

DISTRIBUTION OF LEUCINE-³H DURING AXOPLASMIC TRANSPORT WITHIN REGENERATING NEURONS AS DETERMINED BY ELECTRON-MICROSCOPE RADIOAUTOGRAPHY

THOMAS L. LENTZ

From the Department of Anatomy, Yale University School of Medicine,
New Haven, Connecticut 06510

ABSTRACT

The distribution of leucine-³H in neurons was determined by electron-microscope radioautography after infusion of label into the spinal cord or sensory ganglia of regenerating newts. In the nerve cell bodies 3 days after infusion, the highest concentration of label per unit area occurred over the rough-surfaced endoplasmic reticulum. In the large brachial nerves, the silver grains were not distributed uniformly in the axoplasm, indicating that the labeled materials are restricted in their movement to certain regions of the axon. Almost all of the radioautographic grains observed in myelinated nerves could be accounted for by the presence of a uniformly labeled band occupying the area 1500–9000 Å inside the axolemma. This region of the axon was rich in microtubules and organelles while the unlabeled central core of the axon contained mainly neurofilaments. This observation supports the hypothesis that microtubules are related to axonal transport. In small, vesicle-filled nerve terminals in the blastema, labeled material was restricted to a thin zone a short distance beneath the plasma membrane while the central region of the terminal was largely unlabeled. The peripheral pattern of labeling in the nerve endings is consistent with successive addition of newly synthesized proteins at the periphery of the growth cone and release of substances such as trophic factors at the nerve terminal.

INTRODUCTION

Proximodistal flow of axoplasm from nerve cell bodies down the axon has been demonstrated by a variety of techniques (Weiss, 1963, 1969; Lubiąska, 1964; Barondes, 1967). Substances believed to be carried by axoplasmic flow include part or all of the axoplasmic constituents such as proteins, enzymes, and phospholipids, transmitter substances, and trophic materials. Radioactive tracer methods have been used to study axoplasmic transport. A high level of incorporation of labeled precursors into nerve cells can be achieved by injecting the labeled substances directly into

the nerve tissue (see Lasek et al., 1968). With this method, it has been shown that tritiated amino acids injected into the spinal cord or dorsal root ganglia are incorporated into proteins in the nerve cell body and subsequently transported down the axon (Ochs et al., 1967; Lasek, 1968).

In the present study, axoplasmic transport of leucine-³H was determined by electron microscope radioautography after infusion of label into the spinal cord or sensory ganglia of regenerating newts. During limb regeneration of newts, there is rapid outgrowth and regeneration of nerve fibers

into the blastema (Singer, 1949; Lentz, 1967). During regeneration, it can be expected that neurons are engaged in active synthesis of axoplasm and axoplasmic constituents. The nerves, furthermore, are believed to elaborate and release a trophic substance (Singer, 1952, 1960; Lentz, 1971) necessary for limb regeneration. High-resolution radioautography allows the intra-axonic

distribution and routes of transport of labeled materials to be determined while these processes are occurring.

MATERIALS AND METHODS

These studies were performed on adult newts, *Triturus viridescens*, which were maintained in large aquaria in the laboratory. Newts were anesthetized

