STUDIES ON THE BIOSYNTHESIS OF NEUROFILAMENT PROTEINS

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ABSTRACT

To determine whether the triplet polypeptides of neurofilaments arise by degradation of precursor, we studied the biosynthesis of neurofilament polypeptides both in vivo and in cell-free systems. Neurofilament-enriched fractions and polyribosomes were prepared from the same rabbit spinal cord homogenates. At 1 h after intracisternal administration of [^{35}S]methionine, radiolabeled neurofilament proteins were detected in spinal cord homogenates as well as in isolated filaments. When polyribosomes from rabbit spinal cord were allowed to incorporate [^{35}S]methionine into protein, triplet polypeptides were among the proteins labeled. Addition of spinal cord polyribosomes to rabbit reticulocyte lysates led to several cycles of translation of the spinal cord mRNA; the three neurofilament polypeptides were among the proteins synthesized in this system. The results demonstrate that the triplet polypeptides of neurofilaments are synthesized as such in the course of individual translational events and do not arise from degradation of P200 or a larger precursor.

Mammalian neurofilaments appear to be formed of at least three polypeptides (8, 12, 21). These peptides generally are in the range of 200,000 (P200), 150,000 (P150) and 68,000 (P68) daltons. They appear to be sensitive to proteolysis (20, 22) and depolymerize at low ionic strength (19). The triplet polypeptides are readily phosphorylated (23) and are components of the material carried by the slow axonal transport system; newly synthesized triplet polypeptides are transported at ~1–2 mm/d (8, 11).

For a study of dynamic aspects of the biology of neurofilaments, it would be useful to know whether the triplet polypeptides are translated from individual mRNA molecules or whether they result from processing or degradative cleavage of P200 or of a larger precursor. Reports from several laboratories have led to contradictory conclusions. Antibodies raised against any of the triplet polypeptides cross-react, at least to some extent, with the other polypeptides (6, 12; Anderton et al., personal communication). These results have been interpreted to suggest that the triplet polypeptides are derived by degradation of P200 or a larger precursor (24). Peptide mapping, after limited proteolysis or cyanogen bromide cleavage, indicates very little similarity among the three polypeptides (3, 5, 12, 24), suggesting an independent origin for each. However, Julien and Mushynski (9) compared peptide maps of calf-brain filament polypeptides and concluded that each of the triplet polypeptides may include a segment with a com-

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mon amino acid sequence. Shelanski and Liem (24) have suggested the possibility of a large precursor in the range of 410,000–450,000 daltons or larger. Each of the triplet polypeptides would be a different fragment of the parent protein.

We studied the synthesis of neurofilament polypeptides to approach the question of their origin. In this paper, we show that it is possible to prepare both filaments and the protein biosynthetic machinery that makes the filament proteins from the same rabbit spinal cord. With this perparation, we can compare the proteins synthesized in vivo with those made in a cell-free system. This preparation has been used to demonstrate that each of the triplet polypeptides is synthesized in vitro as well as in the live animal, indicating that each of the triplet polypeptides is the product of an individual translational event; they are not degraded from precursor protein.

MATERIALS AND METHODS

Buffers

Buffer A includes 50 mM Tris (pH 7.6), 25 mM KCl, and 10 mM $MgCl_2$. Buffer B is 1 mm dithiothreitol in Buffer A. Buffer C is 0.5% Triton X-100 in 50 mM Tris (pH 7.6) and 200 mM KCl.

Simultaneous Preparation of Polyribosomes and Filaments

The rabbits used in these studies were all 7-wk-old males. After the rabbit was killed, the spinal cord was quickly removed and transferred to ice-cold 0.5 M sucrose/buffer B. The meninges were removed and the tissues homogenized in 7 ml of 0.5 M sucrose/buffer B per gram of tissue wet weight. The homogenate was made 1.1 M sucrose/buffer B by addition of 2 M sucrose/buffer B. We placed 10 ml of homogenate beneath a discontinuous sucrose gradient consisting of three 8-ml layers of 0.9, 0.8, and 0.67 M sucrose/buffer A, with an upper layer of 2 ml of buffer A. After centrifugation of each gradient at 26,000 rpm for 90 min in a SW27 rotor (Beckman Instruments, Inc., Spinco Div., Irvine, California) (77,000 *g* avg), four bands of material were found at the interfaces of the different sucrose layers (M_1–M_4). This material was used to prepare filaments. In addition, a pellet was formed at the bottom of the tubes. These pellets were suspended in 0.25 M sucrose/buffer A and centrifuged for 10 min at 1000 *g*. The supernate was centrifuged at 7700 *g* for 10 min. The resulting supernate was layered on a discontinuous sucrose gradient consisting of 5 ml of 2 M sucrose/buffer A overlaid with 8 ml of 1.35 M sucrose/buffer A. The gradients were centrifuged for 3 h in a 50.2 Ti rotor (Beckman Instruments, Inc.) at 50,000 rpm (225,000 *g* avg). The pellet of free polyribosomes was suspended in 0.25 M sucrose/buffer A and stored at −80°C. Properties of these polyribosomes are described in another communication (4).

