NEUROFILAMENT PROTEINS OF RAT PERIPHERAL NERVE AND SPINAL CORD

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ABSTRACT

Intact neurofilaments were isolated in parallel from rat peripheral nerve and spinal cord by osmotic shock into hypotonic media containing divalent cation chelators. Isolated neurofilaments were washed and separated by multiple centrifugations in 0.1 M NaCl. Abundant intact neurofilaments were identified in the washed pellets by negative staining techniques. Their origin from neurofilaments was confirmed by immune electron microscopy. Washed neurofilaments were extracted from lipid and membranous components with 8 M urea.

Analyses of neurofilament isolates on sodium dodecyl sulfate gels showed that proteins of 200,000, 150,000, and 69,000 mol wt were the major components of intact neurofilaments derived from rat peripheral and central nervous systems. These same proteins were identified in whole tissue homogenates of both sources and became enriched during the isolation of intact neurofilaments. A minor component of 64,000 mol wt arose during isolation. Other proteins were identified as contaminants. Small amounts of proteins with electrophoretic migration of tubulin and actin remain in neurofilament isolates.

KEY WORDS intermediate filaments · mammalian neurofilaments · protein subunits gel electrophoresis · immune electron microscopy

Although neurofilaments are abundant within large axons (13, 14, 39, 40) and dendrites (38, 40, 41), they have remained a poorly understood organelle. Attempts to identify the neurofilament subunit protein from different sources have resulted in conflicting data. The 50,000–54,000 mol wt neurofilament protein isolated from mammalian brain (8, 9, 18, 34–36, 42) is not seen in the electrophoretic profiles of other neurofilament-rich tissues such as mammalian peripheral nerve (17, 20, 24) or invertebrate axoplasm from squid (20, 23) and *Myxicola* (15, 17, 20). Conversely, the larger neurofilament proteins of peripheral nerve (17, 20, 24), squid axoplasm (20, 23), and *Myxicola* axoplasm (15, 17, 20) have not been identified as major components in neurofilaments isolated from brain (8, 9, 18, 34–36, 42).

Differences in protein composition among neurofilament isolates may arise from an inherent lability of neurofilament proteins. Mammalian neurofilament proteins are rapidly altered during Wallerian degeneration (33), concomitant with a postulated calcium influx into the axoplasm (25, 26, 29, 31). A similar breakdown of neurofilament proteins occurs when invertebrate neurofilaments are exposed to calcium (15). Some alterations of neurofilament proteins could occur during the prolonged preparative procedures used to isolate neurofilaments from mammalian brain tissues. A relationship between the 50,000–54,000 mol wt neurofilament proteins and higher molecular weight proteins in brain neurofilament iso-

lates has been demonstrated upon re-electrophoresis of proteins (8, 9, 35).

The present study addresses the issue concerning the apparent differences between neurofilament proteins isolated from mammalian central and peripheral nervous systems. Intact neurofilaments have been isolated in parallel from rat peripheral nerve and rat spinal cord by a recently developed method. Comparative analysis of these neurofilament-enriched preparations reveals an identical set of proteins. These same proteins are present in fresh tissue homogenates and in all isolates containing intact neurofilaments.

MATERIALS AND METHODS

Isolation of Intact Neurofilaments and Extraction of Their Proteins

The isolation of neurofilaments was carried out at 18°-20°C as previously described. Neurofilament-rich supernates (PN I) were obtained from osmotically shocked rat peripheral nerves (28). Neurofilaments were stabilized by the addition of 80 μl of 1 M NaCl immediately before centrifugation. ~400–450 μl of PN I was obtained.

The spinal cord-medulla oblongata (700–800 mg) of the same rat was placed in isotonic saline and stripped of its pachy- and leptomeninges. This tissue was separated into four or more longitudinal strips and osmotically shocked as above (28). The swollen tissues (1,800–2,200 mg), without dilution, were homogenized in a 7-ml Dounce homogenizer (Kontes Co., Vineland, N.J.) with five strokes of the "A" pestle. After adding 120 μl of 1 M NaCl, the tissues were placed in 1-ml centrifuge tubes and spun at 12,000 rpm for 30 min in a Sorvall RC2-B centrifuge (DuPont Instruments-Sorvall, DuPont Co., Wilmington, Del). ~750 μl of a slightly cloudy, neurofilament-rich supernate (SC I) was carefully decanted.

