Isolation and Characterization of Neurofilaments from Mammalian Brain†

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ABSTRACT: A procedure is described for rapid isolation of neurofilaments in large quantity from mammalian brain, and evidence is presented to establish their identity with the neurofilaments of peripheral nerve. Neurofilaments were purified from high-speed supernatants of bovine and rat brain by differential centrifugation combined with gel filtration on Bio-Gel A-150m. Their protein composition was examined by sodium dodecyl sulfate gel electrophoresis. The major polypeptides of neurofilaments from bovine brain had molecular weights of 210 000, 160 000, 69 000, and 55 000, while those of neurofilaments from rat brain had molecular weights of 200 000, 145 000, 68 000, and 55 000. The largest three of these polypeptides in each species appear to correspond to the "triplet" of proteins that are components of neurofilaments [Lasek, R. J., & Hoffman, P. N. (1976) *Cold Spring Harbor Conf. Cell Proliferation 3*, 1021–1049; Micko, S., & Schlaepfer, W. W. (1978) *J. Neurochem. 30*, 1041–1049]. The smallest was found to be indistinguishable from tubulin in amino acid composition and in electrophoretic mobility under several conditions. No protein with properties similar to those of glial filament proteins was seen. Antiserum against peripheral nerve neurofilaments was used to test the identity and homogeneity of the brain neurofilaments. Reaction with antibody was assessed by electron microscopic examination of negatively stained filaments that had first been incubated with antiserum. The brain neurofilaments reacted uniformly with antiserum. After the antiserum had been preabsorbed with peripheral nerve neurofilaments, the brain neurofilaments showed no reaction. It was concluded that the preparation yields authentic neurofilaments from brain in large quantity.

In mammalian nervous tissue, two prominent classes of intermediate filaments have been identified, neurofilaments and glial filaments (Goldman et al., 1978; Liem et al., 1978; Schlaepfer & Freeman, 1978; Wuerker, 1970). They are morphologically similar (Wuerker, 1970), and until recently major disagreement existed concerning their protein compositions (Davison & Hong, 1977; Davison & Winslow, 1974; Goldman et al., 1978; Iqbal et al., 1977; Yen et al., 1976). Early biochemical studies from several laboratories seemed to show both glial filaments and mammalian brain neurofilaments to consist primarily of a single polypeptide of approximately 47 000–51 000 molecular weight (Davison & Hong, 1977; Davison & Winslow, 1974; Goldman et al., 1978; Iqbal et al., 1977; Liem et al., 1977; Schook & Norton, 1975). In constrast, mammalian neurofilaments from peripheral nerve were shown to consist of three major polypeptides with subunit molecular weights of approximately 210 000, 160 000, and 68 000 (Hoffman & Lasek, 1975; Lasek & Hoffman, 1976). It has recently been demonstrated that neurofilaments from mammalian brain and peripheral nerve are in fact very similar to each other, each consisting primarily of three polypeptides of 210 000, 160 000, and 68 000 molecular weight in human and bovine systems (Lasek & Hoffman, 1976; Liem et al., 1978) and of 200 000, 145 000, and 68 000 molecular weight in rabbit (Liem et al., 1978) and rat (Micko & Schlaepfer, 1978; Schlaepfer & Freeman, 1978; Schlaepfer & Micko, 1978). It is not known at present which of these proteins are essential structural proteins of the neurofilaments, nor is it known which of the numerous minor proteins that accompany the major triplet are true constituents of the neurofilament.

Present published methods (Liem et al., 1978; Schlaepfer, 1977a) allow neurofilaments to be isolated from peripheral nerve, but they are too tedious and time consuming to yield the tens of milligrams of protein required for many biochemical analyses. Isolation from brain has been hindered by the difficulty of establishing the identity of the isolated filaments. We report here a rapid procedure for isolation of large quantities (~80 mg per preparation) of filaments from mammalian brain. Examination of these filaments shows that they are morphologically, immunologically, and biochemically very similar to neurofilaments prepared by others from mammalian peripheral nerve (Liem et al., 1978; Micko & Schlaepfer, 1978; Schlaepfer & Freeman, 1978; Schlaepfer & Micko, 1978) and leads to the conclusion that they are authentic neurofilaments. In the accompanying paper (Runge et al., 1981), we describe the properties of a protein kinase that is firmly associated with these neurofilaments from brain. A phosphodiesterase has also been described (Runge et al., 1979b) that is associated with these neurofilaments from brain. Parts of this work have been presented in preliminary form (Runge et al., 1978), and a simpler version of this preparation has been described (Runge et al., 1979a).