Each of the myelin-rich fractions that floated above the homogenate in the first centrifugation (M_1-M_4) was extracted with 0.5% Triton X-100 in 50 mM Tris-200 mM KCl for 1 h at 4°C. The filaments were collected from these treated fractions by sedimentation through a continuous sucrose gradient of 0.4–1.3 M sucrose in buffer C, suspended in 0.25 M sucrose/buffer A, and stored at −80°C.[1]

Cell-free Protein Synthesis

HOMOLOGOUS SYSTEM: The system for cell-free protein synthesis (at 37°C) included 82.5 mM Tris; 6.25 mM sucrose; 125 mM NH_4Cl; 0.5 mM GTP; 2 mM ATP; 10 mM creatine phosphate; 0.15 μg creatine kinase/μl; a mixture of 19 amino acids (no methionine), 25 μM each; 0.1 μCi/μl [^{35}S]methionine (Amersham Corp., Arlington Heights, Ill.; >700 Ci/mmol). The optimal $MgCl_2$ concentration varied with each preparation of polyribosomes. Accordingly, the $MgCl_2$ was adjusted to yield maximal amino acid incorporation for each preparation before the initiation of experiments.

HETEROLOGOUS SYSTEM: Polyribosomal mRNA was also translated by incubating polyribosome preparations in a cell-free translation system derived from rabbit reticulocyte lysate prepared according to Pelham and Jackson (16).

Radiolabeling of Spinal Cord in Vivo

We injected 100 μCi of [^{35}S]methionine (in 50 mM Tris pH 7.6–0.9% NaCl) into the cerebrospinal fluid via the cisterna magna. At 1 h after the injection, the animal was killed and the spinal cord was removed and homogenized as described above. Homogenates were rapidly frozen in liquid N_2 and stored at −80°C.

Electrophoresis of Proteins on Polyacrylamide Gels

For analysis on one-dimensional SDS gels, samples were made 2% (wt/vol) SDS, 1% (wt/vol) β-mercaptoethanol, 10% (wt/vol) glycerol, 0.001% (wt/vol) bromphenol blue and 20 mM Tris-phosphate, pH 7.6, and heated for 5 min at 95°C. The electrophoresis was carried out on 0.75-mm thick, 5–20% acrylamide gradient gels, employing the discontinuous buffer system of Laemmli (10). The gels were stained with Coomassie blue R-250 and destained with 10% acetic acid/20% methanol. For two-dimensional analysis, samples were prepared and separated in the first dimension according to O'Farrell (14). In the second dimension, the isoelectric focusing tube gels were applied on 0.75-mm thick, 5–20% acrylamide gradient slab gels, and samples were run, using the buffer system of Laemmli (10). The gels were stained and destained as described above. For autoradiography, 1-D or 2-D slab gels were dried. Kodak X-Omat film was exposed to the gels and developed in Kodak D-19.

Analytical Techniques

Protein was estimated using Coomassie blue G-250 (1). Radioactivity of hot 5% trichloroacetic acid-insoluble material was determined according to the procedure of Mans and Novelli (13).

Electron Microscopy

Subcellular fractions were fixed by adding an equal volume of 8% glutaraldehyde/0.2 M phosphate buffer, pH 7.0, pelleted

[1] Czosnek, H., D. Soifer, and H. M. Wisniewski. Heterogeneity of neurofilament proteins from rabbit spinal cord. *Neurochem. Res.* In press.

in an Airfuge (Beckman Instruments, Inc.), postfixed in 1% OsO_4, dehydrated, and embedded in Spurr's resin (25). The pellets were cut into slices before embedding. The slices were oriented so that sections of them were in fact sections through the whole pellet. Ultrathin sections were prepared and stained with uranyl acetate and lead citrate (18). Sections were examined and photographed in a Philips EM 300 electron microscope operating at 80 kV and fitted with a 25-μm objective aperture.