Intact neurofilaments within PN I and SC I fractions were washed three times by admixture with 0.1 M NaCl in 5-ml centrifuge tubes and pelleted in a Model L centrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) using a SW 50.1 rotor at 25,000 rpm for 30 min. The final washed neurofilament pellets (PN II and SC II) were resuspended in 150–200 μl of H_2O, dialyzed overnight against 8 M urea using miniature dialysis boats (28), and centrifuged in 0.8-ml tubes (Beckman Instruments, Inc., Spinco Div.) at 22,000 rpm for 30 min in a SW 50.1 rotor. The clear supernates (PN III and SC III) were very carefully aspirated with a no. 27 needle through the side of the tubes, leaving behind cloudy, viscous, membranous pellicles (PN M and SC M). These pellicles were resuspended in H_2O or 0.1 M NaCl and pelleted by centrifugation at 25,000 rpm for 30 min in a SW 50.1 rotor.

Examination of Samples by Negative Staining and Immune Electron Microscopy

Fixed and unfixed specimens were examined as described (28). Immune electron microscopy was carried out by flotation of neurofilament-laden grids on phosphate-buffered saline (PBS) containing rabbit antineurofilament immunoglobulin (IgG) (0.25 mg/ml) or normal rabbit IgG (0.25 mg/ml), as previously described (27). Experimental antisera were raised to neurofilament protein derived from rat peripheral nerve (27). Both experimental and control rabbit IgG were prepared by column chromatography, frozen (−70°C), and stored (27).

Comparative examinations by immune electron microscopy were conducted on neurofilaments isolated from rat spinal cord (SC I) and rat peripheral nerve (PN I) and subjected to the same incubational conditions. Immunoabsorptive capacity of neurofilament-rich extracts from spinal cord was studied by addition of 0.5 mg/ml of SC I to some incubational solutions containing rabbit antineurofilament IgG.

Preparation of Samples for Electrophoresis

25–50-μl aliquots of samples were added to equal volumes of a solution containing 2% sodium dodecyl sulfate (SDS), 0.1 M dithioerythritol (DTE), and 10 M urea, vortexed, and heated to 100°C for 5 min. Whole tissue homogenates of peripheral nerve (PN H) and spinal cord (SC H) were prepared from nerve roots and caudal spinal cord of the same rat by homogenization in 20-fold dilution (wt/vol) of a solution containing 1% SDS, 0.05 M DTE, and 5 M urea using Duall homogenizers (Kontes Co). Tissue samples were heated to 100°C for 5 min, rehomogenized, and centrifuged at 40,000 rpm for 60 min in a SW 50.1 rotor to float insoluble membranous or lipid components. Protein standards were prepared from stock solutions of bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.), phosphorylase A (Worthington Biochemical Corp., Freehold, N.J.), porcine brain tubulin purified by the method of Shelanski et al. (37) and rabbit muscle myosin.

Protein assays were conducted on aliquots of different samples by the method of Lowry et al. (22).

Polyacrylamide Disc Gel Electrophoresis

Proteins were analyzed by disc gel electrophoresis in SDS in general accordance with the method of Laemmli (19), as previously described (27), with the following modifications. Gel polymerization was performed with 0.04% *N,N,N′,N′*-tetramethylethylenediamine (TEMED) electrode buffer contained 0.1% SDS in 0.025 M Tris and 0.192 M glycine, pH 8.3, and samples containing 15–25 μg of protein were added to sample buffer with the following constituents (at final concentration): 2% SDS, 20% sucrose, 80 mM DTE, 2 mM EDTA, 0.001% bromphenol blue, 3.2 mM Tris, and 25

mM glycine, pH 8.3. Samples were underlaid atop the stacker gels. Fixation, staining, destaining, scanning, and determination of relative mobilities were performed as previously described (27).