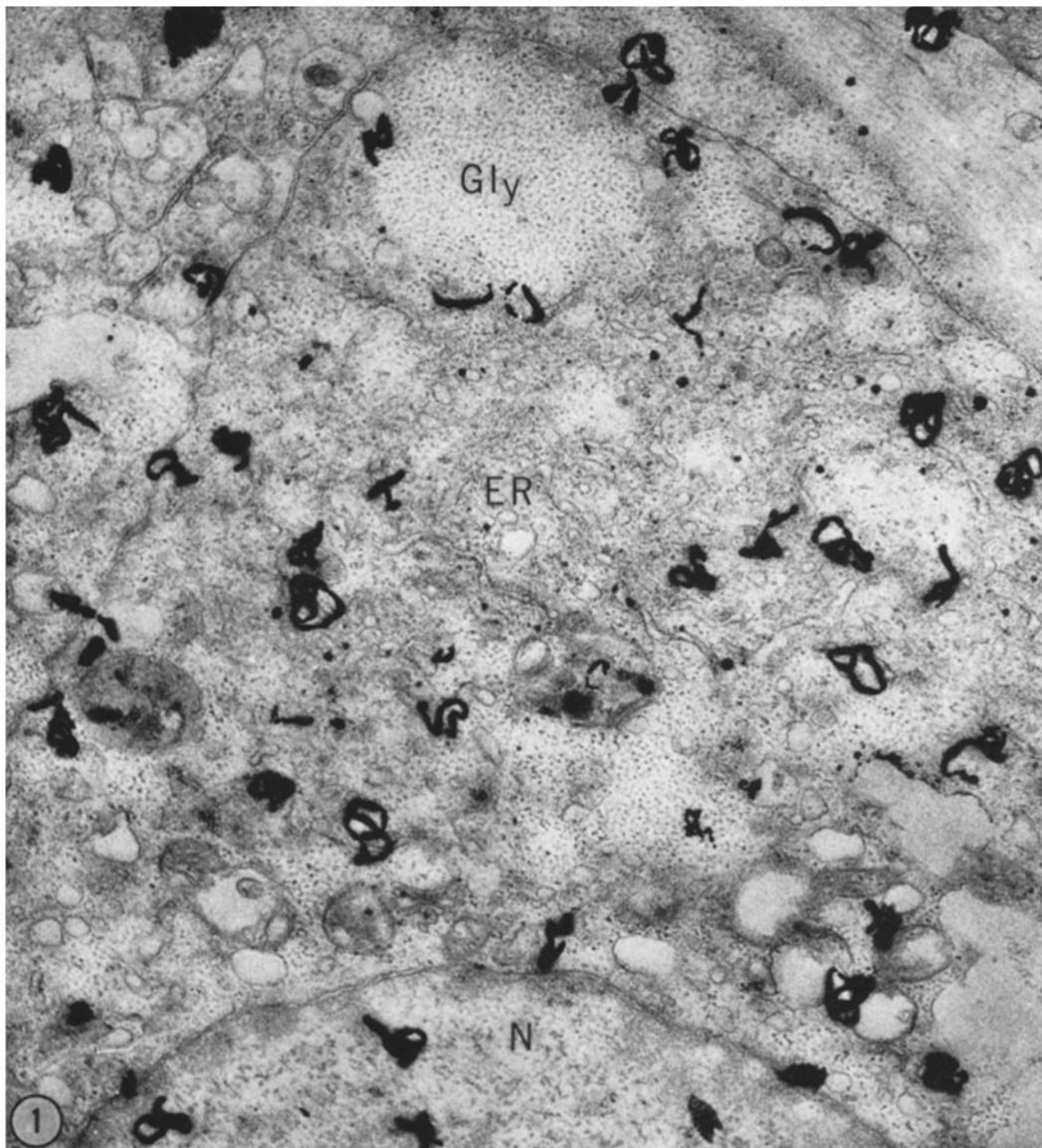


FIGURE 1 Radioautograph of a sensory ganglion cell 3 days after infusion of leucine-³H into the ganglion. Silver grains occur throughout the cell, indicating effective infusion and incorporation of labeled leucine. *N*, nucleus; *ER*, endoplasmic reticulum; *Gly*, glycogen. $\times 17,000$.

in 0.1% Chloretone and the limb was amputated at the level of the lower third of the upper forelimb. The newts were then placed on damp paper in covered finger bowls and fed chopped beef liver three times a week. The limbs were allowed to regenerate for periods of 2–22 wk before infusion of tritium-labeled leucine into sensory ganglia or spinal cord.

Infusion of Leucine-³H

Tritium-labeled leucine (L-leucine-4,5-³H) with a specific activity of 38.5–58 Ci/mmmole was obtained from New England Nuclear Corp., Boston, Mass. A solution of 0.9% saline containing 6 μ Ci leucine-³H/ μ l was prepared for infusion.

The regenerating newts were anesthetized and the spinal cord or sensory ganglia exposed. The spinal cord was exposed by making an incision parallel to the vertebral column, dissecting away the spinal muscles, and removing the vertebral laminae. The ganglia were exposed by making an incision down to the transverse processes of the vertebrae and by chipping away the transverse process and portion of the rib that overlie the ganglion (Singer, 1942). After infusion, the wound edges were approximated and the newts kept in finger bowls. Newts tolerated this procedure remarkably well with little mortality and were active and feeding within 1 day after the operation. Animals in which there was subsequent paralysis of an arm were not used.

The labeled leucine solution was infused into the exposed spinal cord or sensory ganglia by using the procedure described by Lasek (1968). The infusion apparatus consisted of a 50 μ l Hamilton syringe (Hamilton Co., Whittier, Calif.) connected to a glass micropipette by polyethylene tubing. Injection of the solution was performed under a dissecting microscope slowly over a period of about 5 min. Injections were made into the spinal cord at the third to fifth cervical segments or into the fourth dorsal root (sensory) ganglion. 5–10 μ l (30–60 μ Ci leucine-³H) were infused into each animal.

