# INTERMEDIATE FILAMENTS IN NERVOUS TISSUES

RONALD K. H. LIEM, SHU-HUI YEN, GARY D. SALOMON,
and MICHAEL L. SHELANSKI

From the Department of Neuropathology, Harvard Medical School, Boston, Massachusetts 02115.
Dr. Liem's and Dr. Shelanski's present address is the Department of Pharmacology, New York
University Medical Center, New York 10016. Dr. Yen's present address is the Department of
Pathology, Albert Einstein College of Medicine, Bronx, New York 10461

## ABSTRACT

Intermediate filaments have been isolated from rabbit intradural spinal nerve roots by the axonal flotation method. This method was modified to avoid exposure of axons to low ionic strength medium. The purified filaments are morphologically 75–80% pure. The gel electrophoretogram shows four major bands migrating at 200,000, 145,000, 68,000, and 60,000 daltons, respectively. A similar preparation from rabbit brain shows four major polypeptides with mol wt of 200,000, 145,000, 68,000, and 51,000 daltons. These results indicate that the neurofilament is composed of a triplet of polypeptides with mol wt of 200,000, 145,000, and 68,000 daltons. The 51,000-dalton band that appears in brain filament preparations as the major polypeptide seems to be of glial origin. The significance of the 60,000-dalton band in the nerve root filament preparation is unclear at this time. Antibodies raised against two of the triplet proteins isolated from calf brain localize by immunofluorescence to neurons in central and peripheral nerve. On the other hand, an antibody to the 51,000-dalton polypeptide gives only glial staining in the brain, and very weak peripheral nerve staining.

Prolonged exposure of axons to low ionic strength medium solubilizes almost all of the triplet polypeptides, leaving behind only the 51,000-dalton component. This would indicate that the neurofilament is soluble at low ionic strength, whereas the glial filament is not.

These results indicate that neurofilaments and glial filaments are composed of different polypeptides and have different solubility characteristics.

KEY WORDS neurofilaments glial filaments

Isolation of intact intermediate filaments (8–10 nm diameter) from the mammalian central nervous system has depended on the axonal flotation technique (19), which uses the presence of the myelin around the axons to float neuronal material away from the other brain tissue. One can then remove the myelin from the axons by exposure to hypotonic solution and obtain purified intermediate filaments by applying the nonmyelin material to a sucrose gradient (22). The filament-rich fraction obtained from the gradient appears to be over 90% 8–10-nm filaments by electron microscopic examination. They are present mostly as loose bundles resembling neurofilaments, although some tight bundles resembling glial filaments are also observed. When this fraction is

analyzed by sodium dodecyl sulfate (SDS)-gel electrophoresis, it is found to migrate with a major band of mol wt 51,000 daltons. This polypeptide appears to be distinct from either of the tubulin subunits by biochemical and immunological criteria (13).

To test whether this protein is indeed the subunit of the neurofilament, an antibody to this polypeptide purified by gel electrophoresis has been raised (22). This antibody was used at the light microscopic level to localize the antigen in central and peripheral nervous tissue. By using a fluorescein-conjugated second antibody, we have found at the light microscopic level that the antibody to the 51,000-dalton component of the isolated filament localizes primarily to glial cells in the brain. Bergmann glial and radial glial fibers are found to stain very brightly, as do the astroglial cells. No convincing neuronal staining can be discerned in the central nervous tissue; however, some staining of peripheral nerve, both inside the axons as well as in the sheath, was observed (20).

The staining of the central nervous tissue was similar to the staining reported by Bignami et al. (2) for an antibody made against the glial fibrillary acidic protein (GFA), a soluble protein isolated from multiple sclerosis plaques and presumably arising from the glial filaments. More recently, an antibody raised against a protein isolated from normal brain, which is reported to be antigenically related to the multiple sclerosis plaque GFA protein (4), was found to localize to the glial filaments at the electron microscopic level (14).

These conflicting results, as well as the recent report of a 68,000-dalton protein associated with neurofilaments (16), have led us to reexamine the possibility that, instead of purifying neurofilaments, we have succeeded in purifying glial filaments with a small amount of neurofilament co-purifying. This mixed antigen might give rise to an antibody with a strong reaction against glial filaments and little or no reaction against neurofilament-containing cells and axons.

This possibility appears, at first, to be unlikely because we are using neurofilament-rich axons as our starting material. However, reports by Schlaepfer (15–17) have pointed to a possible flaw in the purification scheme. These reports have shown that peripheral neurofilaments from the rat sciatic and radial nerves are soluble upon prolonged exposure to low ionic strength medium such as is used during the demyelination procedure of Yen et al. (22). If brain neurofilaments

behave in a manner similar to rat peripheral neurofilaments, most, if not all, of the filaments could be solubilized or degraded during this procedure, leaving us with a fraction enriched in glial filaments.