Materials and Methods

Pipes,[1] EGTA, GTP, colchicine, and DTE were obtained from Sigma Biochemicals, St. Louis, MO. Bio-Gel A-150m and pH 3–10 ampholines (Bio-Lyte) were from Bio-Rad Laboratories, Richmond, CA. Sephadex G-25 was from Pharmacia, Inc., Piscataway, NJ, and urea ("Ultrapure" grade) was from Mann Research Laboratories, New York.

All experiments involving neurofilaments isolated from bovine or rat brain were performed in 0.1 M Pipes (pH 6.9), 2 mM EGTA, 1 mM $MgSO_4$, and 2 mM DTE (referred to as PM buffer) at 4 °C, unless otherwise specified. Experiments

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[1] Abbreviations used: anti-NF serum, rabbit antiserum raised against rat peripheral nerve neurofilaments; DTE, dithioerythritol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; IgG, γ-globulin; GTP, guanosine 5′-triphosphate; PC, phosphocellulose; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

0006-2960/81/0420-0170$01.00/0

involving rat peripheral nerve neurofilaments were performed in 0.1 M NaCl, 2.5 mM EDTA, and 2.5 mM EGTA at 25 °C or at 4 °C, as specified. Protein concentrations were measured by the method of Bradford (1976). Phosphocellulose-purified tubulin (PC-tubulin) was prepared by a modification of the method of Weingarten et al. (1975), as described by Detrich & Williams (1978). Amino acid analyses were performed, on duplicate samples, by A.A.A. Laboratories, Seattle, WA.

Isolation of Neurofilaments from Peripheral Nerve. Neurofilament-enriched extracts were obtained from saline-perfused, desheathed, osmotically shocked, and minced rat peripheral nerve tissues as previously described (Schlaepfer, 1977a) with the following modifications. All steps subsequent to the removal of the intact peripheral nervous tissue were performed at 4 °C. Additional purification of the neurofilaments was obtained by resuspending them by gentle homogenization in 0.1 M NaCl, 2.5 mM EDTA, 2.5 mM EGTA, and 0.5 M sucrose, in a Dounce homogenizer, and then subjecting them to centrifugation at 95500*g* in a Beckman T35 rotor for 90 min. Pelleted neurofilaments were resuspended into the same medium, but without sucrose, in a Dounce homogenizer and stored at −30 °C.

Isolation of Neurofilaments from Brain. Fresh brain tissue was cooled on ice, meninges were carefully removed, and the tissue was minced with surgical scissors. This minced tissue was homogenized at 4 °C with PM buffer, in the ratio of 1 g of tissue to 0.75 mL of buffer, in a Sorval Omni-Mixer for 50 s at speed 3 and 10 s at speed 9. The homogenate was subjected to centrifugation at 6000*g* for 15 min. The supernatant was removed and subjected to centrifugation at 95500*g* for 75 min to remove particulate matter. The resulting high-speed supernatant (40–50 mL, containing ~1 g of total protein) was applied to a 5 × 40 cm column of Bio-Gel A-150m and eluted with PM buffer at a flow rate of ~15 mL/h. Void volume fractions were pooled and centrifuged in a Beckman T35 rotor at 35000 rpm (95000*g*) for 75 min to pellet the neurofilaments. Pellets were resuspended in ~6 mL of PM buffer by 10–15 strokes of a Dounce homogenizer. The suspension was layered on top of 50 mL of PM buffer containing 20% sucrose in a thick-walled T-35 centrifuge tube. The mixture was centrifuged at 35000 rpm for 90 min to pellet neurofilaments. A hazy layer was visible at the top of the supernatant and was discarded. The pellet was resuspended into PM buffer by means of a Dounce homogenizer, and the suspension was frozen dropwise in liquid nitrogen. Before use, neurofilaments were rapidly thawed and centrifuged for 10 min at top speed in an International clinical centrifuge to remove small amounts of aggregated material. The yield obtained was 50–80 mg of purified neurofilament protein from a single bovine brain.

Electron Microscopy. Samples for electron microscopy were applied to freshly carbon-coated collodion-covered grids and negatively stained. One drop of sample was deposited on a grid and allowed to stand for 30 s. Excess solution was drawn off with filter paper and several drops of 1% aqueous uranyl acetate were pipetted onto the grid. The last drop was absorbed with filter paper, and the grid was allowed to dry in air. Samples were examined with a Hitachi HU-11B electron microscope.

