ISOLATION AND CHARACTERIZATION OF GLIAL FILAMENTS FROM HUMAN BRAIN

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ABSTRACT

Intermediate (8–9 nm) filaments of human central nervous system astrocytes were isolated from the gliosed white matter of cases of adrenoleukodystrophy (ALD). This hereditary lipidosis is characterized pathologically by demyelination, loss of axons, and replacement of the white matter of the caudal cerebrum by a glial scar. Glial filaments were composed largely of a single protein component with a mol wt of about 49,000 daltons. Smaller components (44,000–39,000 daltons) were detected in some samples, and appear to represent degradation products of the filament protein. Human neurofilaments were isolated from the normal frontal white matter of ALD cases by the standard myelin-free axon technique. Isolated glial and neurofilament proteins comigrated during acrylamide gel electrophoresis in SDS. Polypeptides resulting from cyanogen bromide cleavage of the two filament proteins were the same. Both proteins reacted with rabbit antisera raised against isolated bovine neurofilament protein and human glial fibrillary acidic protein.

KEY WORDS glial filaments · neurofilaments · GFA protein · astrocytes · adrenoleukodystrophy

Bundles of parallel filaments, 8–9 nm in diameter, are characteristic organelles within the cell bodies and processes of astrocytes (26). In various pathological states, both hereditary (23, 29, 33) and acquired (19), these filaments increase greatly in number to become prominent components of gliosed scar tissue. The composition and properties of these filaments are not well understood, largely because their isolation has not been demonstrated. Indirect evidence from studies with the glial fibrillary acidic (GFA) protein, originally isolated as a soluble component of multiple sclerosis plaques and other gliosed tissues (13, 38), and with brains of mouse mutants in which fibrous gliosis has occurred (23), has suggested that the major protein of glial filaments is similar or identical to that of the filaments isolated from CNS axons (neurofilaments), which have been characterized biochemically (11, 31, 32). Other evidence, however, has suggested that glial filament protein is immunologically different from neurofilament protein (7, 14, 24, 28) or that it is similar to tubulin (21).

Since the isolation of glial filaments is a requisite for biochemical characterization, heavily gliosed CNS tissue without contamination from axons would provide the most suitable starting material for such a study. Gliosed tissues such as multiple sclerosis plaques and postleukotomy scars usually contain varying degrees of residual axonal material. For this reason, we chose to examine white matter from cases of adrenoleukodystrophy

(ALD), a hereditary lipidosis of childhood, characterized initially by CNS myelin destruction followed by inflammation and loss of axons, with subsequent replacement of white matter by a glial scar (29). Most cases demonstrate a 2-4 yr, caudal-rostral progression in the pathological process. The initial changes are in the occipital pole, with cortical blindness a common early finding. In heavily gliosed early affected areas, such as the occipital pole, few or no axons have survived by the time of death, and this tissue has largely become a network of filament-rich astrocyte processes. We have isolated the filaments from this material and performed biochemical and immunological characterizations of the major filament protein, attending in particular to its relationship to the GFA protein.

This work has been presented in preliminary form (18).

MATERIALS AND METHODS

Isolation of Human Glial Filaments

We studied three cases of ALD, in which unfixed brains had been sliced coronally, sampled serially for electron microscopy, and frozen within 30 min postmortem and stored at −75°C for 6 mo to 2 yr. Gliosed white matter (0.5–1 g) from occipital lobes or from parietal or temporal lobes at the lateral geniculate level was minced and homogenized in 20 ml of 0.9 M sucrose, 0.05 M Na phosphate pH 7.6, 1 mM $MgCl_2$ in a Dounce homogenizer (Kontes Co., Vineland, N.J.) (a tight-fitting pestle was used to produce a fine suspension). The volume was brought to 25 ml, and the homogenate was centrifuged at 39,000 *g* for 20 min on a discontinuous gradient (5-ml steps) of 1.1 M, 1.5 M, and 1.9 M sucrose in 0.05 M Na phosphate pH 7.6, 1 mM $MgCl_2$. The gradient had been allowed to stand for 1 h at 4°C so that the interfaces blurred. Fractions were examined under the light microscope at each step of isolation procedures. The filament-filled astrocyte processes were found at the 1.5/1.9 M interface.

Phosphate Buffer Extraction of Gliosed ALD White Matter

Frozen gliosed white matter was homogenized at a wet weight-to-volume ratio of 1:10 in 0.05 M Na phosphate, pH 8.0, in a ground glass grinder (Micro-Metric Instrument Co., Cleveland, Ohio) at 4°C. The homogenate was centrifuged at 105,000 *g* for 30 min. The resulting pellet was ground in an additional volume of buffer, the centrifugation was repeated, and the supernates were combined. Further buffer extractions did not release additional soluble protein. Soluble proteins were precipitated from supernates by the addition of cold acetone to 94% (1), collected by low-speed centrifugation, and dried under N_2. Soluble and insoluble proteins were dissolved in 1% sodium dodecyl sulfate (SDS) by heating at 100°C for 15 min.

Isolation of Filaments from Myelin-Free Axons

Filaments were isolated, using a modification of the Schook and Norton procedure (31), from unaffected frozen frontal lobe white matter from the brain of a 6-yr-old male with adrenoleukodystrophy. The white matter was judged normal morphologically by light and electron microscope analyses. A sample of 8 g of white matter was homogenized in 230 ml of 0.9 M sucrose, 0.03 M Na phosphate, pH 6.5, 0.01 M KCl, 1 mM $CaCl_2$ (medium A) in a Dounce homogenizer (eight strokes of loose, six strokes of tight pestle) and centrifuged at 82,000 *g* for 30 min. The floating layer of myelinated axons was rehomogenized in 230 ml of medium A and centrifuged as described above. Homogenization of the floating layer and centrifugation were repeated once again. The floating layer was suspended in 90 ml of 0.32 M sucrose and stirred overnight at 4°C. The suspension was made 1 M in sucrose and centrifuged at 39,000 *g* for 20 min to yield an axon pellet (P_4) and a floating myelin layer. The floating layer was disrupted in a Sorvall Omnimixer (DuPont Instruments-Sorvall, DuPont Co., Wilmington, Del.) in medium A (three 30-s bursts at top speed) and centrifuged again at 39,000 *g* for 20 min, yielding an axon pellet (P_5) which was analyzed apart from the final pellet. The P_4 pellet of crude axons was suspended in medium A in a Polytron homogenizer (three 30-s bursts at top speed; Brinkmann Instruments, Inc., Westbury, N.Y.) and centrifuged at 39,000 *g* for 20 min. One additional Polytron homogenization and centrifugation produced the final pellet (P_7).

