

Filament Proteins in Central, Cranial, and Peripheral Mammalian Nerves

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ABSTRACT Several classes of 10-nm filaments have been reported in mammalian cells and they can be distinguished by the size of their protein subunit. We have studied the distribution of these filaments in nerves from calves and other mammals. From the display on polyacrylamide electrophoretic gels of proteins in extracts from fibroblast and central, cranial and peripheral nerves, we cut the appropriate stained bands and prepared iodinated peptide maps. The similarities between the respective maps provide strong evidence for the presence of vimentin in cranial and peripheral nerves. The glial fibrillary acidic protein was found in axon preparations from the central nervous system, but was not identified in distal segments of some cranial nerves, nor in peripheral nerve.

Electron microscopy has revealed filaments of 10-nm diameter in a wide variety of cell types. Extensive arrays of such filaments have been demonstrated by detergent treatment of cells cultured on thin films for electron microscopy (5, 35). Because the filaments are relatively insoluble, they have been named "cytoskeletal filaments" but the name carries implications of a structural function for the filaments; in fact, their function is at present unknown. Chemical studies have shown that such filaments from different types of cells have different protein subunits (2, 9, 22, 25, 26, 36); it seems that they may have different functions.

In nervous tissue, astrocytes and neurons contain 10-nm filaments but the classes from the two cells have been distinguished morphologically (38) and immunologically (26, 30). The chemical characterization of these filaments has been attended by controversy. Aqueous extracts of areas of brain gliosis yield a protein preparation that was attributed to the glial filament and was called the glial fibrillary acidic protein (GFAP) (18, 37). Direct immunochemical staining subsequently confirmed that the filaments in astrocytes react with antibody to GFAP (17, 29). A protein of similar size (~49,000–51,000 daltons) extractable only with denaturants from isolated segments of myelinated axons (13) was deduced to be the subunit of the neurofilament (12, 33), but subsequent studies have shown that this is a buffer-insoluble form of GFAP and is derived from glial filaments contaminating the axon segments (16). The direct isolation of human glial filaments has been described more recently and the similarity of the subunit to GFAP reported (20). Labeling studies on axoplasmic pro-

teins led Hoffman and Lasek (21, 24) to identify a triplet of proteins (~70,000, 160,000 and 200,000 daltons) as the components of the neurofilament. Recently, further observations have supported this conclusion because the same protein triplet is obtained on the dissolution of both peripheral and central nerve neurofilaments (1, 26, 27, 32).

Thus, it appears at present that in the mammalian nervous system the 10-nm filaments in neurons are built from a triplet of proteins while another type of filament built from GFAP, a 50,000-dalton protein, is restricted to fibrous and protoplasmic astrocytes and some ependymal cells (4, 14). Histology has shown that astrocytes are found only in the central nervous system and along the Ist and IInd and proximal reaches of the VIIIth and other cranial nerves (3, 34). However, in a previous study (8) we detected a GFAP-like protein in dog sciatic nerve where astrocytes are not found. At that time we identified that protein with the neurofilament; interpreted from the present viewpoint, that finding was either wrong or it implies the presence of GFAP outside the central nervous system. We describe here experiments reexamining our earlier observation: we have analyzed by peptide mapping a protein having the same electrophoretic mobility as GFAP in bovine IIIrd, Vth, and VIth cranial nerves and the canine sciatic. In addition, we have analyzed in the nerves a protein with the same mobility as the cytoskeletal protein (22) (or vimentin [19]). This protein has been identified repeatedly in cultured fibroblasts but only in a few cells in differentiated tissues (19). We found that GFAP is limited to the proximal segments of some of the cranial nerves, and that the 50,000-dalton protein in distal

segments and the sciatic nerve yields a different fingerprint. Vimentin is present along all the cranial nerves studied.

