IDENTIFICATION OF THE SUBUNIT PROTEINS OF 10-NM NEUROFILAMENTS ISOLATED FROM AXOPLASM OF SQUID AND *MYXICOLA* GIANT AXONS

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ABSTRACT

Neurofilaments were isolated from the axoplasm of the giant axons of *Myxicola infundibulum* and squid. The axoplasm was fractionated by discontinuous sucrose gradient centrifugation and gel filtration on Sepharose 4B. The fractions were monitored for neurofilaments by electron microscopy. When isolated in the presence of chelating agents, the neurofilaments of *Myxicola* are composed almost entirely of protein subunits with mol wt of 150,000 and 160,000. Squid neurofilaments contain two major proteins with mol wt of 200,000 and 60,000. These proteins are compared with other intermediate filament proteins which have been reported in the literature.

KEY WORDS neurofilaments · 10-nm filaments · neurofilament protein · axon

Neurofilaments appear to be members of the class of cytoskeletal structures called intermediate filaments which have been found in a variety of cell types (1–3, 14, 18, 33, 38, 42). Neurofilaments are particularly abundant in the axonal processes of neurons, and it is widely thought that in conjunction with axonal microtubules they provide a supporting lattice which gives the axon its shape (3, 10, 11, 28, 39, 44). To study the structure and composition of neurofilaments, we have developed a method for the isolation of intact neurofilaments from the giant axons of the marine polychaete, *Myxicola infundibulum*, and the squid, *Loligo pealii*. These giant axons are known to be particularly enriched sources of neurofilaments, and axoplasm can be obtained from the giant axons by simple mechanical means (11–13, 20, 28). Although the axoplasm is available only in limited quantities, it provides material for fractionation studies which is free of any significant contamination from extra-axonal sources. The giant axon of *Myxicola* is a particularly valuable source for the study of neurofilaments because neurofilaments are the major structural components of the axoplasm (12, 19). The giant axon associated with the stellar nerve of the squid differs from that of *Myxicola*, in that it contains both neurofilaments and microtubules (4, 27). In fact, except for its size the squid giant axon closely resembles the axons typically found in invertebrates and vertebrates.

Using the electron microscope to follow the fractionation of the axoplasm, we have devised a method for isolating neurofilaments. The proteins comprising the fractions obtained by this method have been analyzed by SDS-polyacrylamide gel

J. CELL BIOLOGY © The Rockefeller University Press · 0021-9525/79/08/0336/11 $1.00
Volume 82 August 1979 336–346

electrophoresis (SDS-PAGE), and the subunit proteins of *Myxicola* and squid neurofilaments are compared with those identified from mammalian brain. In an accompanying report, we describe the fine structure of the isolated neurofilaments (19).

MATERIALS AND METHODS

Isolation of Axoplasm

Axoplasm was obtained from squid giant axons by extrusion as described previously (19). The axons were either extruded immediately after removal from the squid, a process which takes about 10 min for each axon, or were tied at each end and stored in filtered sea water at 0°C so that a number of axons could be extruded at the same time. The axons were left in sea water for at most 2 h. No differences were seen in the neurofilaments isolated from axons which were extruded immediately and those which were left in sea water.

Myxicola were obtained from Maritime Research Associates, Deer Island, New Brunswick, Canada, by air freight. The animals ranged in weight from 2.5–6.0 g. Generally, the animals were used within 1–3 wk of receipt. Axoplasm were obtained from *Myxicola* by dissection, using a dorsal approach to expose the axon. The coelom was flushed out, and the gut and ventral blood vessel were carefully removed.The axon was cleaned of any adhering vasculature, flushed with *Myxicola* Ringer's solution made without Ca^{++}, and was blotted with tissue paper to remove all of the Ringer's. The giant axon was then slit along the dorsal surface with fine scissors, and the axoplasm was immediately sucked up into a 50-μl capillary pipette. The entire procedure, starting with removal of the animal from the tank, takes 10–15 min to complete and yields 10–40 mg of axoplasm depending upon the size of the worm used. Clear, colorless axoplasm which is not contaminated with any significant cellular debris can be obtained by this method. The most important precautions are complete removal of the ventral blood vessels without puncturing the axon, and insuring that the giant axon is free of any excess Ringer's before it is slit open.

Identification of Neurofilaments in Subcellular Fractions

Samples were checked for neurofilaments by negatively staining a drop of the preparation with 2% unbuffered uranyl acetate and inspecting the grids with a transmission electron microscope (19). Neurofilaments from squid and *Myxicola* axoplasm have a characteristic appearance in negatively stained preparations and are easily recognized. However, in a number of the early fractionation experiments the presence or absence of neurofilaments in particular fractions was scored by a microscopist who had no previous knowledge of the character of the sample.

