

OBSERVATIONS ON THE DISASSEMBLY OF ISOLATED MAMMALIAN NEUROFILAMENTS

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ABSTRACT

Intact mammalian neurofilaments were separated by centrifugation of osmotically shocked, desheathed segments of rat peripheral nerve. Neurofilament-rich supernates were incubated in different media with varying dilutions or dialysis of samples. Neurofilaments attached to carbon-Formvar-coated grids were exposed to similar incubations. The relative preservation or disruption of neurofilaments during different incubational conditions was monitored through periodic examinations of neurofilaments by negative staining techniques.

Maximum structural stability of neurofilaments was manifested during incubation in isotonic NaCl or KCl. Decreasing salinity of incubational media led to increasing disruption of neurofilaments, especially in solutions less than 0.01 M. Hypertonic saline solutions were found to be less disruptive to mammalian neurofilaments. Additional studies examined neurofilamentous alterations effected by pH, protein denaturants, mitotic spindle inhibitors, reducing agents, and freeze-thawing procedures.

KEY WORDS neurofilaments · mammalian ·
ultrastructure · disassembly · hypotonicity

Neurofilaments have remained a prominent but poorly understood neuronal organelle. Experimental evidence is lacking which might support speculation that neurofilaments function in intracellular transport (1, 10, 25) or in a structural supportive role within asymmetrical cell processes (7, 14, 26). These functions are also attributed to microtubules (29), an organelle frequently admixed with neurofilaments. It is also unclear whether neurofilaments exist as part of a three-dimensional cytoplasmic network, as suggested by metallic impregnation studies (15-17, 19-21).

The identification of neurofilaments has been limited to morphological criteria, usually applied to fixed, osmicated, and sectioned tissues. The separation of a neurofilament-enriched fraction

from rat peripheral nerve (23), and the demonstration of intact neurofilaments by negative staining techniques (23, 24) have enhanced the prospects for further studies concerning the nature of these organelles from mammalian tissues.

Studies of isolated neurofilaments are critically dependent upon the state of the structural preservation of the filaments. Disassembly of mammalian neurofilaments in low ionic strength buffer enabled the separation of membranous and particulate contaminants by centrifugation (23). The preservation of isolated neurofilaments was essential to studies of their substructure (24) and structural plasticity.¹ The present study has examined the effects of incubational conditions on the pres-

¹ W. W. Schlaepfer. 1977. Deformation of isolated neurofilaments and the pathogenesis of neurofibrillary pathology. *J. Neuropathol. Exp. Neurol.* In press.

ervation of neurofilaments isolated from rat peripheral nerve.

MATERIALS AND METHODS

Neurofilament-enriched extracts were obtained from saline-perfused, desheathed, osmotically shocked, minced, and partially macerated rat peripheral nerve tissues, as previously described (24). Intact neurofilaments of these extracts were attached to carbon-Formvar-coated, 400-mesh grids by flotation. Multiple grids from the same extract preparation were simultaneously incubated by flotation on media of differing consistency for variable intervals at 18–22° C, then rinsed by flotation on 0.1 M NaCl before examination by negative staining with and without fixation. Aliquots (50 μ l) of neurofilament-enriched extracts were also admixed with equal volumes of different solutions and incubated at 18–22° C on a rocker platform. Neurofilaments within these incubated solutions were monitored by comparative examinations of negatively stained grids prepared at periodic intervals. Studies on the exposure of neurofilaments to different solutions were also conducted with lesser dilutions of neurofilament-enriched extracts. Accordingly, 50- μ l aliquots were admixed with 5.5 μ l of M NaCl or with similar volumes of H₂O. Some of these samples were subjected to freeze-thawing before examination. A more controlled incubation of intact neurofilaments in solution was achieved by the placement of neurofilament-enriched extracts into homemade "dialysis boats" (DB) which were subsequently floated on media of differing consistency. DB were constructed by covering the open ends of 3/16" Beckman cellulose nitrate centrifuge tubes with small rectangular sheets of prepared dialysis membrane, sealing the edges with vaseline, and inserting tube and membrane through a truncated plastic #1 Caplug (Tanler and Co., St. Louis, Mo.) whose tapered body served as a sealing O ring and whose outward circular flange acted as a flotation collar. The centrifuge tube was transected above the circular flange, providing ready access to the cylindrical chamber of 150–200- μ l capacity. The assembly floated securely inside a capped 5-ml cylindrical vial (Kimble Products Div., Owens-Illinois, Inc., Toledo, Ohio) atop a rocker platform. If a leak developed in the dialysis membrane, the assembly would sink. DB incubations were conducted on 50- μ l samples of neurofilament-rich supernates admixed with equal amounts of media. Negatively stained preparations were prepared from these samples at periodic intervals during the combined dialysis and incubation of sample. After 24-h incubations, some samples were centrifuged at 25,000 rpm in a Beckman Model L ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The precipitates were fixed in formalin and examined by negative staining. Supernates were assayed by the Lowry technique (18) before and after centrifugation.

