypeptides can link neurofilaments to microtubules and membrane-bounded organelles, because these structures were not preserved under the conditions (detergent treatment without taxol) of our antibody decoration experiments. A second class of cross-bridges served to link microtubules to other microtubules, to membrane-bounded organelles, and to mitochondria. Because these organelles were not preserved in the current experiments, we do not know whether the anti-195-Kd poly-

peptide reacts with these cross-bridges; however, because of the difference in their morphology, it is likely that they are composed of a different polypeptide. A third class of cross-bridges (50–150 nm long) in the frog sciatic nerve linked the axolemma and actin-like filaments with neurofilaments and microtubules. Fodrin, a protein related to erythrocyte spectrin, is a candidate for a component of these cross-bridges, because fodrin is concentrated beneath the axolemma. Furthermore, in the brush border of the intestinal epithelium, anti-fodrin antibodies can decorate 50–150 nm cross-bridges that appear to mediate actin-actin interaction, and in addition may mediate actin-membrane, actin-intermediate filament, and membrane-vesicle interactions (12).

The composition of cross-bridges between neurofilaments in the cell body pose the following alternatives. On the one hand, they could be mediated by 195-Kd polypeptides; their relative scarcity in the cell body compared with the axon is consistent with the paucity of 195-Kd polypeptides and our failure to observe decoration with anti-195-Kd polypeptides in the cell body. However, it seems more likely that many of these cell body and dendritic cross-bridges between neurofilaments are composed of a different protein, because they are generally shorter than the axonal cross-bridges. One possibility would be that they involve the 145-Kd neurofilament polypeptide, as discussed above. The high molecular weight microtubule-associated proteins (HMW MAPs) are prime candidates for cross-bridges between microtubules and neurofilaments as well as other organelles in the cell body and dendrites (1, 10). These proteins appear to have a distribution in certain neurons that is complementary to that of the 195-