Fractionation of the Axoplasm

After a large number of empirical attempts to isolate neurofilaments, two methods were finally adopted as routine procedures: (*a*) sedimentation through discontinuous sucrose gradients and (*b*) gel filtration. Both methods are based upon the fact that intact neurofilaments are very long structures measurable in microns. Thus, neurofilaments can be separated from other axoplasmic constituents on the basis of their relatively large mass. Axoplasm was homogenized in a hypotonic solution (squid body fluids are 1.01 osmol) of either buffer A (0.1 M KCl, 10 mM Tris-HCl, 0.8 mM 2-mercaptoethanol, pH 7.4) or buffer B, in which NaCl was substituted for the KCl. A glass-glass microhomogenizer was employed with several vigorous strokes of the pestle. Gilbert et al. (13) have noted the presence of a Ca^{++}-activated protease in axoplasm from squid and *Myxicola*. Therefore, in certain experiments the buffer was supplemented with 5 mM EGTA. The neurofilaments were isolated by layering the homogenate of axoplasm upon a discontinuous gradient in 0.65-ml cellulose nitrate tubes (Beckman 305528, Beckman Instruments, Inc., Fullerton, Calif.) and centrifuged in a SW 50.1 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 40,000 rpm for 1–4 h. A typical gradient consisted of 0.35 ml of homogenate containing 25–50 mg of axoplasm layered on top of 0.1 ml each of 0.3, 1.0, and 2.5 M sucrose solutions made up in the homogenization buffer. Gel filtration was carried out on either Sephadex G-200 or Sepharose 4B on a 0.9 × 30-cm column at 4°C and fractions were monitored by UV absorbance at 280 nm or protein was determined by the method of Lowry et al. (25).

SDS-PAGE

Disk electrophoresis was carried out on either 7.5% or 4–12% polyacrylamide gels or slab gels (1.5 mm thick) using the method of Neville (29). The samples were made up to 1% SDS, 5% 2-mercaptoethanol, and heated in a boiling water bath for at least 3 min. A white precipitate formed in the samples containing KCl because of the low solubility of K-dodecylsulfate. Comparison of electrophoretograms in which the KCl was absent or removed from the sample indicated that the precipitate had no apparent effect on the migration of proteins in the gel. This problem was avoided by substituting NaCl for KCl in the buffer because we were not able to detect any difference in the behavior of the neurofilaments in these buffers. The gels were stained with Coomassie Blue or Fast Green, and densitometric analyses of the gels were performed to assess the relative contribution of individual bands to the total sample.

RESULTS

Myxicola Neurofilament Protein (NFP)

Analysis of *Myxicola* axoplasm by SDS-PAGE shows that one protein dominates the profile. Densitometric scans of Fast Green-stained gels indicate that this protein represents 40–50% of the total protein on the gels (Fig. 1). These results confirm those of Gilbert et al. (13) and are consistent with the possibility that this protein is the major subunit of *Myxicola* neurofilaments. When *Myxicola* axoplasm was fractionated on sucrose gradients, a white floccular layer was found at the 1.0 M–2.5 M interface. On removing the fractions, a clear gel-like material was found to extend from the 1.0 M–2.5 M interface into the 2.5 M fraction. Electron microscope inspection of the fractions obtained from gradients such as those shown in Fig. 2 demonstrated that most of the neurofilaments were in fraction 3 (Fig. 3*b*). Fraction 1 contained very few short filaments, and fraction 2 contained more of the longer neurofilaments and some membrane fragments resembling exploded mitochondria (Fig. 3*a*). Analysis of the gradient fractions on SDS-PAGE demonstrates that fraction 3 contains two bands with mol wt of 150,000 and 160,000 which are the major proteins of whole axoplasm (Fig. 2). Most of the other proteins of the axoplasm did not sediment with the neurofilaments and remained behind in fractions 1 and 2. A 230,000-dalton protein was consistently found primarily in fraction 2.

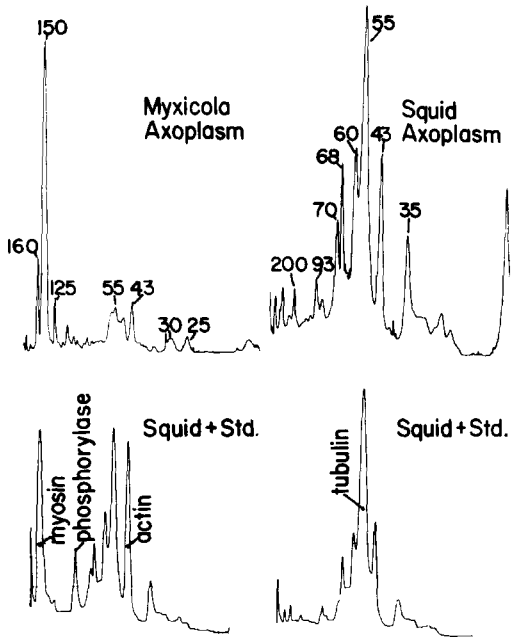

FIGURE 1 Densitometric tracings of Fast Green-stained 7.5% cylindrical SDS-polyacrylamide gels containing *Myxicola* and squid axoplasm illustrate the major proteins of these axoplasms. In the lower panels, several standard proteins were mixed with squid axoplasm and co-migrated on the gels. These proteins are denoted on the figure.