Neurofilament preparations were fixed by flotation of

grids on 5% formalin in 0.1 M NaCl buffered with sodium phosphate (0–1 mM) to pH 6.5–6.8, and subsequently rinsed in 0.1 M NaCl. Potassium salts were substituted in the fixative and the rinse after incubations with KCl. Specimens were generally fixed and rinsed for intervals of 2–6 h, but variations of these intervals of 1–16 h caused no appreciable change in neurofilament appearance. Both fixed and unfixed specimens were negatively stained with unbuffered 1 or 2% uranyl acetate. All preparations were examined and photographed in a Siemens IA Elmiskop equipped with 100 μ m condenser and 15 μ m objective apertures and operated at 80 kV.

RESULTS

Quantitation of Neurofilaments by Examination of Negatively Stained Preparations

Demonstration of neurofilaments after adsorption onto EM grids was facilitated by a relatively high avidity between these organelles and the surface carbon and/or Formvar films. Large numbers of neurofilaments could be demonstrated consistently in neurofilament-rich peripheral nerve extracts. Their high relative density suggested that they represented the overwhelmingly predominant organelle within the supernatant fractions. The density of neurofilaments and other organelles was slightly diminished by increasing the salinity or by fixation of samples before their adsorption onto grids. Some fluctuations in the absolute density of organelles also resulted from variations in the layered thicknesses of sample and/or negative stain.

The large numbers of demonstrable neurofilaments within neurofilament-enriched extracts tended to lessen the importance of preparative variations in assessing the neurofilament composition of different samples. Preparations of fresh extracts generally revealed numerous overlapping neurofilaments which could not be accurately quantitated. The temporal breakdown of neurofilaments within these samples was manifested by a loss of intact neurofilaments as well as an increase in the appearance of fragmented neurofilaments and their irregular globular breakdown products, as previously documented (23). Eventually, intact neurofilaments could not be demonstrated, and the preparations contained only membranous and particulate profiles.

The rate and degree of neurofilament breakdown was dependent upon incubational condi-

tions, a feature which could be readily appreciated by comparative examinations of the same neurofilament-enriched extracts which had been subject to different incubational conditions. Quantitation of neurofilament breakdown was based on these comparative examinations as well as on the ease of demonstrating intact neurofilaments and the presence of neurofilament breakdown products. Categories of neurofilament preservation were designated as relatively intact (2+), moderately disrupted (1+), and severely disrupted (0), which roughly corresponded to neurofilament densities

of >25, 1-25, and <1 neurofilament per grid square (~2,500 μm^2), respectively. Fresh preparations were estimated to contain over 200 neurofilaments per grid square. Table I summarizes the state of neurofilament preservation (or disruption) following a variety of different incubational conditions.

Effects of Tonicity on the Structure of Neurofilaments

Isotonicity appeared to promote the preservation of neurofilaments. Numerous intact neurofil-

TABLE I
Effects of Incubational Media on Preservation of Neurofilaments

Media	Concentration <i>mM/liter</i>	Demonstration of intact neurofilaments*				
		<i>1h</i> ‡	<i>4h</i> ‡	<i>8h</i> ‡	<i>16h</i> ‡	<i>24h</i> §
H ₂ O	—	1+	0	0	0	0
2-mercaptoethanol	50	2+	1+	1+	1+	0
Colchicine	1	1+	1+	1+	0	0
Vinblastine	1	1+	1+	1+	1+	0
NaCl (or KCl)	10	2+	1+	1+	1+	1+
NaCl (or KCl)	20	2+	2+	2+	1+	1+
NaCl (or KCl)	100	2+	2+	2+	2+	2+
Urea (or guanidine HCl)	500	0	0	0	0	0
Urea	500					
NaCl	100	2+	2+	2+	1+	1+
NaPO ₄ -buffer, 5.2	10					
NaCl	100	2+	1+	1+	0	0
NaPO ₄ -buffer, 5.7	10					
NaCl	100	2+	2+	2+	1+	1+
NaPO ₄ -buffer, 6.2	10					
NaCl	100	2+	2+	2+	2+	2+
NaPO ₄ -buffer, 6.7	10					
NaCl	100	2+	2+	2+	2+	2+
NaPO ₄ -buffer, 7.2	10					
NaCl	100	2+	2+	1+	1+	0
MES-buffer, 6.2	10					
NaCl	100	2+	2+	2+	2+	2+
Tris-buffer, 7.2	10					
NaCl	100	2+	2+	1+	1+	1+
NaPO ₄ -buffer, 6.2	10					
MES-buffer, 6.2	10	2+	2+	2+	1+	1+

* Estimation of neurofilament structural integrity based upon ease in which intact neurofilaments could be visualized and prevalence of their breakdown products in negatively stained preparations (see text).

