

Isolation of 10-nm Filaments from Astrocytes in the Mouse Optic Nerve

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ABSTRACT Astroglial filaments ~10 nm in diameter were isolated from degenerated mouse optic nerves by Triton X-100 and DNase I treatments followed by sucrose density gradient centrifugation. 2-4 wk after bilateral enucleation, optic nerves contained virtually a single population of 10-nm filaments (astroglial filaments), free from neurofilaments. In negative-staining and thin-section electron microscopy, the isolated filaments were seen as nonbranching linear structures with smooth contour, and were morphologically identical to those *in situ*. Sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed the isolated filaments to be composed of two major polypeptides with molecular weights of 45,000 and 55,000, present in an approximate molar ratio of 1:1. These findings, together with the results of one-dimensional peptide mapping and solubility study, indicate that the astroglial filaments in the mouse optic nerve are primarily composed of these two polypeptides.

Since the initial discrimination between 10-nm filaments and actin-containing microfilaments *in situ* (7, 8), extensive studies on 10-nm filaments have been made in various cell types. Mammalian brain tissues contain mainly two classes of 10-nm filaments, i.e., neurofilaments and glial filaments. Neurofilaments isolated from the rat peripheral nerve are composed of three polypeptides with molecular weights of 200,000, 160,000, and 68,000, present in relative molar proportion of 1:2:9 (12, 13, 16, 17). This polypeptide triplet is shown to migrate slowly within the axons (6, 11). On the other hand, the glial fibrillary acidic (GFA) protein, with a molecular weight of 54,000 (2, 14) or 47,000 (3), has been intensively studied as the major polypeptide of glial filaments. A possible contamination of neurofilaments makes it less convincing to isolate well-defined glial filaments from the brain tissue. Only from the human gliosed brain, Goldman et al. (5) succeeded in isolating 10-nm filaments which were mainly composed of a single species of polypeptide of 49,000 molecular weight.

Among neuroglial cells, only astrocytes are rich in 10-nm filaments (astroglial filaments). The optic nerve contains a large number of astrocytes, and almost all axons that originate in the retina degenerate after enucleation (22). Thus, the degenerated optic nerves represent a highly enriched source of astroglial filaments, virtually free from contamination with neurofilaments.

Taking advantage of these properties of degenerated optic nerves, we have succeeded in isolating from them a relatively pure population of astroglial filaments. Biochemical analyses showed that the astroglial filaments were composed of two

major polypeptides with molecular weights of 45,000 and 55,000 (referred to as P45 and P55 throughout).

MATERIALS AND METHODS

Isolation of Astroglial Filaments

DEGENERATION OF OPTIC NERVE: For each isolation experiment, degenerated optic nerves from 30 to 40 mice were used. 3-wk-old mice were anesthetized with ether, and both eyes were enucleated after ligation of the stalks at the posterior poles.

TRITON-DNase TREATMENT: 2 wk after the operation, mice were killed by decapitation, and degenerated optic nerves (each ~5 mm long) were excised bilaterally. All the subsequent procedures were carried out at 4°C. Pooled nerves were always washed several times in phosphate-buffered saline (PBS; 6 mM Na⁺-K⁺ phosphate, 171 mM NaCl, and 3 mM KCl, pH 7.0), and muscular and connective tissues around the nerve were carefully removed. According to the method used by Starger et al. (19), nerves were lysed (10 ml for each pair of optic nerves) for 3 h with three changes of lytic solution containing 1% Triton X-100, 0.6 M KCl, 0.1 mM phenylmethyl sulfonyl fluoride (PMSF), and 1 mg/ml *p*-tosyl-L-arginine methyl ester-HCl (TAME) in PBS. Subsequently, nerves were treated with 1 ml (per pair of nerves) of the lytic solution containing 10 mM MgCl₂ and 0.4 mg/ml DNase I for 4 h to extract DNA and actin, and then washed three times in PBS containing 5 mM EDTA. Such optic nerves kept their entire structure but became very white.