RESULTS

Subcellular Fractionation and Characterization of Fractions

The simultaneous preparation of filaments and polyribosomes from rabbit spinal cord is summarized in Fig. 1. The first centrifugation already

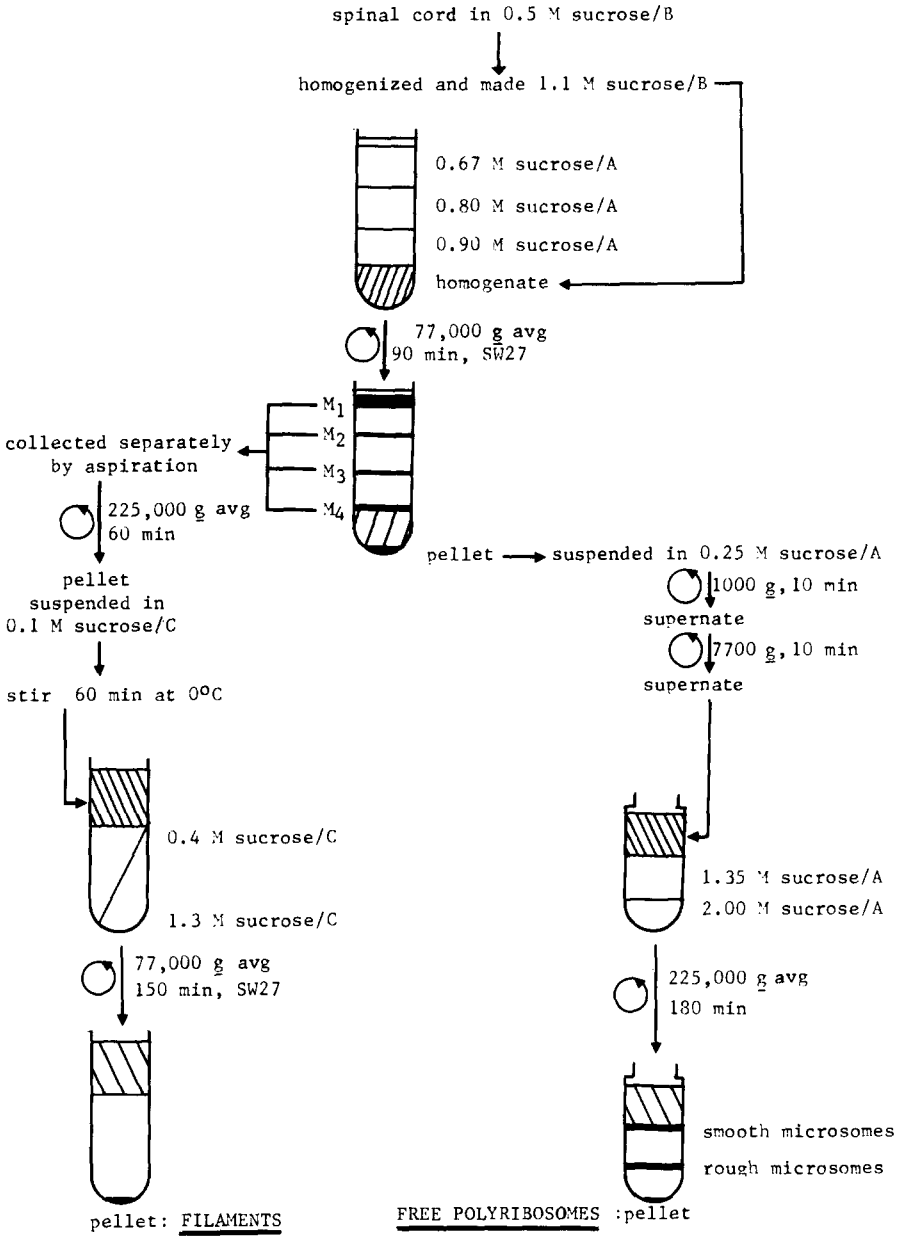

FIGURE 1 Flow diagram for fractionation of rabbit spinal cord.

separated filament-rich fractions (mainly fragmented axons and vesicular material) from a ribosome-rich fraction (which also included mitochondria, nuclei, and unbroken cells). Subsequently, filaments were purified by detergent treatment and ribosomes by differential centrifugation.