To distinguish between these possibilities, we have decided to isolate neurofilaments from the peripheral nervous system. Because of the difficulty of obtaining pure neurofilaments from sciatic nerve free of sizeable amounts of collagen, we have taken as our starting material the collagen-poor intradural spinal nerve roots. This area is also free of astroglia, assuring that the filament preparations obtained from these nerve roots will not be contaminated with glial filaments.

## MATERIALS AND METHODS

### Nerve Root Filament Preparation

Rabbit intradural spinal nerve roots were isolated by exposure of the spinal cord from the lower thoracic to the lumbar region. The dura was carefully cut and pinned back on either side of the spinal column. The roots were then severed at the point of penetration of the dura and traced back to the spinal cord. The nerve roots were dissected out and suspended in a buffer containing 0.1 M NaCl, 10 mM phosphate, 5 mM EDTA, pH 6.5 (solution A), homogenized in 0.85 M sucrose in the same buffer, and the myelinated axons were isolated by the axonal flotation method as described below. To remove myelin, a solution of 1% Triton (Rohm and Haas Co., Philadelphia, Pa.) in solution A (*vide infra*) was used.

### Purification of Brain Filaments

Two methods were used to isolate intermediate filaments without prolonged exposure to low ionic strength demyelinating solution. In the first, calf brains were placed in solution A immediately after slaughter. White matter was carefully dissected from these brains and suspended in a 0.85-M sucrose solution in solution A. The material was homogenized with a Dounce homogenizer (Kontes Glass Co., Vineland, N. J.), and the axons were floated as described by Yen et al. (22). Demyelination was done by brief exposure to a low ionic strength solution (0.01 M phosphate buffer, pH 6.5 for 1 h). After this period of time, the pH of the solution was raised to pH 8.8, and the material was mixed with an equal amount of 0.85 M sucrose in 0.01 M Tris (tris[hydroxymethyl]amino methane)-HCl, pH 8.8 and centrifuged at 10,000 rpm for 15 min in a Beckman SW 27 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.); the pellet was collected and homogenized in 0.01 M Tris buffer, pH 8.8. This homogenate was layered on a 0.85-M sucrose solution, and centrifuged

for 30 min at 270,000 g. The resulting pellet was homogenized with solution A and applied to a sucrose gradient of 1.0 M, 1.5 M, and 2.0 M sucrose, all made in solution A. The gradients were centrifuged at 270,000 g for 60 min, and the material at the 1.5–2.0-M sucrose interface was collected. This material was found to contain the filament-rich material.

A second method employed no exposure at all to low ionic strength medium. Since the neurofilaments appear to be resistant to treatment with 1% Triton, we employed a solution of 1% Triton in solution A to separate myelin from the axons. After flotation on 0.85 M sucrose in solution A, the myelinated axons were homogenized in 1% Triton in solution A; the suspension was then layered on top of 0.85 M sucrose in solution A and centrifuged at 270,000 g for 30 min. The material which floated to the top of the sucrose was rehomogenized in the Triton solution and layered again in 0.85 M sucrose in solution A to maximize the yield. The final pad was resuspended in 1% Triton and applied to a discontinuous sucrose gradient of 1.0, 1.5, and 2.0 M all in solution A at 270,000 g for 60 min. The interface between the 1.5- and 2.0 M sucrose portions contained the filament-rich fraction. All electron microscopy was done on step-sections through the block to determine homogeneity.

## Iodination and Peptide Isolation

The filament preparations obtained were iodinated with Chloramine T. The filaments were solubilized in 8 M urea and protein concentration adjusted to ~1 mg/ml. 20 μl of this solution was then mixed with 1 mCi $^{125}$I. Chloramine T was added to start the reaction to a final concentration of 0.3 μg/ml. The reaction was stopped within 5 s by the addition of sodium metabisulfite. Potassium iodide was then added to remove the excess sodium metabisulfite, and the iodinated protein was separated from the unreacted iodide by chromatography on a Sephadex G-25 column (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.). To obtain purified iodinated polypeptides, the iodinated material was run on 6–15% gradient polyacrylamide gels containing 0.1% SDS, and the bands corresponding to the Coomassie blue (Merck Chemical Div., Merck & Co., Inc., Rahway, N. J.)-stained polypeptides were excised from the gel and eluted electrophoretically into a dialysis bag. This isolation procedure was also applied to obtain nonradioactive, Coomassie blue-stained polypeptides. To eliminate extensive degradation due to exposure to acid, the gels were fixed in 50% methanol without acetic acid for 1 h and stained only long enough to reveal the desired bands. Elution was allowed to proceed overnight at 4 mA per sample. The purity of each polypeptide was ascertained by rerunning the eluted material on gels.