‡ Examinations conducted on neurofilament-rich supernates dialyzed vs. media.

§ Examinations conducted on fixed sediment of samples after centrifugation.

aments were observed in neurofilament-enriched extracts which had been incubated for several days in 100-150 mM NaCl or KCl.¹ Dialysis of samples did not impair the stability of neurofilaments under isotonic conditions.

Parallel incubations of unaltered hypotonic neurofilament-rich extracts and identical samples rendered isotonic (100 mM) by the addition of molar NaCl or KCl revealed striking differences in the preservation of neurofilaments (Figs. 1 and 2). Similar preservation of neurofilaments under isotonic incubational conditions occurred with fourfold dilutions of sample, or with dialysis of sample against large volumes of isotonic saline (Table I). Progressive disruption of neurofilaments was observed in samples diluted fourfold or dialyzed with large volumes of water. Disruption of neurofilaments corresponded to the severity of hypotonicity (Table I). Neurofilaments attached to grids were considerably less susceptible to the disruptive effects of hypotonic media. Flotation of neurofilament-laden grids on 10 mM NaCl or KCl caused minimal alteration, although similar flotation on H₂O lead to subtotal breakdown of neurofilaments within a 4-h interval.

The breakdown of neurofilaments on exposure to hypotonic conditions was not prevented by additions of 2-mercaptoethanol (50 mM), colchicine (1 mM), or vinblastine (1 mM) to water. Nevertheless, these additives retarded the hypotonic disruption of neurofilaments (Table I). The stabilizing effects of these agents upon neurofilaments were more evidenced in moderately hypotonic conditions (10 mM). No alteration in appearance of neurofilaments was noted when these same substances were added to isotonic incubations.

Rendering the neurofilament-rich supernatant fraction hypertonic caused a "salting out" precipitation at concentrations at or above 0.5 M NaCl. A white cloudy precipitate sedimented in solutions of 0.5 or 1.0 M NaCl, but formed a pellicle atop 2.0 M NaCl. Considerable retention of neurofilament structure could be demonstrated within these pellets or pellicles after *in situ* fixation by equal admixtures of 5% formalin. These preparations also revealed considerable neurofilament breakdown, the extent of which was sometimes less manifested in precipitates of higher molar content. Flotation of neurofilaments attached to grids on hypertonic saline solutions caused moderate disruption of neurofilaments when incubated in solutions above molar concentration. Incuba-

tion in high salt concentrations also tended to dislodge neurofilaments from the carbon-coated grids.

Effects of pH on the Neurofilament Structure

The structural integrity of neurofilaments was not appreciably enhanced by the addition of buffers to isotonic incubational conditions (Table I). In the presence of buffers, optimal structural preservation of neurofilaments occurred in slightly acidic conditions, at pH 6.7 and 6.2. At pH 5.7 and 5.2, neurofilaments tended to aggregate and become progressively disrupted. Lesser states of neurofilament structural preservation were consistently observed in incubations conducted at or above pH 7.2. No differences were detected in neurofilament response to pH changes induced by phosphate buffer or larger organic buffers. Neither phosphate buffer nor MES (2[N-morpholino] ethane sulfonic acid) buffer, pH 6.2, prevented the hypotonic breakdown of neurofilaments which occurred at 10 mM tonicity (Table I).

Neurofilament Disruption by Protein Denaturants

The disruption of neurofilaments in H₂O or in hypotonic solution could be accelerated by the additions of urea or guanidine hydrochloride. Neurofilament breakdown induced by these protein denaturants could be observed among neurofilaments attached to carbon-coated grids or in solution. The rapidity of neurofilament disruption was roughly proportional to the concentration of denaturant.

The disruptive effects of urea upon neurofilaments was progressively reduced by increasing additions of NaCl to the incubational media. Dissolution of neurofilaments by 0.5 M urea occurred within 1 h, but was incomplete after 24 h when 0.1 M NaCl was also present in the medium (Table I).