SUCROSE DENSITY GRADIENT CENTRIFUGATION: After the Triton-DNase treatment, nerves were finely minced with razor blades and homogenized in 1.5 ml of 30 mM Na⁺-K⁺ phosphate buffer containing 10 mM EDTA, pH 6.5 (solution A). The homogenate was mixed with an equal volume of 2.0 M sucrose in solution A, and fractionated by centrifugation at 145,000 *g* for 100 min on a discontinuous gradient consisting of 1 ml each of 1.5 and 2.2 M sucrose dissolved in solution A. In some experiments, degenerated optic nerves were fractionated essentially according to the procedure of Shelanski et al. (18).

Electron Microscopy

For thin-section electron microscopy, samples were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) with or without 0.5% tannic acid at 4°C for 10–20 h. After being postfixed in cold 1% OsO₄ in the same buffer for 2 h, they were stained en bloc with 0.5% uranyl acetate for 2 h. They were then dehydrated in ethanol and embedded in Epon 812. Thin sections were cut, doubly stained with uranyl acetate and lead citrate, and then examined in a Hitachi 11-DS electron microscope at an accelerating voltage of 75 keV.

Negative staining was performed on collodion-carbon-coated grids with saturated uranyl acetate after the sample solution was fixed on grids with 5% formaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2).

Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis

Samples were homogenized or sonicated in medium consisting of 2.3% (wt/vol) SDS, 5 mM β -mercaptoethanol, 10% (vol/vol) glycerol, and 62.5 mM Tris-HCl (pH 6.8). The homogenate was incubated for 30 min at 37°C and then heated in a boiling water bath for 5 min, and an aliquot was subjected to electrophoresis (9, 11) in 10% polyacrylamide slab gels. Gels were stained with 0.25% (wt/vol) Coomassie Brilliant Blue R-250 (CBB). Relative amounts of polypeptides with different mobilities were estimated from densitometric tracings recorded at 560 nm.

One-dimensional Peptide Mapping

Protein bands to be examined were cut out from the gel, subjected to a limited proteolysis with *Staphylococcus aureus* V8 protease (1), and electrophoresed in 15% SDS polyacrylamide gels.

Solubilization of Astroglial Filaments

Degenerated optic nerves from 60 mice, either treated or untreated with Triton-DNase, were minced with razor blades and homogenized in 0.8 ml of 2 mM Tris-HCl (pH 8.6) containing 0.5 mM dithiothreitol (DTT). After stirring at 4°C for 30 h and at 20°C for 30 min to solubilize astroglial filaments, the homogenate was centrifuged at 100,000 g for 60 min at 4°C. To reaggregate the solubilized proteins, 0.05 vol of 2 M imidazole-HCl (pH 6.7) was added to the supernate and incubated at 37°C. These conditions are similar to those used by Rueger et al. (15) in their study on reassembly of the GFA protein. Protein determination (10), SDS gel electrophoresis, and electron microscopic observation (negative staining) were made at appropriate steps during this experiment.

RESULTS

Morphological and Biochemical Changes of Optic Nerves in the Degeneration Process