Tissues were removed for fixation 3 days after in-

fusion of leucine-³H. Spinal cord, fourth sensory ganglion, segments of large brachial nerves approximately 2 mm from the spinal column, and the blastema of the regenerating limb were removed. The tissues were fixed for 1 hr in cold 3% glutaraldehyde in 0.5 M cacodylate buffer (pH 7.2). The blocks were rinsed briefly in cold buffer and fixed for an additional hour in cold 1% osmium tetroxide in 0.05 M cacodylate buffer at pH 7.2. The tissues were dehydrated in ethanol, infiltrated with Maraglas (Freeman and Spurlock, 1962), and polymerized at 49°C. Thick, 1–2- μ sections were cut with glass or diamond knives on a Sorvall MT-2 microtome (Ivan Sorvall, Inc., Norwalk, Conn.) and stained with 0.1% toluidine blue for light microscope orientation.

Radioautographic Procedures

Sections were prepared for radioautography according to the methods of Salpeter and Bachmann (1964). Silver-gold thin sections were placed on glass slides coated with 1% collodion. The sections were then stained for 2–5 min with 2% aqueous uranyl acetate and 5–10 min with lead citrate (Reynolds, 1963). The slides with sections were vacuum coated with a 50–60 Å layer of carbon. The slides were dipped in Ilford L4 emulsion (Ilford Ltd., Ilford, Essex, England) diluted 1 to 4 and containing 1% glycerol. They were then drained and allowed to dry, producing a purple interference color of the emulsion (~1500 Å thick). The slides were placed in light tight boxes in the presence of Drierite (W. A. Hammond Drierite Co., Xenia, Ohio) and stored in a desiccator at 4°C. The slides were exposed for a period of 14–20 wk.

The slides were developed at room temperature for 4 min with Microdol X (Eastman Kodak Co., Rochester, N. Y.). After fixing and rinsing, the supporting membrane was stripped from the glass slides, copper grids were placed over the sections, and the membrane with emulsion and grids was picked up. The sections were then examined with an RCA EMU 3F electron microscope.

Several controls were performed to determine the

TABLE I
Comparison of the Distribution of Silver Grains in Sensory Ganglion Cells
with the Areas Comprised by the Cellular Constituents

	Nucleus	Endo- plasmic reticulum	Cytoplasm	Mito- chondria	Lyso- somes	Glycogen	Golgi
% of grains	11	23	47.5	8.5	2.5	2	5.5
% of area	17	12.5	54	6.5	2	3	5
% of grains/% of area	0.65	1.84	0.88	1.31	1.25	0.67	1.10

Based on 295 grains and 861 random points.

extent of background radiation and amount of labeled material reaching the nerves by other than intra-axonal routes. Some tissues from regenerating animals were processed for radioautography but without previous administration of labeled material to the animal. Blastemal tissue was taken from animals receiving leucine- ^3H but in which the brachial nerves supplying the limb were severed immediately before infusion. Finally, in animals in which the sensory ganglion was infused with leucine- ^3H , tissues were taken from the limb on the side opposite the infused ganglion. Some sections of nerve and blastema were left uncoated for study of nerve morphology.

Analysis of Radioautographs

The distribution of silver grains in nerve cell bodies was analyzed by comparing the localization of silver grains with the areas comprised by the cellular components (Ross and Benditt, 1965). The center of a circle enclosing the silver grains was used to designate the structure beneath the grain. Areas were determined by applying a transparent screen of regularly spaced points over the micrographs and calculating the percentage of points overlying each structure.

For analysis of the distribution of silver grains in nerve fibers, the procedures of Salpeter et al. (1969) were employed (see also Budd and Salpeter, 1969). Histograms were constructed of the density of silver grains inside and outside the axons. First, the number of grain midpoints per unit perpendicular distance on both sides of the axon membrane were determined. Relative areas were obtained by placing a transparent sheet with regularly spaced points over the micrographs and determining the number of points per unit distance from the membrane. The density distribution (grains per unit area) was obtained by dividing the number of silver grains per unit distance from the axolemma by the number of points from the uniform grid in the same unit distance. Distances were tabulated in units of half distance (HD, the distance from a line source in a radioautographic specimen within which half the developed grains fall). For the material used in this study (pale gold sections, monolayer of Ilford L4 emulsion, Microdol X development), HD is approximately 1500 Å.