MATERIALS AND METHODS

Cranial nerves were dissected from calf brains that were obtained within 2 h of slaughter and from dogs and rabbits obtained immediately after exsanguination under anesthesia. The nerves were dissected at, or 2–3 mm beyond, the brain or brain stem surface, placed in iced, phosphate-buffered saline (0.14 M NaCl, 0.01 M phosphate, pH 7.4) containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and cleaned of blood vessels and loose connective tissue. Sciatic and peroneal nerves were taken from dogs and rabbits; these nerves and the optic nerves were dissected free of epineurium.

The nerve segments were frozen on solid carbon dioxide, transversely sliced, and then crushed in a Dounce homogenizer (Kontes Co., Vineland, N. J.) first with 0.1 M NaCl, 0.01 M Tris, 0.01 M EDTA, 0.01 mM PMSF, pH 7.0. The extract was centrifuged at 30,000 g for 15 min. The precipitate was then extracted in 3 M guanidine hydrochloride, 0.01 M EDTA, pH 8.0, again with homogenization, and the mixture was centrifuged. The insoluble residue was homogenized in 6 M guanidine hydrochloride, 0.01 M mercaptoethanol, 0.01 M phosphate pH 8.0, and the dissolved components in this third extract were recovered by centrifugation. The extracts were centrifuged repeatedly (diluted with water if necessary) until clear of lipid, and then all the guanidine salts were eliminated by dialysis. SDS was added to all the extracts to 1% by weight for electrophoresis.

For the isolation of the protein from the cytoskeletal filaments of fibroblasts (5, 22), primary explants were established from fetal bovine dermis and rabbit corneas. They were grown under 5% CO₂ in air in Dulbecco's modification of minimum Eagle's medium, 10% newborn calf serum with antibiotics. After the fourth splitting of the cultures they appeared to contain solely fibroblasts, and single T25 flasks with confluent cells were used for each preparation. The cells were washed in physiological saline, then treated with the same saline with 0.2% Triton X-100, 2 mM EDTA, 1 mM PMSF. The cells were scraped from the flask in this medium and the suspension was centrifuged for 10 min at 20,000 g. The supernatant solution was discarded and the precipitate was extracted with 3M guanidine hydrochloride or 1% SDS to provide a sample for electrophoresis.

Two iodination procedures were used. The 0.5- to 1.0-mg samples in 1% SDS, 0.1 M sodium phosphate, pH 8.2, were reduced and carboxymethylated with mercaptoethanol and iodoacetic acid, dialyzed, and then treated with 100–400 μ Ci ¹²⁵I (New England Nuclear, Boston, Mass.) and chloramine T, followed by sodium metabisulfite; they were then dialyzed against 0.1% SDS. The samples were adjusted with Tris buffer and glycerol before applying them to polyacrylamide gel slabs. These procedures have been described previously (7). The appropriate stained bands were cut from the gel and either the radioactive protein was eluted, precipitated, and then digested with trypsin or chymotrypsin (7), or the slices were washed for 30 min in 50% methanol and then lyophilized before being rehydrated in 0.1% trypsin or chymotrypsin solutions in 0.1 M ammonium bicarbonate, pH 8. After 3- to 8-h proteolysis at 37°C, the slices were extracted with ammonium bicarbonate and the solutions were lyophilized. The ¹²⁵I level in the peptide digests was determined and appropriate aliquots were applied to thin-layer plates of silica gel.

The second iodination procedure involved the iodination of the protein in bands cut from a gel. The procedure was modified only in detail from those described by Elder et al. (15) and Bryant et al. (6).

Resolution of the iodinated peptides on the plates was improved by running them first in 30% ammonia in *n*-propanol, drying the plate, and then rerunning in the same solvent.

Gel electrophoresis was conducted in SDS-7% polyacrylamide gel slabs in a discontinuous buffer system (23). The gels were stained with Coomassie Blue and scanned with a densitometer (Joyce, Loebel and Co., Ltd., Gateshead-on-Tyne, England).