Ribosome and filament preparations were routinely characterized by electron microscopy (Fig. 2). The polyribosome fraction appeared nearly homogeneous (Fig. 2 *a*); its properties are the subject of a separate communication (4). The filament fraction was formed of large masses of separate 10-nm-thick filaments with tightly packed filamentous bundles dispersed among them (Fig. 2 *b*). No other structures were seen in these fractions. The protein composition of the filaments (*F*, Fig. 3) was similar to that of the brain intermediate filaments prepared by Liem et al. (12). The SDS gel electropherogram of the filaments shows the three characteristic neurofilament polypeptides apparently weighing 200,000, 150,000, and 68,000 daltons, and a 50,000-dalton protein that is probably derived from glial cells (12). Also present in small amounts were polypeptides that comigrate with α- and β-tubulin (3 and 4%, respectively, of the total protein, by densitometry) and a doublet of ~240,000 daltons that comigrates with the actin-binding protein, filamin (<2%).

Experimental Design

That we were able to isolate, from the same animal, both the filaments and the protein synthesis machinery that synthesizes the filament proteins was useful in the following experiments to study the origin of the neurofilament proteins. We could detect postsynthetic events affecting neuro-

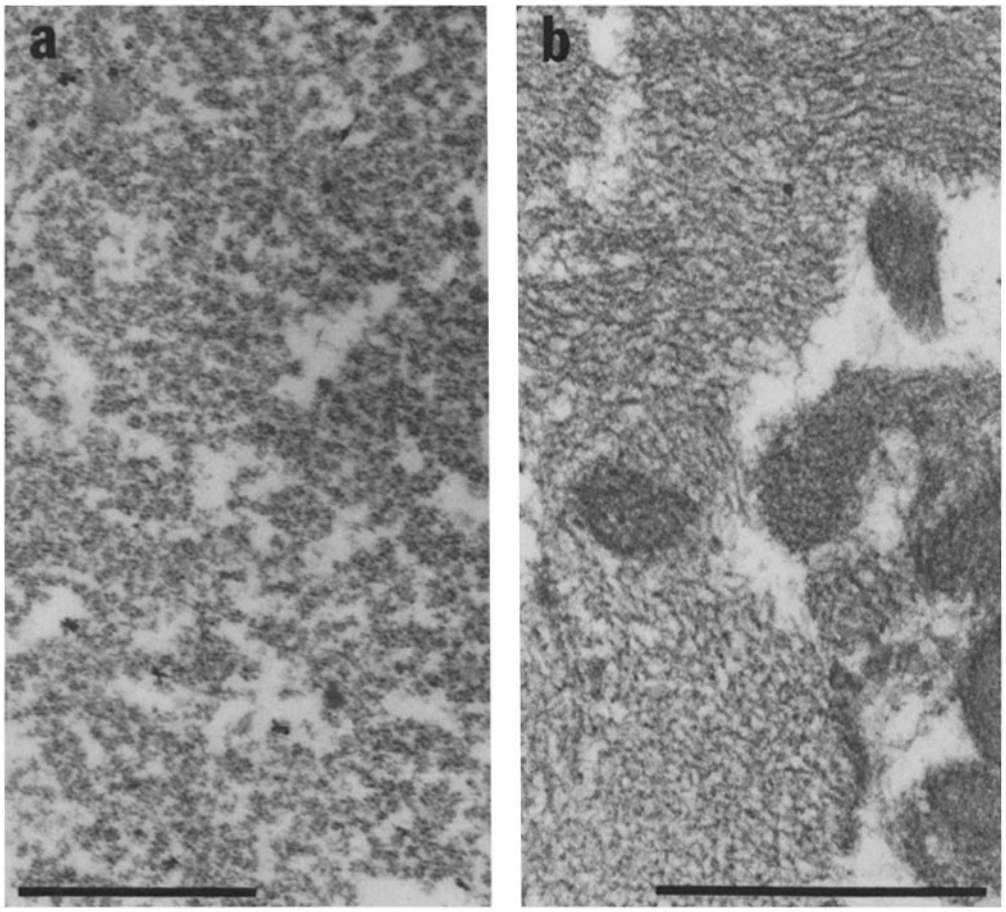

FIGURE 2 Electron micrographs of sections of free polyribosomes (*a*) and filaments (*b*) prepared from spinal cord. Each micrograph is typical of its entire section. Bars, 1 μm.