The normal optic nerve is similar to the white matter of the

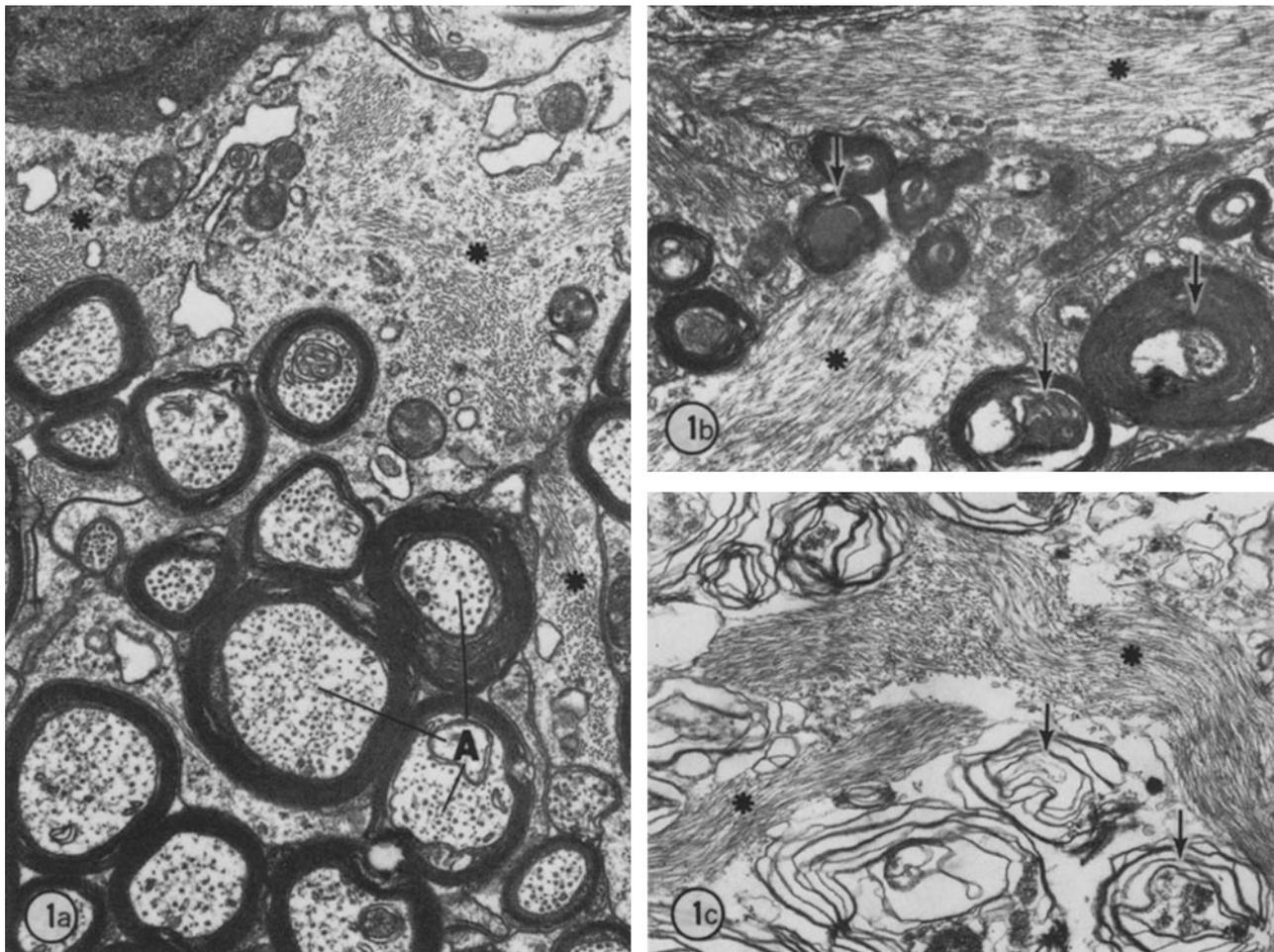


FIGURE 1 (a) Transverse section of the control optic nerve. Myelinated axons (A) are surrounded by the astrocytic processes (*) which contain bundles of astroglial filaments. Inside the myelinated axons are seen transversely cut neurofilaments and microtubules. $\times 18,000$ (b) Transverse section of the degenerated optic nerve 2 wk after enucleation of the eyeball. All myelinated axons are degenerated, leaving dense materials inside the deformed myelin sheaths (arrows). Almost all the 10-nm filaments in the degenerated optic nerve thus represent the astroglial filaments (*). $\times 20,000$. (c) Transverse section of the optic nerve degenerated for 2 wk and then treated with Triton-DNase. Such nerves are characterized by prominent bundles of astroglial filaments (*) with some fragmented myelin sheaths (arrows). $\times 20,000$. All the nerves were prefixed with 2.5% glutaraldehyde without tannic acid.

central nervous system and consists of a large number of myelinated axons, which are kept together by fibrous astrocytes. The astrocytes characteristically contain bundles of 10-nm filaments extending into their processes (Fig. 1*a*). In contrast to the astrocytes, oligodendrocytes contain a small number of 10-nm filaments that do not form any bundles. The myelinated axons contain longitudinally running neurofilaments and microtubules. Thus, 10-nm filaments in normal optic nerves are primarily astroglial filaments and neurofilaments. Enucleation of eyes causes the Wallerian degeneration of myelinated axons and activation of astrocytes (Fig. 1*b*). 2–4 wk after the operation, virtually all axons in the optic nerves underwent degeneration, leaving some dense materials inside the deformed myelin sheaths. Spaces originally occupied by nerve fibers were filled with astrocytes. Oligodendrocytes showed no appreciable change in their filament content for at least 4 wk after the operation. Thus, it can be said that, 2–4 wk after the operation, almost all the 10-nm filaments in the degenerated optic nerves represent the astroglial filaments. When these nerves were treated with Triton-DNase, many structures disappeared but 10-nm filaments remained morphologically unchanged (Fig. 1*c*).