RESULTS

Our initial objective was to extract from peripheral and cranial nerves the subunits of the 10-nm filaments in order to compare their distributions. Neurofilaments are such prominent constituents of axons when viewed in the electron microscope that it was anticipated that the proteins corresponding to them would be major components of the extracts and could be satisfactorily resolved by one-dimensional gel electrophoresis. Therefore we crushed cleaned lengths of various nerves from humans, hogs, dogs, rabbits, and calves and extracted the proteins. In many instances the mixture of proteins was too complex for simple interpretation of the array of bands on the SDS gels. Much of

this complexity is probably contributed by the satellite cells and connective tissue in the endoneurium. A resolution of this difficulty was offered by the solubility characteristics of the 10-nm filaments; it was known that several classes of these filaments are insoluble in physiological saline and nonionic detergents, but are soluble in denaturants. Therefore we adopted a procedure of extracting the sliced and crushed nerves (stripped wherever possible of epineurium) successively with neutral buffer, then 3M and finally 6M guanidine hydrochloride. The proteins in such extracts are exemplified in Fig. 1. These successive extracts have the advantage of displaying separately certain overlapping protein populations; in particular, the aqueous extract removed most of the prominent 68,000-dalton protein described by Schlaepfer and initially attributed by him to the peripheral neurofilament (31). With its removal the 70,000-dalton component of the neurofilament is revealed in the guanidine extracts. Collagen chains were extracted by 6 M guanidine hydrochloride but only minimally by 3 M. They were readily recognized on the gel both by the reddish color the stained band assumes on prolonged destaining and by comparison with authentic acid-soluble or pepsin-solubilized samples (10). Previous experiments had shown that glial filaments, neurofilaments, cytoskeletal protein (vimentin), and desmin (or skeletin) are soluble in 3 M guanidine hydrochloride so this extract, referred to as 3GX, was of greatest interest.

Comparison of SDS-gel electropherograms of the major proteins in the 3GX preparations from the rabbit sciatic nerve and calf axon segments shows several similarities. The triplets

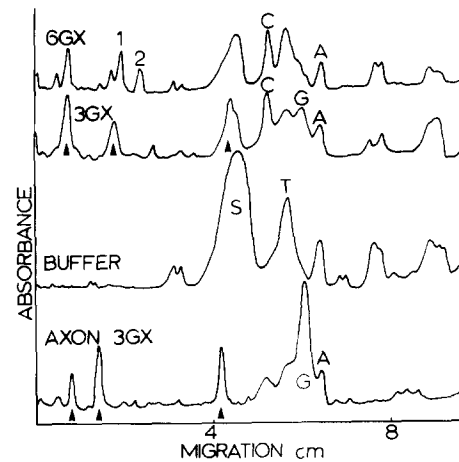


FIGURE 1 Densitometric profiles from a stained, SDS polyacrylamide electrophoresis gel slab. The three upper traces display the proteins in successive extracts of rabbit sciatic nerve. The extracting solutions were a low ionic strength buffer, 3 M guanidine hydrochloride, and 6 M guanidine hydrochloride with mercaptoethanol (see Materials and Methods). The yields were 30, 28, and 22%, respectively, of the lipid-free dried nerve. The lower trace shows the proteins extracted by 3 M guanidine hydrochloride from a preparation of calf "axon segments." The three components ascribed to the neurofilament triplet are marked by arrowheads; the larger two in the rabbit and calf differ in electrophoretic mobility. A component with the same mobility as GFAP (G) is seen in both denaturant extracts of the sciatic nerve. The 68,000-dalton protein described by Schlaepfer (31) is marked as S. In experiments not shown here we have found that S gives peptide maps identical to serum albumin (see also reference 26). The α 1 and α 2 chains of collagen are marked 1 and 2 in the upper trace. The positions of tubulin (T) and actin (A) are marked. The prominent peaks migrating beyond 7 cm are myelin proteins. The presumptive cytoskeletal protein is marked C. It was not obvious in the extracts from axon segments.