RESULTS

Yield of Proteins in Neurofilament Isolates

Average amounts of total proteins in different tissue fractions were approximately as follows (in μg): PN I (2,500), PN II (700), PN III (250), SC I (5,200), SC II (475), SC III (300). The amounts of urea-extracted neurofilament protein (PN III and SC III) were quantitated indirectly because of the interference of urea with the Lowry assay (22). These values were based upon the differences between total protein dialyzed (PN II and SC II) and that recovered in the membranous pellicles (PN M and SC M).

Examination of Neurofilament Isolates by Negative Staining

Neurofilaments identical to those previously described (27, 28, 30) were abundant in the crude supernates of osmotically shocked rat peripheral nerve (PN I) and spinal cord (SC I). Intact neurofilaments, stabilized in 0.1 M NaCl (28), could be readily demonstrated in PN II and SC II pellets (Figs. 1 and 2). Neurofilaments were by far the most conspicuous component of these preparations. Some membranous and particulate profiles were also encountered, especially in PN II. Small particulate debris with occasional intact neurofilaments were seen in saline washes of the pelleted neurofilaments.

Intact neurofilaments were not seen in PN II and SC II preparations which had been dialyzed overnight against 8 M urea. These preparations and the membranous pellicles (PN M and SC M) contained only membranous and particulate debris.

Examination of Neurofilaments by Immune Electron Microscopy

Neurofilaments of PN I and SC I were decorated by antineurofilament IgG, as previously described (27). Neurofilament decoration was consistently and widely observed in all preparations. A population of undecorated filaments could not be identified in preparations from nerve or from spinal cord.

Neurofilaments of PN I and SC I were not decorated after control incubations in PBS containing normal rabbit IgG. Neurofilament-decorating capacity of antineurofilament IgG was completely absorbed by the addition of 0.5 mg/ml of SC I to the incubational media. Decoration of neurofilaments by antineurofilament IgG remained unaltered after equivalent additions of tubulin or bovine serum albumin (27).

The decoration of neurofilaments by immune electron microscopy could be reproduced in PN I and SC I preparations which had been frozen and stored.

SDS Polyacrylamide Gel Electrophoresis of Neurofilament Isolates

Densitometric tracings of gels showed the same proteins in fresh homogenates of peripheral nerve (Fig. 3*A*) and in neurofilament isolates of nerve (Fig. 3*B–E*). Nerve proteins of 200,000 and 150,000 mol wt were enriched during neurofilament isolation. A 69,000 mol wt protein was also a major component of isolated neurofilaments. This protein was also prominent in PN I fractions (Fig. 3*B*) and in the saline washes of pelleted neurofilaments (not shown). A protein which comigrated with porcine brain tubulin (55,000 mol wt) was also enriched in the PN I fraction (Fig. 3*B*) but was found predominantly in the saline washes rather than in the neurofilament pellet (Fig. 3*C*). Myelin proteins (15,000–30,000 mol wt), which comprise the predominant proteins of peripheral nerve homogenates (16, 24), were present in all neurofilament isolates from peripheral nerve, especially in the membranous pellicle (Fig. 3*E*). A minor nerve protein of 45,000 mol wt remained a small component in the neurofilament isolates.

Electrophoresis of whole spinal cord homogenates (Fig. 4*A*) presented a banding pattern much more complex than that seen in peripheral nerve homogenates (Fig. 3*A*). Protein bands were observed in spinal cord homogenates that corresponded to the major proteins in neurofilament isolates (Fig. 4*B–D*). Spinal cord proteins of 200,000, 150,000 and 69,000 mol wt became enriched during neurofilament isolation. A 55,000 mol wt protein was very prominent in the SC I fraction (Fig. 4*B*) but not in SC II pellets of intact neurofilaments (Fig. 4*C*). This protein was especially prominent in the saline washes of intact neurofilaments (not shown). A less intensely stained band of 51,000 mol wt was seen in spinal