Acrylamide Gel Electrophoresis

Proteins were resolved in polyacrylamide slab gels in SDS with a discontinuous buffer system. The separating gel (10 cm long, 15 cm wide) constructed in a vertical slab gel apparatus (Hoefer Scientific Instruments, San Francisco, Calif.) was made of a linear gradient of 5–15% or 12.5–20% acrylamide, containing also a linear sucrose gradient of 5.6–17.3% for stabilization (5). Acrylamide concentration in the stacking gel, 1 cm long, was 4.5%. *N,N'*-Methylenebisacrylamide comprised 2.6% of total acrylamide in stacking and separating gels. Both gels contained 0.1% SDS. Polymerization was effected with 0.025% ammonium persulfate, added as a freshly made 10% solution, and 0.03% *N, N, N, N*-tetramethylethylenediamine. A discontinuous buffer system was used with a Tris-borate upper reservoir buffer, pH 8.64, Tris-sulfuric acid stacking gel buffer, pH 6.1, and a Tris-HCl separating gel and lower reservoir buffer, pH 9.18 (25). Just before electrophoresis, sample buffer

(0.05 M Tris HCl, pH 6.8; 1% SDS; 0.2% β-mercaptoethanol; 10% glycerol; 0.001% phenol red) was added to protein samples (usually 5-50 μg) to a total vol of 50 μl. Samples were then heated for 90 s at 100°C. Electrophoresis was performed for 3-3.5 h at 30 mA/gel (5-15% gradient) or 3.75 h at 30 mA/gel (12.5-20% gradient) at room temperature. Gels were fixed and stained in 0.25% Coomassie blue in 50% methanol, 7% acetic acid, and destained by diffusion in 30% methanol, 7% acetic acid.

The molecular weights of proteins were estimated by reference to standard proteins, which included β-galactosidase (130,000 daltons, Boehringer Mannheim Biochemicals, Indianapolis, Ind.), phosphorylase *a* (96,000 daltons, Sigma Chemical Co., St. Louis, Mo.), bovine serum albumin (monomer 68,000 daltons, dimer, trimer; Sigma), porcine brain tubulin (55,000 daltons, a gift from Dr. Felicia Gaskin, Department of Pathology, Albert Einstein College of Medicine), rabbit muscle actin (45,000 daltons, Worthington Biochemical Corp., Freehold, N.J.), ovalbumin (43,000 daltons, Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N.J.), ribonuclease A (13,700 daltons, Pharmacia), cytochrome *c* (12,000 daltons, Sigma), and rat brain myelin (basic proteins 14,000 and 18,000 daltons, proteolipid protein 24,000 daltons). The 5-15% gradient gels gave a linear relationship between log molecular weight and migration for proteins of mol wt of between 25,000 and 100,000 daltons, with nonlinear deviations observed in the high and low molecular weight ranges.

Cyanogen Bromide (CNBr) Cleavage of Filament Proteins

Several bands, containing a total of 200-400 μg of filament proteins, were cut out of Coomassie blue-stained gels and eluted with 1.5 ml of 0.05 M Na phosphate, pH 7.6, 0.1% SDS, 0.1% β-mercaptoethanol, 1 mM EDTA for 3.5 h at 37°C. Gel pieces were incubated with 1 ml of fresh buffer for an additional 1 h, and the eluates were combined. A 3.75-ml portion of 10% acetic acid in cold methanol was added to each sample to precipitate protein (10), and samples were placed at −20°C overnight. Protein was collected by low-speed centrifugation and the pellet was dried under N_2.

Pellets were dissolved in 70% formic acid at a protein concentration of between 0.5 and 1 mg/ml. CNBr, as a 20 mg/ml solution in 70% formic acid, was added to give a CNBr/protein (wt/wt) ratio of 3.0. This is approximately equivalent to a 150:1 molar ratio of CNBr/methionine residues, given a methionine content of 2.1 residues/100 amino acid residues for calf neurofilament protein (11). Samples were incubated at room temperature for 48 h, then lyophilized, dissolved in 50 μl of electrophoresis sample buffer and electrophoresed on 12.5-20% gradient gels. Controls were incubated in formic acid in the absence of CNBr. Gel electrophoresis demonstrated that the undigested filament proteins were completely stable for 48 h in formic acid.

Ouchterlony Immunodiffusion

Plates for Ouchterlony immunodouble diffusion, made on glass microscope slides, contained 1% agarose, 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.3 0.5% Triton X-100, 0.1% SDS, and 0.1% NaN_3. Addition of detergents prevented the formation of precipitin artifacts observed when samples containing an SDS concentration of 0.5% or greater were run against antiserum, up to a 1:10 dilution. Since filament samples were dissolved in SDS, this became an important consideration. Wells, containing 3 μl or 5 μl of sample or antiserum, were formed 4 mm apart on plates. Plates were used within a few hours after they had been made. Antigen samples that were turbid were sonicated at 37°C for several hours until clear before they were applied to plates. Plates were incubated in a closed, humidified container at 37°C overnight, washed at 4°C in a large vol of 0.15 M NaCl, 0.05 M Tris HCl, containing detergents as above, and photographed after washing or after drying and staining with Coomassie blue.

Electron Microscopy

Subcellular fractions were washed by high-speed centrifugation through H_2O and fixed in 5% buffered glutaraldehyde at 4°C overnight, postfixed in Dalton's solution at room temperature for 90 min, dehydrated in a series of increasing ethanol concentrations and embedded in Epon. Thin sections were mounted on carbon-coated copper grids. After staining with 4% uranyl acetate and saturated lead citrate, grids were examined under a Siemens 1 or 1a electron microscope.

Small pieces of gliosed white matter, immediately adjacent to the sections sampled for filament isolation, were obtained fresh, at autopsy, or frozen, and were fixed and embedded in the same manner. One-micrometer sections were stained with toluidine blue for light microscopy; thin sections were cut for electron microscopy.

Antifilament Antisera

Antiserum to the 51,000-dalton bovine neurofilament protein (isolated from myelin-free axons) was raised in rabbits by Dr. William Schook. The purified filament protein, resolved as a single band by polyacrylamide gel electrophoresis (31), was cut from gels, ground with Freund's complete adjuvant in a 2:1 ratio, and injected into rabbits at 2-wk intervals, using 150-200 μg of protein per injection.

Antiserum raised in rabbits against human GFA protein was kindly provided to us by Dr. Elisabeth Bock (4).

RESULTS

Morphology of ALD Tissue

Extensive fibrous gliosis of white matter, with loss of myelin and axons, is characteristic of ALD

(29). Gliosis is most severe in the occipital white matter (Fig. 1*a*) while the white matter of the frontal lobes may not show pathological changes (Fig. 1*b*). Electron microscopy of the occipital lesion demonstrated many astrocyte processes, filled with parallel arrays of 8–9 nm filaments (Fig. 2). Like the filaments of normal fibrous astrocytes of the white matter (36), these structures are not connected by thin cross-bridges, as are axonal filaments (36). Filaments were readily discernible in ALD tissue that had been fixed in a fresh state (Fig. 2). In gliosed tissue that had been frozen at −76°C before fixation, however, many filament bundles appeared as granular material filling astrocyte processes (Fig. 3). In some areas, distinct filament structures could be seen (Fig. 3).