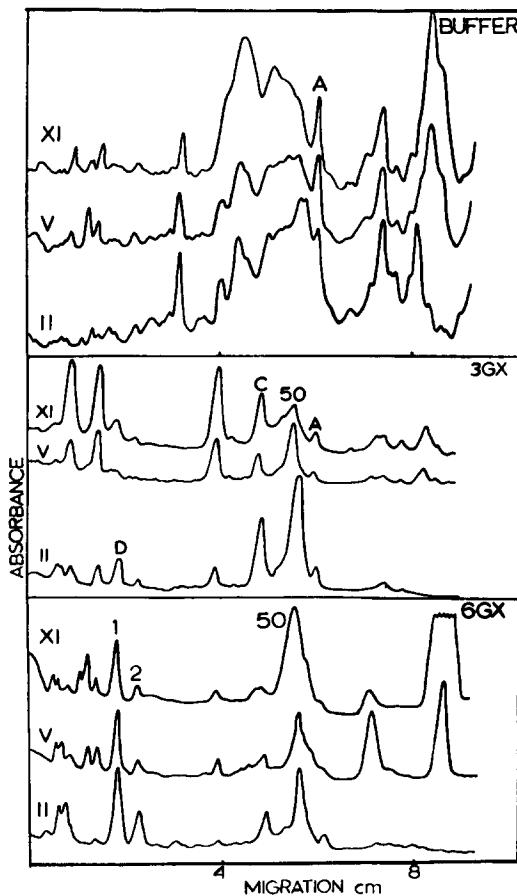


FIGURE 2 A comparison of densitometer traces from gel electropherograms of proteins from calf cranial nerves. Successive extracts with buffer, 3 M (3GX) and 6 M (6GX) guanidine hydrochloride are shown. The 50,000-dalton protein (50) is marked in the 3GX trace, actin is marked with A. The neurofilament triplet is prominent (at 1.0, 1.5, and 3.9 cm) in the 3GX traces of the Vth and XIth nerves. The 50 peak is large in the IInd nerve 3GX trace; that nerve is heavily invested with astrocytes containing GFAP. The band is overloaded and migrated slightly faster than usual. The presumptive cytoskeletal protein is marked C. The dimer of GFAP which is produced by oxidation is marked D.

of neurofilament proteins are marked in Fig. 1 by arrowheads; the differences in the mobilities of the "200,000" and "160,000"-dalton proteins of rabbit and calf belie their homology that is revealed by their peptide maps (11). Both extracts also contain: actin (a protein present in each extract, and whose identity has been confirmed by peptide mapping that is not shown here); a smaller peak that may be tubulin, incompletely extracted by the treatment with neutral buffer (compare to the buffer extract); and a peak between the latter two and labeled G. In the 3GX preparation from the axon segments, this G is the 50,000-dalton protein identified as GFAP (17). The presence of a protein in the sciatic nerve extract with this mobility we reported previously (8). We obtained from extracts of dog sciatic nerve electropherograms essentially similar to those from the rabbit nerve shown in Fig. 1.

Similar successive extracts were made from calf cranial nerves, and the electrophoretic distribution of the proteins in these extracts is shown in Fig. 2. Again, the 3GX samples show a relatively simple pattern with prominent bands which include the neurofilament triplet, the 50,000-dalton protein, actin (A) and component C whose mobility matched that of the cyto-

skeletal protein, vimentin, extracted from bovine fibroblasts cultured in vitro.

The objective of our experiments now became the identification of protein band C and the 50,000-dalton proteins from the cranial and peripheral nerves. We elected to use thin-layer chromatography of peptide digests of the iodinated proteins as a criterion for the similarity of proteins migrating at the same rates on gel slabs. This peptide mapping procedure yields autoradiograms that can be compared between different proteins and is sensitive enough to be used with ~1 μ g of protein, the content of a faint band on a Coomassie Blue-stained gel (7, 15).