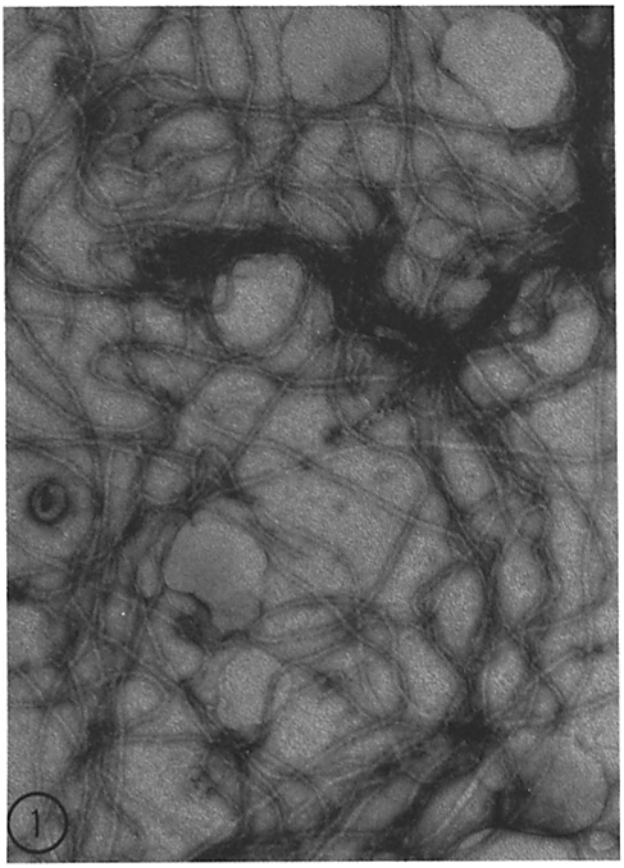

FIGURE 1 Numerous overlapping intact neurofilaments isolated from rat peripheral nerve by osmotic shock and washed by multiple centrifugations in 0.1 M NaCl (PN II). Formalin-fixed preparation stained with 1% unbuffered uranyl acetate. × 90,000.

cord homogenates and in neurofilament isolates but was obscured in the SC I fractions. A 45,000 mol wt protein remained a minor component of neurofilament isolates. Electrophoresis of the membranous pellicle (SC M) revealed a multiplicity of minor polypeptide bands between 40,000–150,000 mol wt (not shown).

A similar set of polypeptides was extracted from washed neurofilament pellets of nerve and spinal cord (Fig. 5). Polypeptides of 200,000, 150,000, 69,000, 64,000, 55,000, and 45,000 mol wt were consistently present in all neurofilament preparations. Occasionally, the two largest polypeptides appeared as doublets with the slower migrating component of each doublet corresponding to the single band of 200,000 or 150,000 mol wt in fresh homogenates of peripheral nerve. The splitting of high molecular weight bands could be seen in crude neurofilament-rich supernates (PN I and SC I) but occurred most often in neurofilament preparations from peripheral nerve. The 64,000 mol wt polypeptide was also more prominent in neurofilament extracts from peripheral nerve. It did not correspond to a polypeptide band of whole nerve homogenates but appeared most conspicuously after urea extraction. In many preparations, the 64,000 mol wt polypeptide appeared as a doublet. The 55,000 mol wt polypeptide of neurofilament extracts co-migrated with the tubulin standard (a singlet band when unalkylated and run in SDS gels without urea [7]) and a corresponding band in whole tissue homogenates. The 45,000 mol wt band constituted a minor component of whole tissue homogenates and neurofilament extracts.

Some proteins were seen in either nerve or spinal cord preparations. Peripheral nerve myelin proteins (15,000–30,000 mol wt) were present in neurofilament isolates from nerve (Fig. 5*A*) but

not from cord (Fig. 5*B*). A 51,000 mol wt protein was present in neurofilament preparations from spinal cord (Fig. 5*B*) but not from nerve (Fig. 5*A*).

