IMMUNOLOGICAL AND ULTRASTRUCTURAL STUDIES OF NEUROFILAMENTS ISOLATED FROM RAT PERIPHERAL NERVE

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ABSTRACT

Neurofilaments were isolated from desheathed and minced segments of rat peripheral nerve by osmotic shock into 0.01 M Tris-HCl buffer, pH 7.2. Freshly isolated neurofilaments were observed to undergo disassembly by progressive fragmentation upon exposure of dilute tissue extracts to this buffer. Low- and high-speed centrifugations of these tissue extracts separated membranous and particulate constituents and produced a progressive enrichment of 68,000-dalton polypeptide band in successive supernates, as determined by analyses of soluble proteins by SDS-polyacrylamide electrophoresis. The final high-speed supernatant fractions (S3) of nerve extracts, which were predominately composed of 68,000-dalton polypeptide, were used to raise a specific experimental antisera in rabbits.

Utilizing techniques of immune electron microscopy, experimental rabbit antisera was shown to contain antibodies against neurofilaments. Intact neurofilaments isolated from rat nerves and attached to carbon-coated grids became decorated when exposed to experimental rabbit antisera or purified gamma globulin (IgG) derivatives. The decoration of neurofilaments closely resembled the IgG coating seen in immune electron microscopy. Antibody absorption techniques were used to identify the biochemical constituency of neurofilamentous antigenic determinants. The decoration of neurofilament by experimental IgG was not altered by additions of tubulin or bovine serum albumin, but was prevented by additions of S3 fractions as well as the 68,000-dalton polypeptide of this fraction which was eluted and recovered from polyacrylamide gels.

These findings are indicative that a 68,000-dalton polypeptide is a constituent subunit of rat peripheral nerve neurofilaments.

Neurofilaments occur abundantly in neurites, representing the predominant structural constituency of large myelinated axons (20, 21, 49) and some large dendrites (57, 58). Yet, neurofilaments remain a poorly understood organelle, their functional significance is still obscure, and their functional relationship, if any, to accompanying microtubules has not been clarified. Similar organelles have been recognized as 100 Å intermediate-sized filaments in other cell types, some of which are also associated with asymmetrical cell processes (7, 19, 23, 33). Both neurofilaments (8, 12, 55) and intermediate-sized filaments (4, 5, 11, 14, 23, 25, 26, 27, 29) become more prevalent in tissues treated with colchicine or Colcemid, but neither neurofilaments (9, 10) nor intermediate-sized filaments (30, 48) are decorated by exposure to heavy meromyosin (HMM).

The abundance of neurofilaments in neurites has enabled their isolation for biochemical analysis (13, 31, 46, 59). Nevertheless, the elucidation of the biochemical nature of neurofilaments has proceeded very slowly. Major discrepancies exist concerning the polypeptide composition of neurofilaments from mammalian brain (13, 31, 46, 59), from mammalian peripheral nerve (24), and from invertebrate giant axons (22, 28, 42). Furthermore, no biochemical characteristics have been identified which could serve as a marker for neurofilaments during their isolation and purification.

The present studies have utilized immunological reactions to localize biochemical components within the structural constituency of neurofilaments. Immune electron microscopy, a useful virological procedure (2, 43), has been modified in order to demonstrate antigenic sites within intact neurofilaments. Antibody absorption techniques have been employed to define more precisely the biochemical components which contain the neurofilamentous antigenic sites.

MATERIALS AND METHODS

Extraction of Nerve by Osmotic Shock

Male Sprague-Dawley albino rats weighing 300–350 g were anesthetized with ether and perfused through the heart with approx. 200 ml of isotonic saline. Major peripheral nerves of hind- and fore-limbs were excised, placed in isotonic saline, and desheathed. Each rat yielded approx. 300 mg (wet weight) of desheathed nerves. These desheathed nerves were rinsed in 0.01 M Tris-HCl buffer, pH 7.2, and transversely minced into 0.2–0.4-mm nerve segments with no. 11 blades and fine eye forceps under a dissecting microscope. Minced nerves were diluted with 25–50 vol of Tris-HCl buffer (20–30 mg desheathed nerves/ml buffer) and agitated with a 5-mm magnetic stirrer for 90 min at 20–23°C. Nerve preparations were spun in a Sorvall RC2-B centrifuge (DuPont Instruments, Sorvall Operations, Newton, Conn.) at 1,000 rpm × 10 min and at 12,000 rpm × 30 min and in a Beckman model L ultracentrifuge (Beckman Instruments, Spinco Div., Palo Alto, Calif.) at 45,000 rpm × 120 min, all centrifugations carried out at 18°–20°C. All precipitates were discarded; the final high-speed supernates (S3) were frozen (−70°C), stored, and utilized for immunological studies.

The extraction and isolation procedure was also carried out with higher initial dilutions of desheathed and minced nerves (2–3 mg nerve/ml buffer). The very dilute high-speed supernates of these preparations were concentrated in an Amicon model 8MC micro-ultrafiltration system behind an Amicon UM20 filter (Amicon Corp., Scientific Sys. Div., Lexington, Mass.), yielding a modified high-speed supernatant fraction (S3′).

Preparation of Antisera and Gamma Globulin (IgG) Derivatives

Antisera was raised in rabbits by multiple footpad and subcutaneous injections of S3 fractions (0.2–0.3 mg/rabbit) admixed with complete Freund's adjuvant. Monthly and bimonthly booster injections were administered over a 6–12-mo period. Antisera was pooled, frozen (−70°C), and stored. Small amounts of antibody which precipitated rat sera on Ouchterlony plates were completely absorbed by affinity chromatography, using activated cyanogen bromide to couple rat sera to a sepharose column (39). IgG was obtained from absorbed antisera. Both experimental and control rabbit IgG were prepared by precipitation of rabbit sera with 40% NH_4SO_4, dialysis of the precipitate with 15 mM potassium phosphate buffer, pH 8.0, and chromatographic separation using a 2.4 × 10 cm column packed with Bio-Rad Cellex-D (Bio-Rad Laboratories, Richmond, Calif.) (17). The sharp initial protein peaks which were eluted with 15 mM buffer were frozen (−70°C) and stored.

Polyacrylamide Gel Electrophoresis

Polyacrylamide disc gel electrophoresis in sodium dodecyl sulfate (SDS) was performed in general accordance with the method of Laemmli (36). Resolving gels of 12% acrylamide and 0.22% *N,N′*-methylene-*bis*-acrylamide were prepared from admixtures of stock solutions containing 30% (wt/vol) acrylamide, 0.8% (wt/vol) *bis* and 30% acrylamide, 0.35% *bis* in 0.375 M Tris-HCl, pH 8.8 and 0.1% SDS. 76-mm gels were prepared in clean glass tubes of 5 mm ID and 125 mm length. During polymerization, the concentration *N,N,N′,N′*-tetramethylethylenediamine (TEMED) was 0.06% (vol/vol) and of ammonium persulfate was 0.035% (wt/vol). The 3% stacking gels contained 0.08% *bis* and were polymerized in 0.0625 M Tris-HCl, pH 6.7, and 0.1% SDS by additions of 0.05% TEMED and 0.105% ammonium persulfate. The electrode buffer contained 0.1% SDS in 0.05 M Tris and 0.384 M glycine, pH 8.3. Samples containing 5–10 μg protein were added to sample buffer with the following constituents (at final concentration): 0.75% SDS, 12.5% sucrose, 6.7 M urea, 4.8 mM β-mercaptoethanol, 0.0006% Bromphenol Blue, 4.2 mM Tris, and 32.25 mM glycine, pH 8.3. Samples kept at room temperature gave the same results as those immersed in boiling water bath for 3–5 min. After pipetting the samples atop the gels and overlying with electrode buffer, electrophoresis was performed at 1 mA/gel to achieve stacking (1 h) and then at 2.5 mA/gel until the tracker dye reached the bottom of the resolving gels (2.2 h). The polypeptides were fixed by incubating the gels for approx. 16 h in several changes of 12% trichloracetic acid and 50% ethanol at 50°C, stained for 3–4 h at 50°C in 0.1% Coomassie blue, 10% acetic acid, and 50% ethanol, and then destained in 10% acetic acid. After destaining, the gels were scanned for OD at 565 mm with a Gilford spectrophotometer