Iodine reacts with thiol and tyrosyl groups most readily. To block reaction with thiols (the content of which could vary with the state of oxidation of the protein), the nerve extracts were reduced and carboxymethylated. In many but not all instances this treatment resulted in the appearance of two bands instead of one in the 70,000-dalton neurofilament band, band C, and the 50,000-dalton band. Both bands were analyzed when they appeared.

The proteins were iodinated either by reacting the whole 3GX samples with 125 I and chloramine T and then applying the mixture to the electrophoresis gel, or by iodinating the proteins in each band sliced from the gel. The latter procedure gave the higher specific labeling. The two methods did not yield identical peptide maps. The conditions of iodination differed so that tyrosyl residues presumably were exposed to substitution to different degrees. Moreover, both methods introduced artifact bands that were derived from reagents or impurities in the gels (15). Nevertheless, similar proteins could be effectively compared by one or the other procedure.

Most of our experiments involved calf proteins. Fig. 3 compares the one-dimensional peptide maps obtained from GFAP (extracted with 3 M guanidine hydrochloride from an axon segment preparation) with the protein in the 50,000-dalton band from mixed extracts from calf Vth and VIth cranial nerves. Two or three artifact bands were found to be common to all peptide maps, and these were not eliminating by using ultrapure reagents at all stages of the electrophoresis. They are indicated by arrowheads on each figure. The remaining bands

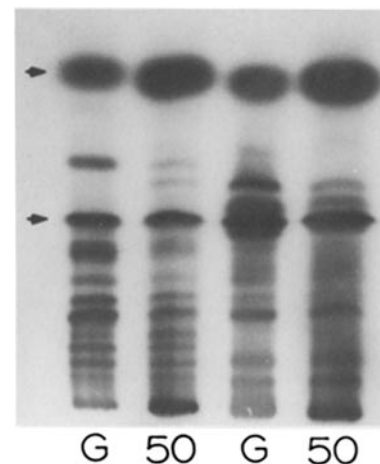


FIGURE 3 The autoradiograph of a thin-layer plate on which was run the 125 I-iodinated tryptic (left) and chymotryptic (right) peptides of calf brain GFAP (G) and the 50,000-dalton protein (50) from the Vth and VIth calf cranial nerves. Two artifact bands common to all the digests are marked by arrows.

may be used to assess qualitatively the similarities between the proteins under comparison.

The tryptic and chymotryptic maps from the two proteins are very similar and suggested that GFAP was present in segments of cranial nerve beyond 2 mm from the brain stem. To check our conclusion that GFAP was not restricted to the central nervous system, we repeated the analysis of proteins in successive segments of the Vth cranial nerve of the calf. Fig. 4 shows densitometric traces from a gel loaded with the 3GX proteins from such an experiment. The content of the 50,000-dalton protein is significantly diminished somewhere between 5 and 12 mm from the brain stem. Histology showed a zone of transition in this region where the cell populations changed abruptly in their staining characteristics, despite the obvious continuity of the axons. This is the region of glial/Schwann cell transition described by Berthold and Carlstedt (3) and others. Nevertheless there was present in the extracts from more distal nerve segments a protein band similar to GFAP. This protein appeared as two bands on reduction and carboxymethylation. A similar pair of bands was present in dog and rabbit sciatic nerve 3GX extracts.

Both of these pairs of bands were treated to prepare peptide maps. The results are illustrated in Fig. 5. Both bands that are generated from the single band by reduction and carboxymethylation give the same maps but they are not identical with the maps from GFAP.