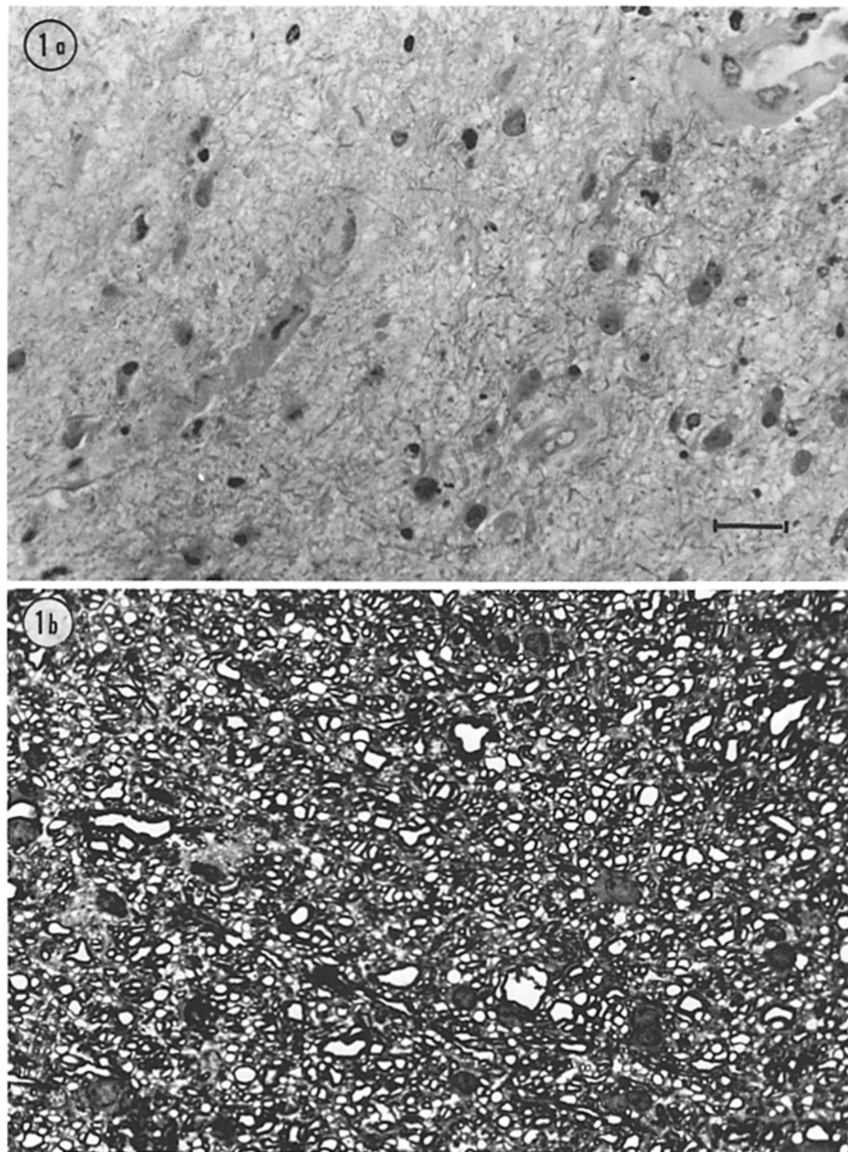

FIGURE 1 (*a*) Light micrograph of gliosed occipital white matter of ALD brain showing astrocyte nuclei embedded in a dense network of astrocyte processes. Axons are not observed. × 100. Bar, 0.1 mm. (*b*) Light micrograph of frontal white matter of ALD brain. The tissue appears normal, composed mainly of myelinated axons. Astrocytes are present, but there is no indication of glial proliferation. The sections were stained with toluidine blue. × 100. Bar, 0.1 mm.

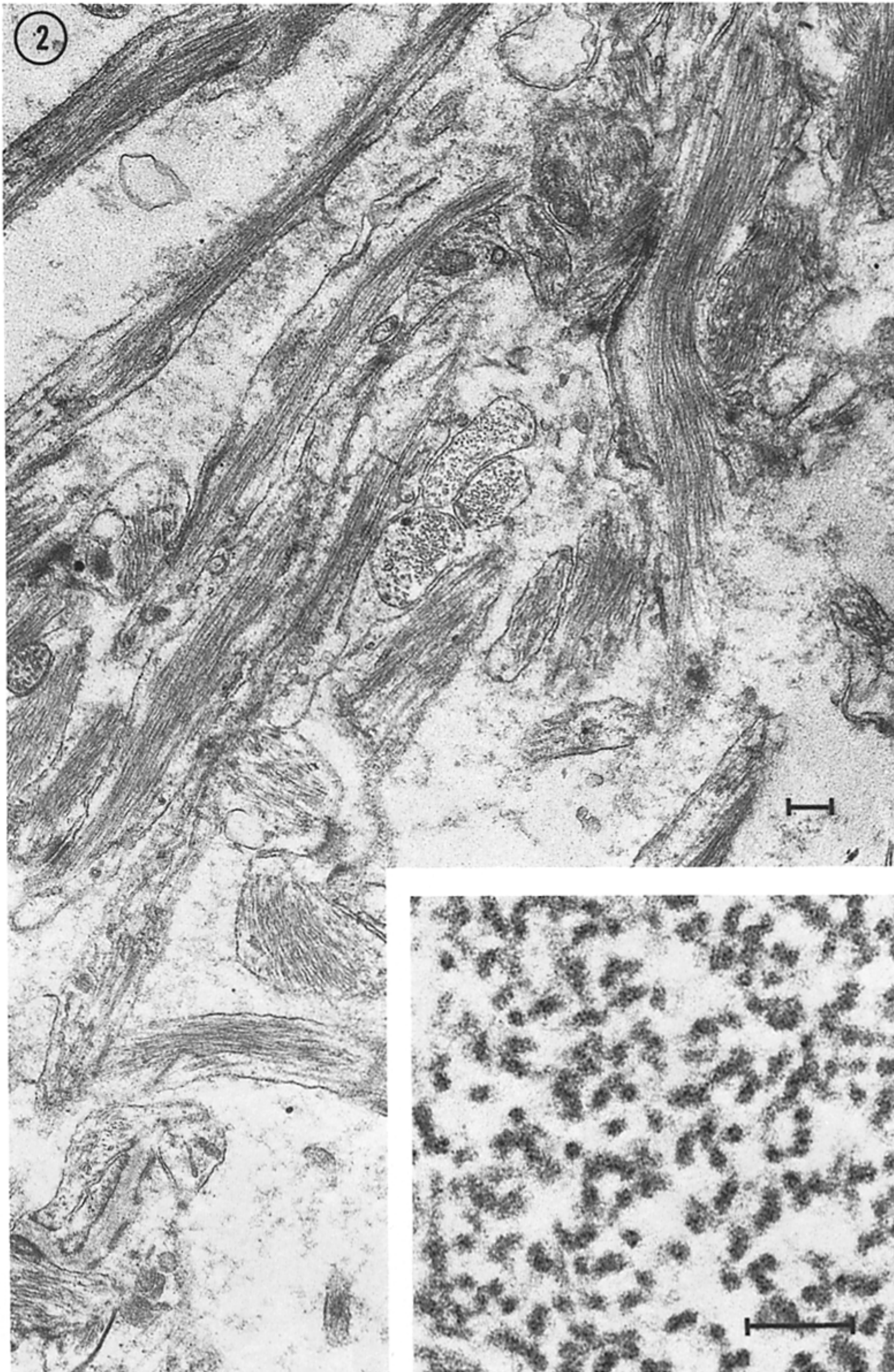

FIGURE 2 Electron micrograph of gliosed occipital white matter adjacent to the area shown in Fig. 1*a* fixed in glutaraldehyde immediately postmortem. Astrocyte processes, seen longitudinally and in cross section, are filled with filaments. × 15,000. Bar, 0.4 μm. *Inset:* Glial filaments cut transversely are seen at higher power to be tubular organelles. Distinct cross-bridges are not appreciated. Filaments measure 8–9 nm in diameter. × 150,000. Bar, 0.1 μm.