The experimental grain-density distributions were then compared with the universal density curves provided by Salpeter et al. (1969) for radioactive sources of different shapes. Curves for other sources (hollow circle labeled at its periphery superimposed on a solid disc, uniformly labeled circular band) were constructed by combining the theoretical curves for hollow circles or solid discs. The theoretical distribution for a uniformly labeled circular band was determined by numerical integration of density functions for hollow circles with radii from the inner



FIGURE 2 Radioautograph of a myelinated nerve from the brachial plexus 3 days after infusion of leucine- ^3H into the spinal cord. Note the dense concentration of silver grains within the axoplasm and absence of grains elsewhere. $\times 11,000$.



FIGURE 3 Radioautograph of a myelinated nerve and Schwann cell from the brachial plexus 3 days after infusion of leucine- ^3H into the spinal cord. Grains occur in the axoplasm and appear to be concentrated in a zone roughly midway between the axolemma and center of the axon. Little label is seen over the Schwann cell and extracellular space. $\times 22,000$.

to outer circumference of the band. In all cases, the theoretical and experimental distributions were normalized to 1.0 at the same point on the x-axis.

RESULTS

Control Experiments

Approximately one to two silver grains were seen per grid square in tissues processed for radioautography but not receiving label and in the portions of the sections of experimental blocks not containing tissue. Only an occasional silver grain was observed in cells of tissues which were taken from animals in which the brachial nerves were severed before infusion or which were taken from the side opposite the infused ganglion (Fig. 10). These findings rule out the presence of significant background radiation, chemography, distant diffusion of label from the injected site, or widespread distribution of labeled material via the bloodstream. Thus, the great majority of radioautographic grains observed within the nerve fibers are attributed to transport of labeled material from the infusion site.

Sensory Ganglion and Spinal Cord

After administration of tritium-labeled leucine, heavy concentrations of label were found over many sensory ganglion cells and spinal cord neurons. Radioautographic grains were located over all of the cellular constituents (Fig. 1). Analysis of the grain distribution (Table I) in sensory ganglion cells showed that the frequency of silver grains over the rough-surfaced endoplasmic reticulum was greater than if the grains were distributed randomly. Fewer grains occurred over the nucleus than expected with a random distribution.

Few grains occurred over the intercellular spaces in the sensory ganglia and spinal cord. Other cellular constituents (pigment cells, Schwann cells, satellite cells, neuroglia, fibroblasts), however, contained radioautographic grains. Both myelinated and unmyelinated nerve fibers were heavily labeled.

Some of the nerve cells and fibers, particularly in the sensory ganglia, showed evidence of degeneration. Cell bodies were disrupted and disorganized with degeneration or lysis of organelles. Nerve fibers showed degeneration of axoplasm and breakdown of myelin sheaths. Most cells and fibers in the ganglion did not show these changes and

were normal in appearance. Disrupted cells and fibers tended to be clustered in localized regions and were not usually labeled to the extent of the normal cells.

Nerves of the Brachial Plexus

In the large nerve trunks, label was largely confined to the nerve fibers. More nerves contained radioautographic grains after infusion of the spinal cord than the sensory ganglion. A greater number of degenerating axons were seen after ganglion injection. The greatest number of grains occurred over the axoplasm of the large myelinated nerve fibers (Figs. 2, 3) while a smaller number were found over small unmyelinated fibers. Intercellu-