(Gilford Instrument Laboratories, Inc., Oberlin, Ohio). Relative mobilities (Rfs) of the stained bands were measured relative to the mobility of the Bromphenol Blue tracking dye on each gel.

SDS-polyacrylamide slab gel electrophoresis was performed using a Hoefer SE500 assembly (Hoefer Scientific Instruments, San Francisco, Calif.) with a gel width of 0.75 mm. Preparation and composition of samples, buffers, stacking gels, and resolving gels were the same as those used for disc gels. Stacking of samples was achieved with a current of 1 mA/gel, which was increased to 2 mA/gel while the tracker dye traversed the resolving gel. Fixation and staining of gels were carried out at room temperature on a rocker platform for 16 and 24 h, respectively.

Isolation and Demonstration of Intact Neurofilaments

Approx. 300 mg (wet weight) of desheathed rat peripheral nerves were soaked for 60 min in 0.01 M Tris-HCl buffer. The swollen nerve fascicles were then transversely minced and diluted with an equal volume of buffer. Minced nerve segments were gently spun with a 5-mm magnetic stirrer for 60 min at 20°–23°C, avoiding bubble formation. The resulting tissues of mushy consistency were placed in a 1-cc Sorvall conical glass centrifuge tube and spun in a Sorvall RC2-B centrifuge at 15,000 rpm × 30 min at 18°–20°C. The clear viscous supernate was carefully decanted and placed directly on a sheet of dental wax. Carbon-Formvar-coated 400-mesh grids were touched to the surface of the drop of supernate, excess liquid was removed with filter paper and the wet grid immediately fixed for 1 h at 20°–23°C by flotation on a drop of 5% formalin in phosphate-buffered saline (PBS). After rinsing in several drops of PBS, the preparations were stained with 1% unbuffered uranyl acetate. Specimens were also stained without fixation.

Immune Electron Microscopy of Neurofilaments

Fixed neurofilaments adherent to carbon-coated grids were incubated by flotation on PBS-containing control IgG (0.25 mg/ml) or experimental IgG (0.25 mg/ml). Incubations with experimental IgG were also undertaken in the presence of S3 or S3′ fractions (0.1 mg/ml), S3 fraction eluted from G-100 Sephadex (0.1 mg/ml), S3 fraction eluted with 0.4 M NaCl from *O*-(diethylaminoethyl)cellulose (DEAE-cellulose) (0.1 mg/ml), bovine serum albumin (BSA, 0.5 mg/ml) or tubulin (0.5 mg/ml). Tubulin was prepared from porcine brain by the method of Shelanski (47), frozen (−70°C) and stored. Additional absorbants of experimental antibodies included polypeptides, which had been eluted and recovered from SDS-polyacrylamide disc gels (53). The acrylamide fractions corresponding to 68,000-dalton polypeptide band positions of the S3 fractions and BSA were selectively excised from unfixed and unstained gels, macerated, and eluted with 0.05 M NH_4HCO_3 containing 0.05% SDS and lyophilized. SDS was removed by passage through an anion exchange column containing Dowex-1 resin. The eluent was lyophilized, frozen (−70°C), and stored.

Standardized incubational conditions consisted of an initial 2-h wash of formalin-fixed neurofilament preparations in PBS, a 4-h incubation in PBS-containing control or experimental IgG, and an overnight wash in PBS. All incubations were carried out in a 4°C refrigerator within small petri dishes atop a rotating platform. Washed preparations were negatively stained with 1% unbuffered uranyl acetate. All negatively stained preparations were examined and photographed in a Siemens IA Elmiskop operated at 80 kV.

All protein concentrations were assessed by the Lowry technique (38), using BSA as standard reference.

RESULTS

Examination of Neurofilaments by Negative-Staining Techniques

The breakdown of neurofilaments was found to be reduced when minced nerve segments were osmotically shocked in small dilutions of buffer. Modification of the extraction procedure in this manner yielded negatively stained preparations with optimal preservation of neurofilaments admixed with variable numbers of membranous and large particulate profiles. Microtubules and collagen fibers were very infrequently seen.

In their morphology, the rat nerve neurofilaments (Fig. 1) closely resembled the profiles of neurofilaments from mammalian brain (13) and invertebrate giant axon (22, 28), as well as those of intermediate filaments (IF) from embryonic muscle (30). Individual neurofilaments coursed in discrete and unbranching patterns. Some neurofilaments could be followed for a distance of at least 10 μm. Their profiles were slightly accentuated by small accumulations of electron-dense stain along their lateral margins. The contour of neurofilaments was that of a uniform cylindrical structure; lateral sidearm appendages were not seen. The parallel lateral margins of neurofilaments were usually 80–110 Å apart, although thinner segments could be seen. In fact, the terminations of neurofilaments were sometimes tapered into a single thin strand (Fig. 1). More often, neurofilaments terminated abruptly. Multiple protofilaments or other subunit structures of the neurofilaments were not apparent. Some neurofilaments revealed irregular lateral margins and small axial