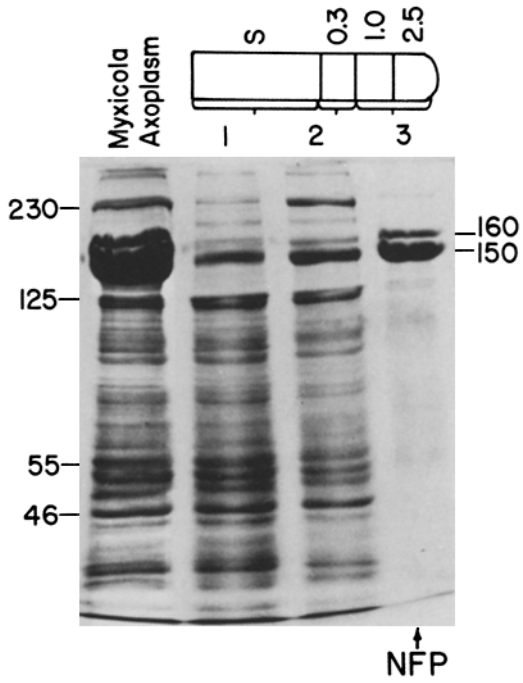

FIGURE 2 Fractions of *Myxicola* axoplasm obtained by sedimentation of homogenized axoplasm on a discontinuous sucrose gradient are illustrated in this 7.5% slab gel stained with Coomassie blue. The first gel column shows the proteins of whole axoplasm before fractionation. The protein compositions of the three fractions obtained from the sucrose gradient are shown in the designated gel columns. The bulk of the neurofilaments sediment to fraction 3 which contains two proteins with mol wt of 150,000 and 160,000. In this experiment, all of the solutions were made up in Buffer B containing 5 mM EGTA.

Ca^{++} and Myxicola Neurofilaments

In the experiment illustrated in Fig. 2, all of the solutions contained EGTA to inhibit the Ca^{++}-activated protease identified by Gilbert et al. (13). Fig. 4 shows an example of an earlier experiment in which neurofilaments were purified at 20°C without added EGTA. Bands with mol wt of 100,000 and 55,000 daltons were found along with