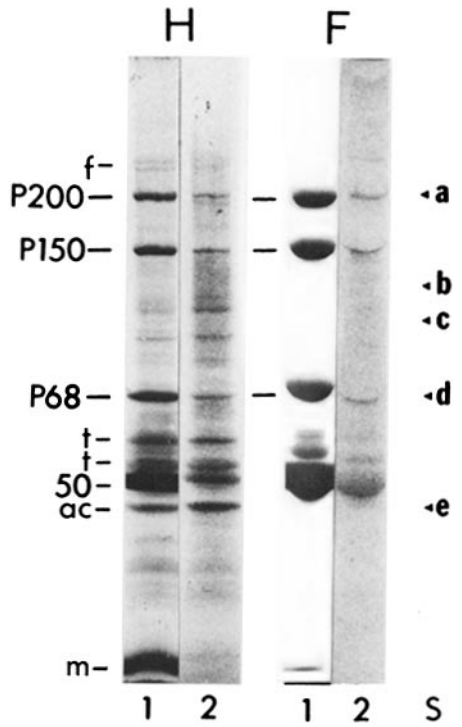

FIGURE 3 In vivo synthesis of filament proteins. Analysis by electrophoresis on SDS-polyacrylamide gels followed by autoradiography. *H*, homogenate of rabbit spinal cord prepared 1 h after administration of [^{35}S]-methionine into the cerebrospinal fluid; 30 μg of protein including 5,000 cpm applied to gel. *1*, Coomassie blue stain; *2*, autoradiogram. *F*, filament fraction prepared from the same radiolabeled spinal cord homogenate; 30 μg of protein including 4,000 cpm applied to gel. *1*, Coomassie blue stain; *2*, autoradiogram. *S*, standards of molecular weights; *a*, myosin (200,000); *b*, β-galactosidase (130,000); *c*, phosphorylase *b* (94,000); *d*, bovine serum albumin (68,000); *e*, ovalbumin (43,000). Markers on left indicate polypeptides that comigrate with filamin (*f*), α- and β-tubulins (*t*), actin (*ac*), and myelin basic protein (*m*). The positions of the neurofilament proteins (*P200*, *P150*, and *P68*) and of the 50,000 dalton protein are also indicated.

filament proteins by comparing the proteins synthesized in the living animal, as visualized by radiolabeling with [^{35}S]methionine, to the proteins synthesized in vitro by polyribosomes in cell-free systems containing [^{35}S]methionine. We could determine whether the triplet polypeptides were synthesized as three distinct polypeptides or whether they arose by the degradation of a precursor or of P200, by comparison of autoradiograms of the SDS electropherograms of radiolabeled proteins synthesized in vivo and in vitro. If each of the triplet neurofilament proteins were detectable among the radioactive proteins synthesized in vivo and in cell-free systems, each of the peptides would have to have arisen from the translation of a different mRNA. If the complete triplet were not detectable among the proteins synthesized in the cell-free systems, the triplet would have appeared to have arisen by degradation of a large precursor. Absence of the triplet from the proteins synthesized in both in vivo and cell-free systems would suggest that the triplet was an artifact of purification of filaments. Some possible origins of the triplet polypeptides are considered in Table I, which indicates identifiability of triplet neurofilament proteins on autoradiographs of gels of in vivo and cell-free synthesized proteins. The basic procedure calls for identification of newly synthesized triplet in homogenates, confirmation, by analysis of isolated filaments, that the newly synthesized triplet is really neurofilament protein and demonstration of whether the triplet polypeptides may be detected among the products of cell-free translation of spinal cord polyribosomal mRNA.

In Vivo Protein Synthesis

When [^{35}S]methionine was administered to the spinal cord 1 h before sacrifice of the rabbit, proteins synthesized during that hour were radiolabeled. Analysis of the polypeptide composition of the homogenate by Coomassie blue staining of SDS gel electropherograms shows that the homogenate included considerable amounts of material with apparent molecular weights similar to those of isolated neurofilaments (*H*, Fig. 3). The triplet-like polypeptides are among the radiolabeled poly-