Immunological Studies. Immunological investigation of the isolated neurofilaments was carried out by the electron microscopic methods described previously (Schlaepfer, 1977b). Rabbit antiserum to an extract of rat peripheral nerve neurofilaments was used as a source of anti peripheral nerve neurofilament IgG, and preimmune serum was used as a source of control IgG. The primary protein in the immunizing preparation was the 68000 molecular weight protein of the neurofilaments. Neurofilaments were applied to an electron microscope grid, fixed with 5% formalin, and then incubated with IgG by floating the grid for 4 h at 4 °C on a 0.25 mg/mL IgG solution. Incubations with preabsorbed IgG were carried out by mixing the IgG with either rat peripheral nerve neurofilaments, rat brain neurofilaments, or bovine brain neurofilaments before floating the grid. After incubation, grids were negatively stained and examined in the electron microscope.

Polyacrylamide Gel Electrophoresis. $NaDodSO_4$–polyacrylamide gel electrophoresis was performed according to Laemmli (1970) on 7.5% cylindrical or slab gels. Gels were stained with Coomassie Brilliant Blue R (Detrich & Williams, 1978). For separation of α- from β-tubulin, samples were reduced and alkylated according to Crestfield et al. (1963) and subjected to electrophoresis by the method of Yang & Criddle (1970) on 7.5% slab gels. $NaDodSO_4$–urea–polyacrylamide gradient slab gel electrophoresis was carried out as described by Kim et al. (1979). This method is similar to that described by Laemmli (1970) except that the gels contained a 4–16% gradient of polyacrylamide concentration, a 1–8 M gradient of urea, and no $NaDodSO_4$ in either the stacking or the running gel. Estimates of the molecular weights of filament proteins were made from the positions of molecular weight standards: rabbit muscle myosin (212000), β-galactosidase (116000), bovine serum albumin (66500), catalase (58000), phosphocellulose-purified tubulin (55000), and ovalbumin (43000).

Results

Isolation of Neurofilaments. Results of gel filtration of the high-speed supernatant of bovine brain are shown in Figure 1. The first peak to emerge (indicated by the arrow in Figure 1A) contains ~10% of the total protein and is visibly turbid. Since its elution position corresponds to the void volume of the column, it must contain primarily those structures with molecular weights > 150 million, the exclusion limit of the gel. Its protein composition is different from that of the included peak, as shown in Figure 1B. The void volume peak is substantially enriched in proteins of molecular weight 55000 and above. Although numerous other polypeptides are present, those corresponding to the neurofilament triplet and tubulin are prominent in fractions 5, 6, and 7.

Figure 2A is an electron micrograph of a negatively stained sample of the pooled void volume peak. Numerous filamentous structures are seen, which have the typical appearance of isolated neurofilaments. In addition, substantial amounts of globular material, both densely stained and stained in outline, are seen. Figure 2B shows the preparation after its sedimentation in the sucrose-containing buffer. It is evident that the amount of globular material is greatly reduced, although not completely eliminated, by this step. The inset to Figure 2B shows a short length of neurofilament at high magnification. An appearance of a twisted structure is evident. Comparison of the images in Figure 2B with those of known neurofilaments, as reported by Schlaepfer (1977a), reveals no discernable differences in apparent diameter or in general appearance and staining characteristics. The one qualitative difference observed was that these filaments were shorter than those prepared from peripheral nerve, probably due to different degrees of breakage accompanying the two preparative methods.

Protein Composition. The protein composition of neurofilaments, after the final sedimentation step, is shown in Figure