The protein components of the normal and degenerated optic nerves (at days 0, 3, 7, 14, and 28 after the operation) were analyzed using SDS polyacrylamide gel electrophoresis (Fig. 2). The triplet polypeptides with molecular weights of 200,000, 160,000, and 68,000, which are considered to be the neurofilament subunits, disappeared within 1 wk after the operation. This loss of the triplet was more clearly shown in the Triton-DNase treated nerves. The majority of actin, which has the same electrophoretic mobility as the skeletal muscle

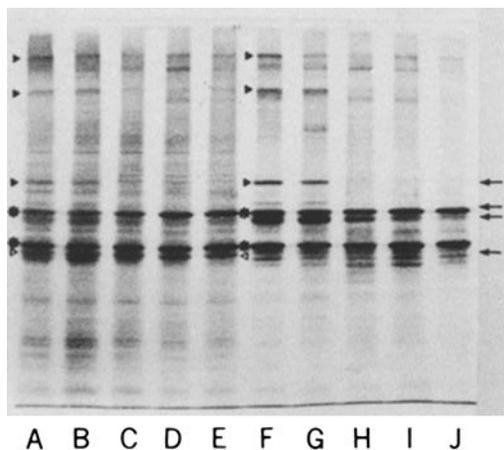


FIGURE 2 Changes in electrophoretic banding patterns of the optic nerve polypeptides during degeneration. Optic nerves removed at days 0 (A and F), 3 (B and G), 7 (C and H), 14 (D and I), and 28 (E and J) of degeneration were analyzed by SDS-polyacrylamide gel electrophoresis. In slots A–E, nerves were directly processed for electrophoresis. In slots F–J, nerves were first treated with Triton-DNase and then processed for electrophoresis. Throughout the degeneration stages, two polypeptides (*) with molecular weights of 45,000 and 55,000 that are present in an approximate molar ratio of 1:1 are seen to predominate. The neurofilament triplet polypeptides (▲) are decreased as the degeneration proceeds and are hardly detectable after day 14 (D–E and I–J). Actin (▶) and many other polypeptides are removed by treatments with Triton-DNase (cf. A–E with F–J). Arrows indicate molecular weights of 68,000, 56,000, 53,000, and 43,000 from the top.

actin, was removed by the Triton-DNase treatment. Irrespective of the degeneration stages, P45 and P55 were seen to exist in an approximate molar ratio of 1:1 and constituted the major polypeptides of the optic nerve. These two polypeptides amounted to >50% in nerves degenerated for 4 wk and subsequently treated with Triton-DNase.

Isolation of Astroglial Filaments

In thin-section electron microscopy, optic nerves that had degenerated for 2 wk and then were treated with Triton-DNase contained astroglial filaments, dense materials, fragmented myelin sheaths, other membranous structures, basement membranes, and collagen fibrils around nerves. Electrophoresis of this nerve preparation revealed the presence of P45 and P55, but in addition, minor polypeptides were seen in both high and low molecular weight regions (Fig. 2*I*).

After sucrose density gradient centrifugation, astroglial filaments were recovered at the 1.0/1.5-M interface mainly as bundles (Fig. 3). At the 1.5/2.2-M interface and at the bottom of the 2.2-M sucrose, the astroglial filaments were also recovered, but these fractions were contaminated by a small number of collagen fibrils, basement membranes, and some dense materials. Membranous structures were recovered almost exclusively at the top of the 1.0-M sucrose, together with a small number of relatively short filaments.

Astroglial filaments isolated as bundles were morphologically identical with those *in situ*. The extent of filament packing varied in different bundles (Fig. 3*a*), and some bundles had membrane fragments and fine meshwork structure around them (Fig. 3*b*). The isolated filaments were seen as discrete, unbranching linear structures ~10 nm in diameter, and their surface appeared to be smooth in both negative-staining and thin-section electron microscopy (Figs. 3*c* and 4).