## Preparation of Antisera

Antisera to the different polypeptides of the brain filament preparation were raised in guinea pigs by using proteins eluted from gels. 100 μg of gel-purified polypeptide was emulsified with complete Freund's adjuvant and injected into the foot pad of each guinea pig. A second injection was performed 3 wk later in the same manner, except that incomplete Freund's adjuvant was used. Blood was collected by cardiac puncture 1 wk after the second injection and tested by radioimmunoassay as described below. Subsequent immunizations were performed until a titer of at least 1:100 was obtained as tested by the radioimmunoassay.

## Radioimmunoassays of the Antisera

The radioimmunoassays were performed as described by Liem et al. (13). Iodinated polypeptides eluted from gels were used at a concentration of ~10,000 cpm/20 μl. Serial dilutions of the antisera were made by using 1:5 diluted preimmune serum in 0.15 M borate buffer, pH 8.5 as diluent. 20 μl of the labeled antigen was added to the same amount of the antiserum dilutions. The antigens were dissolved in borate buffer containing 0.05% SDS. These mixtures were incubated at 4°C overnight. The following day, 20 μl of goat anti-guinea pig IgG or goat anti-rabbit IgG were added to each tube. The mixtures were incubated and centrifuged as described, and the pellets were washed with borate buffer containing 0.01% SDS. The solutions were centrifuged again and the precipitates counted. The titer of the antiserum is described as the point where half of the maximum number of counts is precipitated. Controls were done with preimmune serum alone.

## Indirect Immunofluorescence

Rat or mouse brain and rat sciatic nerve were frozen on dry ice immediately after sacrifice. Sections (10 μm) cut in a cryostat were placed on egg-albumin-coated glass slides and allowed to air dry for a few minutes. They were then incubated for 1 h at room temperature with preimmune serum or with the experimental antiserum. After incubation, the slides were washed several times with phosphate-buffered saline and incubated for 1 h at room temperature with fluorescent labeled goat anti-rabbit IgG (Antibodies, Inc., Davis, Calif.). The slides were again washed several times with phosphate-buffered saline and examined with epifluorescence optics and a Xenon illuminator.

## Immunoprecipitation of Peptides

Proteolysis products of P51 were obtained by digesting the gel-purified iodinated polypeptide with the protease from *Staphylococcus aureus* V8 (25 μg/ml) in the presence of 0.1% SDS (3) for 1 h at 37°C. After this limited proteolysis, a 1:20 dilution of each antiserum in 0.15 M borate buffer, pH 8.5, which contained a 1:5 dilution of pre-immune serum, was mixed with an equal amount of the digested polypeptide solutions. These mixtures were incubated overnight at 4°C, after which

goat anti-guinea pig IgG (Antibodies, Inc.) or goat anti-rabbit IgG was added to a final concentration of 30% vol/vol. These reaction mixtures ware incubated at 37°C for 15 min and at 4°C for 60 min, after which they were treated exactly as the mixtures from the radioimmunoassay. The final pellets were dissolved in a sample buffer containing 1.0% SDS, 0.1 M mercaptoethanol, and 0.35 M Tris, pH 6.8. The samples were analyzed by gel electrophoresis on a 12-20% polyacrylamide-gradient gel containing 0.1% SDS. The resultant gels were fixed with 50% methanol, 10% acetic acid, dried, and exposed to X-ray film to obtain an autoradiogram.

## RESULTS

### Peripheral Nerve Filaments

Purification of peripheral nerve filaments by the axonal flotation method has heretofore been unsuccessful because of the large amount of collagen present in nerve. The intradural nerve roots are collagen-poor and lack astroglia, enabling us to avoid the problems of contamination with collagen and glial filaments. As in the brain filament preparations, the intradural nerve root filaments were found at the 1.5-2.0 M interface on sucrose gradients. The material was examined by electron microscopy (Fig. 1) and appears to be 75-80% pure intermediate (8-10 nm) filaments. On gel electrophoresis, there appear to be four major bands in the intradural nerve root filament preparation with mol wt 200,000 (P200), 145,000 (P145), 68,000 (P68), as well as a polypeptide which has a mol wt of 60,000 daltons (Fig. 2). The rabbit brain filament preparation also shows the first three major bands. but in addition has a major band at 50,000 daltons (P50). This band corresponds to the major polypeptide seen by Yen et al. (22) in calf brain filament preparations. The 60,000-dalton component is present in the nerve root filament preparation and not in the brain filament preparation. The only three polypeptides that are present in both preparations are P200, P145, and P68. All preparations were done in the presence of EDTA to protect against a calcium-activated protease (6, 7) such as is present in squid axoplasm. The addition of the protease inhibitor phenyl methyl sulfonyl fluoride (PMSF) was found to have no effect on the three polypeptides. From protein determinations and densitometric scanning of the gels, the yield of triplet polypeptides before the final sucrose gradient is about 75% of the amount of triplet obtained after axonal flotation, indicating little, if any, breakdown of filaments due to the treatment with Triton. No triplet
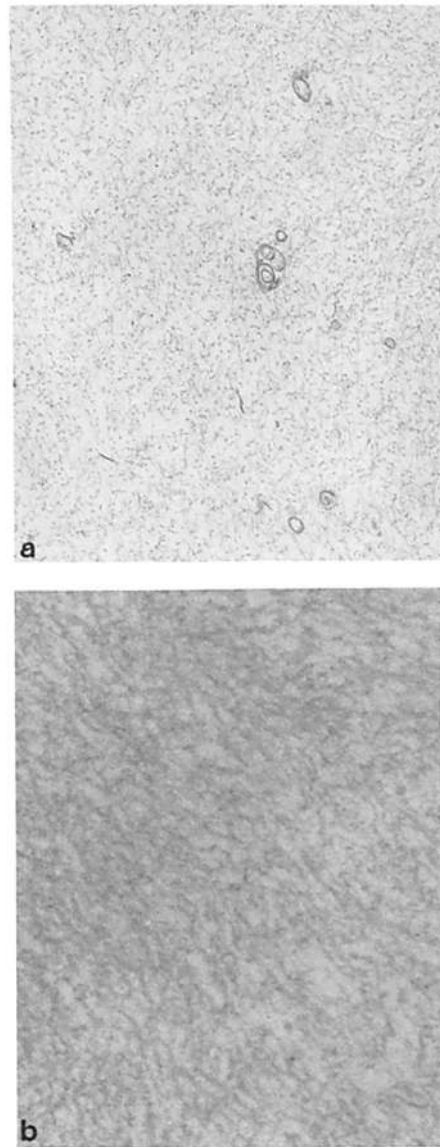