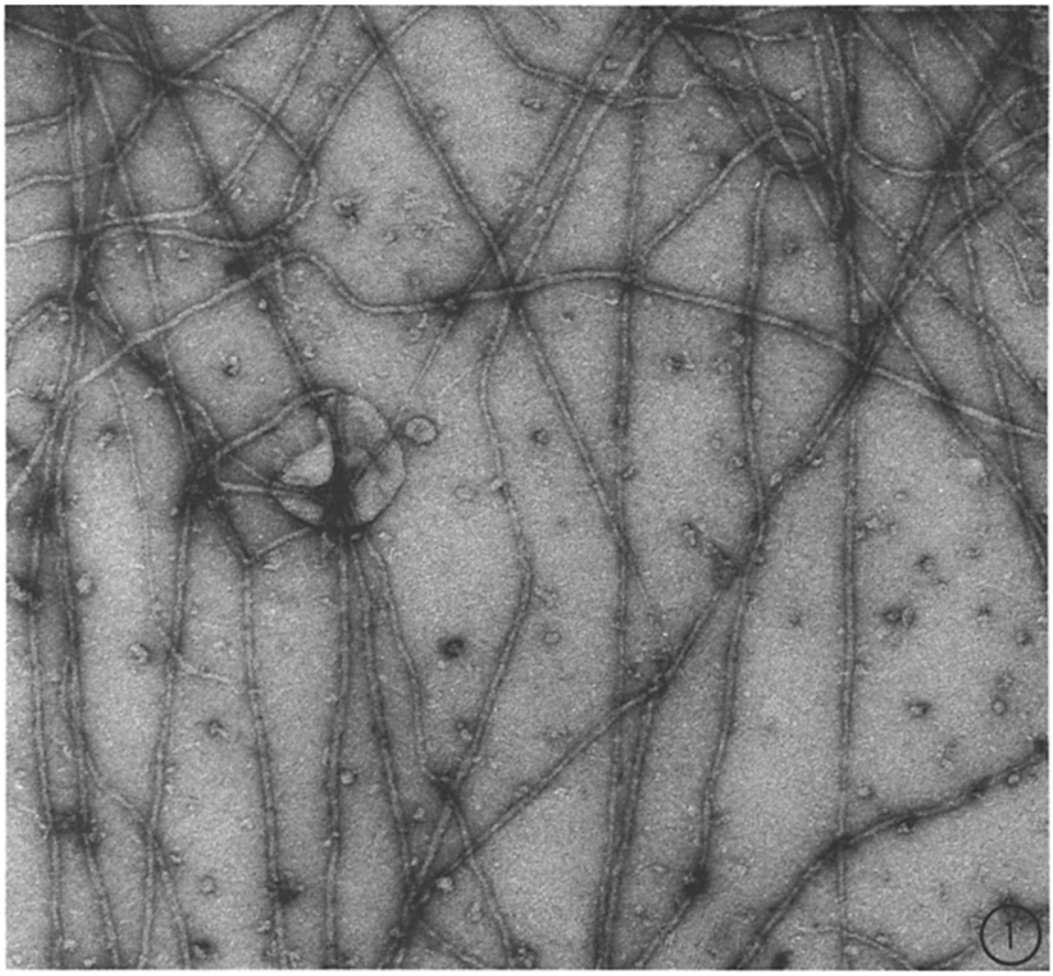

FIGURE 1 Numerous intact neurofilaments isolated from rat peripheral nerve by osmotic shock. Neurofilaments have a cylindrical profile without sidearm appendages. Most neurofilamentous profiles measure 80–110 Å in diameter, but thin and tapered segments can also be seen. Neurofilaments are admixed with globular profiles of irregular shape measuring 100–200 Å in diameter. Large particulate profiles can be seen to the left of center. Fixed preparation, negatively stained with uranyl acetate. × 130,000.

discontinuities, changes which were interpreted as artefacts resulting from lesser states of preservation. Similar neurofilament profiles were seen with fresh and fixed preparations using uranyl acetate or 1% phosphotungstic acid, pH 6.8, as staining media.

Neurofilaments were admixed with variable numbers of irregular globular profiles (Fig. 1). Most of these globules measured about 100–200 Å and had irregular shapes. They were usually randomly scattered over the grid but, occasionally, were arrayed linearly, in single-file fashion. Irregular globules were particularly conspicuous in preparations containing few intact neurofilaments.

Immune Electron Microscope Examination of Neurofilaments

Antigenic reactivity of neurofilaments was tested by examination of neurofilaments adherent to carbon-coated grids after their incubation in experimental and control antisera. Exposure of neurofilaments to experimental antisera or its IgG derivative caused a striking alteration in the appearance of neurofilamentous profiles. These neurofilaments became enveloped by a meshwork of fine lacy material which tended to obscure the visualization of the underlying neurofilamentous axial core (Fig. 2). Constituent elements of the

fine lacy material measured 30–50 Å in diameter. The aggregation of this material formed the meshwork which covered and extended from the lateral margins of the neurofilaments, thereby providing a total diameter of 500–700 Å for the decorated neurofilament. The fine lacy material also seemed to possess an increased affinity for uranyl acetate stain, thus imparting an enhanced electron density to the widened image of the decorated neurofilament. The resulting accentuation of the negatively stained image enabled the decorated neurofilament to be readily detectable at low magnification. Furthermore, the decoration of neurofilaments with experimental IgG was a very reproducible phenomenon, occurring widely and uniformly among different neurofilament preparations. The decoration of neurofilaments was consistently observed in fresh and fixed neurofilament preparations after 1–4-h incubations at 4°C or 23°C in concentrated or diluted samples of experimental antisera or IgG derivative. The same decoration of neurofilaments was observed using experimental antisera or IgG which had not been adsorbed with rat serum proteins.

Simultaneous control incubations of identical neurofilament preparations in the same concentrations of nonspecific rabbit antisera or IgG were always undertaken and consistently failed to show a similar decoration of neurofilaments (Fig. 3). Neurofilaments remained clearly outlined after exposure to control IgG. The background of these control preparations often contained a fine lacy material which was diffusely scattered over the grid and resembled the coating material of decorated neurofilaments. This fine lacy material of control preparations was not associated with accu-

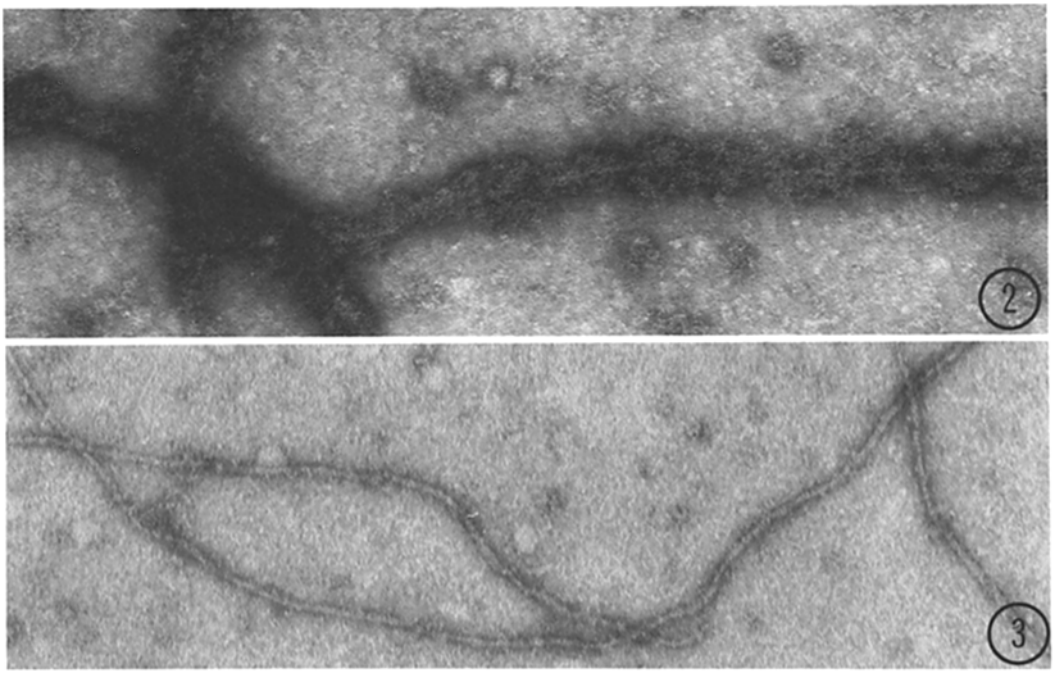

FIGURE 2 Exposure to experimental rabbit IgG has resulted in the envelopment of overlapping neurofilaments by a meshwork of fine lacy material with affinity for electron-dense uranyl stain. The resulting neurofilamentous images are broadened and accentuated, obscuring the visualization of the underlying neurofilamentous axial core. Components of the fine lacy meshwork measure 30–50 Å, while the decorated neurofilaments have a variable diameter of 500–700 Å. Fixed preparation, negatively stained with uranyl acetate. × 100,000.