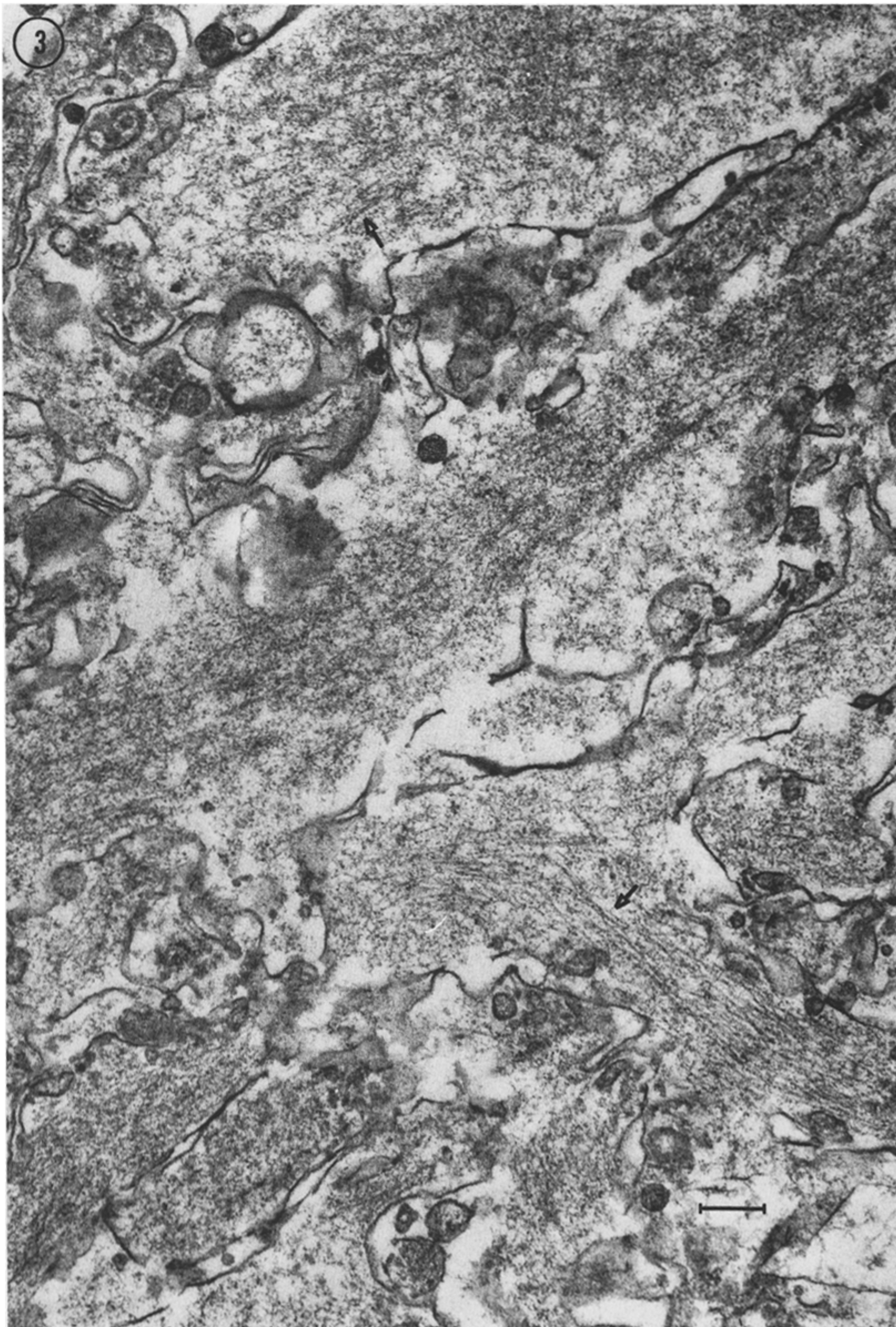

FIGURE 3 Electron micrograph of gliosed white matter from an ALD brain which had been frozen at −76°C postmortem. Astrocyte processes are filled with a granular material, in which distinct filaments can be seen (arrows). × 34,300. Bar, 0.3 μm.

Isolation of Filaments from Gliosed Tissue

We wished to isolate glial filaments from areas of gliosed white matter that contained no axons. Sections of tissue adjacent to those used for biochemical analyses were examined by light microscopy to insure little or no axonal contamination. Homogenates of gliosed white matter were also examined under the light microscope, where myelinated axons could easily have been distinguished from the thin glial processes. The lack of a floating layer of myelin after sucrose density centrifugation and the lack of characteristic myelin proteins from the gliosed samples attested to the completeness of the demyelination.

Gliosed ALD tissue, homogenized in buffered 0.9 M sucrose (see Materials and Methods), appeared as a suspension of thin processes, nuclei, and capillaries. Centrifugation of the suspension through a discontinuous sucrose gradient fractionated the various tissue components. Any myelin present would have been found floating at the top of the gradient. The interfaces between 0.9 and 1.1 M sucrose and 1.1 and 1.5 M sucrose contained suspensions of amorphous granular material, which, when examined in the electron microscope, were found to be heterogeneous collections of membranes. The 1.5-1.9 M interface was made up almost entirely of the thin astrocyte processes. The pellet was composed of nuclei, capillaries, and astrocyte processes. The 1.5-1.9 M interface contained no nuclear or capillary contamination. Examination of the astrocyte process fraction under the electron microscope revealed processes filled with granular material (Fig. 4) similar to that seen in whole tissue frozen before fixation (Fig. 3).