Effects of Freeze-Thawing on Neurofilament Structure

Neurofilaments showed remarkable structural preservation after the freezing and thawing of unaltered samples of neurofilament-rich extracts. The breakdown of neurofilaments which slowly occurs in these samples at 18-22° C was markedly retarded by freezing and storage at -20° C.

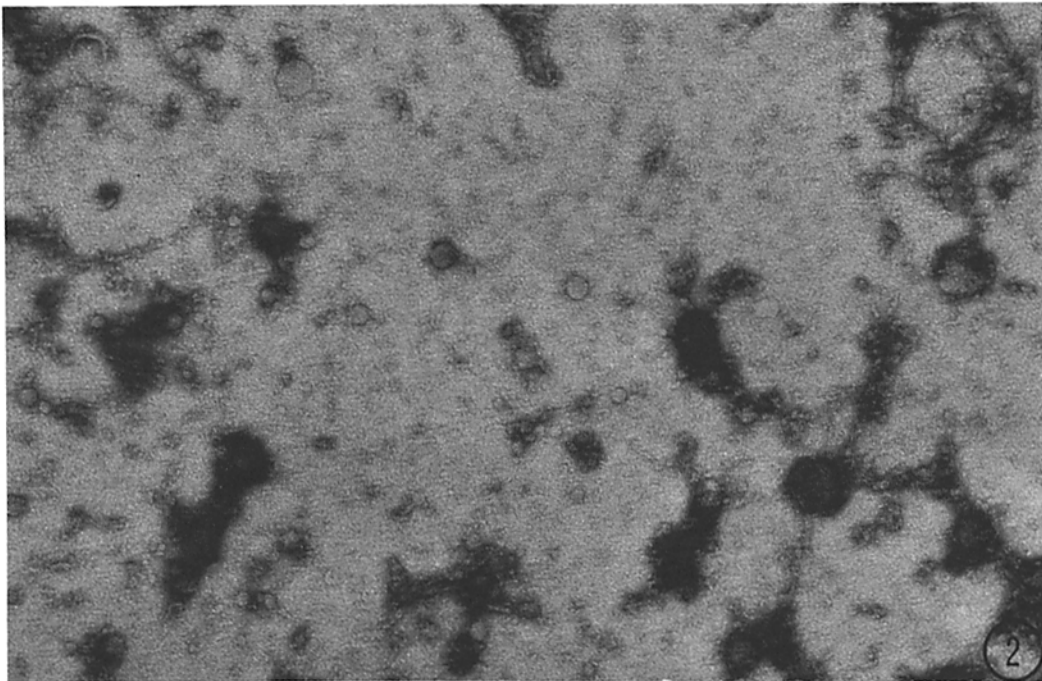
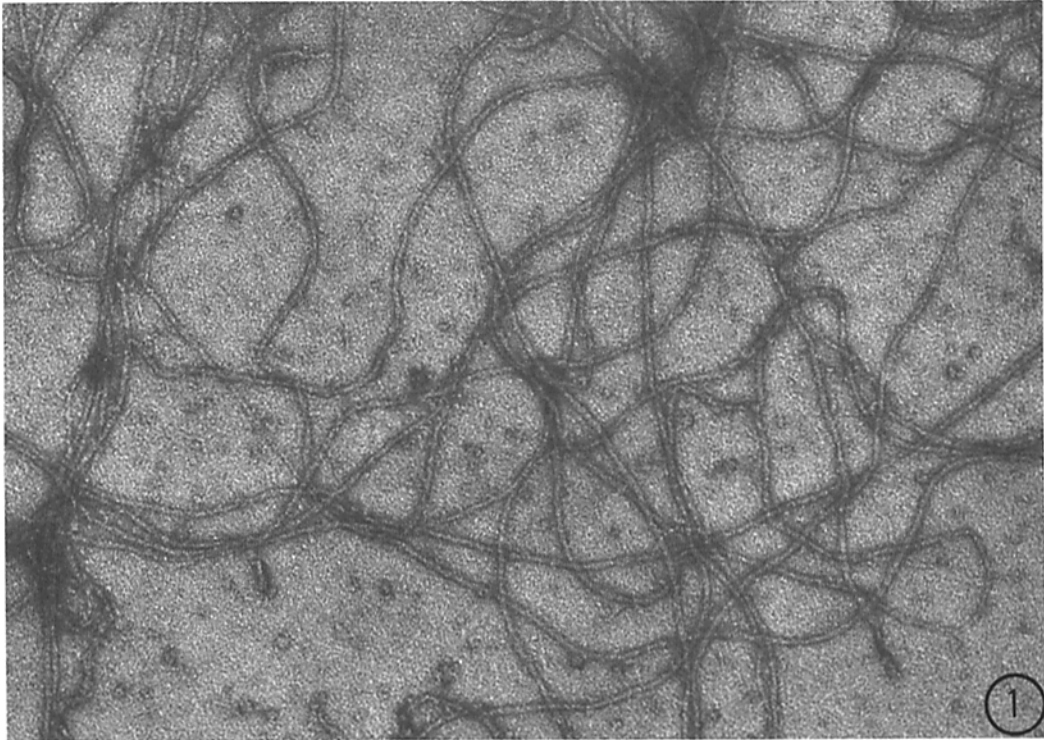