FIGURE 3 Simultaneous exposure of identical neurofilament preparations to the same concentrations of control rabbit IgG did not alter or obscure the visualization of overlapping neurofilamentous profiles. A fine lacy material is present diffusely in the background which resembles the coating substance of decorated neurofilaments but also blends with the background granularity of the preparation. Fixed preparation, negatively stained with uranyl acetate. × 100,000.

mulated uranyl stain and was not always clearly differentiated from the background granularity inherent in negatively stained preparations (6).

The decoration of neurofilaments was always accompanied by a similar decorative alteration of small irregular globules which were scattered among the neurofilaments of negatively stained preparations. The decoration of irregular globules was less readily appreciated at high (Fig. 2) than at low magnification (see Discussion and Figs. 5 and 6). This unusual phenomenon was due to the fact that the decoration obscured the underlying globular profiles but marked their positions by the focal aggregations of fine lacy material which tended to accumulate uranyl stain. The resulting increases in size and electron density accentuated the presence of decorated globules in negatively stained preparations.

The negatively stained profiles of larger particulate or membranous components or the occasionally encountered microtubule or collagen fiber remained unaltered after incubation in experimental IgG.

Extraction of Peripheral Nerve Proteins

Evidence that the axonal constituency of peripheral nerve fibers could be extracted by osmotic shock was provided by previous studies in this laboratory (44). Ultrastructural examinations of finely minced segments of rat sciatic nerve exposed to low ionic strength media revealed swollen myelin sheaths with widely splayed lamellae which were associated with very little enclosed axonal or surrounding Schwann cell constituents. Quantitative protein analyses of media in which these nerve segments had been immersed disclosed that increasing amounts of protein were liberated into media of decreasing ionic strength. Furthermore, the peripheral nerve proteins liberated by osmotic shock became separated from the high lipid content of the nerve tissue which remained largely with the residue fraction.

The release of proteins from axonal sources would also account for the large amount of peripheral nerve protein liberated by osmotic shock. Approx. 0.6 mg/ml of protein was extractable from 20 mg/ml (wet weight) of desheathed and finely minced peripheral nerve. This represents about 40% of the total protein of desheathed peripheral nerve, as determined by NaOH digestion (my unpublished data). Most of the protein extracted by osmotic shock was liberated during the initial 10–20 min of exposure and was associated with a cloudy discoloration of the media. Differential centrifugation of the tissue extracts with an average force of 20,000 $g \times 30$ min and 150,000 $g \times 120$ min removed particulate and membranous constituents, producing clear supernates with protein concentrations of approx. 0.2 and 0.15 mg/ml, respectively.

Examination of Nerve Extracts by Negative Stain

Sequential examinations of the extracting media during exposure of nerve to osmotic shock were indicative of both an extensive liberation of neurofilaments from the tissues and the instability of the released neurofilaments in low ionic strength buffer. The latter phenomenon was evidenced by the progressive alterations of neurofilaments which occurred during incubation in 0.01 M Tris-HCl, pH 7.2. An extensive linear fragmentation of neurofilaments into irregular globular subunits was prevalent in samples taken during the initial 30-min extraction interval (Fig. 4). After 90-min incubations, almost all neurofilamentous arrays were dispersed, the irregular globular subunits were no longer aligned in linear assembly, but similar irregular globules were scattered throughout the preparation. The disseminated globules became indistinguishable from nonspecific particulate matter or even the background granularity of the negatively stained preparation.

Globular neurofilamentous subunits retained the antigenicity of the parent neurofilament, a feature which could be demonstrated by immune electron microscopy. Accordingly, incubations with experimental IgG served to visualize the neurofilamentous constituency of peripheral nerve extracted by osmotic shock. These preparations revealed a decorative coating which covered and surrounded intact neurofilaments and their globular subunits, whether in linear arrays or disseminated over the grid (Fig. 5). The decoration was characterized by a fine lacy material which appeared to possess some affinity for the uranyl stain, thereby imparting focal increases of electron density in the areas of decoration. The large numbers of decorated globular subunits of neurofilaments indicated an abundance of neurofilamentous components in low ionic strength extracts of peripheral nerve.

Similar decoration by experimental IgG using

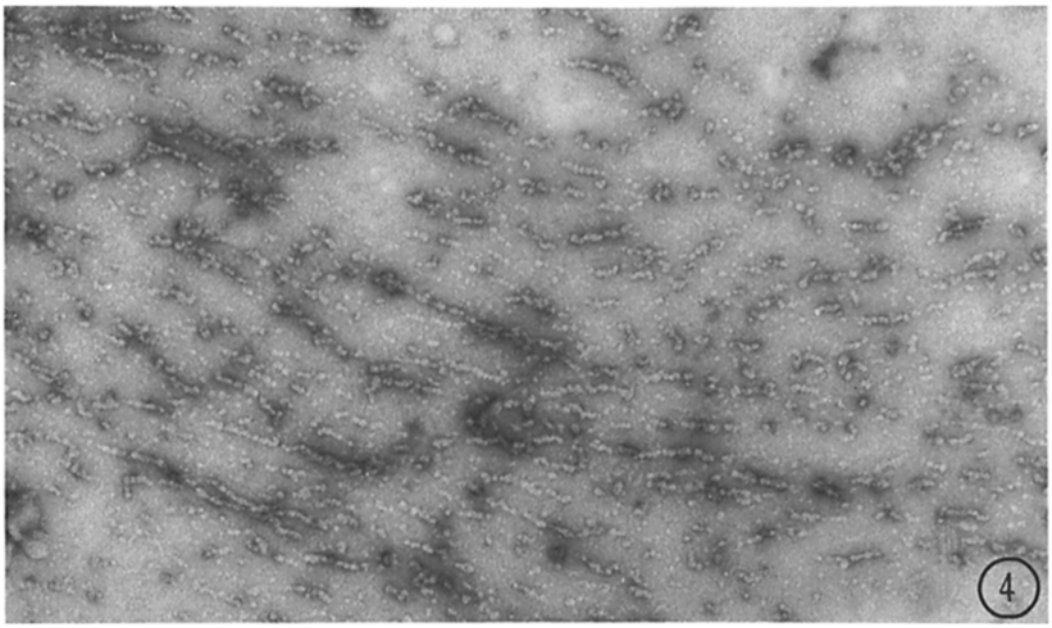

FIGURE 4 Instability of neurofilaments extracted from rat peripheral nerve by osmotic shock is evidenced by their linear fragmentation into globular subunits after 30-min incubation in 0.01 M Tris-HCl buffer, pH 7.2. The alignment of globules in linear arrays is suggestive of their neurofilamentous origin. Other irregular globules have identical profiles, but are haphazardly scattered over the grid. Fixed preparation, negatively stained with uranyl acetate. × 45,000.

immune electron microscopy was not associated with large membranous profiles, occasional collagen fibers, or infrequent microtubules (Fig. 6) in preparations from rat peripheral nerve extracts.