This glial filament fraction was composed almost entirely of a single protein component, re-

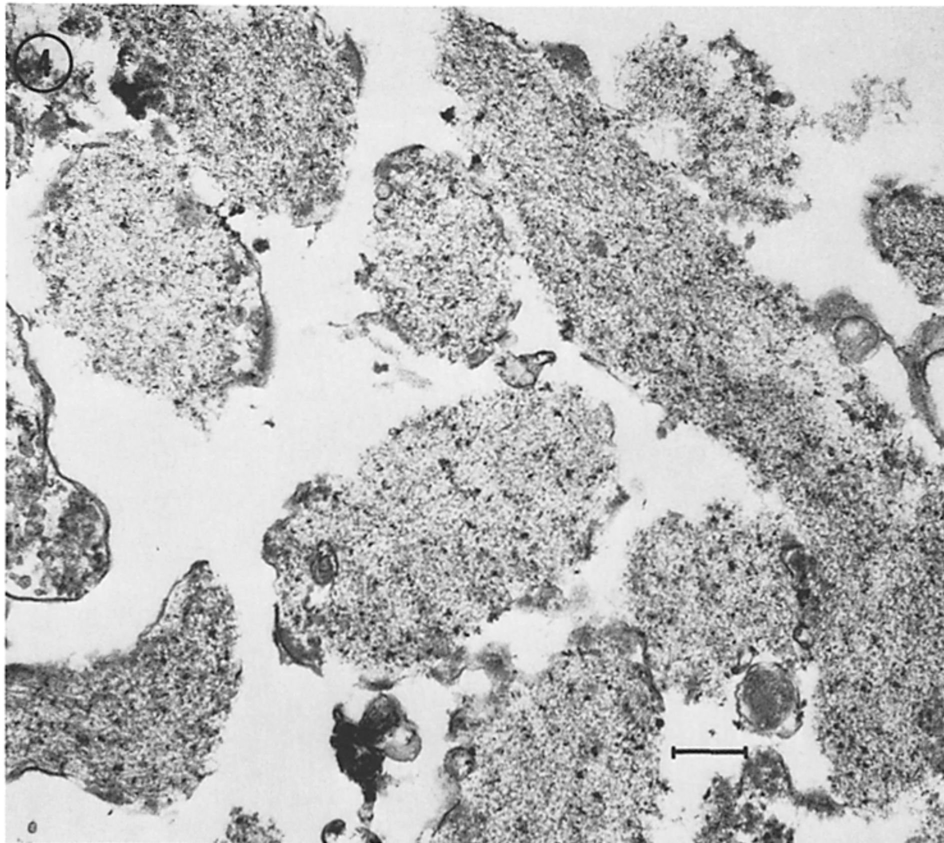

FIGURE 4 Electron micrographs of the glial filament fraction after sucrose gradient centrifugation (1.5/1.9 M interface). Many processes containing a granular material are seen. In many areas, a filamentous structure can be appreciated. × 42,000. Bar, 0.25 μm.

solved by polyacrylamide gel electrophoresis in SDS (peak *A*, Fig. 5 A), with a mol wt of approx. 49,000 daltons (49,500 ± 130, mean ± SEM, n = 6). A minor component with a mol wt of approx. 44,000 daltons (44,500 ± 150, n = 6) was also seen (peak *B*, Fig. 5 A). Actin and the α- and β-subunits of tubulin were separable from the major filament component.

Some differences in protein composition were found in different ALD brains and in different areas of the same brains. For example, in the glial filament fraction from a second ALD brain, the protein of 44,000-dalton mol wt was more prominent, and two other, smaller components appeared (Fig. 5 B). In the filament fraction from a different area of this second brain, the smaller components (mol wt 43,000, 42,000, 40,000, and 39,000 daltons) were more prominent (peaks *C*–*F*, Fig. 5 C). Biochemical evidence (see below) suggests that all components are related to the 49,000-dalton protein, probably representing degradation products. No differences were observed microscopically among the various areas of white matter with different proportions of these proteins.

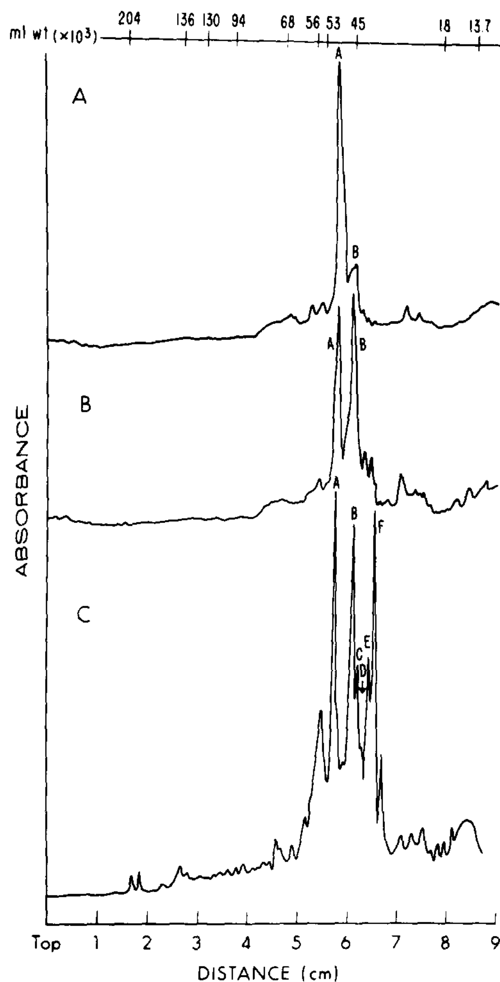

FIGURE 5 SDS-polyacrylamide gel electrophoretograms (5–15% gradient gel) of glial filament fractions from gliosed areas of ALD brains. Densitometric scans of Coomassie blue-stained gels were performed at 560 nm. Samples were electrophoresed on the same gel slab. (A) Glial filament fraction from one ALD brain. (B and C) Fractions from two different areas of a second ALD brain. Filament components are marked peaks *A*-*F*.

The 49,000-dalton filament protein was less soluble than the smaller components. When gliosed tissue containing the smaller proteins was extracted with 0.05 M Na phosphate, pH 8, little of the largest component was solubilized, compared to the smaller components (Fig. 6 A). In the insoluble residue, the larger proteins (49,000 and 44,000 daltons) made up a larger proportion of the total (Fig. 6 B). It appeared that the smallest components were the most soluble.

Isolation of Filaments from Myelin-Free Axons

We isolated filaments from frontal white matter from one of the ALD brains used for glial filament extraction by the standard procedure involving flotation of myelinated axons followed by stripping of the myelin (31, 32) (see Materials and Methods). This white matter was normal, as judged by light microscope and electron microscope examination. In particular, there were no macrophages, myelin destruction, inflammation, abnormal numbers of astrocytes or axonal degeneration. The filaments so isolated were morphologically identical to those isolated from bovine myelin-free axons (32). Under phase microscopy, they consisted largely of clearly identifiable axons having diameters of up to 10 μm, and of lesser amounts of thin processes. Bundles of 10-nm filaments were seen on electron microscope examination (Fig. 7).

Gel electrophoresis of these filament samples demonstrated a protein of mol wt of approx. 49,000 daltons (Fig. 8). Variable, but smaller, amounts of a smaller component, of mol wt of about 44,000 daltons, were also seen. The P_5 and P_7 pellets (see Materials and Methods) gave similar electrophoretic profiles.