The identity of the C band was pursued by isolating the 58,000-dalton band from a gel electropherogram of the cytoskeletal proteins from calf fibroblasts. Peptide maps from this protein and the C band from calf cranial nerve are shown in Fig. 6. To give a sense of the discrimination that the mapping procedure can provide, we placed maps of bovine brain actin and bovine and canine desmin (smooth muscle filament protein [25]) on either side of the cytoskeletal protein map. The two desmin maps are very similar, indicating homology between the species; protein C and fibroblast vimentin give essentially identical maps. A similar comparison of rabbit corneal fibroblast vimentin and protein C from rabbit sciatic nerve is shown

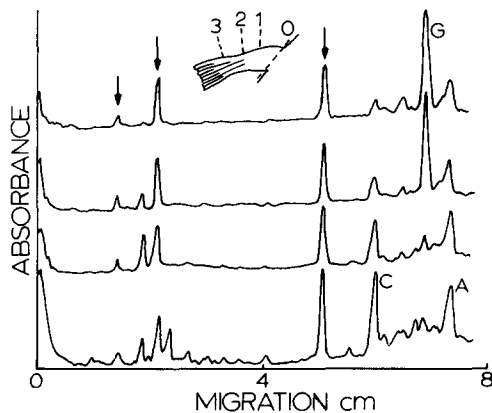


FIGURE 4 Densitometric profiles from a stained polyacrylamide slab gel after electrophoresis of 3 M guanidine hydrochloride extracts of successive segments of a calf Vth cranial nerve. The diagram shows the way the segments were cut. 1-mm sections were taken for histology at 0, 4, 9, and 15.5 mm from the brain surface (because the nerve emerges obliquely these distances refer to the shortest path to the surface) and the intervening segments 1-3.5, 4.5-8.5, 9.5-15, and beyond 16 mm yielded the profiles displayed from top to bottom. The neurofilament triplet is indicated by arrows, vimentin by C, GFAP by G, and actin by A.

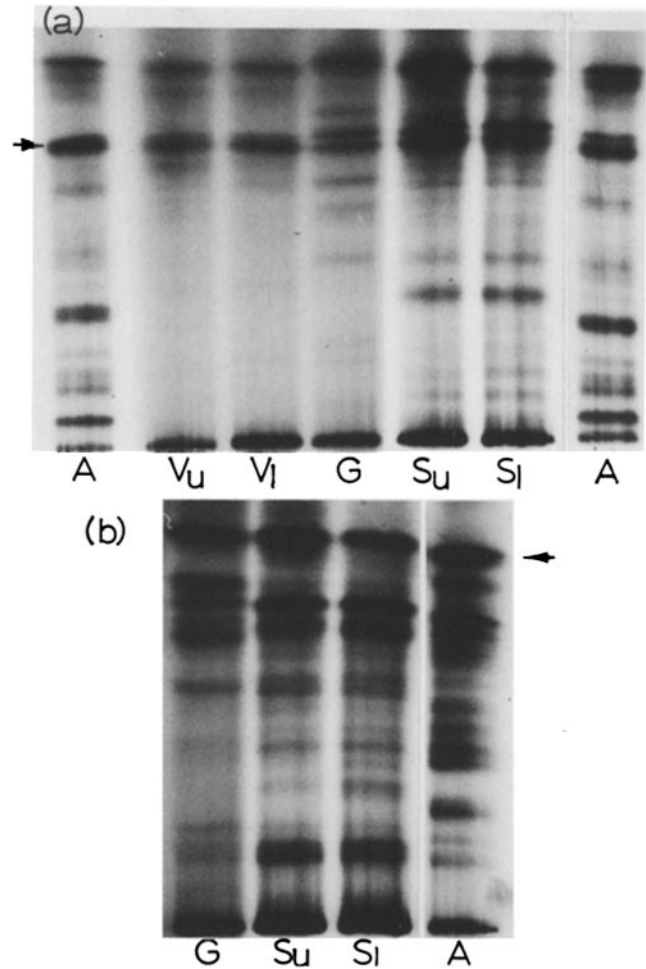


FIGURE 5 Autoradiographs of peptide maps from (a) tryptic digests of the following iodinated proteins (left to right): calf brain actin (A); the upper (V_u) and lower (V_l) bands from the 50,000-dalton protein in the distal segment of the calf Vth cranial nerve; GFAP from calf axons segments (G); the upper (S_u) and lower (S_l) bands from the 50,000-dalton protein in the dog sciatic nerve; and calf brain actin (A). The poor resolution in tracks 2 and 3 is because of the small amount of protein analyzed, but enough detail is visible to show that the proteins from either nerve do not match the GFAP. Artifact bands are indicated by arrows. The autoradiographs from the chymotryptic digests of the latter proteins are shown in b.