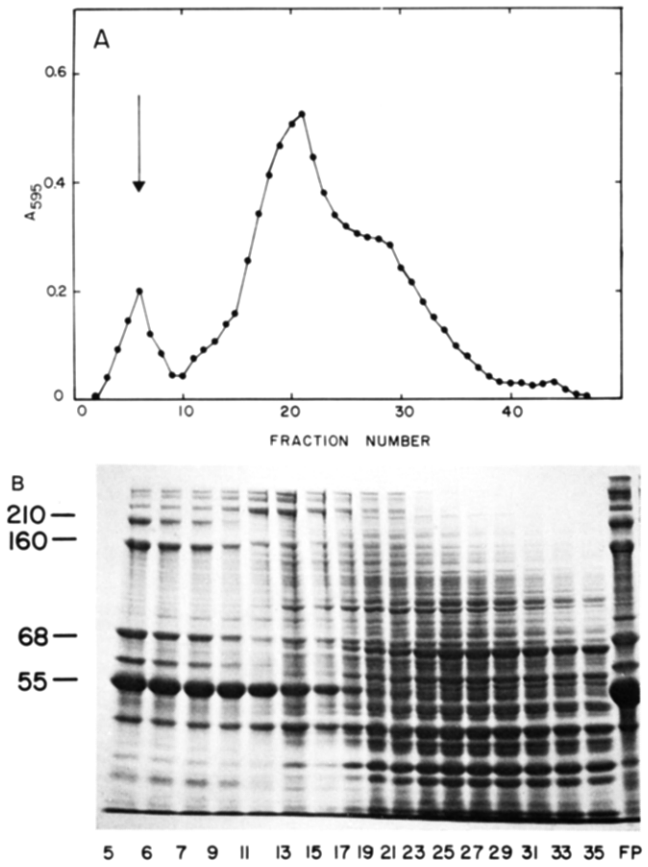

FIGURE 1: Isolation of bovine brain neurofilaments by gel filtration. Supernatant from the high-speed extract of brain homogenate (15 mL at ~20 mg/mL) was eluted from a column (2.5 × 30 cm) of Bio-Gel A-150m. The column profile is shown in (A). An aliquot of each sample was diluted 10-fold for protein assays. One unit of absorbance corresponds to a protein concentration of ~11 mg/mL. (B) Fractions from the column described in (A) were separated on a 7.5% $NaDodSO_4$–polyacrylamide slab gel. Fraction numbers are shown.

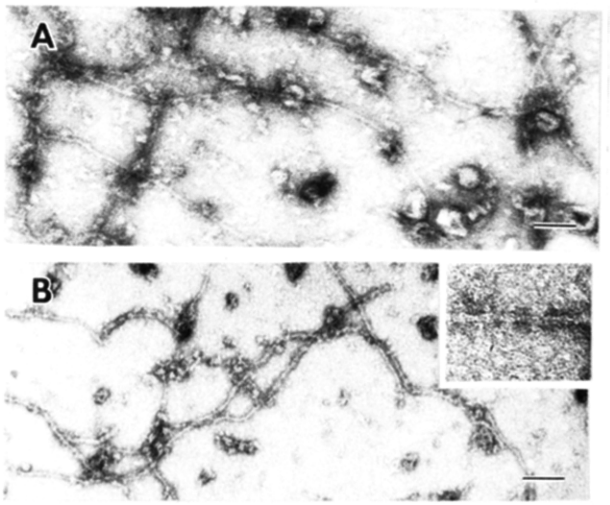

FIGURE 2: Electron micrographs of negatively stained neurofilament preparations from bovine brain. (A) Neurofilament preparation after gel filtration on Bio-Gel A-150m but before further purification. Note membranous contamination. Bar = 0.1 μm. (B) The same preparation after further purification by differential centrifugation. Bar = 0.1 μm. (Inset) Single neurofilament profile at 3.5-fold higher magnification.

3. Tubulin is shown as a control in lane A, neurofilaments from bovine brain are in lane B, and neurofilaments from rat brain are in lane C. The $NaDodSO_4$–urea–polyacrylamide gradient gel separates tubulin into its α and β chains. It is

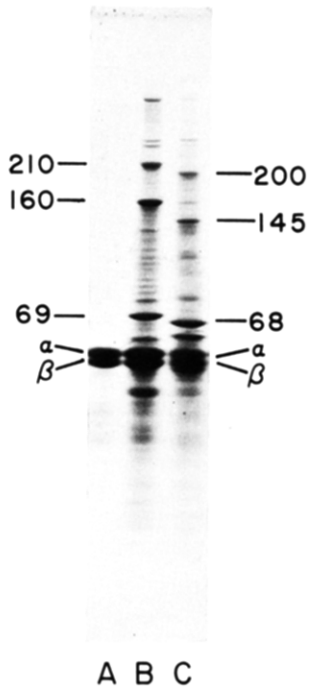

FIGURE 3: $NaDodSO_4$–urea polyacrylamide gradient gel electrophoresis of neurofilament proteins and tubulin. (A) PC-tubulin. The protein is resolved into its α and β subunits (as marked) in this electrophoretic system. (B) Bovine brain neurofilament preparation. The 55000 molecular weight material has been resolved into two bands that comigrate wth α- and β-tubulin. The band below tubulin has an apparent molecular weight of 44000. (C) Rat brain neurofilament preparation.

clear that the neurofilament-associated polypeptides of ~55000 molecular weight (labeled α and β) are also separated into two components and that these components comigrate with the two chains of tubulin. The minor protein of molecular weight ~44000 is of unknown identity but is possibly actin. The other minor protein present between tubulin and the 68000 or 69000 molecular weight band is also of unknown identity.