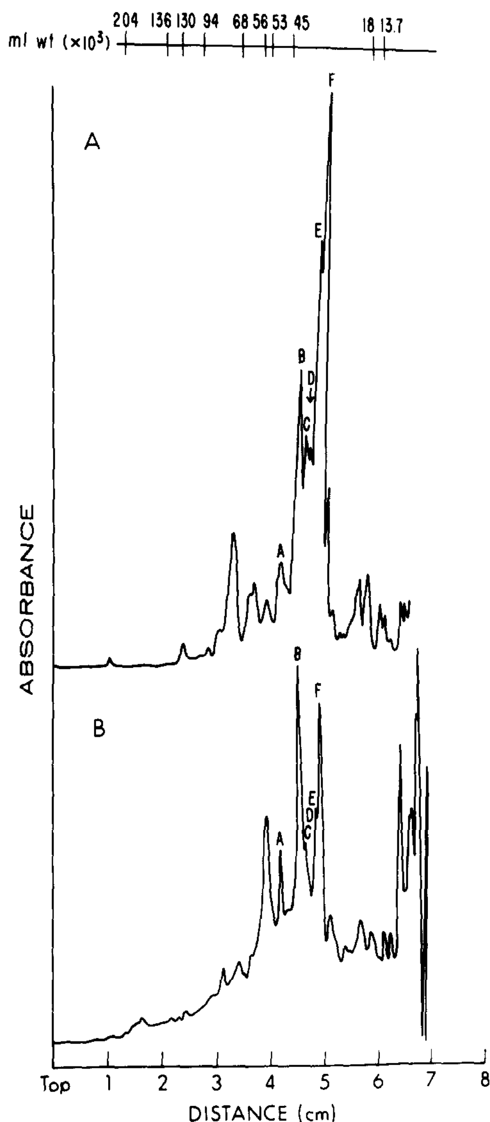

FIGURE 6 Densitometric scans of SDS-polyacrylamide gel electrophoretograms (5–15% gradient gel) of soluble and insoluble proteins from gliosed white matter. (A) Material extracted with buffer as described in Materials and Methods. (B) Material insoluble in buffer.

The filaments so isolated have been interpreted historically as being of axonal origin (11, 31, 32, 38), but immunologic studies suggest that some are derived from astrocytes and represent glial filaments (16). For this and other reasons, there is controversy regarding the nature of CNS neuronal filaments (see Discussion). We will demonstrate (see below) that the glial filament protein and the protein isolated by the conventional neurofilament (myelin-free axon) isolation are similar if not identical. The term "neurofilaments" as used below will thus denote filaments from myelin-free axon preparations.

Comparison of Glial Filament and Neurofilament Proteins

We obtained evidence for the identity of the two filament proteins by several biochemical and immunological methods. The major components of these filaments co-migrated during gel electrophoresis in SDS (Fig. 8A). The minor components (44,000-dalton protein) also co-migrated during electrophoresis.

A further indication for the electrophoretic identity of the filament proteins came from an analysis of ALD white matter which had undergone partial gliosis. Myelinated axons were present, but a proliferation of fibrous astrocytes had taken place. Electrophoretic analysis of the proteins of the whole white matter revealed only one component at 49,000 daltons, in what must be a mixture of glial filament and neurofilament proteins (Fig. 8B).

A more sensitive biochemical comparison was obtained by studying the polypeptides of filament proteins produced by CNBr digestion. Cleavage of the 49,000-dalton filament proteins yielded identical polypeptide patterns (Fig. 9A). The digestions had gone almost to completion, as judged by the small amounts of undegraded and partially degraded material. The approximate mol wt of the major CNBr cleavage product is 21,000 daltons. The sizes of the major smaller products are subject to some error because of gel nonlinearity, but their mol wts are about 9,000 and 4,000 daltons.

Immunological studies also provided evidence for the identity of the two filament proteins. We tested the ability of glial and neurofilament proteins to react with antiserum raised in rabbits to isolated bovine neurofilament protein. The human neurofilament fraction and all glial filament fractions reacted with this antiserum, with precipitin lines of antigenic identity (Fig. 10*A*). No reactions were observed with pre-immune serum or with rabbit anti-bovine serum albumin or anti-ovalbumin antisera. Furthermore, all filament fractions reacted with antiserum raised in rabbits to human GFA protein (Fig. 10*B*), with lines of antigenic identity. Neither anti-bovine neurofilament nor anti-GFA antisera reacted with porcine brain tubulin, rabbit muscle actin, bovine serum albumin, or rat or bovine myelin proteins at the

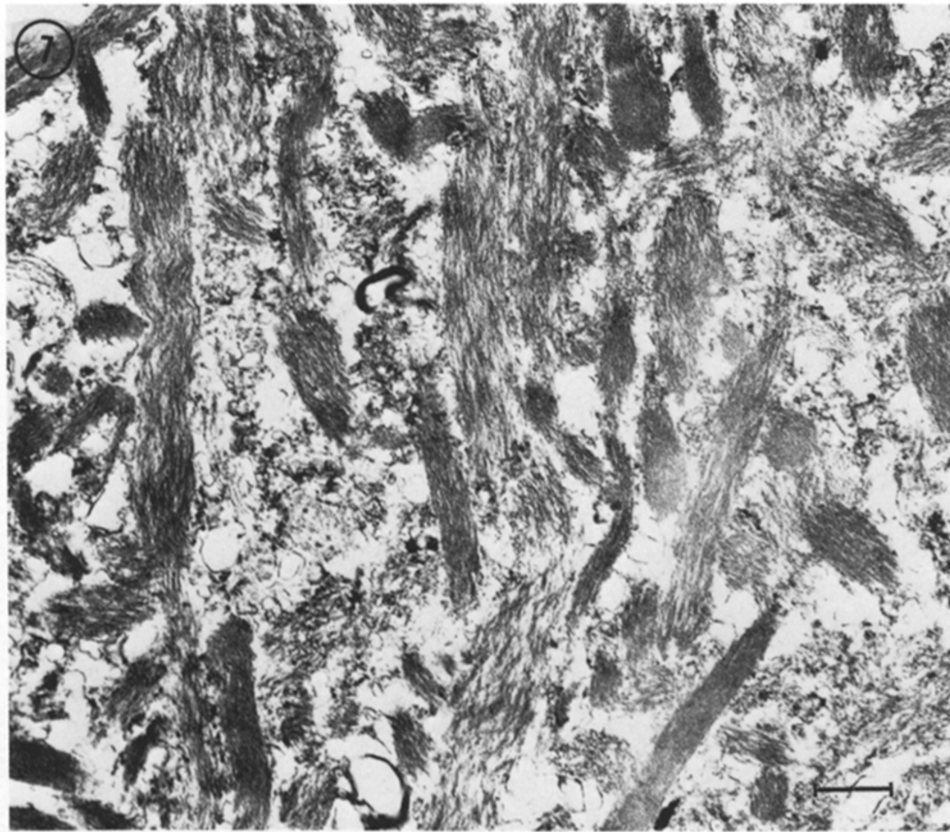

FIGURE 7 Electron micrograph of human neurofilaments from normal (frontal) white matter of ALD brain. (P_7 pellet). × 17,500. Bar, 0.6 μm.

same concentrations as those used for filament proteins.

Relationship of the Smaller Components to the Glial Filament Protein

With CNBr digestion, we obtained evidence that the proteins smaller than the major glial filament protein observed in some samples (Fig. 5 B and C) were all structurally related and represent degradation products of the filament protein. Electrophoresis of cleavage products of these components revealed major similarities, with a few small differences, as would be expected for a series of related proteins of decreasing size (Fig. 9 B).