DISCUSSION

The present study has demonstrated a similar protein composition among neurofilaments isolated from the rat peripheral (PNS) and central (CNS) nervous systems. These proteins closely resemble the neurofilament subunits which have been postulated from studies of mammalian PNS. Neurofilament proteins of 212,000, 160,000 and 68,000 mol wt were initially suggested on the basis of their prominence in slow axonal transport and the synchrony of their distad movements (17, 20). Proteins of 200,000, 150,000, and 69,000 mol wt were subsequently identified as the major components of purified PNS axonal fractions containing an abundance of neurofilaments (24). More recently, the same three proteins were noted to disappear selectively from transected peripheral nerve during the time interval in which neurofilaments underwent granular disintegration (33).

The demonstration of 200,000, 150,000, and 69,000 mol wt proteins in neurofilament isolates and in fresh tissue homogenates of rat PNS provides evidence that these proteins are present within neurofilaments *in situ*. The same proteins were enriched in CNS neurofilament isolates. Their lack of prominence in CNS homogenates may be due to the large admixtures of different proteins in CNS tissues and to the relative prominence of gray matter in caudal rat spinal cord (43) with attendant reduction of white matter rich in neurofilaments and their antigens (32).

The identity of neurofilaments from rat spinal cord was established by immune electron micros-

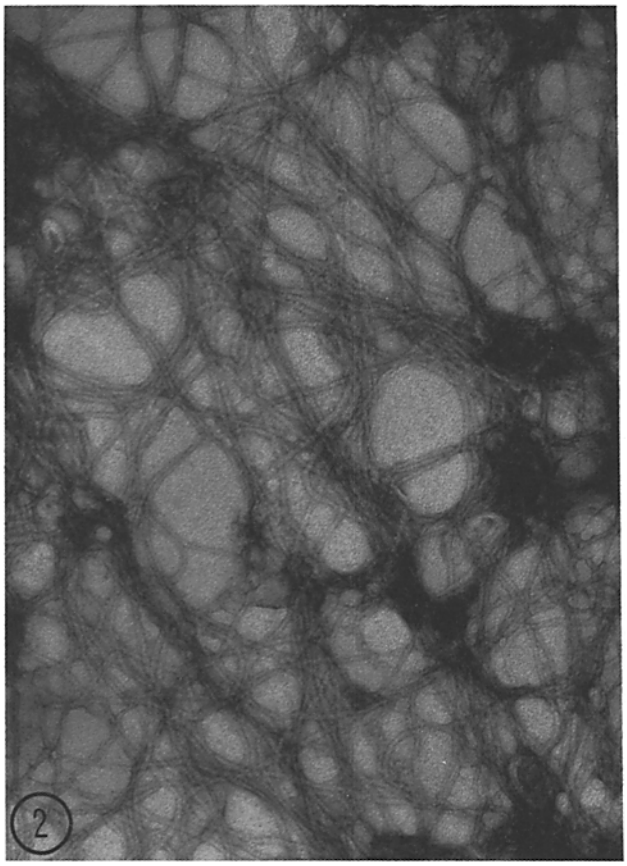

FIGURE 2 Numerous overlapping intact neurofilaments isolated from rat spinal cord by osmotic shock and washed by multiple centrifugations in 0.1 M NaCl (SC II). Formalin-fixed preparation stained with 1% unbuffered uranyl acetate. × 90,000.