FIGURE 1 Electron micrograph of intermediate filaments obtained from the intradural spinal nerve roots. (a) × 13,000, (b) × 64,800.

polypeptide is found in the Triton-soluble fraction. Final recovery from the gradient exceeds 65%.

To reconcile these data with those of Schlaepfer, we prepared the 68,000-dalton soluble protein from rat sciatic nerve following exactly the procedure he described (16). This low ionic strength extraction resulted in a single major band co-migrating with serum albumin and comprising approximately 35% of the total protein of the

nerve, or about 50 times the amount of our P68 protein detectable in similar specimens. The 68,000-dalton protein extracted from nerve under these conditions reacted strongly with antisera against both rat serum albumin and bovine serum albumin, and a protein of identical electrophoretic mobility and reactivity against anti-albumins could be isolated by exposure of the tail of the saline-perfused rat to identical extraction. The yield from rat tail was identical to the yield from peripheral nerve.

These results suggest that the majority of the protein purified by Schlaepfer is serum albumin. However, anti-rat serum albumin does not give the neuronal staining seen by Schlaepfer with his antibody against the 68,000-dalton material. Indeed, he has made every effort to remove any trace of anti-albumin from his antisera (17). Therefore, it is likely that his antiserum is directed against the material that we call P68 and that P68 itself is obscured by albumin. This is supported by experiments in which we have presoaked the intact nerve in low ionic strength medium before dissection. Electron microscopy shows shrinkage of the axolemma and preservation of the filaments while a large amount of 68,000-dalton material is solubilized. When these nerves are then cleaned and extracted again at low ionic strength after mincing, one can see clearly the presence of P160 and P210, though P68 is still obscured by albumin. This contamination of peripheral nerve extracts by albumin has been previously noted by Eylar (5).

## Purification of Brain Filaments

The purification of brain filaments by either a 1-h exposure at low ionic strength or treatment with 1% Triton to remove myelin yields filament-rich fractions at the 1.5–2.0-M interface on sucrose step gradients. This material is morphologically similar to the filaments obtained after overnight demyelination in low ionic strength medium, but gives a much altered pattern on polyacrylamide-gel electrophoresis. In addition to the major band at 51,000 daltons (P51), both preparations are enriched in proteins with apparent mol wt of 68,000 (P68), 160,000 (P160), and 210,000 (P210) daltons (Fig. 2). These bands are also present in the filaments prepared by overnight low ionic strength demyelination, but comprise less than 5% of the protein compared to 50% or more in these preparations. It should be noted that the differences in molecular weights of these polypep-
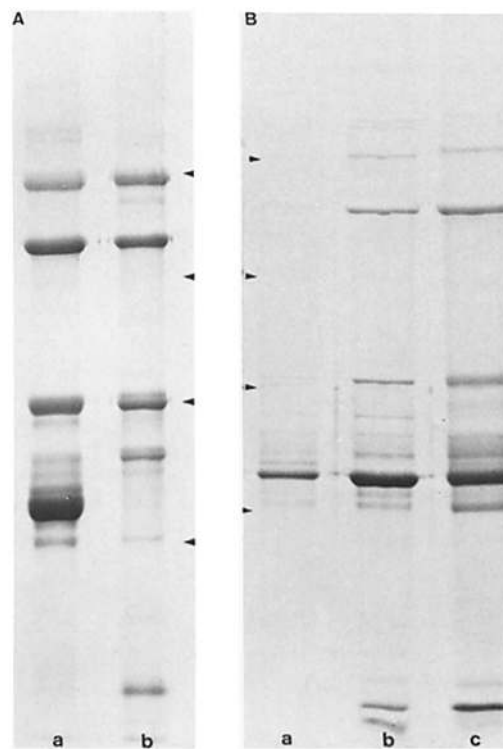


