THE SLOW COMPONENT OF AXONAL TRANSPORT

Identification of Major Structural Polypeptides of the Axon and Their Generality among Mammalian Neurons

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ABSTRACT

This study of the slow component of axonal transport was aimed at two problems: the specific identification of polypeptides transported into the axon from the cell body, and the identification of structural polypeptides of the axoplasm. The axonal transport paradigm was used to obtain radioactively labeled axonal polypeptides in the rat ventral motor neuron and the cat spinal ganglion sensory neuron.

Comparison of the slow component polypeptides from these two sources using sodium dodecyl sulfate (SDS)-polyacrylamide electrophoresis revealed that they are identical. In both cases five polypeptides account for more than 75% of the total radioactivity present in the slow component. Two of these polypeptides have been tentatively identified as tubulin, the microtubule protein, on the basis of their molecular weights. The three remaining polypeptides with molecular weights of 212,000, 160,000, and 68,000 daltons are constitutive, and as such appear to be associated with a single structure which has been tentatively identified as the 10-nm neurofilament. The 212,000-dalton polypeptide was found to comigrate in SDS gels with the heavy chain of chick muscle myosin. The demonstration on SDS gels that the slow component is composed of a small number of polypeptides which have identical molecular weights in neurons from different mammalian species suggests that these polypeptides comprise fundamental structures of vertebrate neurons.

The axonal transport system is a mechanism designed to convey newly synthesized proteins originating in the cell body to the axon and its terminals (2, 27, 39, 41, 73). Radioactive tracer studies have revealed that these proteins can be divided into a number of components on the basis of their differing rates of migration within the axon (6, 16, 32, 33, 38, 39, 44, 54, 80). Such studies have demonstrated that by far the largest single fraction of transported proteins, more than half of the total proteins entering the axon, is present in the 1–2 mm per day slow component (33, 37, 38, 44). In their original demonstration of axonal transport, Weiss and Hiscoe (76) suggested that the material moving at a rate of 1–2 mm per day served a structural role in the axoplasm. Such a concept is supported by electron microscope autoradiographic evidence which suggests that the slow component contains microtubules and 10-mm neurofilaments (13, 14, 16) which are among the most prominent structures of the axon. More direct evidence for the movement of microtubules in the

slow component has been provided by the demonstration that the slow component contains tubulin (31, 34, 45), a constituent protein of microtubules (35, 72). At present the identification of the 10-nm neurofilament in the slow component is necessarily based upon morphological grounds since the composition of the neurofilaments has yet to be elucidated.

Evidence suggesting the transport of a relatively small number of axonal structures in the slow component augured well for the possibility that the polypeptide composition of the slow component would be relatively simple. Unlike previous studies of the fast and intermediate components where a remarkably larger number of polypeptides were revealed (80), the results of the present study demonstrate that the polypeptide composition of the slow component is surprisingly simple. In fact, five polypeptides constitute 75–85% of the protein transported in the slow component.

Evidence suggesting a structural role for these axonal polypeptides as well as their general occurrence in mammalian neurons is discussed. Two of the slow component polypeptides have been tentatively identified as tubulin, a major structural protein, on the basis of their molecular weights. The hypothesis that the three remaining slow component polypeptides are associated with 10-nm neurofilaments is also discussed.

MATERIALS AND METHODS

Labeling Axonally Transported Polypeptides in Rat Ventral Motor Neurons and Cat Dorsal Root Ganglion Cells

Numerous studies have clearly demonstrated that the local injection of labeled amino acid precursors into the nervous system results in the labeling of neuron cell bodies contained within the vicinity of the injection site and subsequent transport of labeled proteins from these neuron cell bodies into their axons (3, 6, 9, 16, 18, 19, 27, 32, 33, 37–39, 44, 45, 54, 73). Autoradiographic analysis of such preparations indicates that the diffusion of labeled precursors from the injection site into associated nerves is minimal (37–39). Thus, essentially all of the labeled proteins which are present in the nerve at distances in excess of a few millimeters from the injection site are confined within axons as a result of axonal transport (16, 37, 38). With these considerations in mind, axonally transported polypeptides in both the rat ventral motor neuron (37) and cat spinal sensory neuron (38) were labeled by the local injection of labeled amino acids into the ventral horn region of the rat spinal cord segments L5 and L6 and the cat L7 dorsal root ganglion. A complete description of the labeling procedure and the anatomy of these systems is published elsewhere (37, 38). Briefly, animals (300–400-g male Wistar rats and adult cats of both sexes) were anesthetized with pentobarbital, and the rat spinal cord or cat dorsal root ganglion was surgically exposed by laminectomy. Labeled amino acids were then locally introduced by means of a glass micropipette (tip diameter 70 μm) positioned by means of a micromanipulator. A volume of 1 μl of labeled amino acid concentrated to an activity of 10 μCi/μl was introduced at each injection site over a period of 5 min. In the case of the rat, a total of six injections was made, three on each side of the spinal cord in the L5-L6 region. A total of four injections was made in the cat L7 dorsal root ganglion.

Labeled Amino Acids

[^{3}H]LEUCINE AND [^{3}H]LYSINE MIXTURE: L-[4,5-^{3}H(N)]leucine (30–50 Ci/mmol) and L-[4,5-^{3}H(N)]lysine (20–40 Ci/mmol) were obtained from New England Nuclear, Bostin, Mass., at a concentration of 1 mCi/ml. After Millipore filtration (Millipore Corp., Bedford, Mass.), equal volumes of these isotopes were mixed, and then taken to dryness under a stream of nitrogen gas and brought to a final concentration of 5 mCi/ml each in [^{3}H]leucine and [^{3}H]lysine, through the addition of distilled water.

[^{35}S]METHIONINE: L-[^{35}S]Methionine (201 Ci/mmol) was obtained from New England Nuclear at a concentration of approximately 1 mCi/ml. After Millipore filtration, this label was also taken to dryness under a stream of nitrogen gas, and brought up to a final concentration of 10 mCi/ml by the addition of distilled water.

Determining the Distribution of Labeled Material in the Rat Sciatic Nerve

The distribution of labeled material in the rat sciatic nerve at various postlabeling intervals was determined essentially as described by Lasek (37). After removal of the sciatic nerve and its L5 and L6 roots from Formalin-perfused animals, the nerve and roots were cut into consecutive 3-mm segments starting at the point of attachment of the roots to the spinal cord. Each segment was dissolved in Soluene 100 (Packard Instrument., Inc., Downers Grove, Ill.) and counted in toluene-base cocktail by standard scintillation-counting techniques.