On electrophoresis of the purified filament fraction (1.0/1.5-M interface), P45 and P55 were seen to dominate, but minor polypeptides were invariably present in the molecular weight range from 55,000 to 40,000 (Fig. 5*A*). Though the fractions at the 1.5/2.2-M interface and at the bottom of the 2.2-M sucrose contained structures other than filaments, P45 and P55 were seen to dominate also in these fractions. Densitometric tracings indicated that the ratio of P45 to P55 was ~1:1 in all these fractions (Fig. 5*A–C*).

In some experiments, degenerated optic nerves were not treated with Triton-DNase, and were fractionated essentially as described by Shelanski et al. (18). This yielded a less purified filament preparation. Still, this filament-rich fraction was again dominated by P45 and P55, present in an approximate molar ratio of 1:1 (Fig. 5*E*).

One-dimensional Peptide Mapping

P45, P55 (in the astroglial filament preparation), the 68,000-dalton component of the neurofilament triplet (P68, see reference 12), α -tubulin (from rat brain), and actin (from rabbit skeletal muscle) were subjected to a limited proteolysis with V8 protease (1). Digestion patterns of these polypeptides clearly differ one another (Fig. 5*F–J*), indicating that P55 and P45 are distinct from the α -subunit of tubulin and actin, respectively, and that P45 is not a degradation product of P55. Also, clear differences shown in peptide maps of P45, P55, and P68 preclude the possibility that P45 and P55 are degradation products of the neurofilament component P68.