Electrophoresis of Extracted Nerve Proteins

Polypeptides which were released from peripheral nerve by osmotic shock and separated by differential centrifugation were analyzed by SDS-polyacrylamide slab gel electrophoresis (Fig. 7). Several major polypeptide bands were noted in nerve extracts and nerve homogenates which corresponded to those seen in published electrophoretic profiles of whole sciatic nerve homogenates (23, 35). A prominent constituent polypeptide band with a migrational rate of 68,000 daltons became progressively enriched in successive supernatant fractions (Fig. 7). Other polypeptide bands were separated from the 68,000-dalton moiety by differential centrifugation. A prominent broad polypeptide band with an Rf indicative of 28,000-dalton mol wt was largely sedimented at 20,000 g × 30 min, while a faint band which comigrated with tubulin was preferentially sedimented at 150,000 g × 120 min (Fig. 7).

The high-speed supernates (S3 and S3′) of nerve extracts revealed electrophoretic profiles which were dominated by a single polypeptide band of 68,000-dalton migrational rate (Fig. 8). Similar banding patterns were noted in electrophoretic profiles of S3 and S3′ fractions carried out on different SDS-polyacryamide systems, including the phosphate buffer method of Weber and Osborn (54), and using Tris-glycine buffer containing 8 M urea. Minor polypeptide bands of the S3 electrophoretic profile were more difficult to characterize since they were often faint and varied somewhat among different preparations. The minor polypeptide bands of the high-speed supernates were reduced, but not eliminated, when nerve extractions were performed under very dilute conditions, yielding a modified supernatant fraction (S3′). Densitometric tracings of electrophoretic profiles from standard (S3) and modified (S3′) supernatant fractions revealed that approx. 70% and 90% of Coomassie blue-staining constituency of the gels were contained in the 68,000-dalton polypeptide bands, respectively.

The 68,000-dalton polypeptide in high-speed supernate (S3) of peripheral nerve extracts was separated by elution from polyacrylamide gels.

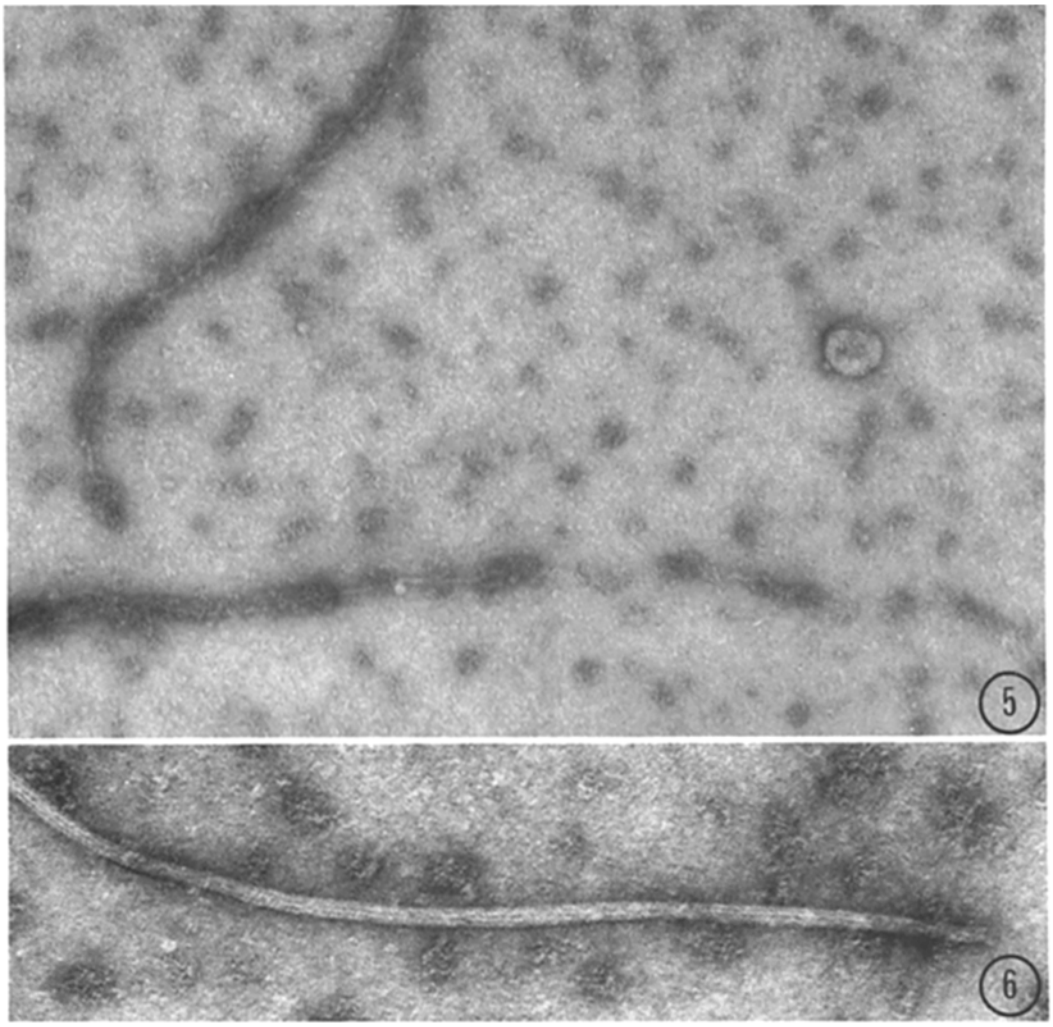

FIGURE 5 Localization of antigenic sites reactive with experimental antisera in osmotically shocked nerve extracts, after incubation of grid content with experimental IgG. Antigenic reactivity is characterized by a decorative coating consisting of fine lacy material with increased affinity for electron-dense uranyl stain which covers and largely obscures the decorated structures. Neurofilaments, their fragmented terminals, and disseminated structures with the distribution of irregular neurofilamentous globular subunits appear to be decorated. Fixed preparation, negatively stained with uranyl acetate. × 60,000.

FIGURE 6 Preparation identical to that in Fig. 5 containing a microtubule. The microtubule is not decorated by experimental antisera; its profile remains clearly discernible, even its protofilaments can be visualized. The decoration of scattered globules in the background consists of fine lacy material associated with focal increases of electron-dense stain. Fixed preparation, negatively stained with uranyl acetate. × 170,000.

Most of the recovered polypeptide reproduced the original migrational pattern upon re-electrophoresis (Fig. 9). In addition, minor polypeptide bands appeared with Rfs corresponding to apparent mol wt of 47,000 and 20,000 daltons. Reelectrophoresis of gel-eluted fractions of BSA simulated the original electrophoretic profile of this protein (Fig. 9).