Procedure for Obtaining Nerve Segments Containing Labeled Polypeptides

Immediately after sacrifice by decapitation (rats) or cardiac puncture (cats) the sciatic nerve and roots were rapidly dissected out. These freshly removed, unfixed nerves were then placed on ice and subjected to further analysis or frozen and stored at −20°C.

Trichloroacetic Acid (TCA) Fraction of Labeled Slow Component Material

Unfixed rat sciatic nerves and roots were obtained 33 days after labeling of the L5-L6 ventral motor neurons. The region of the nerve containing the slow component peak (a region extending 25–55 mm from the spinal cord as indicated by the cross-hatched region in Fig. 3) was homogenized in 5 vol of ice-cold 10 mM Tris, pH 7.6. The homogenate was then extracted with cold 5% TCA, hot 5% TCA, ethanol, ether-ethanol (1:1), and ether, by standard procedures. The radioactivity in each of the resulting supernatant fractions (5) as well as in the final pellet was determined.

Solubilization of Labeled Axonal Polypeptides

Nerve segments containing labeled polypeptides were homogenized in 8 M urea which was made 5% in 2-mercaptoethanol and 1% in SDS. After 5 min of incubation in a boiling water bath and rehomogenization, samples were centrifuged at 200,000 *g* (40 K) for 1 h in an SW 50.1 rotor. After centrifugation, the sample consisted almost entirely of a clear supernatant fraction which was overlaid by a small, well-defined lipid layer. An extremely small white pellet was also present. This procedure was found to solubilize 98% of the total radioactivity in the sample. Aliquots of these supernatant fractions were analyzed by SDS-polyacrylamide gel electrophoresis.

SDS-Polyacrylamide Gel Electrophoresis

Labeled polypeptides were analyzed by electrophoresis on SDS-polyacrylamide gels in a discontinuous Tris buffer system essentially as described by Neville (53). Either 5% or 10% acrylamide gels were employed depending on the molecular weight of the polypeptides to be resolved. The 10% gels were made 0.25% in bisacrylamide, and the 5% gels were 0.125% in bisacrylamide. A 3% stacking gel was employed in all cases. The running gels ranged 70–120 mm in length and 7–11 mm in diameter. Samples ranging in volume from 20 to 200 μl were analyzed. After electrophoresis, gels were stained in 0.25% Coomassie blue in 50% methanol, 7% acetic acid, and destained by diffusion in 50% methanol, 7% acetic acid. After destaining, the gels were scanned for optical density at 565 nm with a Gilford spectrophotometer (Gilford Instrument Laboratories, Oberlin, Ohio). The relative mobilities (Rfs) of the stained bands were measured relative to the mobility of the bromphenol blue tracking dye run on each gel. The amount of protein analyzed on each gel was adjusted so that none of the gel bands was overloaded. All of the gels analyzed were comparable to those illustrated in Fig. 5, with regard to the total quantity of protein loaded.

Molecular Weight Polypeptide Standards for SDS-Polyacrylamide Gels

In order to determine the apparent molecular weights of labeled polypeptides, their electrophoretic mobilities were compared to those of the following polypeptides (with their molecular weights in parentheses): chick muscle myosin (212,000), *Myxicola* neurofilament polypeptide (175,000) (Lasek, unpublished observation), rabbit muscle phosphorylase *a* (94,000), bovine serum albumin (68,000), pig brain tubulin (72) (57,000 and 53,000), rat muscle actin (67) (46,000), and chymotrypsinogen (25,000). ^{3}H- and ^{14}C-labeled pig brain tubulin were prepared by the in vitro labeling technique of Rice and Means (61) which has been shown not to affect the electrophoretic mobilities of polypeptides in SDS gels.

Liquid Scintillation Counting

Liquid scintillation counting was carried out with a Packard 3320 scintillation spectrometer. All samples were counted for 10 min or until less than 2.5% counting error was achieved. Disintegrations per minute were determined with either internal or external standardization.

The Identification of Labeled Polypeptides in the Gel

In order to identify labeled polypeptides in the gel, gels were cut into 1-mm slices on a microtome, and the radioactivity in each slice was determined. Each gel slice was dissolved in H_2O_2 and counted in xylene-Triton x-114 scintillation cocktail (1). As the gels were being sliced, the slices containing the prominent stained bands were noted and their positions recorded. In this way it was possible to correlate the positions of the labeled peaks with the positions of the major peaks of the optical density tracing (Figs. 6, 7). In double-label experiments employing ^{3}H in conjunction with either ^{35}S or ^{14}C, there was less than 10% spillover of either ^{35}S or ^{14}C counts into the ^{3}H channel; ^{3}H counts were corrected for such spillover.

RESULTS

The Labeled Slow Component Peak

The somatofugal movement of the slow component at a rate of 1.0–1.2 mm per day is illustrated by the presence of a labeled peak 20 mm from the spinal cord at 20 days, 40 mm at 33 days, 60 mm at 60 days, and 85 mm at 85 days (Fig. 1). At 135 days, only the trailing portion of the slow component is present in the nerve, indicating that no major component is present which moves at a rate less than that of the slow component.

The presence of this moving peak of radioactiv-

ity in the nerve is consistent with the evidence that labeled amino acids are available as precursors for protein synthesis for only a short period of time after their injection into the central nervous system (15, 36). Thus, in effect, the newly synthesized proteins of the neuron are pulse labeled.

Examination of the distributions of radioactivity along the nerve at 33, 60, and 85 days (Fig. 1) reveals that the slow component appears to be composed of two or three subpeaks. Examination of the individual profiles used to generate the mean profiles shown in Fig. 1 confirms that these subpeaks are a real and consistent feature of the slow component. This finding could be explained if the rate of movement of the slow component varied in different motor axons of the sciatic nerve. Such differences in the transport rate might be related to differences in axonal caliber.

The slow component moves at a rate less than that of other components transported within the axon (33, 37, 39, 80) and is separated from faster-moving labeled material during the course of its movement. Thus, the axon can be envisioned as a biological chromatographic column which separates groups of labeled axonal proteins on the basis of their differing rates of somatofugal movement in the axon. Since the labeled slow component peak is the predominant feature of the distribution of labeled material in the motor axons of the rat sciatic nerve 33 days after labeling (Fig. 1), initial characterization of the slow component was carried out on material obtained at this time period from a region of the sciatic nerve extending 25–55 mm from the spinal cord (Fig. 3).