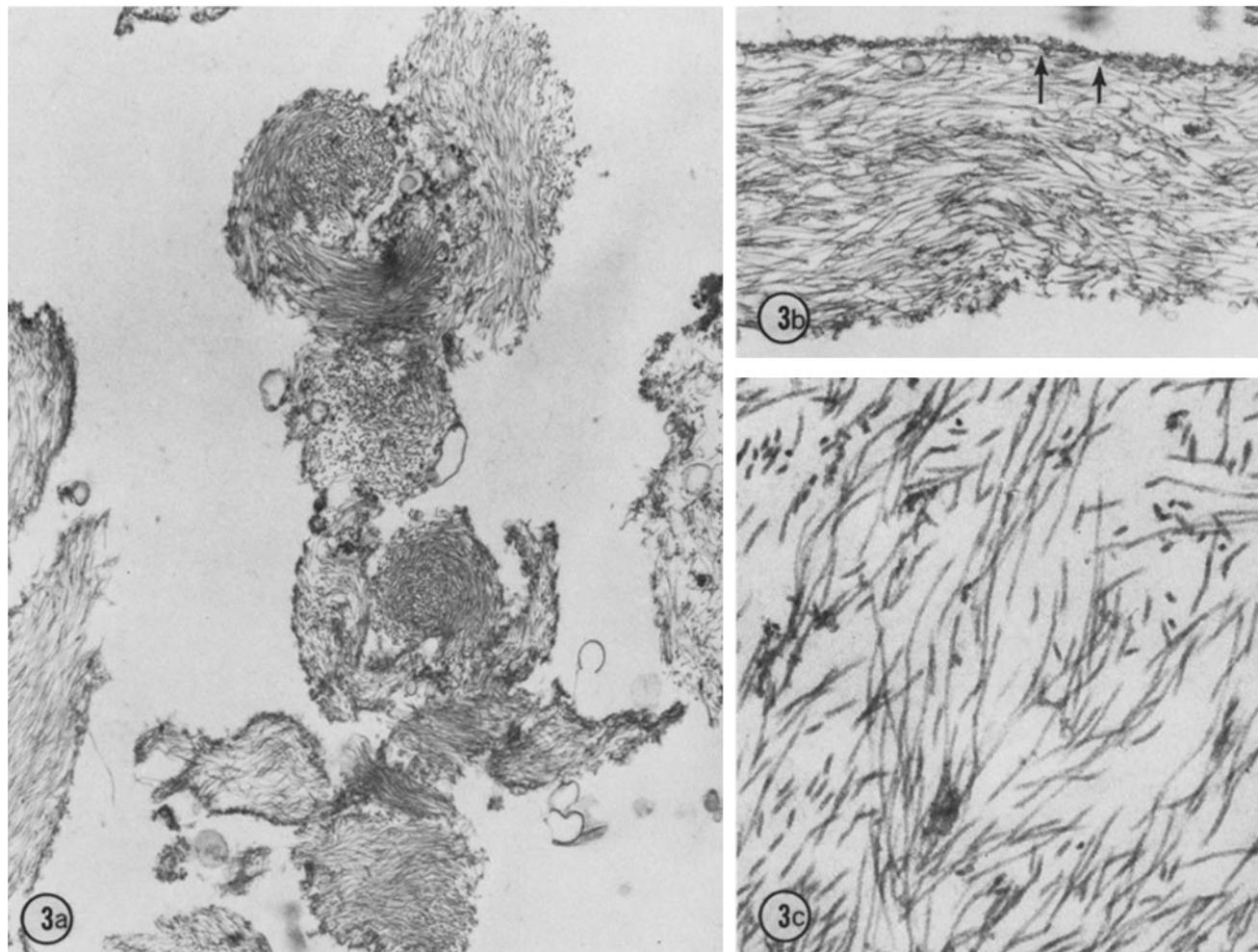


FIGURE 3 Thin-section electron microscopy of isolated astroglial filaments recovered at the interface between the 1.0- and 1.5-M sucrose layers. Samples were prefixed with 2.5% glutaraldehyde containing 0.5% tannic acid. (a) Astroglial filaments were isolated as bundles. Note that the extent of filament packing varies according to bundles. $\times 18,000$. (b) Some bundles are delineated with a layer of fine meshwork structures (arrows). $\times 30,000$. (c) The filaments are discrete, unbranching linear structures ~ 10 nm in diameter. No associated substructures are seen on their surface. $\times 50,000$.

Solubilization and Reaggregation of Astroglial Filament Proteins

The degenerated nerves with or without Triton-DNase treatment were homogenized and incubated in a hypotonic medium. After the homogenate was centrifuged, $\sim 80\%$ of both P45 and P55 was recovered in the 100,000 *g* supernate, together with some minor polypeptides (Fig. 5 *K* and *L*). The molar ratio of P45 to P55 in the supernate was $\sim 1:1$. In negative-staining electron microscopy, no 10-nm filaments were detected in the supernate (Fig. 6 *a*).

When the supernate (0.1–0.5 mg protein/ml) was incubated in 100 mM imidazole (pH 6.7) at 37°C for 3 h, both P45 and P55 precipitated almost completely in an approximate molar ratio of 1:1 (Fig. 5 *M* and *N*). During incubation, a large number of filamentous structures became visible within 20 min by negative staining. These filamentous structures were variable in diameter ranging from ~ 10 to 50 nm and grew longer with time, forming bundles (Fig. 6 *b*).

These results indicate that P45 and P55 can be solubilized and reaggregated in a constant molar ratio, but further detailed studies are needed to optimize and quantitate this solubilization-reaggregation process.

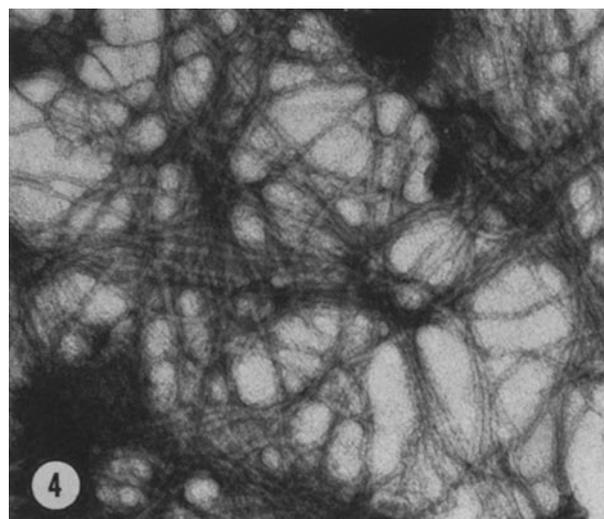


FIGURE 4 Negative staining of the isolated astroglial filaments recovered at the 1.0/1.5-M sucrose interface. The astroglial filaments are seen as nonbranching linear structures with smooth contour ~ 10 nm in diameter. $\times 80,000$.