Studies of Antibody Absorption by Components of Nerve Extracts

A series of incubations were conducted on iden-

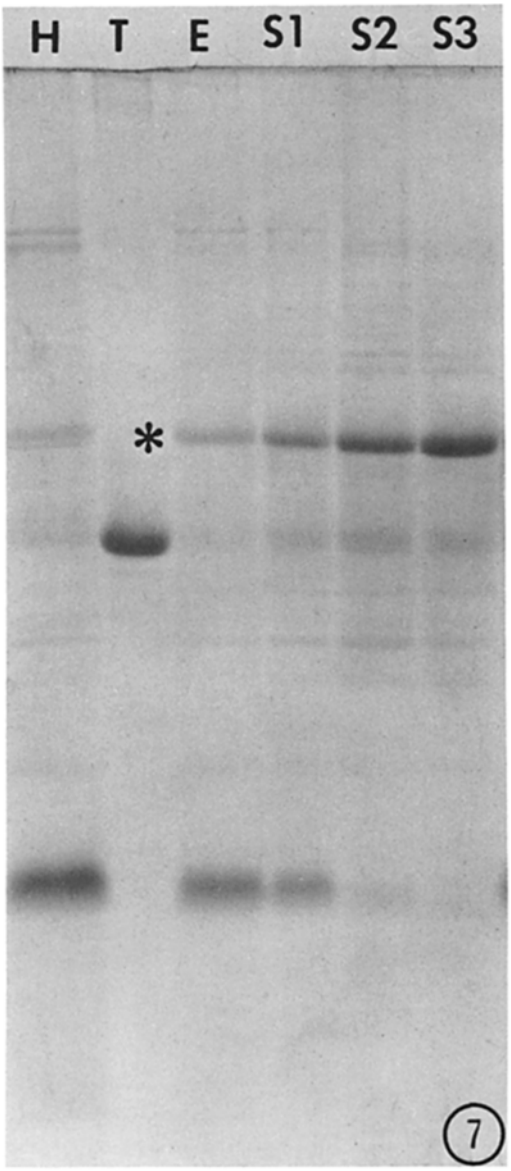

FIGURE 7 SDS-polyacrylamide slab gel electrophoretic profile of polypeptides in extract (*E*) of osmotically shocked rat peripheral nerve and in successive supernatant fractions (*S1*, *S2*, and *S3*) after centrifugation of extract at approx. 100 *g* × 10 min, 20,000 *g* × 30 min and 150,000 *g* × 120 min, respectively. Enrichment of 68,000-dalton polypeptide (*) in successive supernatant fractions is evidenced by the increasing percentage of stain occurring in this band. Each sample contained 5 μg of protein. Adjacent samples were comprised of 5 μg of purified porcine brain tubulin (*T*) and 10 μg of rat peripheral nerve homogenate in 0.01 M Tris buffer (*H*). Faint bands comigrating with tubulin appear in nerve fractions, but the enrichment of this band in nerve supernate was less than that of 68,000-dalton polypeptide. Prominent polypeptide band at approx. 28,000-dalton migrational level in *H*, *E*, and S1 is probably a myelin protein.

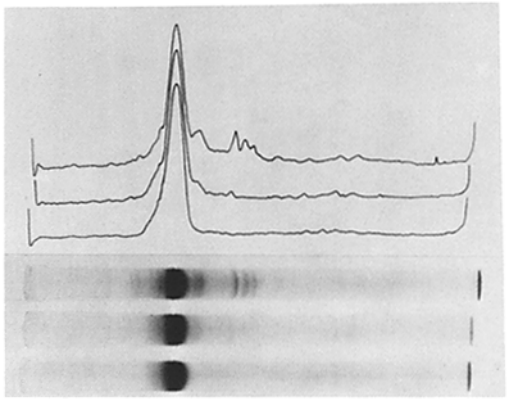

FIGURE 8 SDS-polyacrylamide gels and densitometric tracings of high-speed supernatant fractions (S3 and S3′) of nerve extracts and BSA, demonstrating predominance of single polypeptide band. Equal admixture of S3′ and BSA samples (bottom gel and tracing) produces a co-migrational peak, corresponding to the 68,000-dalton mol wt of BSA. Approx. 90% of the polypeptide constituency of the S3′ fraction (middle gel and tracing) was localized to the 68,000-dalton peak. Minor banding patterns were more apparent in S3 fractions (top gel and tracing). The top of the gels is to the left, producing initial tracing deflections. The terminal deflection of tracings is representative of small molecular weight components which migrated immediately behind the tracking dye.

tical neurofilament preparations, using antibody absorption techniques to prevent decoration of neurofilaments incubated in experimental IgG. This ability to prevent the decoration of neurofilaments was used as a measure of shared antigenicity between neurofilaments and the test substances. High-speed supernatant fractions (S3) of nerve extracts consistently prevented the decoration of neurofilaments, as exemplified by the absence of neurofilamentous coating which occurred upon the addition of S3 protein (0.1 mg/ml) to standard incubations of neurofilament preparations in experimental IgG (Fig. 10). Identical results were obtained when standard incubations containing experimental IgG were admixed with modified high-speed supernatant fractions (S3′), or with S3 proteins eluted from DEAE-cellulose or passed through a Sephadex G-100 column to remove nonprotein contaminants.

Control absorption experiments were simulta-

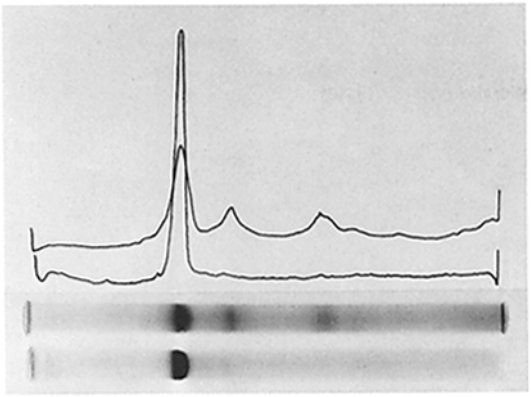

FIGURE 9 SDS-polyacrylamide gels and densitometric tracings of 68,000-dalton polypeptide bands which had been eluted and recovered from gels of the S3 fraction and BSA. Most of the recovered 68,000-dalton polypeptide of the S3 fraction (top gel and tracing) re-electrophoresed at the same migrational rate; however, additional bands appeared with Rfs corresponding to polypeptides with apparent mol wt of 47,000 and 20,000 daltons. Small molecular weight breakdown products can be seen at the end of the gel and are represented by the terminal deflection in the tracing. Recovered BSA (bottom gel and tracing) reproduced the original electrophoretic pattern. The BSA peak overlaps and coincides with the 68,000-dalton peak of the S3 fraction.

neously conducted on identical neurofilament preparations incubated in experimental IgG containing other test additives. Neither tubulin (0.5 mg/ml) nor BSA (0.5 mg/ml) was able to prevent the decoration of neurofilaments. The typical neurofilament decoration in the presence of tubulin is illustrated in Fig. 11.

Additional antibody absorption experiments were undertaken to define more precisely the component of the S3 fraction which prevents decoration of neurofilaments. Accordingly, the ability to prevent the decoration of neurofilaments was localized to the 68,000-dalton polypeptide which had been eluted and recovered from polyacrylamide gels. Re-electrophoresis of this fraction reproduced the 68,000-dalton polypeptide band (Fig. 9), and the addition of this fraction (0.1 mg/ml) to experimental IgG generally prevented the decoration of neurofilaments (Fig. 12). The "de-decorative" capacity of the gel-eluted polypeptide fraction was less than that of the parent S3 fraction at a similar concentration. It is possible that some loss of antigenicity accompanied the partial breakdown of this polypeptide which occurred during its recovery and was exemplified by the appearance of subunit polypeptides on re-electrophoresis (Fig. 9). Control absorption experiments were also conducted with BSA which had been eluted and recovered from polyacrylamide gels in parallel with that of the 68,000-dalton polypeptide of the S3 fractions. A normal decoration occurred around neurofilaments exposed to experimental IgG admixed with gel-eluted BSA (Fig. 13). This control experiment indicated that the decoration of neurofilaments was not altered by factors other than the polypeptide constituency of eluted fractions.