Defining the Slow Component as Protein on the Basis of Its TCA Insolubility

The nature of the labeled material present in the slow component peak of the rat sciatic nerve 33 days after labeling (the cross-hatched region in Fig. 3) was investigated (see Materials and Methods). It was found that 74% of the total label of the slow component could be defined as protein on the basis of its insolubility in TCA, ethanol, and ether:ethanol (51), while less than 2% of the total label was present as cold TCA-soluble material which includes free amino acids and small peptides. In addition, 16% of the labeled slow component material was found to be ethanol soluble. The nature of this ethanol-soluble material has not been defined; however, it may correspond to ethanol-soluble glycoproteins, lipoproteins, or lipids. Thus, at least 74% of the slow component can be defined as protein on the basis of its insolubility in TCA and lipid solvents. This is not surprising, as previous reports have identified the labeled material of the slow component as protein, by the same criteria (6, 32).

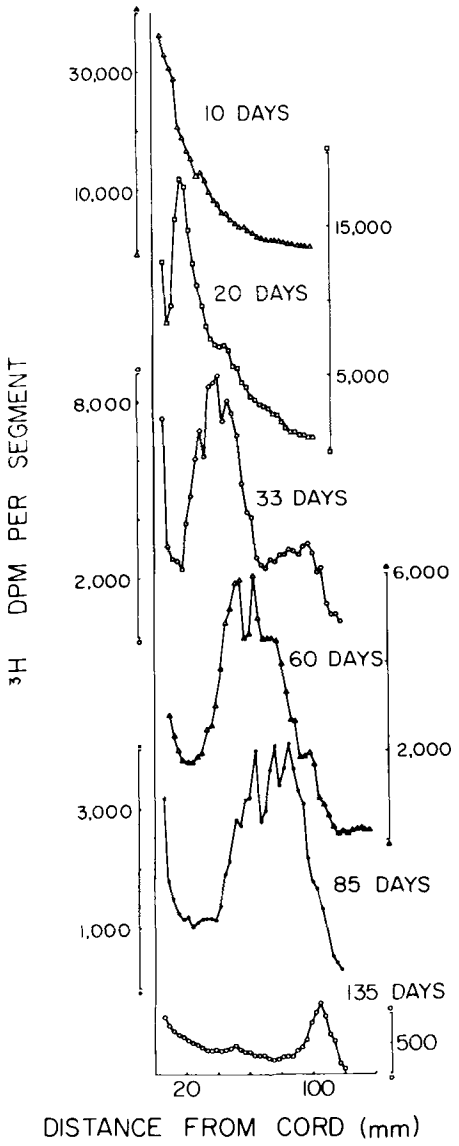

FIGURE 1 Illustrates the somatofugal movement of the rat slow component peak at a rate of 1.0–1.2 mm per day. The ^{3}H content of 3-mm segments of the L5 and L6 roots and their extensions into the sciatic, tibial, and peroneal nerves were plotted against the distance of each segment from the spinal cord. The time intervals in days represent the interval between the injection of ^{3}H-labeled amino acids and sacrifice of the animals. Each data point represents the mean of five values.

Polypeptides Associated with the Slow Component

A complex pattern of labeled polypeptides is present in rat spinal cord 1 day after labeling (Fig. 2). In contrast, analysis of the labeled slow component polypeptides from rat motor axons reveals the most of the label is present in five prominent peaks (Fig. 3), which on the average constitute 75% (64–87%) of the total labeled material on the gel (see also Fig. 9).

The presence of these five labeled peaks was found to be a consistent feature in more than 30 gels of labeled slow component from over 30 rats. The electrophoretic profile of the labeled slow component polypeptides from the cat dorsal root ganglion cell was found to be very similar to that of the rat ventral motor neuron (Fig. 4). For this reason, rat ventral motor neuron slow component polypeptides labeled with [^{35}S]methionine and cat dorsal root ganglion cell slow component polypeptides labeled with [^{3}H]leucine and [^{3}H]lysine were coelectrophoresed on a 10% SDS gel in order to compare the electrophoretic mobilities of the labeled polypeptides from these two sources. Fig. 4 *b* illustrates that the labeled slow component polypeptides from two different types of neuron in two different species, the cat and rat, have identical electrophoretic mobilities.

It is of interest to note that each labeled peak corresponds to a prominent Coomassie blue-stained gel band as shown in the case of the rat sciatic nerve in Fig. 5. The correspondence of labeled peaks to prominently stained bands suggests that these labeled peaks represent major polypeptide constituents of the nerve. This finding is consistent with the view that the slow component may represent the movement of major structural elements of the axoplasm. In addition to the five heavily labeled peaks present on 10% gels, a number of peaks containing less radioactivity were found. For example, peaks with Rfs of 0.41 and 0.61 were consistently found, as well as several minor peaks with Rfs ranging between 0.13 and 0.25 (Figs. 3 and 8). It should be noted that the pattern of labeled peaks on both 5% and 10% gels after labeling with [^{35}S]methionine is identical to that found after labeling with a mixture of tritiated L-leucine and L-lysine.

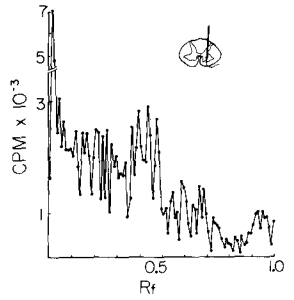

FIGURE 2 Is an electrophoretic analysis on a 10% SDS gel showing the profile of labeled polypeptides present in rat spinal cord at 1 day after the injection of [^{3}H]leucine and [^{3}H]lysine. A schematic drawing of a cross section through the rat spinal cord illustrates the location of the pipette tip used to label ventral motor neurons in the lumbar spinal cord. This procedure leads to the localized labeling of elements contained within the spinal cord without significant labeling of the roots and nerves through the direct diffusion of labeled precursor.

The apparent molecular weights of the major labeled peaks of the rat slow component and the corresponding heavily stained bands were determined by comparing their electrophoretic mobilities to those of standard polypeptides (Figs. 6, 7). The five slow component polypeptides were found to have apparent molecular weights of 212,000, 160,000, 68,000, 57,000, and 53,000 daltons. The electrophoretic mobilities of the 57,000 and 53,000 dalton polypeptides were identical to those of the polypeptides of pig brain tubulin (Figs. 3 and 6). The 212,000 dalton polypeptide was found to have an electrophoretic mobility identical to that of the heavy chain of chick muscle myosin (Figs. 3 and 7).