DISCUSSION

Astroglial filaments have been successfully isolated as bundles from degenerated mouse optic nerves by Triton-DNase treatment followed by sucrose density gradient centrifugation. The degenerated optic nerves are highly enriched with astroglial filaments and are virtually free of neurofilaments. The degenerated optic nerves thus offer advantageous starting materials for the isolation of well-defined astroglial filaments. Removal of membrane components by Triton-DNase was also an important step in the present isolation procedure.

Highly purified astroglial filaments were composed of P45 and P55. These two polypeptides are not the product of new synthesis in response to axonal degeneration inasmuch as they constitute the major polypeptides consistently found in the control optic nerve. One-dimensional peptide mapping has demonstrated that P55 and P45 are distinct from α -tubulin and actin, respectively, and that P45 is not a degradation product of P55. Nonidentity between P45 and actin is also supported by their different solubility in Triton-DNase treatments (see Fig. 2). Furthermore, the neurofilament component with a molecular weight of 68,000 showed a peptide map distinct from

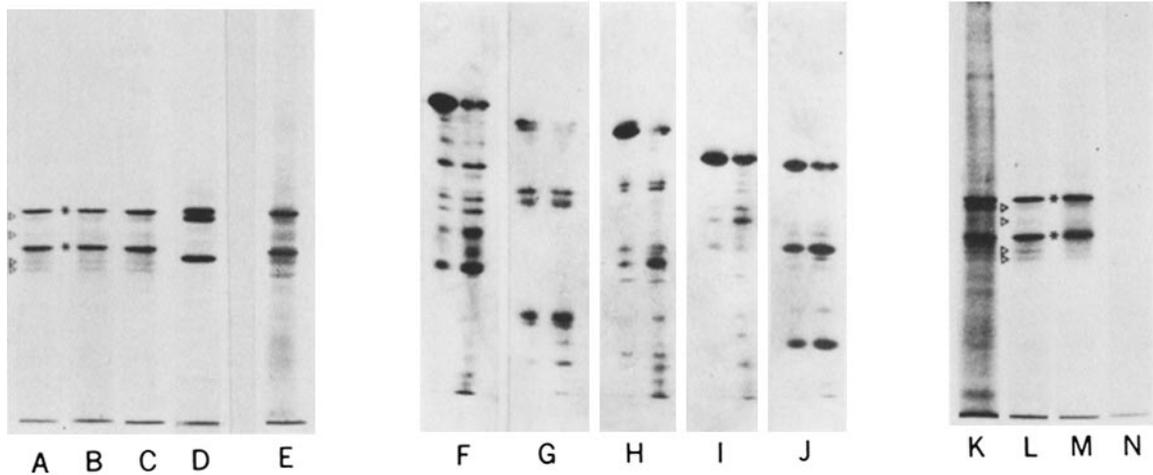


FIGURE 5 Biochemical characterization of isolated astroglial filaments. (A-E) After sucrose density gradient centrifugation, materials recovering at the 1.0/1.5-M interface (A), at the 1.5/2.2-M interface (B), and at the bottom of the 2.2-M sucrose layer (C) were electrophoresed. In all these fractions, two polypeptides (*) with molecular weights of 45,000 (P45) and 55,000 (P55) are seen to predominate, present in a molar ratio of 1:1. In a filament-rich fraction prepared according to the procedure of Shelanski et al. (18), P45 and P55 are also found predominantly (E). D shows the mixture of rat brain tubulins (upper doublet bands) and rabbit skeletal muscle actin (lower single band). (F-J) One-dimensional peptide maps of neurofilament with a molecular weight of 68,000 (F), α -tubulin (G), P55 (H), P45 (I) and actin (J). The amounts of *Staphylococcus aureus* V8 protease used were 0.1 and 1.0 ng in left and right columns, respectively. (K-N) Solubilization and reaggregation studies in vitro. Optic nerves degenerated for 14 d were treated with Triton-DNase, incubated in 2 mM Tris-HCl (pH 8.6), and centrifuged at 100,000 g. K, precipitate; L, supernate. The supernate was then incubated in 100 mM imidazole-HCl (pH 6.7) and centrifuged at 100,000 g. M, precipitate; N, supernate. Note that minor components (\triangleright) are associated with P45 and P55 during these solubilization and reaggregation processes.