The tubulin-like polypeptides, of molecular weights ~55000, were separated from a neurofilament preparation, and from each other, by preparative electrophoresis by the method of Yang & Criddle (1970). The results of a partial amino acid analysis of each are shown in Table I, together with the amino acid composition of control samples of bovine tubulin. The two polypeptides from the neurofilaments are not distinguishable in amino acid composition from α and β-tubulin, within the limits of error.

Reaction of Brain Filaments with Antibodies to Peripheral Nerve Neurofilaments. Figure 4A shows bovine brain neurofilaments that had been incubated with antibody to neurofilaments from rat peripheral nerve. Figure 4B shows (at higher magnification) another sample of the same filaments incubated with the same concentration of a control IgG prepared from rabbits hyperimmune to 2,4-dinitrophenol. The electron-dense, fluffy-looking coat (decoration) formed by the antiserum (Figure 4A) is seen to obscure the visible margins of the neurofilaments and to be readily identifiable. Its absence (Figure 4B) is readily seen also, and an unmistakable distinction can easily be drawn between filaments with adsorbed IgG and those without. Table II gives the results of a series of incubations. Both bovine and rat neurofilaments were decorated by the IgG prepared from rabbits immunized against rat peripheral nerve neurofilaments. No decoration was seen following incubation with the control IgG. Absorption of the

Table I: Amino Acid Compositions of PC-Tubulin and of Tubulin-like Polypeptides from Brain Neurofilaments[a]

	bovine brain tubulin		bovine brain neurofilaments[b]	
	α	β	α	β
alanine	8.5 (±0.8)	8.2 (±0.8)	8.4 (±0.8)	7.8 (±0.8)
arginine	4.3 (±0.4)	4.5 (±0.4)	4.8 (±0.5)	5.0 (±0.5)
aspartic acid	11.1 (±1.1)	12.0 (±1.2)	10.4 (±1.0)	11.0 (±1.1)
cystine/2	ND	ND	ND	ND
glutamic acid	12.3 (±1.2)	13.4 (±1.3)	12.5 (±1.2)	12.5 (±1.2)
glycine	9.4 (±0.9)	9.6 (±0.9)	8.8 (±0.9)	9.4 (±0.9)
histidine	2.8 (±0.3)	2.2 (±0.2)	2.7 (±0.3)	2.1 (±0.2)
isoleucine	6.0 (±0.6)	4.8 (±0.5)	6.1 (±0.6)	4.6 (±0.4)
leucine	8.1 (±0.8)	8.6 (±0.8)	8.8 (±0.9)	9.3 (±0.9)
lysine	4.2 (±0.4)	3.9 (±0.4)	4.4 (±0.4)	3.8 (±0.4)
methionine	1.1 (±0.1)	1.2 (±0.1)	0.8 (±0.1)	1.0 (±0.1)
phenylalanine	4.2 (±0.4)	4.1 (±0.4)	4.0 (±0.4)	4.5 (±0.4)
proline	4.2 (±0.4)	4.0 (±0.4)	4.0 (±0.4)	3.8 (±0.4)
serine	7.5 (±0.7)	8.7 (±0.8)	6.4 (±0.6)	7.2 (±0.7)
threonine	7.1 (±0.7)	7.1 (±0.7)	6.5 (±0.6)	6.3 (±0.6)
tryptophan	ND	ND	ND	ND
tyrosine	3.7 (±0.3)	3.4 (±0.3)	3.3 (±0.3)	3.3 (±0.3)
valine	7.0 (±0.7)	6.6 (±0.6)	6.7 (±0.7)	6.1 (±0.6)

[a] 24-h 6 N HCl hydrolysis at 100 °C; serine increased by 10% and threonine increased by 5% to compensate for destruction by acid. One crystal of phenol was added before acid hydrolysis. Amino acid compositions are expressed in mol %. ND = not determined. Error figures represent maximum probable error. [b] The slower migrating polypeptide is referred to as α in each case. In the case of PC-tubulin, it is α-tubulin.