Comparison of the Slow Component with More Rapidly Transported Polypeptides

Previous investigations have demonstrated the presence of intermediate components moving at rates slightly in excess of that of the slow component (33, 80). Therefore, it was necessary to demonstrate that the labeled polypeptides of the slow component differ from those of more rapidly moving components, and do not represent residual polypeptides left behind by faster-moving components during the course of their journey through the axon. For this reason, rats were sacrificed at 6 days, and the polypeptides of the slow component were compared to the more rapidly transported polypeptides which were present in more distal regions of the nerve (Fig. 8). Note that the profile of labeled polypeptides in region A, which are typical of the slow component, differs from the profiles in more distal regions B, C, and D (Fig. 8). This difference is most obvious in regions C and D where the relative proportion of the five slow

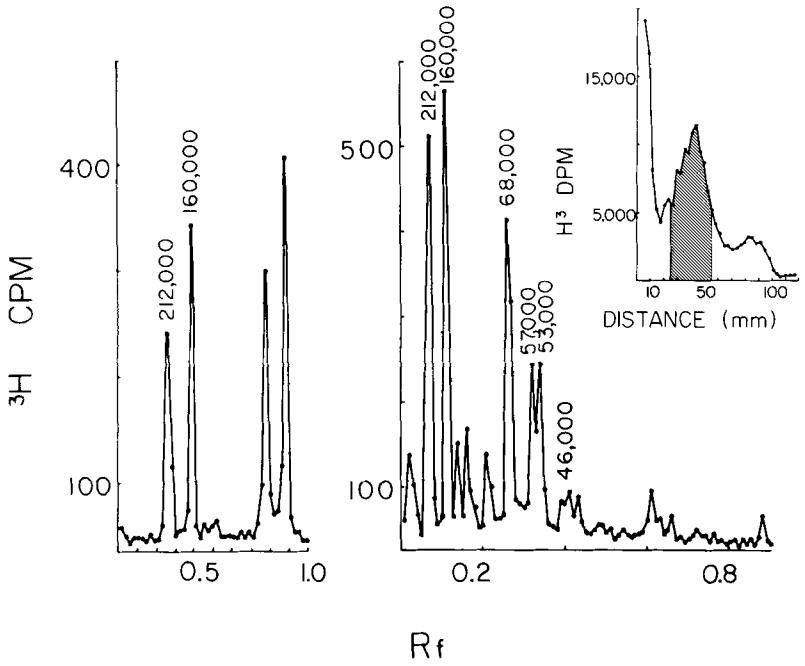

FIGURE 3 Illustrates the profile of labeled, rat ventral motor neuron, slow component polypeptides on SDS-polyacrylamide gels (a 5% gel on the left and a 10% gel on the right). The ^{3}H content of 1-mm slices of these gels was plotted as a function of the relative mobility of the tracking dye (Rf). These gels contain the labeled polypeptides present in a segment of the nerve which was obtained 33 days after labeling ventral motor neurons with a mixture of L-[^{3}H]leucine and L-[^{3}H]lysine. This segment is indicated by the cross-hatched region in the inset. The basic features of the slow component illustrated in these profiles are representative of 30 gels from 30 different animals. The molecular weights of the polypeptides are indicated above the major peaks. Of the original 800-μl homogenate of the segment of sciatic nerve, a 75-μl aliquot was loaded onto the 5% gel, and a 150-μl aliquot onto the 10% gel. Photographs of Coomassie blue-stained gels comparable to these are shown in Fig. 5.

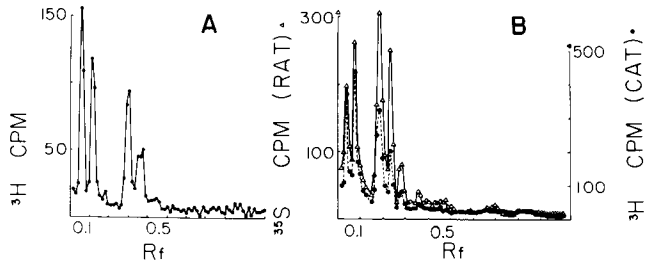

FIGURE 4 A Illustrates the profile of labeled slow component polypeptides obtained from the cat spinal ganglion cells and analyzed electrophoretically on 10% SDS gels. B Shows a comparison of the electrophoretic profiles of the labeled slow component polypeptides obtained from the cat spinal ganglion cells and the rat ventral motor neurons analyzed in the same 10% gel. The difference in Rfs of the slow component polypeptides in these two gels apparently resulted from differences in acrylamide supplied by two different manufacturers. The cat dorsal root ganglion cell slow component was labeled with a mixture of [^{3}H]leucine and [^{3}H]lysine (●-----●), and the rat ventral motor neuron slow component was labeled with [^{35}S]methionine (Δ——Δ). Note that the slow component polypeptides from these two sources have identical electrophoretic mobilities.