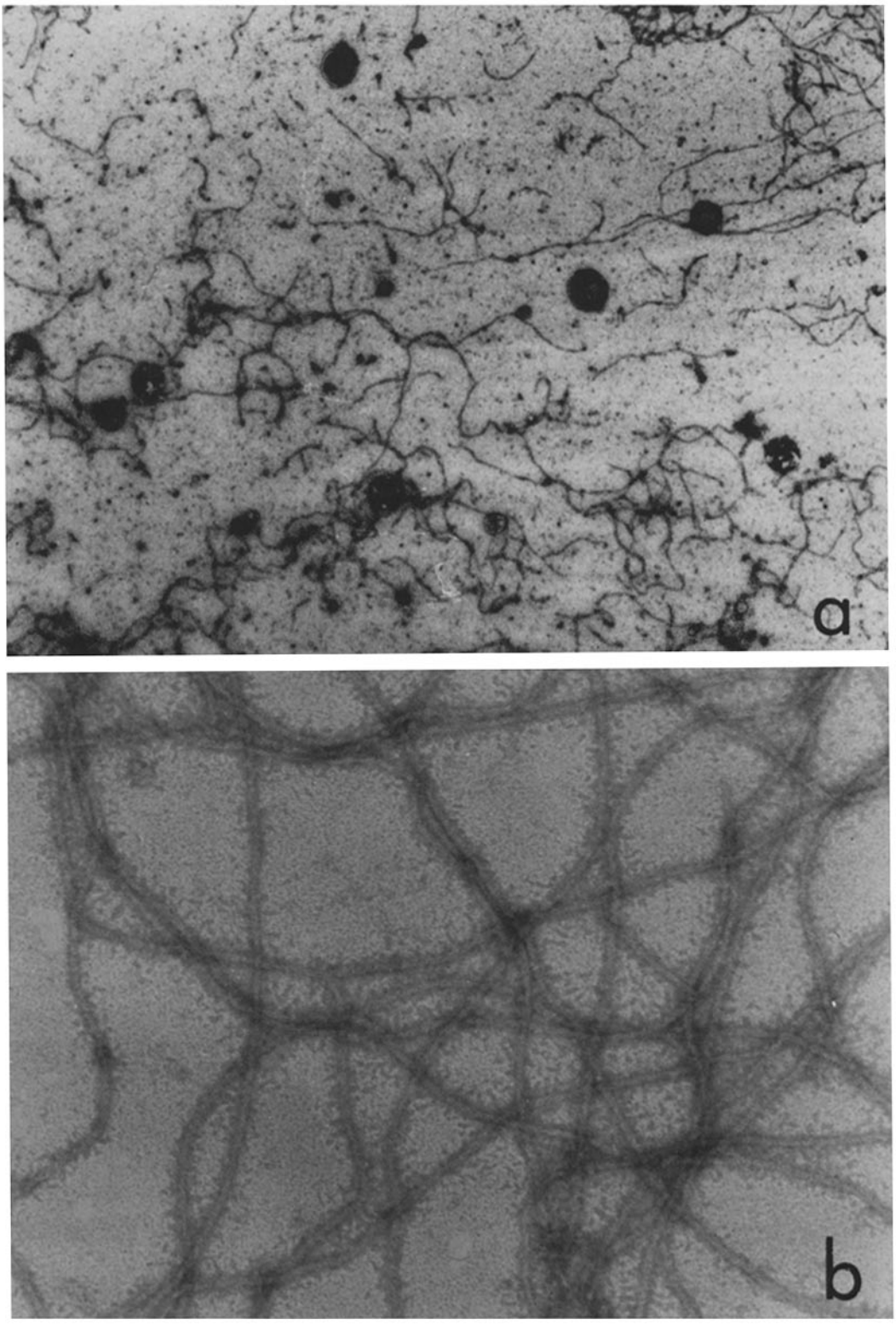

FIGURE 3 These electron micrographs of material, which was negatively stained with uranyl acetate, illustrate the difference between whole axoplasm and the fraction of purified neurofilaments prepared by the method shown in Fig. 2. Fig. 3*a* shows axoplasm obtained from a *Myxicola* giant axon which was homogenized in buffer A. The dense black circular profiles represent mitochondria. Purified neurofilaments from fraction 3 of Fig. 2 are shown in Fig. 3*b*. Mitochondria were rarely found in the purified neurofilament fractions. The purification also eliminated most of the small protein particles which are scattered among the neurofilaments in Fig. 3*a*. These particles were found in fractions 1 and 2. Fig. 3*a* × 18,000; Fig. 3*b* × 143,000.

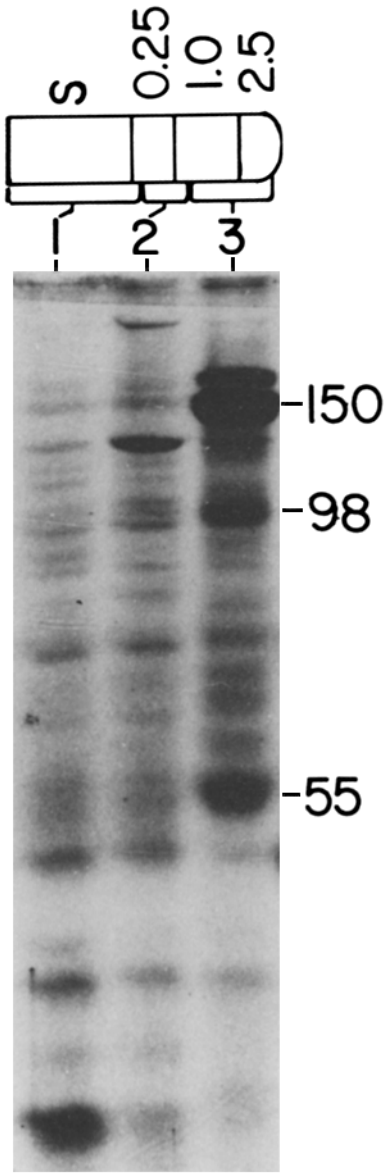

FIGURE 4 Fractionation of *Myxicola* axoplasm at 20°C in buffer A without EGTA. Note that fraction 3 is considerably more complex than in Fig. 2 and contains the 150,000 and 160,000 proteins and two additional proteins with mol wt of 100,000 and 55,000.

the 150,000-dalton bands in fraction 3. These 100,000- and 55,000-dalton proteins are not particularly notable either in fresh axoplasm or in neurofilament-containing fractions isolated with EGTA. These bands also appeared if axoplasm was incubated at 20°C for 30 min in buffer containing 5 or 20 mM Ca^{++} but not when Ca^{++} was omitted and 5 mM EGTA added. When purified neurofilaments obtained from fraction 3 were incubated in the presence of 20 mM Ca^{++} for 30 min at 20°C, there was no observable effect upon the protein profile and it resembled that of fraction 3 seen in Fig. 2. This result is consistent with the suggestion of Gilbert et al. (13) that the protease is soluble and can be separated from the filaments.

To demonstrate that the lower molecular weight bands are conversion products of the 150,000-dalton proteins and to more clearly resolve the cleavage products, purified neurofilaments were iodinated with ^{125}I by the lactoperoxidase method. The iodinated proteins were then mixed with 2 mg of axoplasm in buffer B, with either 5 mM EGTA or 20 mM $CaCl_2$, incubated for up to 60 min at 20°C, and then analyzed by SDS-PAGE and autoradiography. Fig. 5 shows that ^{125}I-NFP incubated for 60 min in the presence of 5 mM EGTA is similar to the starting material, whereas the NFP incubated in the presence of 20 mM Ca^{++} is converted to a number of lower molecular weight products. Several products can be seen at 60 min in the presence of Ca^{++}, and the major ones have mol wt of 55,000–60,000 and 98,000. It may be of interest that similar bands are present as minor constituents of whole axoplasm prepared with EGTA but that these bands do not sediment with intact neurofilaments.

Gel Filtration of Myxicola Neurofilaments

The sucrose gradient fractionation data suggests that the 150,000- and 160,000-dalton proteins are the principal subunits of *Myxicola* neurofilaments. This possibility is fully supported by analyses of the neurofilaments by gel filtration on Sepharose 4B. When fraction 3 from a sucrose gradient preparation was subjected to Sepharose 4B chromatography and eluted with buffer A, all of the protein entered the column and appeared in the void volume. Electron microscope inspection of the void volume showed that only neurofilaments were present. When analyzed by SDS-PAGE, the protein composition of the void volume was indistinguishable from fraction 3 seen in Fig. 2. When neurofilaments (fraction 3) were iodinated by the lactoperoxidase method, further purified by Sepharose 4B chromatography, and analyzed quantitatively by SDS-PAGE, the 150,000- and 160,000-dalton proteins were the primary labeled compo-