Figure 2 (A) Gel electrophoretogram of (a) rabbit spinal nerve root filaments, (b) rabbit CNS filaments. (B) Gel electrophoretogram of filaments obtained after (a) overnight demyelination in low ionic strength medium, (b) 1-h demyelination, and (c) demyelination with 1% Triton. Mol wt indicated by arrows are 200,000, 130,000, 68,000, and 45,000 daltons.

tides compared to the ones in the nerve root preparations are due to species differences. The triplet P68, P160, and P210 from calf brain corresponds to P68, P145, and P200 from rabbit brain. Similarly, P51 (calf) appears to have a mol wt of 50,000 daltons in the rabbit. Further species differences are shown in Table 1.

## Antibody Studies

Antibodies prepared in guinea pigs against P160 and P68 (Ab160 and Ab68) were tested by radioimmunoassay against P210, P160, P68, and P51. It is necessary to distinguish between P51 obtained in this preparation and that obtained by the long low ionic strength extraction (22). This latter polypeptide will be referred to as P51a and the antibody raised against this polypeptide as Ab51a. The titer of Ab160 and Ab68 against all four proteins appears to be similar, although the amount of P68 precipitated was lower than that of

| Component | Species | | | | | |
|---|---|---|---|---|---|---|
| | Calf | Rabbit | Guinea pig | Rat | Squid | Myxicola |
| 1 | 210 | 200 | 215 | 200 | 200 | |
| 2 | 160 | 145 | 145 | 145 | — | 160, 152 |
| 3 | 68 | 68 | 68 | 68, 66 (Doublet) | 60 | |
| 4 | 51 | 51 | 54 | 55, 53 (Doublet) | | 50 (Degradation product) |

Also included are the main proteins from squid (11) and *Myxicola* (7) neurofilaments.

either P160 or P210 in all cases (Table II). The titer of these two antibodies against P51a was, however, significantly lower. Ab51a, on the other hand, was found to have a titer against P51 and P51a only, not against the other three polypeptides. These results suggest that P51 is a mixed polypeptide consisting of both P51a and another polypeptide related to P68 and P160, which apparently solubilized during the long, low ionic strength extraction of the axons. Ab68 did not recognize either rat or bovine serum albumin, and anti-albumins did not react with P68.

## Immunofluorescence Studies

To verify whether P68 and P160 are neuronal, immunofluorescence studies were carried out using Ab68 and Ab160. These two antibodies as well as Ab51a (20) were reacted with sections of cerebellum and sciatic nerve. Preimmune sera and antigen-absorbed sera were used as controls. The results with both Ab68 and Ab160 were the same, and the staining of brain and sciatic nerve by the latter is seen in Fig. 3 (*a* and *b*). Strong peripheral nerve staining is seen, whereas staining in the brain appears to be neuronal. Only minor staining of astrocytes and no staining of Bergmann glial fibers are observed. Staining of the brain obtained with Ab51a is shown in Fig. 3 (*c*). Strong glial staining is observed in the brain sections, with no neuronal staining. Peripheral nerve staining is much weaker and appears to be just slightly stronger than that with the control serum (18). These patterns support the neuronal origin of P68 and P160, and the apparent glial origin of P51a.

## Immunoprecipitation of Peptides

As all three antibodies (Ab160, Ab68, and Ab51a) appear to recognize P51, we have deter-

TABLE II

*Radioimmunoassay Titers of the Different Antibodies against the Radioiodinated Polypeptides from the Brain Filament Preparations*

| Ag | Ab | | |
|---|---|---|---|
| | Ab51a | Ab68 | Ab160 |
| P51 | 1:625 | 1:250 | 1:250 |
| P68 | — | 1:250 | 1:250 |
| P160 | — | 1:250 | 1:250 |
| P210 | — | 1:250 | 1:250 |
| P51a | 1:625 | 1:10 | 1:10 |

Titer is defined as the point where one-half of the maximum precipitable counts are precipitated.

mined antigenic inhomogeneities in this protein by determining what peptides can be precipitated by the antibodies after P51 is digested into smaller fragments by *S. aureus* protease. We can see that Ab68 and Ab160 precipitate exactly the same peptides from P51, showing again the similarities between the two antibodies (Fig. 4). Ab51a, on the other hand, precipitates a major peptide, which is different from any precipitated by Ab68 and Ab160, as well as two peptides which possibly compare with the peptides precipitated by the other antibodies. These differences again point to the mixed nature of P51, which apparently contains a neuronal component recognized by Ab68 and Ab160, as well as a glial component recognized by Ab51a.