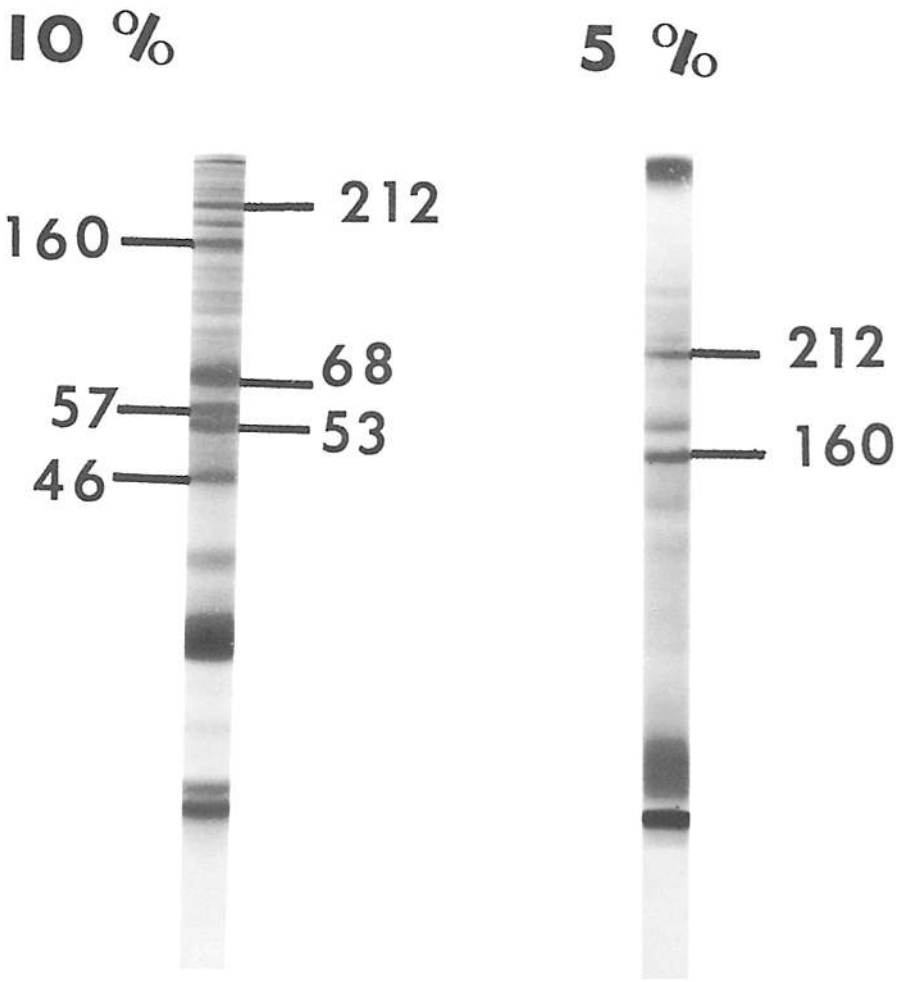

FIGURE 5 Shows photographs of 5% and 10% gels containing protein from the rat sciatic nerve which are stained with Coomassie blue. These gels are representative of those used throughout this study. Note that the heavily stained bands, indicated by their molecular weights (in thousands of daltons), have Rfs corresponding to those of the major labeled polypeptides of the slow component. All gels used in this study were stained and found to be comparable to those shown in this figure, indicating that the gels were not overloaded with protein.

component polypeptides is substantially less than in region A.

It is noteworthy that a labeled peak which has an electrophoretic mobility identical to that of tubulin is the predominant peak in region B (Fig. 8). In fact, the comigration of ^{3}H-labeled pig brain tubulin with [^{35}S]methionine-labeled polypeptides of region B demonstrated that these peaks have identical electrophoretic mobilities (Fig. 8). This demonstrates that a fraction of the labeled polypeptides which have been tentatively identified as tubulin move at a rate slightly greater than that of the other three major polypeptides of the slow component. This finding is consistent with the observation by Karlsson and Sjöstrand (34) that some of the tubulin transported in the axons of the rabbit retinal ganglion cell moves at a rate slightly greater than that of the slow component.

Comparison of the Distribution of the Slow Component Polypeptides

With identification of the five major slow component polypeptides ascertained, it was of interest

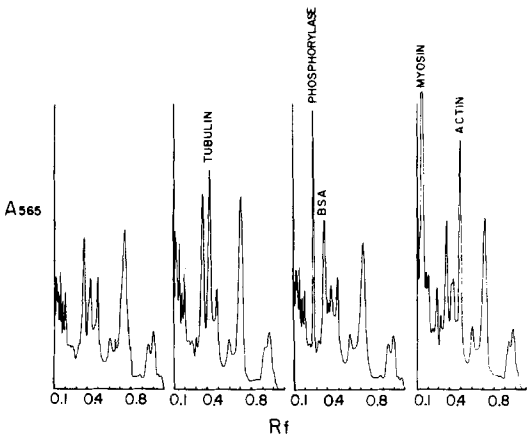

FIGURE 6 Illustrates the optical density tracings at 565 nm of Coomassie blue-stained 10% gels similar to the one illustrated in Fig. 5. The electrophoretic mobilities of the polypeptides present in the rat sciatic nerve are compared to those of polypeptide standards (i.e., actin, myosin, tubulin, bovine serum albumin (BSA), and phosphorylase). Each of these gels contains equivalent amounts of rat sciatic nerve extract in addition to the indicated standards. Note that the rat sciatic nerve contains polypeptides with electrophoretic mobilities (Rfs) identical to those of added tubulin, actin, and BSA standards.

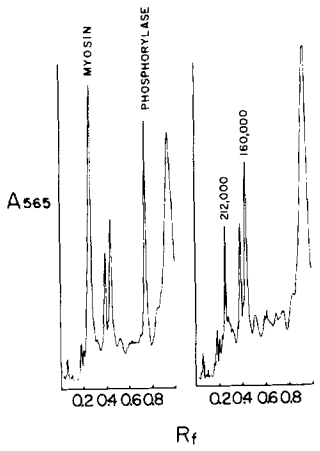

FIGURE 7 Illustrates the optical density scans of Coomassie blue-stained 5% gels. Note the presence of a polypeptide in rat sciatic nerve with an electrophoretic mobility identical to that of chick muscle myosin. See legend of Fig. 6 for details.

to compare the distributions of the individual polypeptides, one to another, during the course of their movement within the axon. Such an analysis was facilitated by the finding that each of the slow

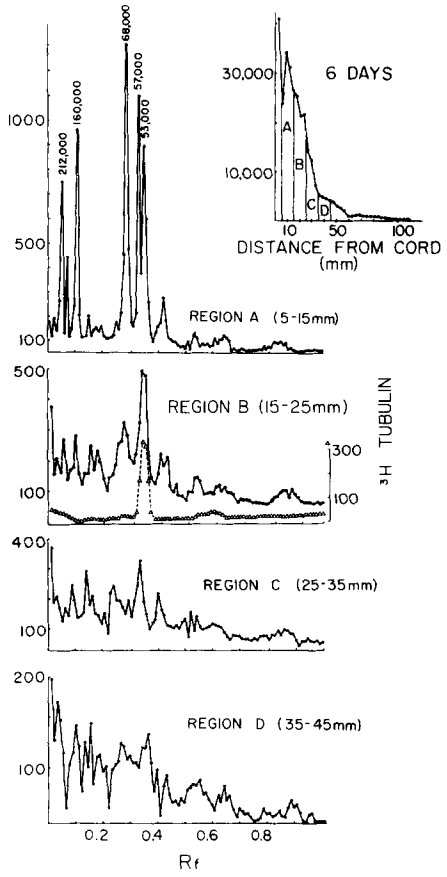

FIGURE 8 Illustrates the profiles of the labeled polypeptides present in different regions of the rat sciatic nerve 6 days after labeling ventral motor neurons with L-[^{35}S]methionine. Each 10% gel contains one-sixth of the total labeled material present in the region of the nerve indicated in the inset. Note that each of the four electrophoretic profiles is different and that three of the major slow component polypeptides that dominate the profile in region A, which contains the slow component peak (see inset), represent a relatively small fraction of the total material in regions B, C, and D, if present at all. The labeled peak which is most prominent in region B has an electrophoretic mobility identical to that of tubulin as demonstrated by the comigration of this peak with ^{3}H-labeled tubulin which was coelectrophoresed with ^{35}S-labeled polypeptides from this region of the nerve. Due to the variability in gel slicing, it is often difficult to obtain a gel slice which symmetrically separates the closely spaced tubulin polypeptides (57,000 and 53,000 dalton polypeptides, Fig. 5). For this reason, the 57,000 and 53,000 dalton labeled polypeptides may be separated by a trough (region A) or appear to form a single broad peak (region B).

component polypeptides corresponds to an easily recognizable, prominently stained band in 10% acrylamide gels (Figs. 3 and 5). The distributions of individual polypeptides were determined by analyzing the labeled material from consecutive 5-mm segments of the nerve on separate 10% gels, and then assessing the radioactivity in each of the gel bands which corresponded to slow component polypeptides. One such gel, sliced in its entirety, is illustrated in Fig. 9.