TABLE I

Formation of Triplets: An Experimental Approach

Possible origin of neurofilament triplet polypeptides	Expected results: triplet demonstrable among proteins synthesized	
	In vivo	In vitro
A. Each peptide direct product of translation	+	+
B. Peptides generated by processing of precursor	+	–
C. Peptides generated by processing of P200	+	P200 only
D. Peptides generated by degradation during isolation of neurofilaments	–	–

peptides visualized by autoradiography of the electropherograms (*H*, Fig. 3). To confirm that the tripletlike polypeptides were, in fact, neurofilament protein, filaments were isolated from the radioactive homogenates and analyzed as were the homogenates (*F*, Fig. 3). All four of the prominent polypeptides of the filament fraction were radiolabeled, including the neurofilament polypeptides. Separation of the filament polypeptides by two-dimensional analysis, followed by autoradiography, confirmed that each of the triplet polypeptides was labeled; each included newly synthesized protein. Nonspecific binding of radioactive soluble filament proteins to the filament is unlikely because the filaments were extracted with detergent and washed by centrifugation through a sucrose gradient containing 200 mM KCl and 0.5% Triton X-100. These results show that, during the hour before sacrifice, the rabbit had not only synthesized triplet polypeptides (seen in the homogenate) but that the newly synthesized triplet polypeptides had been incorporated into filaments.

Cell-free Protein Synthesis

When free polyribosomes were incubated in a cell-free system containing [^{35}S]methionine under standard conditions, most of the translation products were similar to those made by the rabbit in vivo (4). Autoradiography of SDS gels of radioactive proteins synthesized in the cell-free system demonstrates the presence of proteins comigrating with the triplet polypeptides (Fig. 4). The time-dependent increase in the density of the triplet polypeptide bands indicates that these proteins accumulated during the incubation period in vitro.

To confirm that the radiolabeled bands at 200,000, 150,000, and 68,000 daltons corresponded to the triplet polypeptides, samples of protein synthesized in the cell-free system were analyzed in the two-dimensional system of O'Farrell (14). Newly synthesized, radiolabeled polypeptides comigrated with polypeptides of isolated filaments on two-dimensional gels (Fig. 5). Analysis of mixtures of filaments and newly synthesized polypeptides confirmed this observation. When these mixtures were separated on two-dimensional gels, the gels stained, and the autoradiographs prepared, the stained triplet spots were radioactive, confirming that the neurofilament triplet protein was synthesized in the cell-free system.

To eliminate the possibility that the polypeptides synthesized in a run-off system by the spinal-cord free polyribosomes might be incomplete or cleaved proteins, polyribosomes were incubated in a micrococcal nuclease–treated reticulocyte lysate cell-free translation system. In the lysate system, different species of mRNA have been shown to be translated efficiently into full-sized proteins with no detectable accumulation of incomplete products (16). Moreover, precursor proteins were synthesized as nascent polypeptides and did not appear to be processed (cleaved) (15, 17). If there were an mRNA for a triplet precursor protein, it should have been translated in this system; no triplet polypeptides will be formed. If the triplet polypeptides were specified by the mRNA without further processing, they should have been demonstrable among the translation products of the polyribosome-primed reticulocyte lysate system. When rabbit spinal cord polyribosomes were incubated in the reticulocyte lysate buffer system alone, they did not incorporate amino acids into protein (Fig. 6 *c*). Successful incorporation in this buffer system required the reticulocyte ribosomes

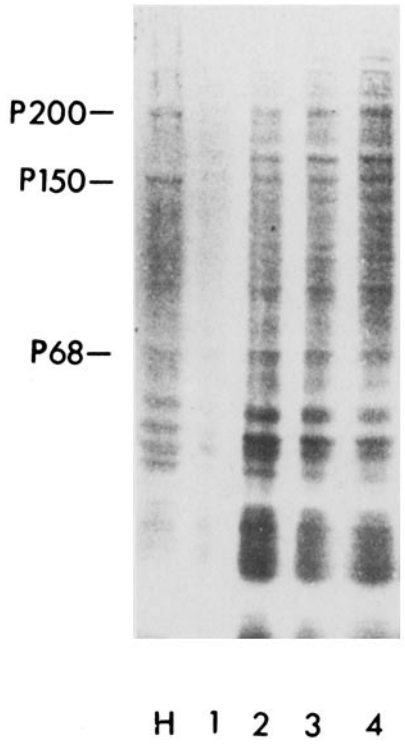

FIGURE 4 Time-course of run-off translation of spinal cord polyribosomes. *H*, spinal cord homogenate labeled in vivo (4,000 cpm). *1*, *2*, *3*, and *4* are radioactive products present after 2.5, 5, 10, and 15 min of incubation. Constant volume of incubation mix applied to each well. Positions of neurofilament polypeptides marked at left.