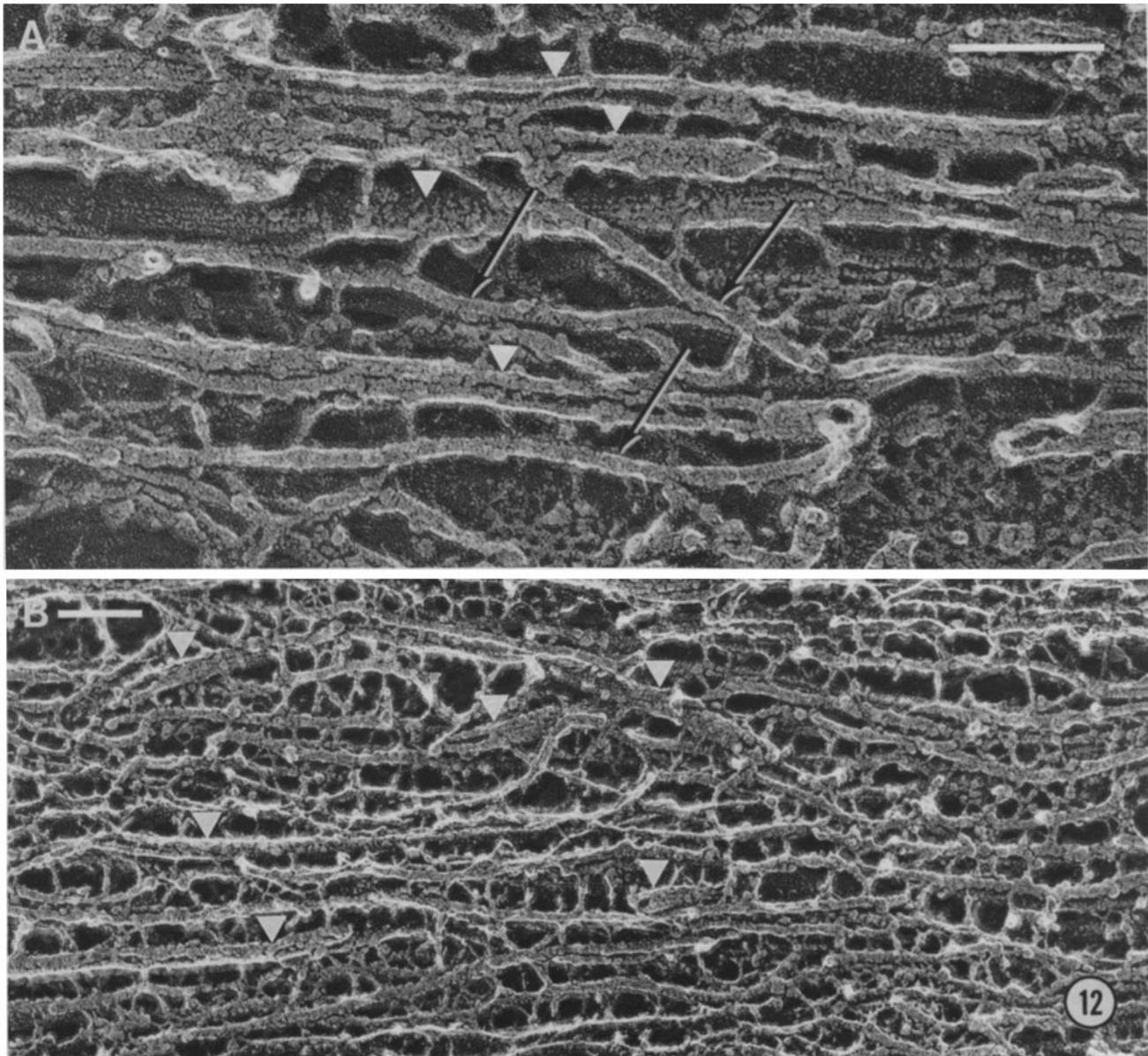


FIGURE 12 Cytoskeleton of 1-d (A) and 29-d (B) postnatal rabbit optic nerve. The axons were made permeable with saponin. Most 11-nm neurofilaments in 1-d optic nerve (arrows) are solitary. There are some cross-bridges between neurofilaments and microtubules (arrowheads), but cross-bridges between neurofilaments are few. At this stage 145- and 73- but not 195-Kd protein are detectable biochemically. In 29-d optic nerve there are numerous neurofilaments extensively cross-linked to each other and at this stage all three neurofilament proteins can be detected biochemically (16, 39). Bar, 0.1  $\mu$ m.  $\times$  223,000 (A);  $\times$  126,000 (B).

Kd cross-linking polypeptide; they are more concentrated in the cell bodies and dendrites than in the axons (21). In addition, HMW MAPs have been observed to participate in interactions between microtubules and actin and neurofilaments *in vitro* (1, 15).

Among intermediate filaments, the axonal neurofilaments are unusual in their high content of high molecular weight polypeptides (195 Kd and 145 Kd) and their extensive cross-linking. Other intermediate filaments are composed primarily of lower molecular weight polypeptides (50 Kd to 70 Kd) and are less extensively cross-linked (e.g., see the glial filaments in Fig. 4*b*). However, synemin, a 230-Kd polypeptide that is a minor component of vimentin intermediate filaments appears to cross-link these filaments, suggesting that they may share with neurofilaments the relationship of a high molecular

weight polypeptide cross-linking a core composed of a 50–70-Kd polypeptide (6).

The experiments reported here illustrate that antibody decoration combined with the quick-freeze, deep-etch technique should be a useful approach toward defining the molecular identity of additional cross-bridges and other cytoskeletal elements of both neurons and non-neuronal cells.

We are grateful for the support of Dr. J. Heuser, and for discussions with Dr. K. Meiri, R. Cheney, C. Baitinger, and C. Simon.

This work was supported by grants to N. Hirokawa and J. Heuser from the National Institutes of Health, and grants EYO2682 and a Muscular Dystrophy Research Center Grant to M. Willard.

Received for publication 23 June 1983, and in revised form 19 December 1983.



FIGURE 13 Neurofilaments adsorbed onto mica. Two protofilaments are arranged as a right-handed helix. Tiny spines project on the surface of protofilaments (arrows). (235,000 $\times$ ) Bar, 0.1  $\mu$ m.  $\times$  235,000.