FIGURE 1 Cluster of overlapping intact neurofilaments after 8-h incubation at 18–22° C in 100 mM NaCl. Neurofilament-rich nerve supernate was rendered isotonic by addition of molar salt solution. $\times 80,000$.

FIGURE 2 Representative negatively stained preparation of unaltered hypotonic neurofilament-rich nerve supernate after 8-h incubation at 18–22° C. Intact neurofilaments have been replaced by floccular aggregates with high affinity for uranyl acetate stain. $\times 80,000$.

Sedimentation of Neurofilaments from Incubated Samples

Intact neurofilaments were readily demonstrated in precipitates of samples incubated for 24 h in isotonic conditions. Similar neurofilaments were not seen in fixed precipitates from parallel samples which had been incubated with H₂O. Nevertheless, comparable percentile amounts of protein were sedimented during the centrifugation of these precipitates which contained such diverse numbers of intact neurofilaments. Precipitates from samples incubated for 24 h in 10 and 20 mM/liter NaCl contained intermediate numbers of intact neurofilaments. Precipitates from samples incubated in H₂O or hypotonic saline showed numerous granular and particulate profiles of neurofilamentous breakdown products.

DISCUSSION

Comparative analysis of neurofilaments by negative staining techniques provides a methodology which has enabled the study of some physical properties of isolated mammalian neurofilaments within a heterogeneous tissue fraction of relatively small size. This technique is indirect and may be limited by some variation in particulate adsorption among different preparations. Yet, variations in adsorptive phenomena seem unlikely to account for the differing rates and extents of neurofilamentous breakdown, as well as the coincident appearance of breakdown products with loss of intact neurofilaments. Furthermore, decreasing tonicity generally favored particulate adsorption, contrary to the observed loss of neurofilaments in markedly hypotonic solutions. Variable preservation of neurofilaments within different incubational media was also apparent in fixed sediments which contained more uniform ionic compositions.

The progressive breakdown of neurofilaments which occurred in differing incubational solutions was not necessarily reproduced when neurofilaments were attached to carbon-Formvar-coated grids and floated on media of identical composition. These observations suggested that the lateral anchoring of neurofilaments through their adsorption to a carbon film provided some structural support, and rendered the attached neurofilaments less susceptible to disruptive factors inherent in incubation solution. Accordingly, some caution should be exercised in the interpretation of nonreactivity among neurofilaments attached

to grids and incubated in media of differing consistencies (21).

The disruptive effects of hypotonicity on mammalian neurofilaments are contrary to experimental observations on squid axoplasmic neurofilaments (12). The relative preservation of mammalian neurofilaments in hypertonic solutions also contrasts with the behavior of invertebrate neurofilaments (8, 12).

The breakdown of mammalian neurofilaments in hypotonic solutions was not dependent upon demonstrable indigenous factors within nerve extracts, as neither dilution nor dialysis of samples significantly altered this phenomenon. Furthermore, simple additions of salt to hypotonic incubational conditions markedly retarded neurofilament breakdown with or without dilution and/or dialysis of sample.

The retardation of hypotonic disruption of neurofilaments which occurred in the presence of colchicine or vinblastine suggests that these mitotic spindle inhibitors can act directly upon neurofilaments in addition to interacting with microtubules. The increase of neurofilaments (4, 22, 27, 28) and 100 Å intermediate-sized filaments (2, 3, 5, 6, 9, 11, 13) which follows exposure of tissue to mitotic spindle inhibitors may relate to a direct interaction with the drugs. Furthermore, functional disturbances brought about by these drugs could be due in part to their interaction with neurofilaments or intermediate-sized filaments.

The relative disruption or preservation of neurofilaments under differing incubational conditions may also reflect some features of the molecular bonds which maintain filamentous structure. The presence of hydrophobic bonds is suggested by neurofilament stability during freeze-thawing procedures and hypertonic precipitation, as well as by neurofilament instability within the hydrating conditions accompanying hypotonicity. The preservation of neurofilaments in the presence of reducing agents would also indicate that accessible S-S bonds are relatively unimportant in the maintenance of filamentous structure.

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