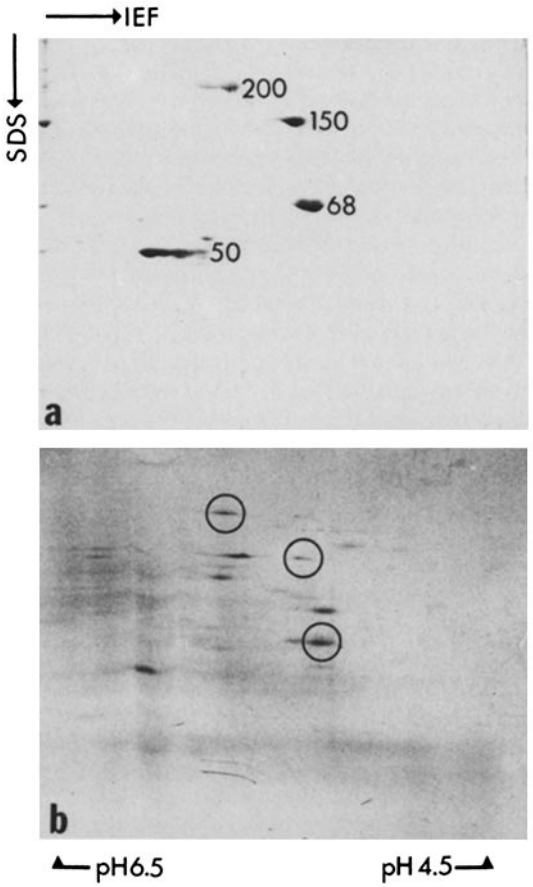

FIGURE 5 Cell-free biosynthesis of filament polypeptides as demonstrated by two-dimensional polyacrylamide gel electrophoresis. *a*, Two-dimensional gel analysis of isolated filaments (20 μg of protein). Numbers indicate the molecular weights of the neurofilament polypeptides and the 50,000-dalton protein complex. *b*, Two-dimensional gel analysis of run-off translation products of spinal cord polyribosomes. Newly synthesized protein is visualized by autoradiography. The positions of the neurofilament polypeptides are circled. The limits of the pH gradients for both gels are indicated at the bottom.

and factors. Addition of rabbit spinal cord polyribosomes to the reticulocyte lysate yielded not only completion of partially synthesized polypeptides but reinitiation of translation and complete synthesis of new polypeptides. Reinitiation continued throughout incubation periods up to 90 min. This was demonstrated by the effect of the inhibitor of initiation, 7-methylguanosine 5′-phosphate (7). Addition of 1 mM 7-methylguanosine 5′-phosphate at the beginning of an incubation stopped incorporation of amino acids into protein within 10 min, by which time run-off translation had been completed. The continued high rate of protein synthesis, in the absence of the inhibitor, represents the translation of the spinal cord mRNA by the reticulocyte ribosomes. Among the readily identifiable proteins synthesized in such a system are the triplet polypeptides (Fig. 6 *d*).

DISCUSSION

The coordinate study of proteins synthesized in vivo and in cell-free systems has enabled us to