## REFERENCES

- Bloom, G. S., and R. B. Vallee. 1983. Association of microtubule-associated protein 2 (MAP 2) with microtubules and intermediate filaments in cultured brain cells. *J. Cell Biol.* 96:1523-1531.
- Burnett, W. N. 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* 112:195-203.
- Ellisman, M. H., and K. R. Porter. 1980. Microtubular structure of the axoplasmic matrix: visualization of cross-linking structures and their distribution. *J. Cell Biol.* 87:464-479.
- Geisler, N., and K. Weber. 1981. *In vitro* self-assembly of the 68,000 molecular weight component of the mammalian neurofilament triplet proteins into intermediate-sized filaments. *J. Mol. Biol.* 151:565-571.
- Geisler, N., E. Kaufmann, and K. Weber. 1983. Protein chemical characterization of three structurally distinct domains along the protofilament unit of desmin 10 nm filaments. *Cell.* 30:277-286.
- Granger, B. L., and E. Lazarides. 1982. Structural associations of synemin and vimentin filaments in avian erythrocytes revealed by immunoelectron microscopy. *Cell.* 30:263-275.
- Griffith, L. M., and T. D. Pollard. 1978. Evidence for actin filament-microtubule interaction mediated by microtubule-associated proteins. *J. Cell Biol.* 78:958-965.
- Heuser, J. E. 1982. A new method for visualizing freeze-dried molecules adsorbed on mica. *J. Mol. Biol.* 169:155-195.
- Heuser, J. E., and S. R. Salpeter. 1979. Organization of acetylcholine receptors in quick-frozen, deep-etched, and rotary-replicated *Torpedo* postsynaptic membrane. *J. Cell Biol.* 82:150-173.
- Hirokawa, N. 1982. Cross-linker system between neurofilaments, microtubules, and membrane organelles in frog axons revealed by the quick-freeze, deep-etching method. *J. Cell Biol.* 94:129-142.
- Hirokawa, N. 1983. Membrane specialization and cytoskeletal structures in the synapse and axon revealed by the quick-freeze, deep-etch method. In *Structure and Function in Excitable Cells*. D. C. Chang, I. Tasaki, W. J. Adelman, and H. R. Leuchtag, editors. Plenum Press, New York. 113-141.
- Hirokawa, N., R. Cheney, and M. Willard. 1983. Location of a protein of the fodrin-spectrin-TW260/240 family in the mouse intestinal brush borders. *Cell.* 32:953-965.
- Hirokawa, N., and J. E. Heuser. 1981. Quick-freeze, deep-etch visualization of the cytoskeleton beneath surface differentiations of intestinal epithelial cells. *J. Cell Biol.* 91:399-409.
- Hoffman, P. N., and R. Lasek. 1975. The slow component of axonal transport. Identification of major structural polypeptides of the axon and their generality among mammalian neurons. *J. Cell Biol.* 66:351-366.
- Leterrier, J.-F., R. K. H. Liem, and M. L. Shelanski. 1982. Interactions between neurofilaments and microtubule-associated proteins. A possible mechanism for intra-organellar bridging. *J. Cell Biol.* 95: 982-986.
- Levine, J., C. Simon, and M. Willard. 1982. Mechanistic implications of the behavior of axonally transported proteins. In *Axoplasmic Transport*. D. G. Weiss, editor. Springer-Verlag, New York. 275-278.
- Levine, J., and M. Willard. 1981. Fodrin: axonally transported polypeptides associated with the internal periphery of many cells. *J. Cell Biol.* 90:631-643.
- Pachter, J. S., and R. K. H. Liem. 1981. Differential appearance of neurofilament triplet polypeptides in developing rat optic nerve. *J. Cell Biol.* 91:86a. (Abstr.)
- Liem, R. K. H., and S. B. Hutchinson. 1982. Purification of the individual components of the neurofilament triplet: filament assembly from the 70,000 dalton subunit. *Biochemistry.* 21:3221-3226.
- Mage, M. 1980. Preparation of Fab fragments from IgGs of different animal species. *Methods Enzymol.* 70:142-150.