The results of this analysis are illustrated in Fig. 10, which shows the normalized distributions of the slow component polypeptides 6, 20, 33, and 60 days after labeling. The polypeptides with molecular weights of 212,000, 160,000, and 68,000 daltons are found to have coincidental distributions within the nerve at all four postlabeling intervals. In contrast, the polypeptides tentatively identified as tubulin on the basis of their molecular weights have a distribution in the nerve which is similar to, but perceptibly different from that of the 212,000, 160,000, and 68,000 dalton labeled polypeptides. A fraction of the transported tubulin appears to run ahead of the 212,000, 160,000, and 68,000 dalton polypeptides at all four postlabeling intervals. The labeled polypeptides in the 46,000 dalton region of the gel were found to have a distribution different from that of any of the slow component polypeptides. Rather than moving in the slow component, these polypeptides appeared to accumulate in the nerve 60 mm from the spinal cord by 20 days, and to remain in this region at 33 and 60 days. It is felt that this may reflect the behavior of a class of axonal polypeptides moving faster than the slow component, which were left behind in this region of the nerve. The accumulation of these polypeptides in this particular region

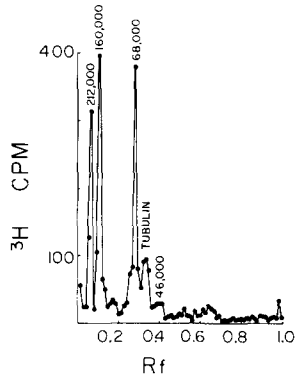

FIGURE 9 Illustrates the electrophoretic profile of a 10% gel containing the labeled proteins obtained from a 5-mm segment of rat sciatic nerve (25–30 mm from the spinal cord at 20 days after injection) containing the most heavily labeled portion of the slow component at this time period. This gel is comparable to those used in the analysis illustrated in Fig. 10 but was sliced in its entirety. Note that this relatively short region of the nerve is particularly enriched in the major slow component polypeptides and that very little radioactivity is present in other polypeptides.

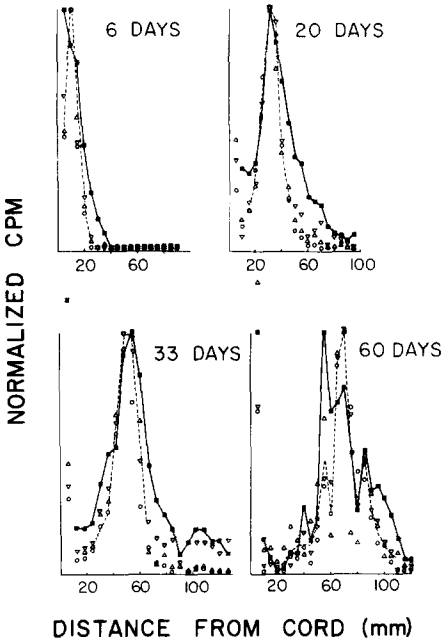

FIGURE 10 Illustrates the distributions of the various slow component polypeptides as a function of distance along the rat sciatic nerve 6, 20, 33, and 60 days after labeling. In order to do this, the labeled protein present within consecutive 5-mm nerve segments was electrophoresed on 10% gels and the stained gel bands corresponding to each of the slow component polypeptides (Fig. 5) were individually sliced out of the gels and counted. In this way, the degree of labeling of each of the slow component polypeptides could be determined within relatively short regions over the entire length of the nerve. Thus, in each case, approximately 20 gels were used to generate the profile at each time period, and each time period represents the analysis of a single nerve. In the diagram, the labeled polypeptides of the slow component are identified by the following symbols: 212,000 daltons (open triangles), 160,000 daltons (open circles), 68,000 daltons (open inverted triangles), and tubulin (solid squares). To facilitate the comparison of the distributions of these polypeptides, they were normalized to the same peak height.

of the sciatic nerve may be due to axonal compression, as it is at this point that the nerve passes through the sciatic foramen.

As we have noted, the 212,000, 160,000, and 68,000 dalton slow component polypeptides have identical distributions within the nerve (Fig. 10). Thus, it is likely that these polypeptides are constitutive, and it seems reasonable to refer to them collectively as the slow component triplet. This nomenclature does not exclude the possibility that other polypeptides belong to this group.

DISCUSSION

A structural role for the material transported in the slow component was first suggested by Weiss and Hiscoe (76) in their classical studies of axonal transport. The results of this study demonstrate that five labeled polypeptides account for 76% of the labeled protein transported in the slow component. As the slow component constitutes more than half of the radioactively labeled protein transported within the axon (33, 44), these five polypeptides account for at least 30% of the total transported protein. Since this small number of long-lived polypeptides accounts for a relatively large fraction of the transported protein, it seems reasonable to suggest that these polypeptides may represent structural constituents of the axon. This suggestion is reinforced by the finding that each of these polypeptides corresponds to an intensely stained band in gels of the rat sciatic nerve (Fig. 5).

In addition to suggesting a structural role for the slow component, the results of this study also suggest the generality of the slow component polypeptides within mammalian neurons. Investigations now in progress have also revealed the presence of the slow component polypeptides in the guinea pig hypoglossal neuron moving at a rate of 1.5 mm/day (M. Black, unpublished observations). In the future, it will be of interest to determine the degree to which the slow component polypeptides have been conserved through the course of evolution of the neuron.

The demonstration by other investigators that tubulin, the microtubular protein (35, 72), is transported in the slow component (31, 34, 45) also suggests a structural role for the slow component. The identification of tubulin in the slow components of the rabbit (34) and goldfish (45) retinal ganglion cell and the chicken ventral motor neuron (31) is based upon the following criteria: (*a*) colchicine binding (34); (*b*) molecular weight (34, 45); (*c*) copurification with tubulin, employing standard purification techniques (34); (*d*) vinblastine precipitation (34, 45); and (*e*) isoelectric point (31). On the basis of these criteria, there is little doubt that tubulin is transported in the slow component, and it appears that we are justified in concluding that the two slow component polypeptides in this study which comigrate with purified tubulin in the SDS gel system are, in fact, tubulin. Investigations currently in progress are designed to provide the evidence necessary to substantiate this conclusion.