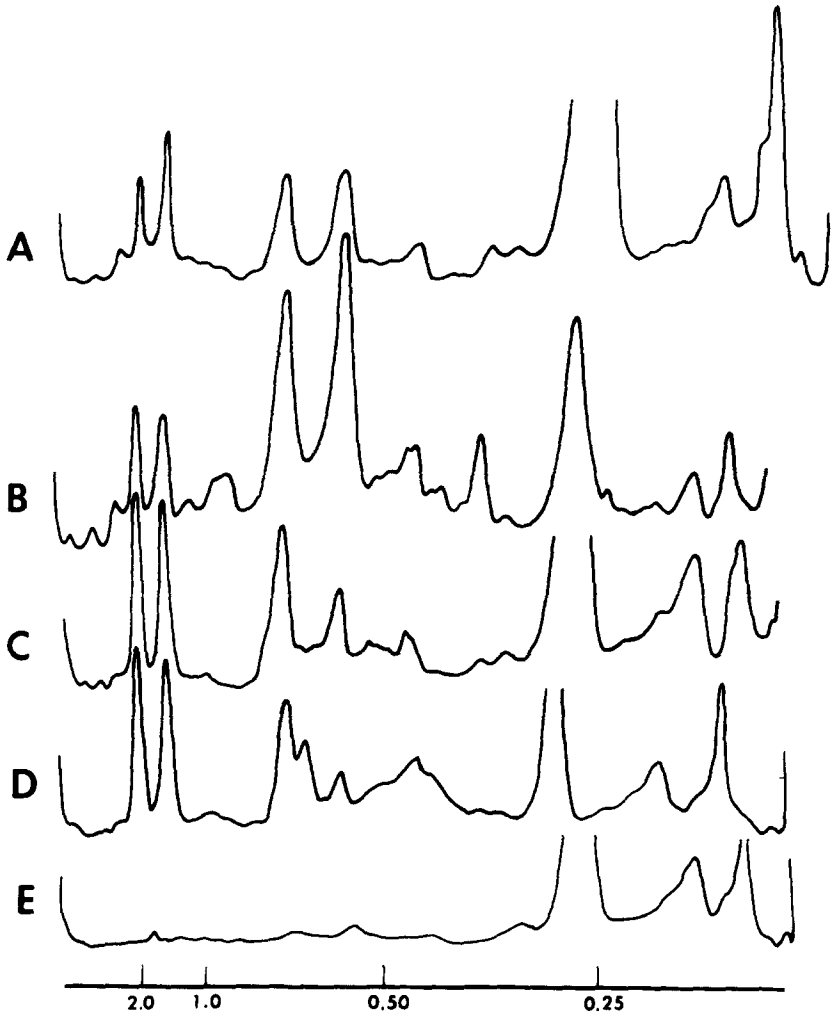

FIGURE 3 Densitometric tracings of protein bands in acrylamide gels of neurofilament isolates from rat peripheral nerve. (*A*) Whole nerve homogenate (PN H). (*B*) Supernate of osmotically shocked tissue (PN I). (*C*) Washed neurofilament pellet (PN II). (*D*) 8 M urea extract (PN III) of neurofilament pellet. (*E*) Urea-insoluble membranous pellicle (PN M) from neurofilament pellet. Scale indicates mol wt $\times 10^5$ constructed from protein standards.

copy. Isolated CNS filaments were decorated by the same antineurofilament IgG which attached to neurofilament-rich areas of tissue sections and was demonstrated by indirect immunofluorescent techniques (32). This antiserum did not cross-react with glial antigens, as evidenced by the absence of stain in the distribution of glial elements (32).

It seems reasonable to assume that similar neurofilaments occur in neurites of the CNS and PNS of the rat inasmuch as many of these cellular processes arise from common neuronal perikarya. This assumption of neurofilament homogeneity within a particular species is supported by the immunological cross-reactivity between PNS and CNS neurofilaments demonstrated in the present and previous (32) studies. The presence of the same proteins in neurofilaments from rat peripheral nerve and spinal cord should not be unexpected, yet these findings do conflict with prevailing concepts regarding subunit-proteins of CNS neurofilaments. Neurofilament preparations from mammalian brain are composed predominantly of a 50,000-54,000 mol wt protein (8, 9, 18, 35,

42). These preparations also contain glial filament proteins (1, 9, 11) whose highly antigenic properties (2, 3) may account for some of the immunological reactivities of these preparations (6, 7, 21, 42). It has been suggested that intermediate filaments of glial and neural origins are very similar, if not identical (6, 42), yet immunofluorescence of neural and glial elements in tissues can be differentially stained and absorbed using nerve tissues not containing glial contaminants (5).