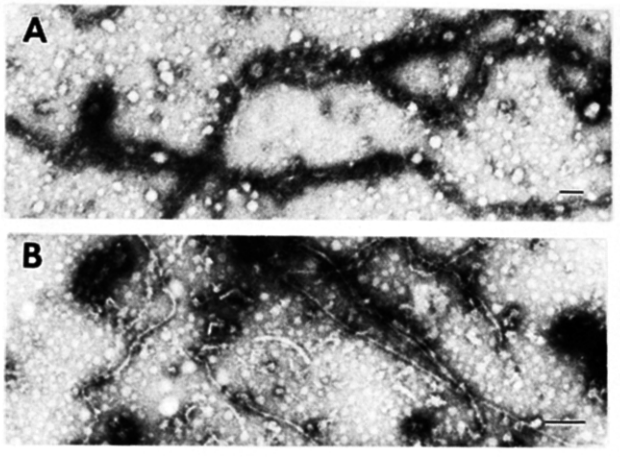

FIGURE 4: Electron micrographs of IgG-incubated neurofilaments. Typical fields. (A) Bovine brain neurofilaments incubated with anti-neurofilament IgG. Neurofilaments are enveloped in a fluffy, densely staining IgG meshwork. The decorated neurofilaments have a variable apparent diameter of 50–70 nm. Bar = 0.1 μm. (B) An aliquot of the same bovine brain neurofilament preparation, incubated in control IgG prepared from a rabbit made hyperimmune to 2,4-dinitrophenol. Bar = 0.1 μm.

IgG with any of the three types of neurofilaments prevented it from decorating either of the other two. These results indicate that the three types of neurofilaments tested here are indistinguishable as antigens. In the three cases where decoration is noted, undecorated filaments were completely absent, and in the cases where no decoration is noted, decorated filaments were likewise absent. The filaments are thus apparently homogeneous in their reactivity with the IgG.

Attempts to Dissociate Tubulin from Neurofilaments. Aliquots of neurofilaments prepared in PM buffer were pelleted by centrifugation (95500*g*, 60 min, 4 °C) and resuspended into PM buffer containing one of the following additions: 0.5 or 1.0 M NaCl, 0.5 or 1.0 M KCl, 0.5 mM colchicine, 0.1% or 1.0% Triton X-100, 2 mM $CaCl_2$, or 0.6 M KI. Each homogenate was dialyzed against two changes (of 4 h each) of the same buffer and was recentrifuged (95500*g*, 60 min, 4 °C) to pellet the neurofilaments. In each case, the supernatant was found to contain negligible protein concentration. Gel electrophoresis of the pellets showed no apparent dissociation of protein. In addition, the inclusion of the above substances in the homogenization buffer did not visibly reduce the amount of tubulin estimated (by gel electrophoresis) to be present in the final neurofilaments.

Table II: Reaction of Neurofilament Samples with Anti-neurofilament IgG[a]

incubation medium	bovine brain NF's[b]	rat brain NF's	rat PN[c] NF's
anti-NF IgG	+	+	+
control IgG	–	–	–
anti-NF IgG, preabsorbed with bovine brain NF's	ND	–	–
anti-NF IgG, preabsorbed with rat brain NF's	–	ND	–
anti-NF IgG, preabsorbed with rat PN[c] NF's	–	–	ND
PBS control	–	–	–

[a] Incubation with IgG and electron microscopy were performed as described under Materials and Methods. In some cases, the anti-NF IgG (against rat peripheral nerve neurofilaments) was preabsorbed with a 0.5 mg/mL neurofilament sample before incubation of the neurofilaments to be tested with the IgG. Decoration present (+); decoration lacking (–); determinations not performed (ND). [b] NF = neurofilament. [c] PN signifies peripheral nerve.

Discussion

The results show that tens of milligrams of filaments of ~10-nm diameter can be conveniently isolated from bovine brain by a series of centrifugation and gel filtration steps. Because of the presence of both glial filaments and neurofilaments (and possibly other co-purifying filaments) in the brain, it was necessary to establish carefully the identity of the isolated structures. This was done by comparing their properties with those of authentic neurofilaments isolated from the peripheral nervous system, where glial cells are largely absent. Is is now established (Lasek & Hoffman, 1976; Schlaepfer, 1977b; Schlaepfer & Lynch, 1977; Liem et al., 1978) that the 10-nm filaments isolated from peripheral nerve (Schlaepfer, 1977a) are authentic neurofilaments.