in Fig. 7. We conclude that vimentin is present in the calf Vth cranial nerve and in greater amount in segments not proximal to the brain. The same protein is present in the sciatic nerve and, because similar protein complements were seen in all the cranial and peripheral nerve extracts, we presume that vimentin is common to them all.

DISCUSSION

We have examined extracts from the IIrd, Vth, VIth and XIth cranial nerves and sciatic nerves. From these nerves from calves, dogs, and rabbits we obtained by 3-M guanidine hydrochloride extraction the proteins that comprise the neurofilament triplet and two proteins with the solubility and the electrophoretic characteristics of GFAP and vimentin. To identify the proteins in these latter bands in the electrophoretic gels, we undertook peptide mapping.

Our experimental strategy should first be justified. O'Farrel (28) has shown clearly that what appears as one band on a

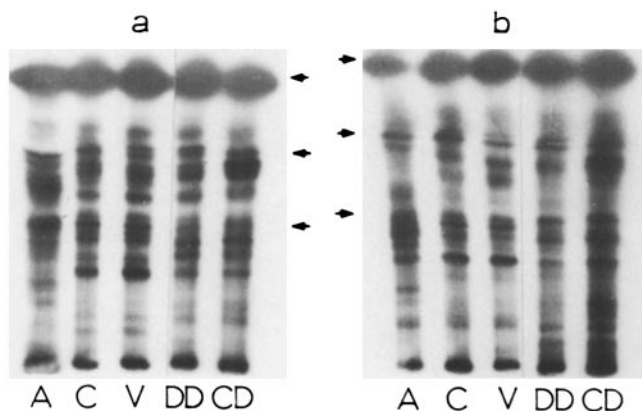


FIGURE 6 Autoradiographs from thin-layer plates on which were run (a) the tryptic and (b) the chymotryptic peptides from: A, calf brain actin; C, protein C from calf Vth cranial nerve; V, vimentin from cultured calf fibroblasts; DD, canine desmin from dog stomach muscle; and CD, bovine desmin from cow uterine smooth muscle. Three artifact bands are marked by arrows. The near identity of the proteins C and V is apparent. There are several features in common with all the maps, but whether this similarity is indicative of a common origin of all these filament proteins is not known.

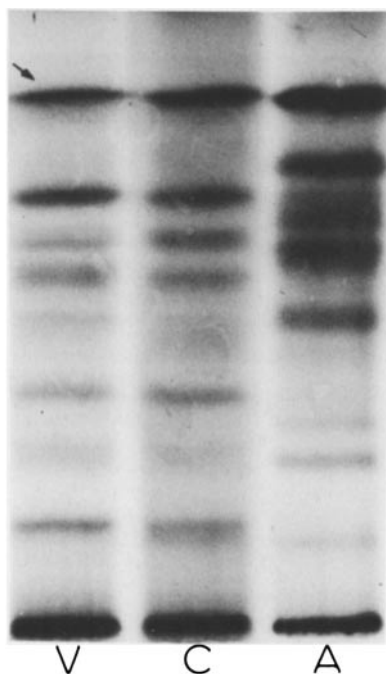


FIGURE 7 Autoradiographs of peptide maps from chymotryptic digests of the following iodinated, rabbit proteins (left to right): fibroblast vimentin (V); sciatic nerve protein C (C); brain actin (A). One row of artifact bands is indicated by the arrow: the remainders at the top of the plate are not reproduced here.