DISCUSSION

The techniques of immune electron microscopy have been utilized in these experiments to demonstrate the presence of antigenic substances against neurofilaments in high-speed supernatant fractions of nerve extracts obtained from osmotically shocked segments of rat peripheral nerve. The specificity of the immune reactions was established by standard immunological controls. An absence of detectable reactivity between neurofilaments and nonspecific rabbit antisera indicated that the decorative coating of neurofilaments in immune electron microscopy resulted from the interaction of specific antibodies of experimental antisera. Furthermore, the prevention of neurofilamentous decoration by absorption techniques provided additional documentation of the underlying antigen-antibody nature of this reaction and served to confirm the presence of neurofilamentous antigenic substance(s) within high-speed supernates of rat peripheral nerve extracts.

The 200–300 Å decorative coating of neurofilaments closely resembled the antibody coating of viruses which have been visualized by immune electron microscopy (1, 2, 16, 37). Individual components of the latticework measured in the 30–50 Å range, corresponding to the dimensions of the IgG molecules in negatively stained imagery (2, 18, 52). Neurofilament coatings also revealed an apparent increased affinity for unbuffered uranyl acetate stain. A similar feature of IgG molecules has been exploited to enable their visualization at the ultrastructural level (50, 51).

Neurofilamentous antigenic sites reactive against experimental antisera were arrayed in linear continuity along the external aspect of intact neurofilaments. These antigenic determinants remained on the external surface of globular neurofilamentous subunits as well as on the exterior of subunit particles present in high-speed supernates of nerve extracts used to raise the antisera. This

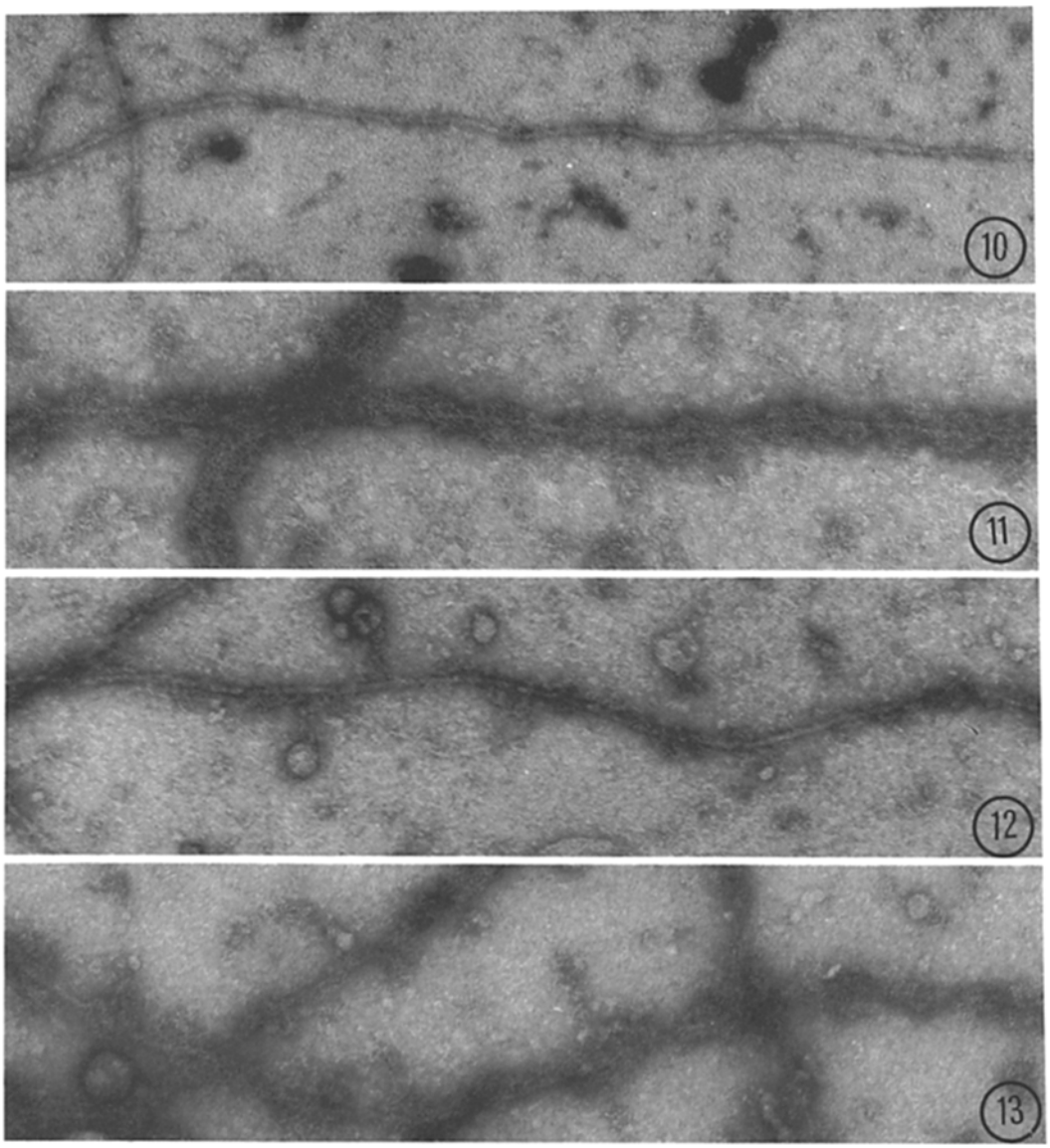

FIGURE 10 The decoration of these overlapping neurofilamentous profiles was prevented by the addition of S3 protein fraction (0.1 mg/ml) to the media containing experimental rabbit IgG in which they were incubated. Successful absorption of antibody decoration was also achieved with S3′ fractions and with S3 proteins eluted from Sephadex and DEAE-cellulose columns. Fixed preparations, negatively stained with uranyl acetate. × 80,000.

FIGURE 11 The addition of tubulin (0.5 mg/ml) to the experimental rabbit IgG media in which these overlapping neurofilaments were incubated did not prevent their envelopment with a decorative coating. In control absorption experiments, the use of BSA also failed to prevent neurofilamentous decoration. Fixed preparations, negatively stained with uranyl acetate. × 80,000.

FIGURE 12 The visualization of this neurofilament profile was preserved, and its decoration by experimental rabbit IgG was largely prevented by antibody absorption with 68,000-dalton polypeptide (0.1 mg/ml) of the S3 fraction which had been eluted and recovered from SDS-polyacrylamide gels. Some focal aggregates of fine lacy material can be seen both adjacent to and overlying this neurofilamentous profile, suggesting that the "dedecorative" capacity of gel-eluted 68,000-dalton polypeptide is less than that of the parent protein fraction at the same concentration. Fixed preparation, negatively stained with uranyl acetate. × 80,000.

FIGURE 13 Characteristic neurofilament decorations surround and obscure these overlapping neurofilaments, which were incubated in experimental rabbit IgG containing BSA (0.1 mg/ml) eluted and recovered from polyacrylamide gels in parallel with the 68,000-dalton polypeptide of the S3 fraction. This control experiment indicated that the prevention of neurofilamentous decoration by the 68,000-dalton polypeptide of the S3 fraction (Fig. 12) was due to the polypeptide constituency of the gel-eluted fraction. Fixed preparation, negatively stained with uranyl acetate. × 80,000.

pattern is consistent with that of a relatively simple and repetitive sequence of antigenic determinants, which may be derived from a commonly exposed area of polypeptide constituency. The degree of immunological heterogeneity among these antigenic sites is, of course, unknown. Nevertheless, the general efficacy in the prevention of neurofilament decoration by absorption with different supernatant fractions of nerve extracts is indicative of a relative homogeneity of antigenic determinants.