## DISCUSSION

Though morphologists have reported differences in diameter between neurofilaments and glial filaments (21), biochemical studies have been complicated by the variety of purification, solubilization, and radiolabeling studies that have been
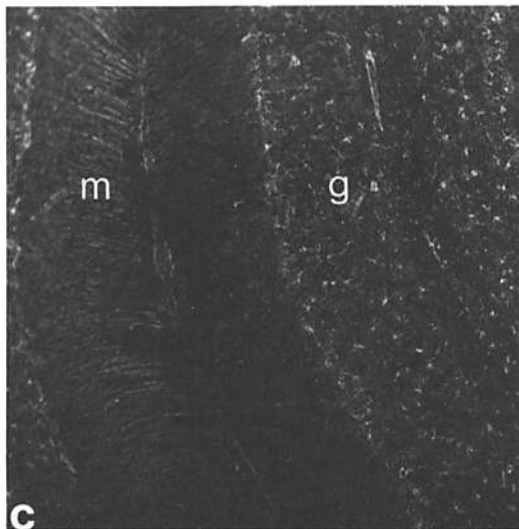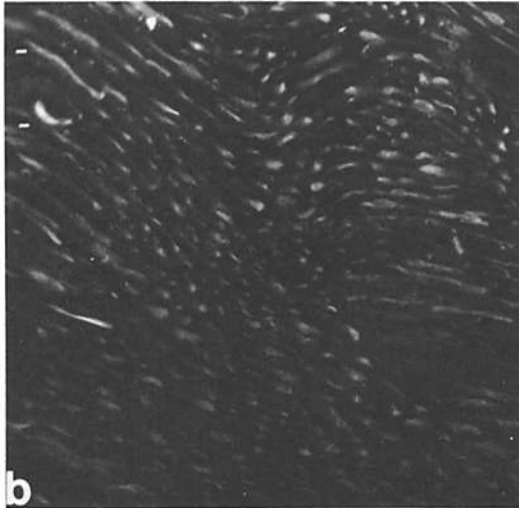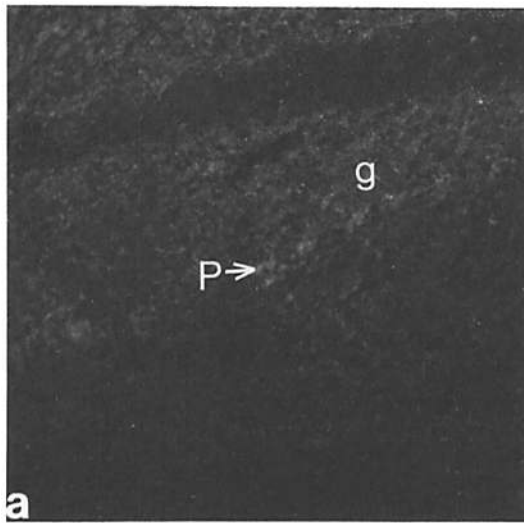
used. Most difficult has been understanding the higher molecular weights in purified invertebrate neurofilaments (7, 10) and in axoplasmic transport studies (9) and the lower weights in purified filament preparations. A second problem has been the reconciliation of the apparent biochemical similarity between the GFA (22) and the brain filaments with the exclusively glial staining obtained with antibodies to either GFA (2, 14) or brain filaments (20). We believe that the results presented here clarify, at least in part, the differences between the intermediate filaments of astroglia and neurons in the mammal.

The data presented above on the peripheral nerve show the absence of the P51 protein in fractions highly enriched in intermediate filaments. That these are neurofilaments is strongly supported by the lack of astroglial processes in the roots, as well as by electron microscopic controls done at each step of the procedure. The polypeptides in this preparation have apparent mol wt of 200,000, 145,000, and 68,000 as well as 60,000 daltons (Fig. 2).

The hypothesis that the neurofilaments are soluble at low ionic strength and were lost from earlier brain filament preparations (22) is supported by the results on central nervous system filaments reported here. When low ionic strength exposure is brief or eliminated by using detergent to remove the myelin, the resulting filament preparation is greatly enriched in the P210, P160, and P68 polypeptides, though a band of 51,000 daltons is always present. These molecular weights differ slightly from those obtained from the rabbit intradural roots, since the central nervous system preparations were routinely done from calf due to species differences (Table I).