Microtubules are among the major structural elements of the axoplasm which, together with 10-nm neurofilaments, appear to form a three-dimensional structural network in the axon (47, 77, 83). Although the actual length of axonal microtubules is unknown, evidence suggests that they may extend uninterrupted over relatively long stretches of the axon (52, 78, 86). The stability of axonal microtubules is yet to be determined; however, their colchicine lability (62) may indicate that they are in a state of dynamic equilibrium with soluble tubulin subunits. In addition to their suggested role in the maintenance of axonal form (83), microtubules have also been implicated to play a role in motile processes within the axon (62). In cilia and flagella, for example, microtubules appear to slide relative to one another, giving rise to the characteristic movement of these structures (68) through the mediation of the ATPase molecule, dynein (25). Although this study does not provide evidence for the transport of dynein in the slow component, it is conceivable that such a protein could exist in association with axonal microtubules (7, 24) and provide a mechanism for their movement.

Although we have tentatively identified two of the slow component polypeptides as tubulin, we can only speculate as to the nature of the remaining polypeptides with molecular weights of 212,000, 160,000, and 68,000 daltons (the slow component triplet). Assuming that these polypeptides constitute identifiable structures, examination of axonal morphology reveals that the candidate structures appear to fall into two broad categories: (*a*) membranous elements, including the agranular reticulum, axolemma, and mitochondria; and (*b*) linear polymers including microtubules, neurofilaments, and their associated matrices (55, 81, 82). At the present time, there is no direct evidence for the movement of membranous structures or membrane-associated substances such as phospholipids (50), gangliosides

(21), or glycoproteins (3, 22) in the slow component. Furthermore, contrary to the original claims that mitochondria move in the slow component (79), all subsequent, more direct evidence suggests that the bulk of mitochondria move at rates greater than that of the slow component (9, 33, 42, 84, 85). In contrast to the apparent absence of membranous structures in the slow component, there is direct evidence suggesting the movement of linear elements such as microtubules and neurofilaments in the slow component. In addition to direct evidence for the movement of tubulin in the slow component (31, 34, 35), electron microscope autoradiographic evidence strongly suggests the movement of neurofilaments as well as microtubules in the slow component (13, 14, 16).

On the basis of these considerations, it appears likely that the polypeptides of the slow component are associated with microtubules, neurofilaments, and their surrounding matrices. The constitutive nature of the polypeptides of the slow component triplet (Fig. 10) would indicate that they are associated with one another, possibly forming a structural complex. The conclusion that these polypeptides are not constituents of axonal microtubules is suggested by: (*a*) the differences in the axonal distributions of tubulin and the slow component triplet (Fig. 10); and (*b*) the finding that intact microtubules isolated from brain are composed primarily of tubulin (35). On the basis of this reasoning, we cannot exclude the possibility that the slow component triplet constitutes a structure intimately associated with the microtubules, i.e. cross-arms connecting them with one another or with neurofilaments (47, 55, 56, 83). However, the differences in the distributions of the polypeptides of the slow component triplet and those tentatively identified as tubulin lead us to propose that the polypeptides of the slow component triplet are associated with the 10-nm neurofilaments and possibly their surrounding maxtrix (55, 56, 81, 82).

If the polypeptides of the slow component triplet are associated with neurofilaments, then the comparison of these polypeptides with those present in preparations of neurofilaments from various sources should reveal similarities. In fact, such comparisons reveal the presence of polypeptides in these preparations with molecular weights similar to those of the polypeptides of the slow component triplet. For example, relatively pure neurofilaments isolated from the giant axon of the polychete worm *Myxicola infundibulum* are composed primarily of a 175,000 dalton polypeptide (Lasek, unpublished observations), which is in the molecular weight range of the 160,000 dalton polypeptide of the slow component triplet. Preparations enriched in mammalian neurofilaments (65) (kindly provided by Dr. George De Vries) were found to contain polypeptides which comigrated with the 212,000 and 68,000 dalton polypeptides of the slow component triplet as well as a 175,000 dalton polypeptide, which is in the molecular weight range of the *Myxicola* neurofilament polypeptide. In addition to these polypeptides, the mammalian neurofilament preparation contains a major polypeptide with a molecular weight of 50,000 daltons (10, 12 64). The significance of this polypeptide is unknown, as it does not appear to be present in the rat sciatic nerve (Fig. 5) which contains numerous neurofilaments. In fact, recent evidence suggests that the 50,000 dalton material may be derived from higher molecular weight polypeptides present in these preparations (64). Another polypeptide which appears to be associated with squid neurofilaments (29) has a molecular weight similar to that of the 68,000 dalton polypeptide of the slow component (49). The identity of the main structural polypeptides of the neurofilament is yet to be established; however, the presence of polypeptides with molecular weights in the range of 160,000–180,000 daltons in neurofilaments from *Myxicola*, squid (unpublished observations), and mammalian sources as well as the mammalian slow component may indicate that the primary structural polypeptides of the neurofilament fall in this molecular weight range.

The available data allow us to estimate the relative proportions of each of the labeled polypeptides of the slow component triplet. If it is assumed that these polypeptides are synthesized at the same rate from equivalent precursor pools, and if one adjusts for the level of incorporation as a function of polypeptide chain length, the relative proportions of the 68,000, 160,000, and 212,000 dalton polypeptides are found to be 10, 5, and 3, respectively.

It is of interest that polypeptides in both the slow component and preparations of mammalian neurofilaments were found to have molecular weights identical to that of the heavy chain of chick muscle myosin (212,000 daltons). Since both actin (4, 20, 58, 59) and myosin (4, 58, 59) appear to be neuronal proteins, this finding is especially tantalizing in that it raises the possibility that neuronal myosin is present in the slow component. The slow

component also appears to contain several minor peaks (Rf of 0.6 in Fig. 3) which have apparent molecular weights of 25,000 daltons, which is similar to that of myosin light chains from both cardiac and skeletal muscle (71). Immunological investigations currently in progress are designed to evaluate critically the hypothesis that myosin is transported in the slow component.