Morphology. The isolated filaments are indistinguishable from isolated neurofilaments in their appearance in the electron microscope (Schlaepfer, 1977a,b). As seen in negative stain, they are characteristically sinuous filaments, bearing "bumps" or "knobs" at irregular intervals along their length. These projections are not seen in fixed and sectioned preparations, and they may arise as an artifact of the isolation procedure.

Protein Composition. The protein composition of the isolated filaments (Figure 3) from bovine and rat brains shows the neurofilament triplet of proteins (Lasek & Hoffman, 1976; Micko & Schlaepfer, 1978). No detectable amount of the glial filament protein, characterized by a molecular weight in the range 47 000–51 000 (Bignami et al., 1972; Dahl & Bignami, 1973; Bignami & Dahl, 1973; Benitz et al., 1976; Schachner et al., 1977; Goldman et al., 1978), is present. These facts together show that the filaments are not glial filaments and suggest strongly that they are neurofilaments.

It is uncertain whether the tubulin present in the preparation was part of the neurofilaments in vivo or whether it simply adhered to them in the course of preparation. One report of protein composition of neurofilaments prepared by axonal flotation (Liem et al., 1978) shows those structures to lack

tubulin, although they contain a protein similar to that of glial filaments. It is possible that tubulin might have been removed during one or more of the steps of the two alternative preparations described there. Other reports of the protein composition of neurofilaments (Micko & Schlaepfer, 1978; Lasek & Hoffman, 1976) concerned filaments that had not been thoroughly isolated from microtubules. The large amounts of tubulin observed in those studies were ascribed to the microtubules present and not to the neurofilaments, but its association with neurofilaments could not have been ruled out by the data presented. Neurofilaments prepared by methods similar to that described here (Wang et al., 1980) have tubulin tightly associated with them, as do neurofilaments isolated as contaminants of cycled microtubule preparations (Berkowitz et al., 1977; Runge et al., 1979a). The possibility that the tubulin could be contributed to the preparation by contaminating cold-stable microtubules (Webb & Wilson, 1980) is exluded by the absence of microtubular forms from electron micrographs of the neurofilament preparations. The failures of the attempts, reported in this paper, to remove tubulin from the neurofilaments prepared from brain indicate its strong association with them. There is insufficient evidence at present, however, to allow one to decide whether tubulin is an intrinsic part of neurofilaments. The possibility remains that it may be.

The significance of the presence of minor protein components, particularly the ones of molecular weights near 44 000 and between 55 000 and 60 000, is not clear at present. Although the data do not suggest that the preparation contains more than one type of filament, no strong demonstration of the homogeneity of the preparation has been made. On their face, the separation methods employed here do not seem to be highly selective. Hence, it is conceivable that any of the proteins could be a constituent (either an essential structural component or an adherent protein) of a minority class of intermediate filaments, isolated together with the neurofilaments. Such a group of filaments would have to appear roughly similar to the majority class of brain neurofilaments and have at least one antigen in common with peripheral nerve neurofilaments in order to give rise to the observed uniformity of appearance and of staining with anti-neurofilament IgG. This question of possible heterogeneity can best be answered by future subfractionation efforts.

The molecular weights of rat and bovine neurofilament proteins have been separately reported (Liem et al., 1978; Wang et al., 1980; Micko & Schlaepfer, 1978; Lasek & Hoffman, 1976). The small molecular weight differences (Figure 3) in apparently homologous proteins are here confirmed by the direct parallel isolations of filaments from the two species.

Immunological Properties. Schlaepfer & Lynch (1977) have shown that the preparation of rabbit anti-neurofilament IgG employed here does not cross-react with glial filaments, and Schlaepfer (1977b) has shown that it does not cross-react with tubulin or with bovine serum albumin. Hence, although it is not known with certainty which of the proteins of the neurofilament preparation bear the determinants to which the IgG is raised, it is clear that neither tubulin nor possible trace amounts of contaminating glial filament protein are responsible. The results of Table II show that the filaments from bovine and rat brains have the same set of antigenic determinants as rat peripheral nerve neurofilaments. The determinants are not associated with the tubulin that is present in each preparation. This similarity between the filaments of peripheral nerve and those of brain is the main conclusion to be drawn from the immunological experiments. In addition, the homogeneity of decoration of filaments with antibody is evidence against the possibility of contamination with glial filaments. If significant numbers of glial filaments had been present, significant numbers of undecorated filaments would have been seen.