Therefore, the elements which are common to the neurofilaments isolated from the intradural root and to the brain filaments isolated under conditions which will not cause neurofilament degradation are the P210, P160, and P68 triplet. Further support for the identification of these polypeptides with the neurofilament comes from enrichment in these peptides in isolated spinal neurons that have aluminum-induced neurofibrillary proliferation (18). The localization of antisera to both P68 and P160 to neurons and to the axons in peripheral nerve also supports the neuronal

Figure 3 Indirect immunofluorescence localization of (a) cerebellum antigens with Ab160, (b) sciatic nerve antigens with Ab160, and (c) cerebellum antigens with Ab51a. In all cases, preimmune serum controls were completely negative. (a) and (b) × 3,260, (c) × 100. p = Purkinje layer, g = granule layer, m = molecular layer.
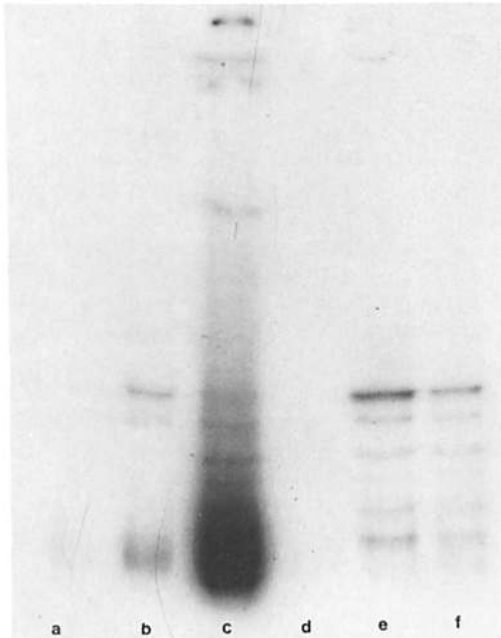
FIGURE 4 Autoradiogram of precipitated protease peptides from P51 obtained using different antisera: (a) preimmune rabbit serum, (b) Ab51a, (c) digestion mixture control, (d) preimmune guinea pig serum, (e) Ab160, and (f) Ab68.

the 51,000-dalton protein obtained after Triton treatment or 1 h low ionic strength extraction shows, in addition to reactivity with Ab51a, strong reaction against Ab68 and Ab160. We suspect that this is caused by proteolytic degradation of one or more of the triplet proteins to 51,000 daltons during the preparation. Some further support is given to this hypothesis by the observation that if a brain filament preparation is allowed to sit over a period of days at 4°C, there is an increase in the 51,000-dalton material.

It is likely that this low residual level of neurofilament-derived material accounts for the faint peripheral nerve staining and the neuronal staining at the electron microscopic level (20) seen with anti-P51a. The immunoprecipitation results on the radiolabeled peptides of P51 also give evidence of the mixed nature of the material, with identical peptides being precipitated by Ab68 and Ab160, while the major peptide precipitated by Ab51a is more rapidly migrating than any in the other precipitates. The precipitation of two peptides by Ab51a which do appear to migrate close to two precipitated by Ab68 and Ab160 is probably a reflection of the low anti-neurofilament titer in Ab51a.

The immunological studies presented, though clearly of a preliminary nature, suggest the intriguing possibility that the members of the triplet are derived one from another. While the molecular weights do not add up exactly, the error in such determinations on gels in the high molecular weight region would allow a mechanism where the P210 is cleaved into P160 and P68. Proof of this will depend on careful study of the peptides in each of these proteins. If this is the mechanism, one can infer from axoplasmic transport studies (9) that the cleavage is the result of cellular processing rather than proteolysis since the ratios of the transported triplet proteins to each other remain constant along the proximo-distal axis.

Radioactively labeled polypeptides migrating with or near the neurofilament triplet reported here have been seen in the slow component of axoplasmic transport and postulated to be associated with the neurofilament (9). Proteins of the molecular weight of the triplet are also seen associated with filaments which co-purify with microtubules (1) and are present in other preparations of intermediate filaments (12).

Preliminary studies on reassembly of the triplet in our laboratory have shown selective precipitation of the triplet proteins by return to physiolog-

origin of these peptides, as does the intense staining of aluminum-induced neurofibrillary bundles in the spinal cord (18).

The lack of P51 in filaments isolated from the intradural root which lacks astroglia, and its intensity in the central nervous system preparations which are from areas rich in astroglia, argues for an astroglial origin for this protein. Further support for this argument comes from the intense localization of an antiserum against the 51,000-dalton protein obtained by the overnight demyelination procedure (P51a) to astroglial processes with no neuronal staining and from the recent observation (8) that a preparation of pure glial filaments shows predominance of a protein with a mol wt of 51,000 daltons. The origin of the 60,000-dalton protein in the intradural root preparations is uncertain.

The apparent contradictions in the immunological data on the 51,000-dalton protein can be partially rationalized by considering the manner of preparation. The P51a that has been extracted at low ionic strength overnight gives only a low titer against Ab68 and Ab160, but is highly reactive against Ab51a, as expected. On the other hand,

ical salt concentrations, and these precipitates can be collected at the same sucrose gradient interface as the original neurofilaments. (Reaggregates of both 2-h brain extracts and sciatic nerve extracts are lacking in 51,000-dalton material.)