A 50,000–54,000 mol wt protein may be derived from preparative alterations of peripheral nerve. Although not present in homogenates of freshly excised nerve (17, 20, 24), a small and variable amount of this protein was identified in nerve extracted with 6 M guanidine hydrochloride or 50% formic acid before solubilization in SDS (6). A similar protein was also isolated from peripheral nerves subjected to prolonged and complex extraction procedures developed for CNS proteins (4). It is also possible that the presumptive neurofilament proteins of 50,000–54,000 mol wt occur at other locations in different electrophoretic systems (12).

The lack of 50,000–54,000 mol wt protein in our neurofilament isolates is probably due to methodology. Our methods rapidly separate neurofilaments into a calcium-free external compartment whereas the isolation of CNS neurofilaments (9, 18, 35, 36, 42) by flotation of myelinated axons (10) maintains the intraaxonal localization of neurofilaments for prolonged periods. Individual neurofilaments isolated from axonal fractions are poorly visualized by negative stain (8). Our experiences suggest that negative staining procedures represent a more sensitive index of neurofilament integrity than the procedures of fixation, osmication, dehydration, and pelleting of samples, as used in previous studies (9, 18, 35, 36, 42). Preparative alterations of neurofilaments may occur during prolonged isolation procedures and remain undetected when monitored by examination of sectioned material.

Proteins which were limited to neurofilament

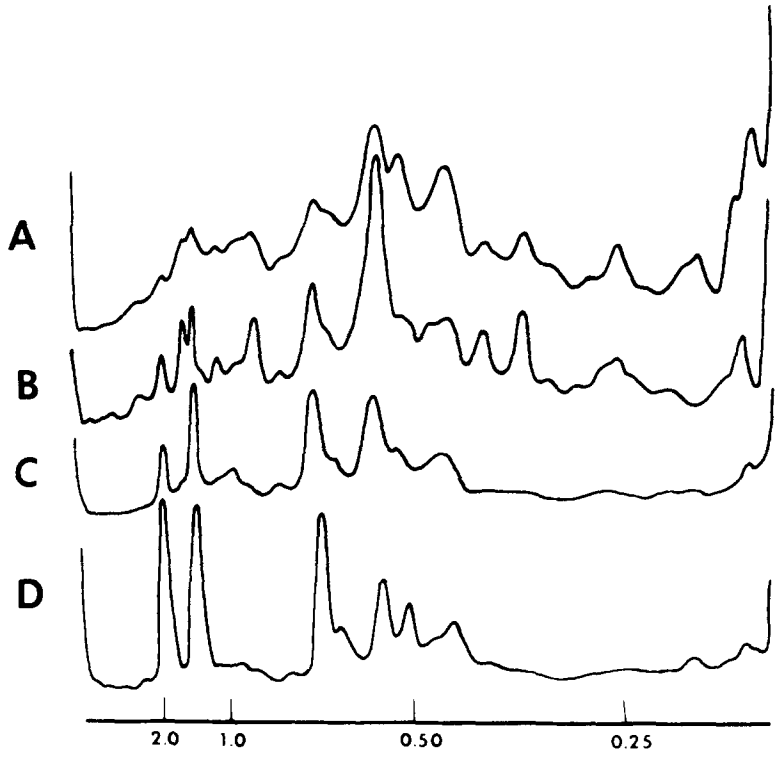

FIGURE 4 Densitometric tracings of protein bands in acrylamide gels of neurofilament isolates from rat spinal cord. (*A*) Whole spinal cord homogenates (SC H). (*B*) Supernate of osmotically shocked tissue (SC I). (*C*) Washed neurofilament pellet (SC II). (*D*) 8 M urea extract (SC III) of neurofilament pellet. Scale indicates mol wt $\times 10^5$ constructed from protein standards.