Success of the Protocol. It appears that the simple isolation scheme described here selectively yields neurofilaments primarily because the glial filaments remain bundled together in the relatively high ionic strength employed (Liem et al., 1978) and are pelleted in conditions where neurofilaments do not sediment. Microtubular elements remain depolymerized because of the low temperature employed and are removed with the other soluble proteins in the gel filtration step. Neurofilaments might be expected to sediment in the first high-speed centrifugation step (95500*g* for 75 min). That they do not is attributable to bound lipid and to the relatively high viscosity and density of the solution, which has a protein concentration > 20 mg/mL. They sediment and form a pellet in a later step in the protocol, in 0.5 M sucrose.

The protocol described here is adequate for the preparation of neurofilaments for many purposes. It has been employed in an abbreviated version (Runge et al., 1979a,b, 1981; Wang et al., 1980) to prepare neurofilaments for biochemical investigations. It is not, however, a final or fully optimized scheme. From Figure 2B it is clear that small numbers of nonfilamentous structure remain in the solutions. The presence of small amounts of many proteins, shown in Figure 3 and discussed above, most likely represents either adventitious material that adheres to the filaments or components of copurifying aggregates. The yield of this method, however, is great enough to allow future fractionation efforts.

Acknowledgments

We thank Dr. H. William Detrich, III, for helpful discussions. Expert technical assistance was rendered by Leslie Milam.

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A MAP-2-Stimulated Protein Kinase Activity Associated with Neurofilaments†

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ABSTRACT: Preparations of mammalian neurofilaments purified from brain consist primarily of four proteins: a triplet of molecular weights 210000, 160000, and 69000, and tubulin. Incubation of bovine brain neurofilaments in the presence of adenosine 5′-[γ-^{32}P]triphosphate ([γ-^{32}P]ATP) brought about phosphorylation of each of these four proteins, indicating that a protein kinase was associated with the neurofilaments. The activity was not removed by centrifugal washing of the neurofilaments in a number of different buffers. The pattern, rate, and extent of phosphorylation were found to be unaffected by adenosine cyclic 3′,5′-monophosphate (cAMP), by preincubation with adenosine 5′-diphosphate, or by addition of a protein kinase inhibitor. The protein kinase is thus cAMP independent. When the high molecular weight microtubule-associated protein MAP-2 was added to neurofilaments and the mixture was incubated with [γ-^{32}P]ATP, the MAP-2 became extensively phosphorylated. In addition, the inclusion of MAP-2 brought about an increase in the extent of phosphorylation of the neurofilament proteins. These results show that neurofilaments possess a firmly associated protein kinase activity and that this activity is stimulated by the presence of MAP-2. Microtubule preparations made by cycles of assembly and disassembly can contain both protein kinase activity and small amounts of contaminating neurofilaments. Two distinct protein kinases were resolved from such microtubule protein by gel filtration on Bio-Gel A-150m. The cAMP-independent protein kinase activity was found in the void volume fractions, together with the contaminating neurofilaments. A cAMP-dependent protein kinase activity was found in the included peak, together with the major proteins of microtubules. The neurofilament-associated protein kinase is thus distinct from, and separable from, the microtubule-associated protein kinase.

Neurofilaments are found in axons in close proximity to microtubules [for reviews, see Dustin (1978) and Lasek & Hoffman, (1976)]. They contain a triplet of proteins of molecular weights approximately 69000, 160000, and 210000 (Hoffman & Lasek, 1975; Lasek & Hoffman, 1976; Schlaepfer, 1978; Micko & Schlaepfer, 1978). In addition, tubulin accompanies them in axonal transport (Lasek & Hoffman, 1976) and through isolation under a variety of conditions (Runge et al., 1979a,b, 1981). Hence, tubulin may be a part of neurofilaments. The functions of neurofilaments are not known, although they are clearly transported in the slow component of axonal transport (Hoffman & Lasek, 1975; Lasek & Hoffman, 1976).

The ability to prepare neurofilaments in tens of milligrams (Runge et al., 1981) has allowed investigation of their biochemical properties. A phosphodiesterase has been found (Runge et al., 1979b) to be firmly associated with neurofilaments purified by the methods employed in the present study. The protein kinase activity reported below was found in a search for other neurofilament-associated enzymic activities, undertaken with the eventual intention of elucidating the functions of these organelles.

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0006-2960/81/0420-0175$01.00/0