one-dimensional electrophoresis gel can often be separated into several components by applying isoelectric focusing. However, some of this heterogeneity can be a methodological artifact and some may arise from the presence of various substituents on a common polypeptide chain. We reasoned that if any protein predominated in any band, then peptide mapping should permit its identification, because large peptides from the major protein should dominate the map, and peptides from one or more minor proteins should contribute little more than a diffuse background. The peptides recovered from the denatur-

ant extracts of the nerves were few enough to allow us to expect that in the case of major bands little confusion should arise from overlapping proteins. However, we recognize that our technique may not reveal on the peptide chain possible substituents (e.g., various levels of phosphorylation) that may have functional significance.

We believe the experimental results have justified our choice of method. The peptide maps in Figs. 3, 5, and 6 illustrate both the discrimination between dissimilar proteins and suggestive resemblances between others. Indeed the similarities between the peptide maps from the nerve protein C and fibroblast vimentin are so great that we conclude confidently that the same protein (or overlapping proteins) is present in fibroblasts and in cranial and peripheral nerve. There is a protein with the solubility and electrophoretic characteristics of GFAP that is present in distal segments of the IIIrd and Vth calf and dog cranial nerves and the dog and rabbit sciatic nerves, but its peptide map is not the same as GFAP. This may be the 50,000-dalton protein whose antibody stained neurons selectively in experiments reported by Schachner, et al. (30). Our findings do not substantiate our earlier report that indicated the presence of GFAP in the sciatic nerve by the criterion of peptide mapping.

In successive segments of the calf Vth cranial nerve the content of GFAP and vimentin appear to vary reciprocally (Fig. 4) (the band labeled G in the proximal segments must be GFAP to produce peptide maps shown in Fig. 3). We have not localized vimentin in the sciatic nerve (in which extracts it is a prominent component) or cranial nerves; immunofluorescent staining with our rather weak guinea pig antibody to calf fibroblast vimentin gave equivocal results. However, S.-H. Yen and K. L. Fields (manuscript submitted for publication) have found intense reaction with antivimentin in the Schwann cell sheaths. There is a large protein band with the mobility of vimentin in extracts of the optic (IInd cranial) nerve (Fig. 2). We have not yet examined its peptide map. We conclude tentatively that the 10-nm filaments observed in Schwann cells are, at least in part, vimentin.

These experiments confirm that GFAP is largely restricted to the proximal segments of cranial nerves and are compatible with the common conclusions that GFAP is restricted to astrocytes and with the repeated histological observation that astrocytes are restricted to the central nervous system, the olfactory and optic nerves, and the proximal regions of the other cranial nerves. The presence of GFAP could not be demonstrated by peptide mapping of proteins from more distal segments. Nevertheless, immunofluorescent staining with rabbit anti-GFAP on longitudinal cryostat sections of cranial and sciatic nerves showed that patches of fluorescent streaks were visible and that bundles of such streaks penetrated beyond the glial/Schwann cell cone in the calf Vth nerve, for example. Whether this staining is artifactual (e.g., selective adsorption or cross reaction with a different protein) or signifies the local presence of GFAP in other cells or in astrocytes penetrating beyond the zone of transition, is not clear. Our failure to produce a peptide map from GFAP in peripheral nerve does not mean that it is absent, only that it is not a major component of the band analyzed.

In summary, the cell populations in cranial and peripheral nerves contain at least three types of 10-nm filaments, glial, cytoskeletal, and neurofilaments. The triplet of proteins associated with the neurofilaments was found in all the extracts; the GFAP and vimentin show, at least in part, a reciprocal distribution.

The peptide mapping technique identified an experimental artifact of unknown origin. The appearance of two bands on the gel in place of one after reduction and carboxymethylation is not indicative of the resolution of two proteins that before substitution were overlapping, because they yield identical peptide maps. The two bands appeared inconsistently and could not be caused by carbamylation because they appeared whether urea or SDS was used to denature the protein before reaction with iodoacetamide. At present we have no explanation for the observation.

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