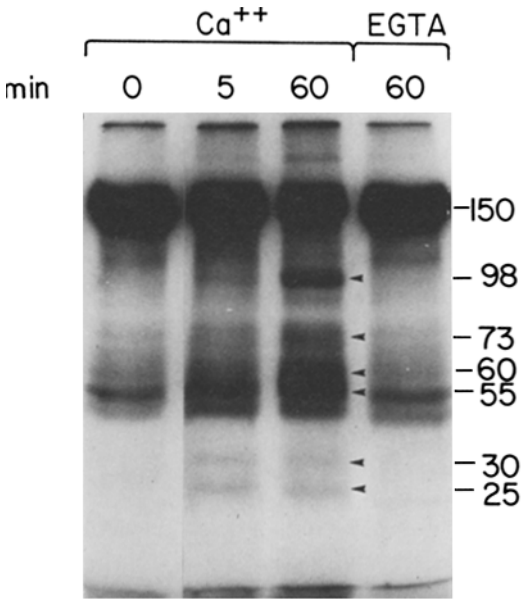

FIGURE 5 The possibility that lower molecular weight proteins are derived from the 150,000- and 160,000-dalton proteins was tested by incubating *Myxicola* ^{125}I-NFP in the presence of Ca^{++} or EGTA and subsequent analysis of the proteins by SDS-PAGE and autoradiography. *Myxicola* NFP was purified by sucrose gradient fractionation (see Fig. 2) and Sepharose 4B chromatography. The NFP was iodinated by the lactoperoxidase method. Aliquots of ^{125}I-NFP in buffer A, each containing 100,000 cpm, were mixed with 2 mg of homogenized axoplasm in 0.05 ml of buffer A. Either $CaCl_2$ was added to produce a final concentration of 20 mM Ca^{++} or EGTA was added to a final concentration of 5 mM. The samples had a final volume of 0.1 ml and were incubated for 0, 5, and 60 min at 20°C as denoted on the figure. The incubation was terminated by making the samples 1% with respect to SDS, 5% with 2-mercaptoethanol, and heating for 10 min at 95°C. The samples were analyzed by slab PAGE and autoradiography. Note that the incubation of ^{125}I-NFP in the presence of axoplasm and 20 mM Ca^{++} results in a significant alteration in the protein profile and the appearance of a number of bands indicated by arrows. This banding pattern resembles that in neurofilaments prepared at 20°C in the absence of EGTA (Fig. 4). The sample incubated in 5 mM EGTA does not differ significantly from the Ca^{++}-treated sample (0 min) in which the incubation was terminated immediately after the addition of the Ca^{++}.

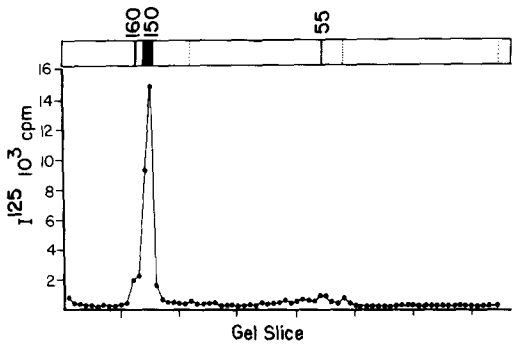

FIGURE 6 Fig. 6 is a quantitative representation of the protein components of purified *Myxicola* neurofilaments labeled with I^{125} by the lactoperoxidase method. The upper figure shows the profile of NFP stained with Coomassie Blue. The gel was sliced into 1-mm pieces and cpm/slice is shown. Note that the 150,000 subunit is by far the major labeled species. The 160,000-dalton component is seen as a shoulder on the 150,000 peak.

nents, representing 65% of the radioactivity (Fig. 6). Of the remaining 35% of the radioactivity, 10% was in the stacking gel and stacking gel/running gel interface, 9% was in the region between 50,000 and 60,000 daltons, and the remainder of the radioactivity was present in the region of the gel containing proteins >160,000 daltons in size and in the regions between 60,000 and 140,000 daltons. Although a significant amount of radioactivity was present in regions of the gel other than 150,000–160,000 daltons, the largest of these other peaks contained only 3% of the radioactivity and had a mol wt of 55,000 (Fig. 6).

Squid Neurofilament Protein

The protein composition of squid axoplasm is very different from that of *Myxicola*. In contrast to *Myxicola* axoplasm, the protein profile of squid axoplasm is dominated by proteins which co-electrophorese with tubulin (Fig. 1). The 53,000- and 57,000-dalton doublet seen in Fig. 7 has been identified as tubulin by its colchicine-binding properties (4). The large amount of tubulin in the giant axon and its paucity in that of *Myxicola* is consistent with the morphology of these two axons. Squid giant axons have a large number of microtubules and the *Myxicola* giant axon has few if any microtubules. As Gilbert et al. (13) have noted, it is surprising to find that none of the major proteins of squid axoplasm correspond in mol wt to the 150,000-dalton protein of *Myxicola* axoplasm (Figs. 1 and 7). Instead, the heaviest stained bands on the SDS-polyacrylamide gels were tubulin and proteins with mol wt of 200,000, 68,000, 60,000, 46,000, and 35,000 (Figs. 1 and 7).