A sharing of antigenic sites along their external surfaces supports the view that globules represent a breakdown subunit of the axial component in neurofilaments. This interpretation was also supported by the observed linear fragmentation of neurofilaments into globular subunits during exposure of neurofilaments to low ionic strength media (Fig. 4). Similar globules in negatively stained preparations of invertebrate neurofilaments were believed to represent condensations of detached neurofilamentous sidearms (22). It has been suggested that neurofilamentous sidearms are the sites of myosin-like proteins associated with neurofilaments (24). These lateral appendages along the neurofilaments have been defined from ultrastructural observations (41, 56, 58), but they are not apparent in negatively stained preparations of neurofilaments (13, 28), possibly due to insufficient preservation during isolation procedures.

Evidence for the liberation of neurofilaments from osmotically shock segments of peripheral nerve was initially based on morphological observations which indicated a marked depletion of these organelles in tissues treated in this manner (44). The inability to demonstrate significant numbers of intact neurofilaments in extracts of osmotically shocked nerve segments was problematic at first, particularly in view of the reported stability of invertebrate (28) and vertebrate (13, 46) neurofilaments in low ionic strength media. The present demonstration of the rapid breakdown of mammalian neurofilaments during osmotic shock attests to their fragility in dilute concentrations in media of low ionic strength at neutral pH. In such a dissembled state, they could be separated from particulate and membranous components in tissue extracts by high-speed centrifugation. A tendency for dissociated neurofilament components to remain in high-speed supernates was evidenced by the findings that dissociated *Myxicola* neurofilaments were retained in supernates at 250,000 *g* for 1.5 h in 0.75 M KCl and could be reassembled by dialysis against 0.1 M KCl (22).

The marked enrichment of the 68,000-dalton polypeptide band in high-speed supernates of nerve extracts was somewhat surprising, considering the probable heterogeneity of proteins released from the tissues by osmotic shock. Some proteins of higher molecular weight, including the dimeric subunit of tubulin, may have been sedimented because of their lesser inherent buoyancy. Other polypeptides which were differentially precipitated may have been derived from heavier particulate or membranous components. On the other hand, it is quite possible that surface adhesive forces between protein moieties resulted in the retention of some of the sedimented proteins or polypeptides in high-speed supernates, a phenomenon which may have accounted for the more homogeneous yields of 68,000-dalton polypeptide in high-speed supernatant fractions of nerve (S3′) which had been extracted into very dilute media. It is also possible that the enrichment of 68,000-dalton polypeptide in isolates of dilute media could have resulted from a milieu which favored the breakdown of larger neurofilamentous components into 68,000-dalton moieties or retarded the disruption of this entity into smaller polypeptide units.

The presence of antigenic determinant(s) of neurofilaments in high-speed supernatant fractions of nerve extracts focused particular interest on the compositional nature of this fraction. The prevalence of 68,000-dalton polypeptide in these fractions provided the likelihood, but no assurance, that neurofilamentous antigenic site(s) were located within this moiety. More substantive identification of neurofilamentous antigen(s) within high-speed supernatant fractions was achieved through antibody absorption experimentation. All absorbants which successfully prevented neurofilament decoration revealed a common predominance of the 68,000-dalton polypeptide band in their electrophoretic profile, whether they were derived directly from high-speed supernates (S3 and S3′) or after recovery of supernatant fractions from DEAE-cellulose or from Sephadex G-100. A more definitive identification of neurofilamentous antigenicity with the 68,000-dalton polypeptide of high-speed supernates was provided by the direct demonstration of antibody absorptive capacity within this polypeptide fraction which had been eluted and recovered from polyacrylamide

gels. The possibility that neurofilamentous antigen(s) were shared by other polypeptides in the S3 fraction could not be excluded. In fact, the presence of smaller polypeptide subunits upon re-electrophoresis of the gel-eluted 68,000-dalton polypeptide (Fig. 9) is indicative of breakdown of this neurofilamentous constituent and raises the question of compositional interrelationship between these and other minor bands seen in electrophoretic profiles.

The identification of neurofilamentous antigenicity of 68,000-dalton polypeptide and the localization of this antigenicity to repetitive surface sites along intact neurofilaments strongly suggestive that this polypeptide is a major constituent of the axial component of mammalian neurofilaments. These findings are supportive of the polypeptide composition of neurofilaments proposed by Hoffman and Lasek on the basis of their analysis of slow axonal flow in rabbit and cat sciatic nerves (24). They found that the radioactivity within the slow-moving wave of labeled axonal protein was localized to five electrophoretic polypeptide bands, corresponding to apparent mol wt of 212,000 160,000, 68,000, 57,000, and 53,000 daltons. The latter two polypeptides were identified as tubulin α and β, confirming previous indications that microtubules partake in slow axonal flow (32, 34, 40). The three larger polypeptides were constitutive, suggesting that they comprise an additional axonal structural component which was tentatively identified as neurofilaments. Among these three polypeptides, the 68,000-dalton moiety was the predominantly labeled species and was characterized by the most intense staining electrophoretic band. A polypeptide of similar molecular weight appeared as the predominant component of neurofilament preparations isolated from squid giant axons (28, 42). Furthermore, electrophoresis of reconstituted neurofilaments from the giant axons of the seaworm, *Myxicola*, yielded multiple polypeptide bands, including a prominent band at the 65,000–68,000 dalton migrational level which became considerably enriched when the filament preparations were exposed to calcium (22). This latter finding is indicative of both a multiplicity and a lability of neurofilamentous polypeptide constituency which may also be dependent upon the conditions prevailing during isolation.

There is additional evidence that the 68,000-dalton neurofilament subunit may represent one of several polypeptides derived from a larger neurofilamentous moiety. Axonal and neurofilament preparations from mammalian brain tissues yield a multiplicity of electrophoretic bands (13, 15, 31, 45, 46, 49). Some interconversion between these bands is evidenced by the generation of both lower (15, 45) and higher (13) molecular weight polypeptides after selective elution and re-electrophoresis of high and low molecular weight polypeptide bands, respectively. Several of the constituent polypeptide bands of axonal preparations show similar amino acid compositions (15). Furthermore, the relative proportion of these polypeptides may be dependent upon the isolation conditions (15). While a polypeptide with an approximate 68,000-dalton migrational rate has been consistently noted or depicted in electrophoretic profiles of axonal and neurofilament preparations of mammalian brains (13, 15, 31, 45, 46, 59), the most prominent polypeptide of these preparations has a migrational rate between 47,000 and 54,000 daltons. Both morphological (15, 59) and immunological (3) evidence indicate that some of the polypeptides within this prominent band may represent the product of glial filaments which co-purify with axonal neurofilaments and have a constituent polypeptide of similar molecular weight. A polypeptide of 47,000–54,000 dalton mol wt is not a constituent of labeled slow axonal transport in neurofilament-rich mammalian peripheral nerve (24), nor is such a polypeptide seen in electrophoretic profiles or pure axoplasmic samples obtained from giant axons of squid (42) or *Myxicola* (22). Yet, the appearance of a minor polypeptide band of this migrational rate after selective elution and re-electrophoresis of the 68,000-dalton neurofilamentous polypeptide from peripheral nerve (Fig. 9) suggests that a polypeptide of this size represents a product of neurofilament breakdown.

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