- Matus, A., R. Bernhardt, and T.-H. Jones. 1981. High molecular weight microtubule-associated proteins are preferentially associated with dendritic microtubules in brain. *Proc. Natl. Acad. Sci. USA* 78:3010-3014.
- Metuzals, J., V. Montpetit, and D. F. Clapin. 1981. Organization of the neurofilamentous network. *Cell Tissue Res* 214:455-482.
- Metuzals, J., and W. E. Mushynski. 1974. Electron microscope and experimental investigations of the neurofilamentous network in Deiters neurons. Relationship with the cell surface and nuclear pores. *J. Cell Biol.* 61:701-722.
- Moon, H. M., T. Wisniewski, P. Merz, J. DeMartin, and H. M. Wisniewski. 1981. Partial purification of neurofilament subunits from bovine brains and studies on neurofilament assembly. *J. Cell Biol.* 89:560-567.
- Peters, A., and J. E. Vaughn. 1967. Microtubules and filaments in the axons and astrocytes of early postnatal rat optic nerves. *J. Cell Biol.* 32:113-119.
- Schiff, P. B., J. Fant, and S. B. Horwitz. 1979. Promotion of microtubule assembly *in vitro* by taxol. *Nature (Lond.)* 277:665-667.
- Schlaepfer, W. W. 1977. Studies on the isolation and substructure of mammalian neurofilaments. *J. Ultrastruct. Res.* 61:149-157.
- Schnapp, B. J., and T. S. Reese. 1982. Cytoplasmic structure in rapid-frozen axons. *J. Cell Biol.* 94:667-679.
- Sharp, G. A., G. Shaw, and K. Weber. 1982. Immunoelectron microscopical localization of the three neurofilament triplet proteins along neurofilaments of cultured dorsal root ganglion neurons. *Exp. Cell Res.* 137:403-413.
- Shaw, G., M. Osborn, and K. Weber. 1981. An immunofluorescence microscopical study of the neurofilament triplet proteins, vimentin and glial fibrillary acidic protein within the adult rat brain. *Eur. J. Cell Biol.* 26:68-82.
- Shaw, G., and K. Weber. 1982. Differential expression of neurofilament triplet proteins in brain development. *Nature (Lond.)* 298:277-279.
- Smith, D. S., U. Jarlfors, and B. F. Cameron. 1975. Morphological evidence for the participation of microtubules in axonal transport. *Annu. NY Acad. Sci.* 253:472-506.
- Steinert, P. M., W. W. Idler, and R. D. Goldman. 1980. Intermediate filaments of baby hamster kidney (BHK-21) cells and bovine epidermal keratinocytes have similar ultrastructures and subunit domain structures. *Proc. Natl. Acad. Sci. USA* 77:4534-4538.
- Sternberger, L. A., and N. H. Sternberger. 1983. Monoclonal antibodies distinguish phosphorylated and nonphosphorylated forms of neurofilaments *in situ*. *Proc. Natl. Acad. Sci. USA* 80:6126-6130.
- Tsukita, S., J. Usukura, S. Tsukita, and H. Ishikawa. 1982. The cytoskeleton in myelinated axons: a freeze-etch replica study. *Neuroscience.* 7:2135-2147.
- Willard, M. 1976. Genetically determined protein polymorphism in the rabbit nervous system. *Proc. Natl. Acad. Sci. USA* 73:3641-3645.
- Willard, M. 1983. Neurofilaments and axonal transport. In *Neurofilaments*. C. A. Marotta, editor. University of Minnesota Press. 86-116.
- Willard, M., and C. Simon. 1981. Antibody decoration of neurofilaments. *J. Cell Biol.* 89:198-205.
- Willard, M., and C. Simon. 1983. Modulations of neurofilament axonal transport during the development of rabbit retinal ganglion cells. *Cell.* 35:551-559.
- Wuerker, R. B., and S. L. Palay. 1969. Neurofilaments and microtubules in anterior horn cells of the rat. *Tissue Cell.* 1:387-402.
- Yamada, K. M., B. S. Spooner, and N. R. Wessells. 1971. Ultrastructure and function of growth cones and axons of cultured nerve cells. *J. Cell Biol.* 49:614-635.
- Zackroff, R. V., W. W. Idler, P. M. Steinert, and R. D. Goldman. 1982. *In vitro* reconstitution of intermediate filaments from mammalian neurofilament triplet polypeptides. *Proc. Natl. Acad. Sci. USA* 79:754-757.