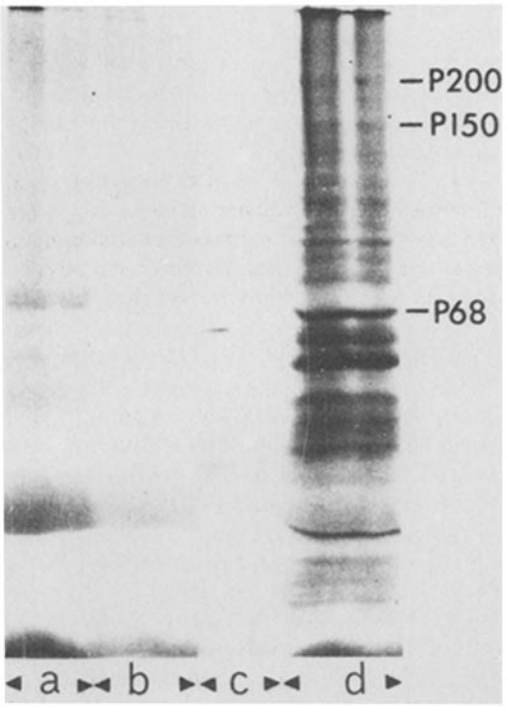

FIGURE 6 Synthesis of filament proteins in a rabbit reticulocyte lysate cell-free system. Analysis by electrophoresis on SDS-polyacrylamide gel followed by autoradiography. *a*, Polypeptides synthesized by endogenous reticulocyte mRNA activity. *b*, Polypeptides synthesized after the lysate has been treated with micrococcal RNAse. *c*, Polyribosomes incubated in the lysate buffer only—no lysate present. *d*, Polyribosomes incubated with RNAse-treated lysate. Position of migration of standard neurofilament polypeptides marked at right. Conditions: *a* and *b*, 85 μl of reticulocyte (nuclease treated in *b*) supplemented with 20 μl of factors as described by Pelham and Jackson (16). *c*, 10 μg (protein) of spinal cord polyribosomes (in 5 μl) are incubated with 85 μl of lysate buffer (without lysate) and 20 μl of supplement. *d*, 10 μg of spinal cord polyribosomes incubated with 85 μl of RNAse-treated lysate and 20 μl of supplement. In all cases, 10-μl samples were taken after 30 min and prepared for electrophoresis.

resolve the question of the origin of the neurofilament polypeptides. All of the triplet polypeptides are synthesized in vivo and in vitro. From the analysis presented in Table I, it is clear that the triplet polypeptides arise as the result of individual translational events, not as the products of the processing of a large parent molecule.

The basic experimental system applied in this study provides a useful approach to the investigation of neurofilament proteins. The ability to isolate, from the same organism, a subcellular structure and the machinery that synthesizes it facilitates the detailed study of the sequence of events from the translation of the message for filament polypeptides to the assembly of the polypeptides into filaments.

The administration of radiolabeled amino acid 1 h before sacrifice of the rabbit made labeled precursor available for the rabbit neurons to synthesize neurofilament polypeptides (and other proteins). Because we prepared polyribosomes and filaments from the same homogenate and because the filaments had hot-acid-insoluble label, we had a preparation that included a population of newly synthesized filament proteins and a population of polyribosomes with the mRNA that specified the labeled filament proteins. The mRNA could be translated in vitro and properties of the direct translation product could be compared with properties of the protein made and processed in the living animal.

The cell-free synthesis of filament polypeptides indicated that these polypeptides are formed directly on ribosomes and do not arise by degradation of a precursor. The run-off translation system that was used for preliminary experiments was not sufficient to demonstrate this unequivocally. The possibility remained that there was a polyribosome-associated factor that might cleave the polypeptides as they emerged from the ribosome. By using the reticulocyte system and confirming that most of the amino acids incorporated in this system were incorporated after reinitiation of translation of the message, we could be confident that we were examining direct translation products and not degraded megaproteins.

The demonstration that the filaments isolated 1 h after administration of [^{35}S]methionine are labeled suggests two possibilities. Either the filaments incorporated protein synthesized within the hour before sacrifice of the animal or there was some nonspecific binding of label to the filaments. Two factors argue against the latter possibility. The filaments were washed by centrifugation through a sucrose gradient containing detergent and 200 mM KCl, a system which should have removed most nonspecific binding substances, including nonassembled triplet. The remaining contaminants should have been separated from the triplet during electrophoresis on SDS gels.

The evidence that the triplet polypeptides of the homogenate, isolated filaments, and in vitro synthesized protein are all homologous is based upon the migration of the triplet polypeptides in the O'Farrell gel system. The same pattern of spots is found in both intact optic nerve and sciatic nerve; these spots disappear when the neurons degenerate (to be published elsewhere). Isolated filaments from spinal cord, brain, and sciatic nerve all include the same polypeptide group as the one that disappears from degenerating nerve. The triplet polypeptides synthesized in vivo and in cell-free systems comigrate with the triplet of isolated filaments and intact nerve.

The synthesis of triplet polypeptides both in vivo and in cell-free systems demonstrated that the triplet is not derived from a larger precursor. There may be common antigenic sites on the different neurofilament polypeptides but each component of the triplet is apparently the translation product of a specific mRNA. These results show that it will be feasible to carry out turnover studies of the individual peptides of the triplet. The incorporation of all three polypeptides into filaments suggests the possibility that the basic subunits of neurofilaments may be multimeric, by analogy to the subunits of microtubules (2).

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