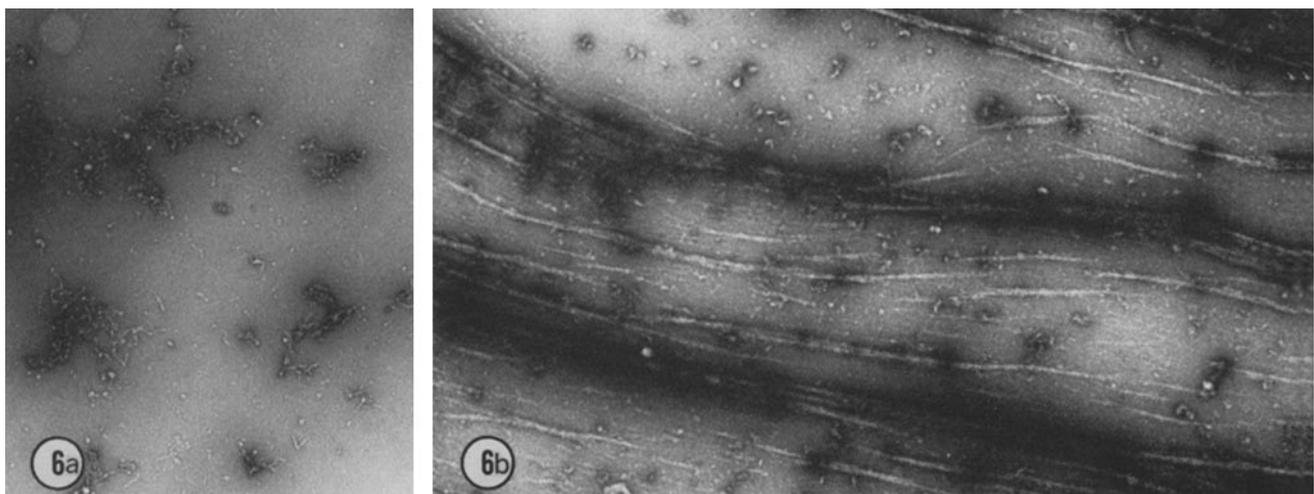


FIGURE 6 Negative staining of reaggregated filamentous structures. The supernate was obtained as in Fig. 5 L, but from the nerve not treated with Triton-DNase. The supernate contains no 10-nm filaments, but many rod-shaped structures are seen (a). 20 min after incubating this supernate in 100 mM imidazole-HCl (pH 6.7) at 37°C, a large number of short filamentous structures \sim 10 nm in diameter become visible. After 90 min, these filamentous structures become longer and tend to form large bundles (b). Filamentous structures at this stage vary in their diameter, ranging from 10 to 50 nm. \times 18,000.

that of either P45 or P55. The approximate molar ratio of P45 and P55 is 1:1 under a variety of circumstances, i.e., in the control and degenerated nerves before and after the treatment with Triton-DNase, in the purified filaments, after being solubilized in a hypotonic medium, and in the reassembled filaments. It is, thus, reasonable to conclude that astroglial filaments in the mouse optic nerve are primarily composed of P45 and P55. It is not uncommon to see that 10-nm filaments are composed of two or more polypeptide subunits, as has been described for prekeratin filaments (4, 20, 21), BHK cell filaments (19, 23), and neurofilaments (12, 13, 16, 17). Another possibility is that either P45 or P55 is the constitutive component of the astroglial filament and that the other is a component closely associated with the filament. This possibility should rigorously be ruled out in further studies, although the negative-staining and thin-section electron microscopy failed to demonstrate any associated substructures on the surface of the astroglial filaments.

Our present findings are in sharp contrast with the demonstrations that the astroglial filaments isolated from the gliosed human brain were composed largely of a single polypeptide subunit with a molecular weight of 49,000 (5) and that 10-nm filaments were assembled in vitro from a single species of bovine GFA protein with a molecular weight of 54,000 (15). At present, it is well known that the molecular weights of 10-nm filament subunits vary among species and cell types. Therefore, it is possible that the variance in the molecular species of astroglial filaments demonstrated in this and previous studies represents species differences or even differences between the astrocyte in the optic nerve and that in brain.

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