DISCUSSION

Isolation of Glial Filaments

Fractions greatly enriched in glial filaments were isolated from severely gliosed white matter, which contained little or no axonal material. The major sources of contamination in the filament fraction were removed by density gradient centrifugation: capillaries and astrocyte nuclei sedimented to a lower density region than the filaments, and membranes remained in the lighter steps of the gradient. During fractionation, the filaments tended to remain in bundles. This may have resulted from plasma membrane enclosing the bundles, but in most cases a membrane boundary could not be demonstrated. While glial filaments were readily observed in tissue fixed while fresh (Fig. 2), their appearance was somewhat altered after freezing (Fig. 3), becoming indistinct in many areas. The preponderance of structures of granular material (Fig. 3) and the appearance of filaments within them lead us to conclude that they are filament bundles. Previous studies with isolated astrocytes (27) have demonstrated a granular material, similar to that observed in this study, within the astrocytes. This material was

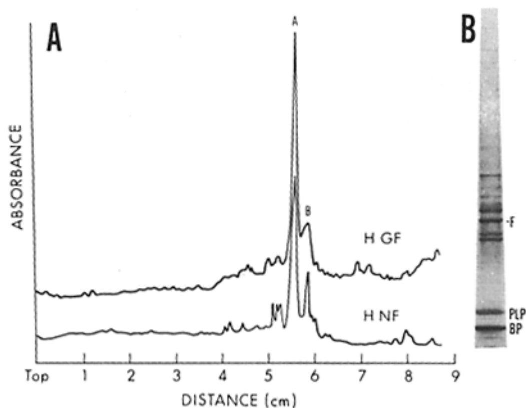

FIGURE 8 SDS-polyacrylamide gel electrophoretograms (5-15% gel) of (A). Human neurofilaments (*NF*) and glial filaments (*GF*) components *A* and *B* are marked. Samples were run in adjacent wells of the same gel. (B) Whole white matter from partially gliosed ALD tissue. Frozen tissue was extracted directly into 1% SDS. Note the single component at 49,000 daltons (*F*), and the presence of myelin basic protein (*BP*) and proteolipid protein (*PLP*). Stained with Coomassie blue.

thought to represent filaments in some altered state (27).

The better preservation of filaments from the myelin-free axons (Fig. 7) may indicate differences between glial filaments and neurofilaments. Differences in the pH of isolation are unlikely to account for this difference since myelin-free axons isolated from bovine brain at pH 7.6 still show a distinct filamentous morphology (A. Chiu and W. T. Norton, unpublished observations).

Protein Components of Glial Filaments

The filament fraction was composed of one major protein, with an approximate mol wt of 49,000 daltons (Fig. 5). In some gliosed white matter samples, other protein components were observed with mol wts between 44,000 and 39,000 daltons (Fig. 5). CNBr cleavage demonstrated that these components are all structurally related, and presumably reflect degradation of the 49,000-dalton filament protein. Degradation was probably ante-mortem, since brains were frozen 30-45 min postmortem, and varying amounts of these components were present in different areas of the same brain. Furthermore, electrophoresis of frozen gliosed tissue solubilized directly in SDS demonstrated the same proportions of these components as electrophoresis of filaments after homogenization and density gradient isolation. In an

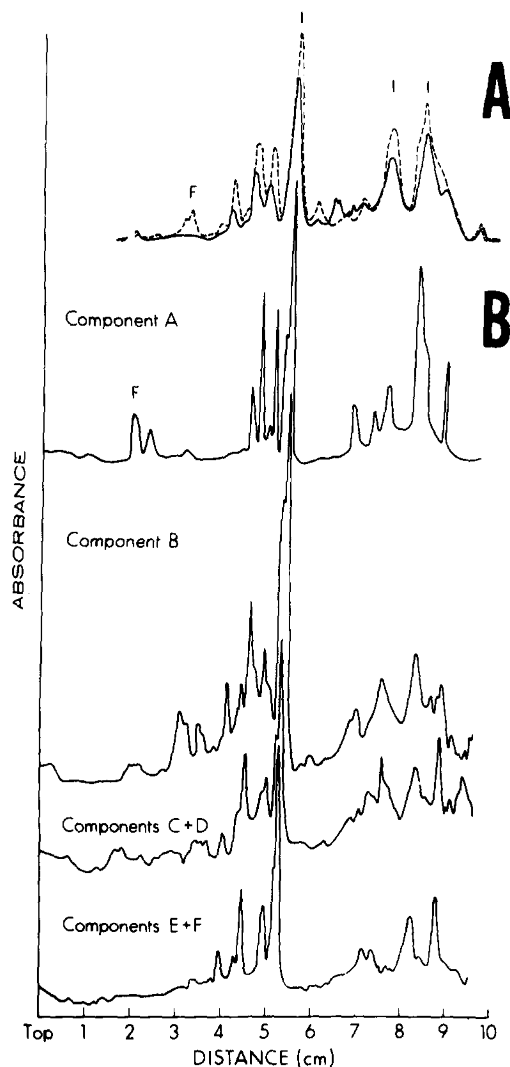

FIGURE 9 Densitometric scans of polyacrylamide gel electrophoretograms (12.5-20% gel) of (A) polypeptides from human neurofilament (—) and glial filament (- - -) proteins resulting from CNBr digestion. Peptides were electrophoresed on the same gel slab. *F*: position of undigested filament protein. Three major peptides are marked (see text). (B) CNBr digests of the several components of glial filament fractions (*A-F*). Undigested filament protein (*F*) is marked. Peptides were electrophoresed on the same gel slab. Parts A and B of figure represent two different electrophoresis runs.

attempt to produce degradation in vitro, gliosed white matter was homogenized in 0.05 M Na phosphate, pH 8, and incubated at 37°C for up to 24 h. No change in electrophoretic profile of white-matter proteins was observed (J. Goldman,

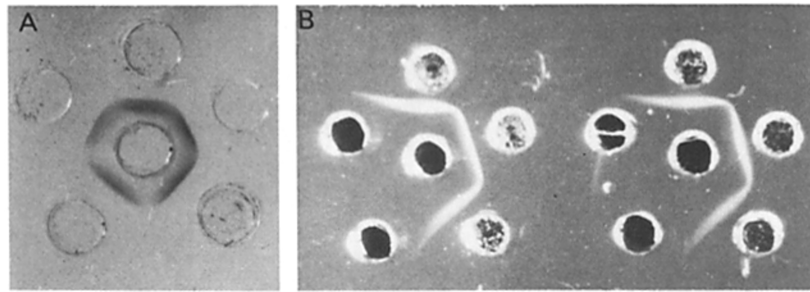

FIGURE 10 (*A*) Ouchterlony double-diffusion plate showing reactions between rabbit antiserum against bovine neurofilament protein (center well) and clockwise from top well human neurofilaments (P_5 fraction), buffer soluble material from gliosed white matter (see Fig. 7), buffer insoluble material from gliosed white matter, total protein of gliosed white matter, purified glial filaments (A in Fig. 6). The plate was dried and then stained with Coomassie blue. (*B*) Reactions between rabbit antiserum against human GFA protein (center well left) and purified IgG fraction from the rabbit anti-GFA antiserum (center well right) and clockwise from top human neurofilaments, glial filaments (*B* in Fig. 6), glial filaments (*A* in Fig. 6), ovalbumin and bovine serum albumin. Plate was photographed unstained.

unpublished observations).