It is interesting to note that neurofilaments appear to share certain similarities with the thick filaments of skeletal muscle. For example, X-ray diffraction studies of *Myxicola* axoplasm, which is composed primarily of neurofilaments (11), indicate the presence of proteins with substantial alpha helical content arranged in a coiled coil (11). Morphological examination of neurofilaments reveals the presence of side-arms (46, 77, 81, 82, 83) which have dimensions similar to those of the arms of skeletal muscle thick filaments (30). This comparison is not meant to suggest that neurofilaments and skeletal muscle thick filaments are homologous, but rather that these structural similarities may be indicative of functional analogy.

Although the myosin hypothesis is attractive, one should not ignore the possibility that the 212,000 dalton peak may represent a trimer of covalently cross-linked 68,000 dalton polypeptides. The covalent cross-linking of polypeptide chains has been shown to occur in processes such as clot stabilization (40, 43, 57) and collagen maturation (60, 69, 70). Such covalent bonds would not be disrupted by the agents used to solubilize the slow component polypeptides in this study.

The results of this study are consistent with the view that the slow component represents a mechanism for the continuous renewal of major structural elements of the axoplasm, possibly the microtubules and neurofilaments. Essentially the same view was originally proposed by Weiss and Hiscoe (76) in their classic studies of the slow component, even though they lacked information concerning the ultrastructural features of the axon. As the microtubules and neurofilaments are among the major structural elements of the axoplasm, regulating their number in the slow component could play a direct role in determining axonal volume (23, 77). One might envision that the slow component polypeptides are assembled into structures such as microtubules and neurofilaments while they are still in the cell body, followed by the transport of these structures "en masse" toward the axon terminals. In order to maintain the relatively constant volume characteristic of the mature axon, it would appear necessary for these structures to be continuously disassembled in the region of the axon terminals.

In addition to serving a major structural role in the axon, microtubules and neurofilaments transported in the slow component might also play a role in the mechanisms of axonal transport (62, 63). Their linear organization makes them ideal candidates for serving as the substrate along which other axonal structures such as vesicles and mitochondria may be rapidly transported (54, 63). Furthermore, the microtubules and neurofilaments may exhibit specificity with regard to the structures in whose transport they participate. In fact, evidence suggests the specific participation of microtubules in the transport of mitochondria (66). The molecules comprising the microtubules and neurofilaments may themselves also participate in generating the forces responsible for their own movement in the slow component.

A Hypothetical Model Involving Actin-Myosin Interactions in the Transport of the Slow Component

These new findings raise the possibility that myosin, a molecule which is known to play a role in cellular motility, is present in the slow component possibly as a constituent of neurofilaments. The presence of myosin in the slow component would make possible the involvement of actin-myosin interactions in the movement of the slow component (63). Both actin (4, 20, 58, 59) and myosin (4, 58, 59) appear to be neuronal proteins, and with the heavy meromyosin binding technique it has been possible to demonstrate the presence of a network of actin-like filaments attached to the inner surface of the axolemma (8). Such an arrangement of actin filaments interacting with myosin may play a role in a number of motile processes (17). Before examining how such interactions in the axon could lead to the movement of the slow component, we will first examine some structural properties of the major organelles transported in the slow component, microtubules, and neurofilaments.

Axonal neurofilaments are longitudinally oriented structures, which by virtue of their helical character (11, 26, 47) appear to be somewhat elastic (26). In contrast to the neurofilaments, axonal microtubules are straight, relatively rigid structures which appear to act as skeletal elements

in the axon (83). Consistent with such a skeletal role, individual microtubules appear to extend as uninterrupted structures over relatively long distances within the axon (52, 78, 86). Groups of axonal neurofilaments are often interconnected via crossbridges to form longitudinally oriented fascicles (47, 56, 77, 83). Neurofilaments within these fascicles are frequently connected via cross bridges with microtubules as well, giving rise to a three-dimensional network of microtubules and neurofilaments within the axon (47, 48, 83). The coherence of the slow component peak, which is exhibited throughout the course of its movement (15), is consistent with the movement of a network consisting of strongly interacting elements whose relative positions are maintained throughout the course of their movement (74).

Given these observations, the following speculative model is proposed. Myosin-containing neurofilaments of the axonal matrix contact actin filaments which are attached to the axolemma, allowing them to participate in generating forces exerted between the axolemma on the one hand, and the axoplasmic network of microtubules and neurofilaments on the other. These radially directed forces could be translated into longitudinal forces if they were largely exerted on the relatively rigid microtubules. Since these forces acting upon the microtubules would be circumferentially directed from many sites of interaction, any forces tending to bend the microtubules in one direction would be balanced by opposing forces directed in the opposite direction; and the net direction of movement would be longitudinal rather than radial. Moreover, the elastic properties of the neurofilaments and the axolemma would allow the energy expended during repetitive actin-myosin interactions to be stored as kinetic energy, allowing the forces developed at a number of sites of interaction to act in concert. As these forces developed, they might first lead to a distortion of the axolemma. Subsequently, when these forces are great enough to exceed those which tend to resist the movement of the axoplasmic network of microtubules and neurofilaments, this network would move, allowing the axolemma to return to its original configuration. In fact, wavelike distortions of the axolemma have been observed, which are propagated somatofugally at a mean rate of 1 mm per day (75), which is identical to the rate of movement of the slow component. The apparent unidirectionality of the slow component in the proximodistal direction could reflect the unipolar orientation of myosin molecules in the neurofilament. By placing the neurofilament in a pivotal position in the generation of forces which more the slow component, we are confronted by the unusual situation of axons in crustacea which do not contain demonstrable neurofilaments but are packed instead with microtubules (18, 52, 62). Since the absence of neurofilaments in crustacea represents an unusual case in phylogeny, it seems premature to rule out the neurofilament as a pivotal element in axonal transport. Instead, we suspect that the crustacea may have to be treated as a special case. This possibility is reinforced by the finding that unlike other axons, crayfish axons can survive for months after they are severed from the cell body (28).

Although these ideas we have developed are speculative, we feel they warrant expression, since they suggest new avenues of research on the mechanisms of axonal transport.

This study was supported by National Institutes of Health grant NS-09299-05, Career Development award HD 28965, and a Sloan Foundation Fellowship to Dr. Lasek and National Institutes of Health traineeship HD 00020 to Dr. Hoffman.

Received for publication 16 January 1975, and in revised form 31 March 1975.

Note Added in Proof: the possibility that the slow component triplet is phylogenetically invariant during vertebrate evolution is supported by the demonstration of these polypeptides in the slow components of the guinea pig retinal ganglion cell and the spinal ganglion sensory neuron of an amphibian *Bufo marinus* (M. Rossi and M. Black, unpublished observations).

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