Our present work leads us to the following hypothesis: (*a*) The neurofilament is composed of a triplet of polypeptides with mol wts of 210,000, 160,000, and 68,000 daltons. These polypeptides are likely to be the same as those seen in the slow component of axonal transport (9). (*b*) The neurofilament in central as well as peripheral nerve is soluble at low ionic strength. All three of the triplet polypeptides are correspondingly solubilized. (*c*) The major constituent of the glial filament also has a mol wt of 51,000, but is not biochemically or immunologically related to the peptides of the neurofilament.

## REFERENCES

1. Berkowitz, S. A., J. Katagiri, H. K. Binder, and R. C. Williams, Jr. 1977. Separation and characterization of microtubule proteins from calf brain. *Biochemistry.* **16:**5610–5617.
2. Bignami, A., L. F. Eng, D. Dahl, and C. T. Uyeda. 1972. Localization of the glial fibrillary acidic protein in astrocytes by immunofluorescence. *Brain Res.* **43:**429–435.
3. Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and U. K. Laemmli. 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J. Biol. Chem.* **252:**1102–1106.
4. Dahl, D., and A. Bignami. 1973. Glial fibrillary acidic protein from normal human brain. Purification and properties. *Brain Res.* **57:**343–360.
5. Eylar, E. H. 1977. The myelin membrane and its basic proteins. *In* The Structure of Biological Membranes. S. Abrahamssen and I. Pascher, editors. Plenum Press, New York. 157–176.
6. Gilbert, D. S. 1977. Axon structure. *In* Proceedings of the International Society of Neurochemistry. International Society for Neurochemistry, Copenhagen. **6:**76.
7. Gilbert, D. S., B. J. Newby, and B. H. Anderton. 1975. Neurofilament disguise, destruction and discipline. *Nature (Lond.).* **256:**586–589.
8. Goldman, J. E., H. Schaumburg, and W. T. Norton. 1978. Isolation and characterization of glial filaments from human brain. *J. Cell Biol.* **78:**426–440.
9. Hoffman, P. N., and R. J. 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.
10. Huneeus, F. C., and P. F. Davison. 1970. Fibrillar proteins from squid axons. I. Neurofilament protein. *J. Mol. Biol.* **52:**415–428.
11. Lasek, R. J., and P. N. Hoffman. 1976. The neuronal cytoskeleton, axonal transport and axonal growth. *In* Cell Motility. R. Goldman, T. Pollard, and J. Rosenbaum, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. 1021–1050.
12. Lehto, V. P., I. Virtanen, and P. Kurki. 1978. Intermediate filaments anchor the nuclei in nuclear monolayers of cultured human fibroblasts. *Nature (Lond.).* 175–177.
13. Liem, R. K. H., S-H. Yen, C. J. Loria, and M. L. Shelanski. 1977. Immunological and biochemical comparison of tubulin and intermediate brain filament protein. *Brain Res.* **132:**167–171.
14. Schachner, M., E. T. Hedley-Whyte, D. W. Hsu, G. Schoonmaker, and A. Bignami. 1977. Ultrastructural localization of glial fibrillary acidic protein in mouse cerebellum by immunoperoxidase labelling. *J. Cell Biol.* **75:**63–73.
15. Schlaepfer, W. W. 1971. Stabilization of neurofilaments by vincristine sulfate in low ionic strength media. *J. Ultrastruct. Res.* **36:**367–374.
16. Schlaepfer, W. W. 1977. Immunological and ultrastructural studies of neurofilaments isolated from rat peripheral nerve. *J. Cell Biol.* **74:**226–240.
17. Schlaepfer, W. W., and R. G. Lynch. 1977. Immunofluorescence studies of neurofilaments in the rat and human peripheral and central nervous system. *J. Cell Biol.* **74:**241–250.
18. Selkoe, D., R. K. H. Liem, S-H. Yen, and M. L. Shelanski. 1978. Biochemical and immunohistological characterization of neurofilaments in aluminum-induced neurofibrillary degeneration. *Brain Res.* In press.
19. Shelanski, M. L., S. Albert, G. H. DeVries, and W. T. Norton. 1971. Isolation of filaments from brain. *Science (Wash. D.C.).* **174:**1242–1245.
20. Shelanski, M. L., R. K. H. Liem, and S. H. Yen. 1977. Microtubules and intermediate filaments of the brain. *In* Mechanisms, Regulation and Special Functions of Protein Synthesis in the Brain. S. Roberts, A. Lajtha, and W. H. Gispen, editors. Elsevier-North Holland Publishing Co., New York. 137–152.
21. Wuerker, R. B. 1970. Neurofilaments and glial filaments. *Tissue Cell.* **2:**1–9.
22. Yen, S-H., D. Dahl, M. Schachner, and M. L. Shelanski. 1976. Biochemistry of the filaments of brain. *Proc. Natl. Acad. Sci. U. S. A.* **73:**529–533.