The 49,000-dalton filament protein was separable from tubulin and actin, which comprise two other major filamentous structures of nervous tissue, microtubules, and microfilaments (37). Glial filament protein migrated as a single component during electrophoresis in several gel systems: SDS in Tris-borate buffer (Fig. 5); SDS in Tris-glycine buffer and SDS in Tris-glycine buffer containing 2 M urea (J. Goldman, unpublished results, buffer as described in reference 31). In particular, it did not split into two components during electrophoresis under conditions in which the α- and β-monomers of tubulin are resolved. Such splitting has previously been reported for presumptive glial filament protein (21).

GFA Protein as the Glial Filament Subunit

The association between GFA protein and astrocytes has been well described. GFA protein was originally isolated from gliosed tissue (multiple sclerosis plaques, leukotomy scars) in both soluble and insoluble forms (13). Antibodies raised against GFA protein stain astrocytes at the light microscope (7, 24) and electron microscope (14) levels, and the increase in GFA immunoreactivity during development correlates with the growth of astrocytes (2). The biochemical nature of GFA protein has been a subject of some controversy. In some studies, this protein has been described as a protein of mol wt of 47,000 daltons (isolated from normal brain) or a mixture of the 47,000-dalton component and a smaller one, 41,000 daltons (isolated from multiple sclerosis plaques) (15). Immunoreactive GFA protein exists in soluble and insoluble forms (15). In other studies, GFA protein has been described as a soluble component of mol wt of 54,000 daltons (6, 8) which is degraded postmortem to a series of proteins with mol wts of between 49,000 and 40,500 daltons (6, 8). The smaller components are structurally related (6, 8), although there is some question as to the identity of the largest component (54,000 daltons) because it is not immunogenic (9).

Thus, while there is indirect evidence that GFA is the protein subunit of glial filaments, this connection has not been directly established by isolation of filaments themselves. Furthermore, the biochemical nature of GFA protein has not yet been clarified. Our characterization of filaments, the similarities between filament proteins and those described by other investigators, and the reactivity with anti-GFA antiserum lead us to suggest a scheme to tie together much work on GFA and glial filaments.

Human glial filaments are composed primarily, or entirely, of a single protein, largely insoluble, with a mol wt of about 49,000 daltons. This protein is probably identical to the larger GFA protein isolated by Eng et al. (15). Glial filament protein will break down *in situ*, first to a component with a mol wt of about 44,000 daltons, probably representing the 41,000-dalton GFA protein isolated from multiple sclerosis plaques by Eng et al. (15), and subsequently to a series of proteins between 43,000 and 39,000 daltons.

These smaller components are, no doubt, the so-called "multiple related polypeptides" of GFA protein described by Dahl and Bignami (8). Thus, GFA protein represents a mixture of molecules, including the filament protein and several degradation products. We found no evidence for a soluble protein of 54,000 dalton size (6, 8) or a protein closely resembling tubulin (21) associated with glial filaments.

Comparison of Glial Filament and Neurofilament Proteins

We have provided evidence for a strong similarity, if not identity, of the glial filament protein and the filament protein isolated by a standard myelin-free axon procedure. The proteins co-migrated during gel electrophoresis. CNBr digestions produced identical polypeptide patterns. Both proteins reacted with antisera to bovine neurofilament protein and to GFA protein, with precipitin lines of antigen identity.

While these results argue for a basic similarity between glial filaments and neurofilaments, we must point out that there is considerable controversy regarding the cellular origin of the filaments isolated by the standard myelin-free axon technique. On the one hand, many axons can unequivocally be identified in the final myelin-free axon preparations. The thin processes also present could, of course, be derived from small axons or astrocyte processes. On the other hand, antibodies raised against the major protein of bovine myelin-free axons (historically called "neurofilament protein" and found to have a mol wt of 47-51,000 daltons) stain astrocytes, rather than axons, by immunohistofluorescent techniques (16). This is the same histofluorescence pattern seen with anti-GFA antiserum (7, 24). Furthermore, bundles of tightly packed filaments appearing much like those isolated from normal human white matter (Fig. 7) stain with anti-GFA antiserum (16). The major protein component of bovine myelin-free axons is biochemically and immunologically strongly similar to GFA protein from multiple sclerosis plaques (15, 16). Nevertheless, antiserum raised against bovine neurofilament protein (from myelin-free axons) does stain neuroblastoma cells in culture, in a distribution corresponding to the distribution of 10-nm filaments, assessed by electron microscopy (22). Reaction between anti-neurofilament antiserum and axons has been reported from work using electron microscopy localization (39).

Studies of the mammalian peripheral nervous system have suggested that axonal filaments are composed of polymers of a 68,000-dalton subunit (30), or made up of three components with mol wts of 68,000, 160,000, and 210,000 (20). Components with these approximate molecular weights do appear, to varying degrees, in myelin-free axon preparations from the CNS (12, 18, 38). Unfortunately, at this time, the problem has not yet been resolved, since a filament protein(s) considered by all investigators unequivocally to have been obtained from axons has not been isolated.

Morphological studies have provided evidence that glial filaments and neurofilaments are not identical. High resolution electron microscopy reveals that glial filaments are slightly smaller than neurofilaments (8-9 nm vs. 10 nm in diameter) (36). Thin bridges connecting the filaments have been described in axonal but not astrocyte filaments (36). Furthermore, neurofilament structures were well preserved in our frozen specimens (Fig. 7), but the glial filaments were not (Fig. 3).

If the two filament types do indeed share a common protein subunit (the 49,000 dalton component described in this study), differences in size and behavior of the two filaments might be explained by assuming that they are more complex than simply polymeric forms of the major protein. For example, lipid may be associated with neurofilaments (31). We do not know whether isolated glial filaments contain significant amounts of lipid. The several proteins larger than the major neurofilament protein and isolated along with it (12, 18, 38) may represent integral parts of the neurofilament organelle (cross-bridges, for example). Such higher molecular weight components were not observed in our glial filament preparations. The complex structure of a neurofilament organelle composed of several proteins in addition to the 49,000-dalton component might prevent antibodies to the major protein from combining with specific antigenic sites in the intact filament. This idea would be consistent with the finding that antisera raised against GFA protein reacts with astrocytes but not with axons in immunohistofluorescence studies (7, 24).

Intermediate (8-10 nm) filaments have been described in a variety of tissues (3, 17, 34, 35, 37) and seem to represent, along with contractile proteins and microtubules, a major polymeric protein system, widespread in its occurrence. Biochemical characterization of filament subunits is in an early stage. Similarities between neurofila-

ments and nonnervous system filamentous proteins have been demonstrated by immunofluorescence techniques (3), but as yet not by biochemical comparisons. The characterization of glial filaments reported here suggests that within the nervous system two major classes of cells synthesize the same major filament protein, but that neurons and glia may assemble this protein into organelles with somewhat different properties.

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