Squid axoplasm was subjected to the same sort

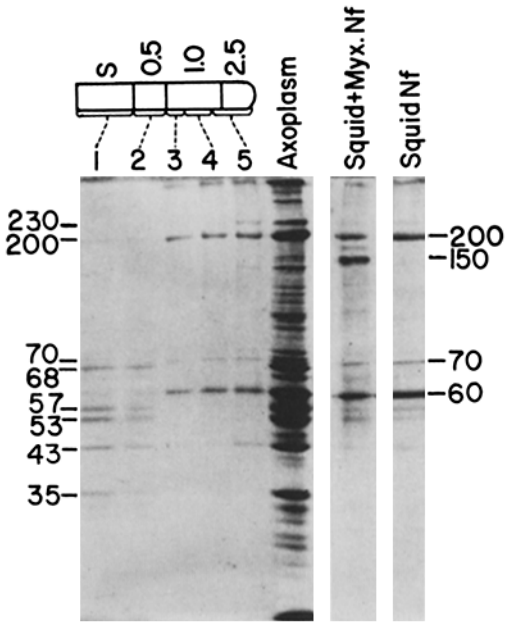

FIGURE 7 The fractionation of squid axoplasm by sedimentation on a discontinuous gradient is analyzed on a 7.5% slab gel. All of the solutions were made up in buffer B. Whole axoplasm is shown for comparison with the gradient fractions. The axoplasmic homogenate was centrifuged at 10,000 *g* for 10 min and the pellet was applied to the gradient and centrifuged for 1 h at 100,000 *g*. Neurofilaments sedimented to fractions 3–5, which contained two major bands with mol wt of 60,000 and 200,000. In the two right-hand columns, squid neurofilaments purified by Sepharose 4B chromatography (see text for details) are compared with purified *Myxicola* neurofilaments. Note that the 150,000-dalton band of *Myxicola* neurofilaments differs substantially in its electrophoretic mobility from the squid NFP.

of fractionation employed to isolate *Myxicola* neurofilaments. However, a low-speed centrifugation (10,000 rpm for 20 min) was added before the sucrose gradient to reduce the amount of tubulin which tended to cofractionate with the neurofilaments. The pellet from the low-speed centrifugation was layered on a discontinuous sucrose gradient and centrifuged at 2×10^5 *g* for 1 h. The results of this fractionation procedure are illustrated in Fig. 7. The 200,000- and 60,000-dalton proteins are the major components of fractions 3–5. These fractions also contained a minor 70,000-dalton band, and fraction 5 contained 230,000- and 46,000-dalton proteins. The latter proteins were always found in the most rapidly sedimenting material. Electron microscope examination of these fractions showed that the neurofilaments were present in fractions 3–5. Whereas fraction 5 contained long aggregated neurofilaments, fractions 3 and 4 contained long but more dispersed neurofilaments. Fractions 1 and 2 contained very few short pieces of neurofilaments. The best separations, such as those shown in Fig. 5, were achieved when the sucrose gradients were loaded with a low-speed pellet from <50 mg of axoplasm. The addition of 1–5 mM EGTA to the buffers had no detectable effect on the protein profile of the fractions when the entire preparation was carried out at 0°–4°C. Pant et al. (31) have also demonstrated that the neurofilament proteins are not rapidly degraded unless Ca^{++} is added to axoplasm. However, because of our experience with the isolation of *Myxicola* neurofilaments, we now routinely include EGTA in all of our buffers.

The enrichment of the 60,000- and 200,000-dalton proteins in the sucrose gradient fractions containing filaments suggested that these proteins might be the major proteins associated with squid neurofilaments. This possibility is supported by further purification of the neurofilaments using Sepharose 4B chromatography. The supernate from a low-speed centrifugation of homogenized axoplasm was analyzed on Sepharose 4B and eluted with buffer A. The low-speed supernate was chosen for the chromatographic experiments because it contained a large amount of dispersed, long neurofilaments. A sharp peak of protein chromatographed with the void volume of the column and the remainder of the protein was retarded by the column and was well separated from this peak. Electron microscope inspection of representative fractions obtained from the column demonstrated that neurofilaments were present only in the void volume. The 200,000- and 60,000-dalton proteins are the primary components of the void volume (Fig. 7). The void volume also contained the 70,000-dalton protein which cofractionated with neurofilaments on the sucrose gradients and a high molecular weight band which is a consistent component of squid neurofilament fractions. Attempts to sediment the NFP from the low-speed supernate demonstrated that much of the NFP remained in solution even after centrifugation at 100,000 × *g* for 2 h. However, we found that it was possible to separate the NFP from the other proteins by gel filtration on Sepharose 4B. The 200,000- and 60,000-dalton proteins were recovered in the void volume, and the remaining proteins of the axoplasm were included by the column (Fig. 8).