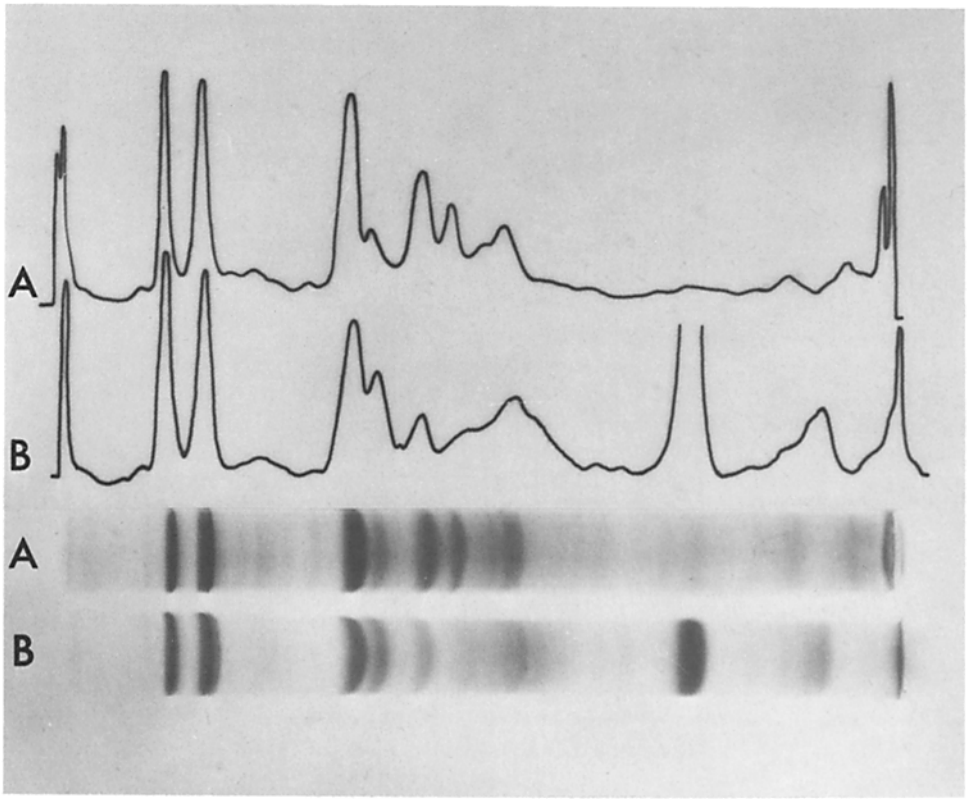

FIGURE 5 Acrylamide gels and their densitometric tracings of neurofilaments from rat peripheral nerve (*A*) and rat spinal cord (*B*). Polypeptides of washed intact neurofilaments extracted with 8 M urea (PN III and SC III).

isolates from either nerve or spinal cord could represent the selective alteration of neurofilament proteins or the co-isolation of contaminants. The latter phenomenon was clearly evidenced by the prominence of myelin proteins in all PNS fractions. Most membranous components in neurofilament preparations remained insoluble in urea and could be separated by centrifugation. Peripheral myelin proteins appear to be more susceptible to urea extraction. Lowering the concentration of urea reduced the amount of myelin protein contaminant but also decreased the density of solution so that the separation of membranous and lipid elements became more difficult.

The 51,000 mol wt protein of CNS preparations may represent glial acidic fibrillary protein, a known contaminant of CNS homogenates (1, 9, 11). The acidic nature of this protein would provide a high affinity for charged components within tissue homogenates. Similarly, other acidic fibrous proteins in neural tissues, such as tubulin and actin, might be difficult to separate during isolation of intact neurofilaments. The presence of minor bands in the position of tubulin (55,000 mol wt) and actin (45,000 mol wt) may represent coincident contamination by these proteins in both CNS and PNS neurofilament extracts. The isolation of tubulin and actin with intact neurofilaments could reflect some integral association of these proteins with neurofilaments *in situ*.

We wish to thank Ms. Mary Hasler for her fine technical assistance in electron microscopy and photography. We are also grateful to Dr. Barry Ashby for his gift of rabbit muscle myosin and Ms. Debby Glazer for her secretarial help.

This study was supported by grant NS 08620 from the National Institutes of Health.

Received for publication 17 January 1978, and in revised form 24 March 1978.

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