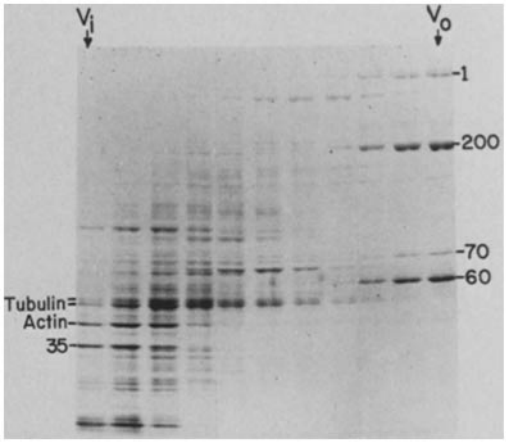

FIGURE 8 Squid NFP purified by gel filtration on Sepharose 4B. A supernate was prepared by centrifuging an axoplasmic homogenate at 100,000 *g* for 2 h. Then this supernate was fractionated by Sepharose 4B chromatography. The 1 × 30-cm column was eluted in buffer B with EGTA. Serial fractions from the column were analyzed on adjacent columns of a 4–12% gradient slab gel and stained with Coomassie Blue. The void volume (V_o) and inclusion volume (V_i) are noted. The 200,000- and 60,000-dalton proteins of squid neurofilaments were present almost exclusively in the void volume (V_o) of the column, which contained all of the neurofilaments. Most of the remaining proteins except the 70,000-dalton band and a high molecular weight band (*1*) were retarded by the column and were clearly separated from the neurofilaments.

DISCUSSION

Myxicola and Squid NFP

By employing axoplasm in our studies, it has been possible to purify neurofilaments in a relatively undenatured state and to identify the major proteins of squid and *Myxicola* neurofilaments. In the case of *Myxicola*, this task was simplified by the relatively small number of other formed elements in the axoplasm, and we have purified the *Myxicola* NFP to a state approaching homogeneity. In fact, the additional minor bands found in the purified *Myxicola* neurofilaments, such as the 55,000-dalton protein, appear to be cleavage products of the 150,000 and 160,000 subunit. The squid axoplasm is more complex, and the neurofilaments have not been purified as completely as in *Myxicola*. However, the results indicate that the 60,000- and 200,000-dalton proteins are the principal proteins comprising squid neurofilaments. This conclusion is supported by the observation that these two proteins are major components of whole axoplasm. In fact, only tubulin exceeds the 60,000-dalton protein in staining intensity. It has also been possible to polymerize neurofilaments from 1×10^5 *g* supernatant fractions of squid axoplasm by adding glycerol and raising the temperature of 37°C. The polymerized neurofilaments contain the 60,000- and 200,000-dalton proteins (22). Recently, we have also noted the presence of a very high molecular weight protein (band 1 in Fig. 7) which co-purifies with squid neurofilaments. This band was first brought to our attention because, like the 200,000-dalton band, it is actively phosphorylated (30). It has such a high molecular weight (>600,000) that it was not well resolved until we switched to 4–12% gradient gels (Fig. 8).

Although some of the other heavily stained bands found in squid axoplasm might be associated with the neurofilaments, it is unlikely that any of these is the principal subunit of neurofilaments because they can be separated from the neurofilaments. The 70,000-dalton protein is one of these proteins which has not been completely removed from the neurofilament fractions. Huneeus and Davison (17) identified a protein in squid axoplasm with a mol wt of 70,000 daltons when measured by sedimentation equilibrium in guanidine hydrochloride. They called this protein filarin and proposed that it was the subunit of squid neurofilaments. It was obtained by first extracting freeze-dried axoplasm with low ionic strength solutions and then with 6 M guanidine hydrochloride, which extracted the filarin. Because our purpose was to obtain native neurofilaments and freeze-drying the axoplasm makes the axoplasm nondispersable in physiological buffers, we have not employed the method of Huneeus and Davison to purify NFP. However, it would appear that the protein which they obtained was the same as the 60,000-dalton protein which co-purifies with neurofilaments in our procedure. In this regard it is interesting that they noted a higher molecular weight component on their polyacrylamide gels which was thought to be an aggregate of the protein filarin. That protein may be equivalent to the 200,000-dalton protein in our preparation.

With regard to strategies for purifying neurofilaments from squid axoplasm, sedimentation on discontinuous sucrose gradients yields relatively pure neurofilaments. However, unlike the *Myxicola* neurofilaments, a substantial fraction (60–70%) of the 60,000- and 200-000-dalton protein did not sediment even at the relatively high cen-

trifugal forces employed (1×10^5 *g* for 2 h at 4°C). This nonsedimenting NFP can be easily separated from the other proteins of axoplasm by Sepharose 4B chromatography (Fig. 8). Gel filtration provides a particularly effective means of isolating NFP from either low-speed or high-speed supernate of axoplasm and insures complete recovery of the proteins.

Comparison of the Proteins of Neurofilament and Other Intermediate-Sized Filaments

It was surprising to find that the subunit proteins of *Myxicola* NFP differed so much from those of squid when compared on SDS-PAGE (Fig. 7). *Myxicola* and squid neurofilaments are morphologically indistinguishable when they are viewed with the electron microscope. The neurofilaments from *Myxicola* and squid have different solubility properties in the 0.1 M buffer. While the *Myxicola* neurofilaments sedimented completely at 100,000 *g* for 2 h in either buffer A or B, only ~30% of neurofilaments from squid axoplasm sedimented under these conditions. However, both *Myxicola* and squid neurofilaments behave alike in denaturing solutions (12, 13, 17, 19). These neurofilaments are partially disrupted by 2 M urea or 0.5 M KCl and completely disrupted by 4 M urea (19). Structural analyses indicate that both are composed of strands twisted together to form a rope (19). Immunochemical studies of squid and *Myxicola* neurofilaments with a monospecific antibody prepared against purified *Myxicola* NFP also indicate that these proteins contain one or more antigenic sites which are similar (23). Using microcomplement fixation to compare the cross reactivity of anti*Myxicola* NFP antibody with squid and *Myxicola* neurofilaments, we obtained complement fixation curves with identical shapes for squid and *Myxicola* neurofilaments (Lasek and Wu, unpublished data). Squid and *Myxicola* neurofilaments share another property in that they are actively phosphorylated by endogenous axoplasmic kinases (30). In the case of the squid, the 200,000- but not the 60,000-dalton subunit is phosphorylated. In *Myxicola*, both the 160,000- and 150,000-dalton subunits are phosphorylated. These results suggest that although molecular weight may not be an important constraint in the evolution of the neurofilament subunit proteins, certain regions of the primary structure of the proteins appear to be conserved during the long span of evolution separating polychaetes and squid.

The proteins which comprise mammalian neurofilaments were first identified in radioisotopic labeling studies of axonal transport (16). These proteins, which have been called the neurofilament triplet, have mol wt of 200,000, 145,000, and 68,000, and have been observed in both central and peripheral neurons of a number of common experimental animals. Fractionation studies and immunochemical analyses of neurofilaments isolated from rat nerves and spinal cord have confirmed that these proteins are the major components of mammalian neurofilaments (24, 35, 36).

Comparison of the major proteins of mammalian neurofilaments with those of squid and *Myxicola* does not reveal any obvious relationship. Such a comparison further supports our contention that molecular weight is not highly conserved in the evolution of neurofilament protein. The wide variation in the subunit molecular weights of these proteins might be attributed to the large amount of evolutionary time separating these animals. However, variation in the molecular weight of NFP may occur within a single species. Two forms of the 200,000-dalton subunit of mammalian neurofilaments, which migrate separately on SDS-polyacrylamide gels, have been identified in rabbits (43). These two proteins appear to be isozymes which differ in their molecular weight. Such variation in molecular weight could result from variation in the actual length of the genes coding for NFP. Another possibility is that the length of the protein is determined by post-translational cleavage of a NFP precursor.

The subunits of mammalian intermediate filaments from non-neuronal cells also differ from those of the mammalian neurofilaments. The principal protein subunits of intermediate filaments from fibroblasts, smooth muscle cells, and glial cells have mol wt ranging from 50,000 to 55,000 (1, 2, 5, 7, 15, 38, 40–42). The difference in the molecular weights of mammalian neurofilaments and glial filaments may be related to the morphological differences which have been noted between these structures. Glial filaments are more densely packed than neurofilaments and have a smaller diameter (19, 44). Immunohistochemical studies have shown that antibody to the mammalian glial filament protein does not crossreact with neurons (6, 9) and that antibody to *Myxicola* NFP does not crossreact with glial filaments in *Myxicola* glial cells (8).

NFP, Tubulin, and Actin

Although we do not have a definitive explanation for the diversity of molecular weights which have been reported for intermediate-sized filament proteins, this literature leads to one important conclusion—that the proteins of intermediate filaments must differ in some fundamental way from the proteins of the other well-characterized primary cytoskeletal elements of nonmuscle cells, i.e., microtubules and actin-containing microfilaments. Both microtubules and actin-containing microfilaments are assembled from globular subunits, tubulin and G-actin, respectively. The molecular weights of these proteins have remained relatively invariant during evolution (26, 32) in marked contrast to the intermediate filament proteins. It seems likely that at least one of the major constraints on the evolution of actin and tubulin results from the need to conserve the positions of the multiple binding sites by which these globular proteins interact with one another to form a complex fibrous structure (32). Although the shape of intermediate filament proteins has not been fully determined, it appears that at least part of the protein is likely to be rod-shaped rather than globular (12, 19).

Ca^{++}-Dependent Cleavage of Neurofilaments

We have confirmed the observation of Gilbert et al. (13) that *Myxicola* axoplasm contains an endogenous protease which cleaves the NFP in the presence of Ca^{++}. The presence of such a protease has been documented in squid axoplasm (31) and in mammalian axons (34, 37). It is interesting that when purified ^{125}I-labeled NFP was treated with axoplasm and Ca^{++}, a number of relatively stable cleavage products appeared. The most prominent of these had a mol wt of 98,000 and 55,000–60,000. This result indicates that the protease acts on a rather specific site in the molecule, resulting in products which are ~⅔ and ⅓ the original size of the protein. Such a protease might function as the first and dependent step in neurofilament degradation during the metabolism of NFP at the end of the axon (21). Another possibility to consider is that the 55,000-dalton product of the proteolytic cleavage is not a degradative product but a physiological product with different functional properties than the high molecular weight "precursor" form of the protein. In this regard, it may be more than coincidental that the non-neuronal intermediate filaments have mol wt in the range of 55,000.

We would like to thank Dr. Gordon Shecket for suggesting the iodination experiment and for his other helpful advice.

This research was supported by National Institutes of Health grants NS09299 and AG00795 to R. J. Lasek.

Received for publication 17 April 1978, and in revised form 2 March 1979.

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