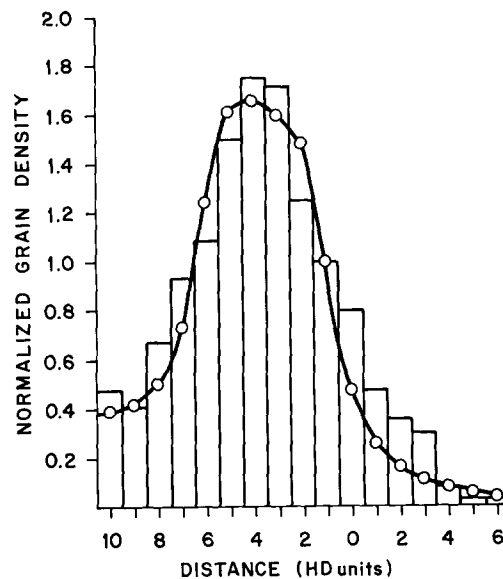


FIGURE 4 Histogram (bars) of the density distribution of silver grains around myelinated axons in the brachial plexus 3 days after infusion of leucine- ^3H into the spinal cord. Distance is measured in units of HD (1500 Å) on both sides of the axolemma (point 0) (left-inside the nerve; right-outside). The grain density was normalized to 1 at 1 HD unit inside the axolemma. The greatest density of grains lies from 3 to 5 HD units inside the limiting membrane. The smooth curve superimposed on the histogram represents the expected distribution if the radioactivity was confined to a solid, circular band 5 HD units wide and with an inner radius of 4 HD and an outer of 9. The outer edge of the band is located 1 HD inside the membrane and normalized at this point. There is a close correspondence between the experimental and theoretical distributions. Histogram based on 19 nerve fibers with an average radius of 1.5 μ , 186 grains, 1474 random points.

lar spaces, Schwann cells, and connective tissue elements were largely unlabeled.

When the distribution of grains within the axons was plotted (Fig. 4), it was found that the density of grains was greatest about 6000 Å (4 HD) inside the axolemma and was less toward the center and the periphery of the axon. Such a distribution was not always readily discernible upon inspection of the radioautographs, emphasizing the importance of actual measurement of the location of large numbers of grains to determine their distribution. The experimental distribution of grains most closely fit the theoretical distribution of a solidly labeled circular band with an inner radius of 6000 Å (4 HD units) and an outer radius of 13,500 Å (9 HD units) located 1500 Å (1 HD unit) inside the axolemma. The experimental grain distribution outside the axon indicates that the myelin sheath and extracellular spaces were not radioactive. The few grains occurring over these structures are accounted for by scatter from the

radioactive nerve. The experimental distribution did not agree with other theoretical curves. For example, if the nerve were uniformly labeled, the grain density would be highest at the center and decrease progressively toward the periphery. A circle of sufficient radius labeled at its periphery would yield a peak of density at the edge of the circle and show a sharp decline on either side.

The structure of typical axons is illustrated in micrographs of uncoated sections (Fig. 5, 6). Neurofilaments are distributed throughout the cross-sectional diameter of the axoplasm. The axons have a variable and generally sparse complement of organelles. However, when present, the organelles tend to be distributed toward the periphery of the axon. Thus, the central core of the axon is largely occupied by neurofilaments, whereas the periphery contains a greater number of microtubules, mitochondria, channels of smooth-surfaced endoplasmic reticulum, and an occasional vesicle as well as neurofilaments.

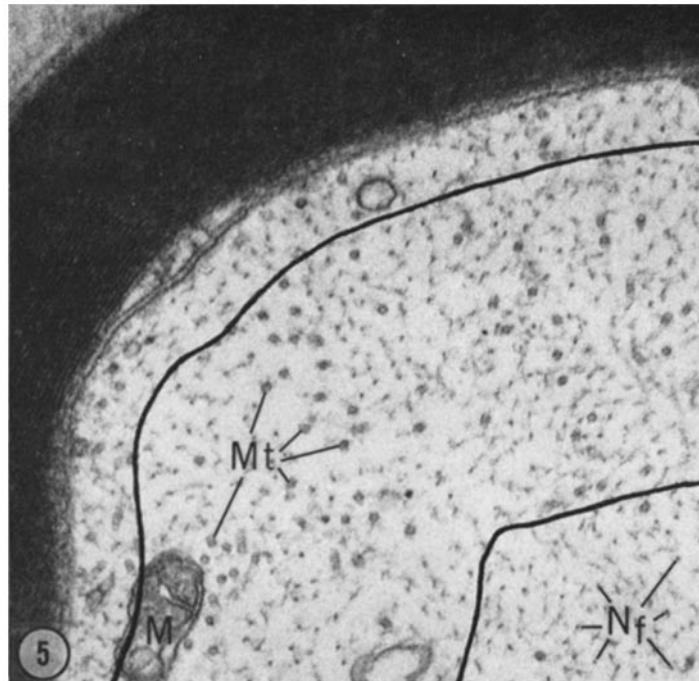


FIGURE 5 Portion of a myelinated nerve in the brachial plexus of the newt. Radii of 4 HD units (6000 Å) and 9 HD units (13,500 Å) are superimposed on the axon. The observed distribution of silver grains in myelinated axons after infusion of the spinal cord with leucine-³H can be accounted for by uniform labeling of the axon between these radii. Note that this region of the axoplasm contains a larger number of microtubules (*Mt*) than elsewhere. Neurofilaments (*Nf*) are found throughout the axon. Note the occurrence of microtubules around the mitochondrion (*M*). $\times 59,000$.

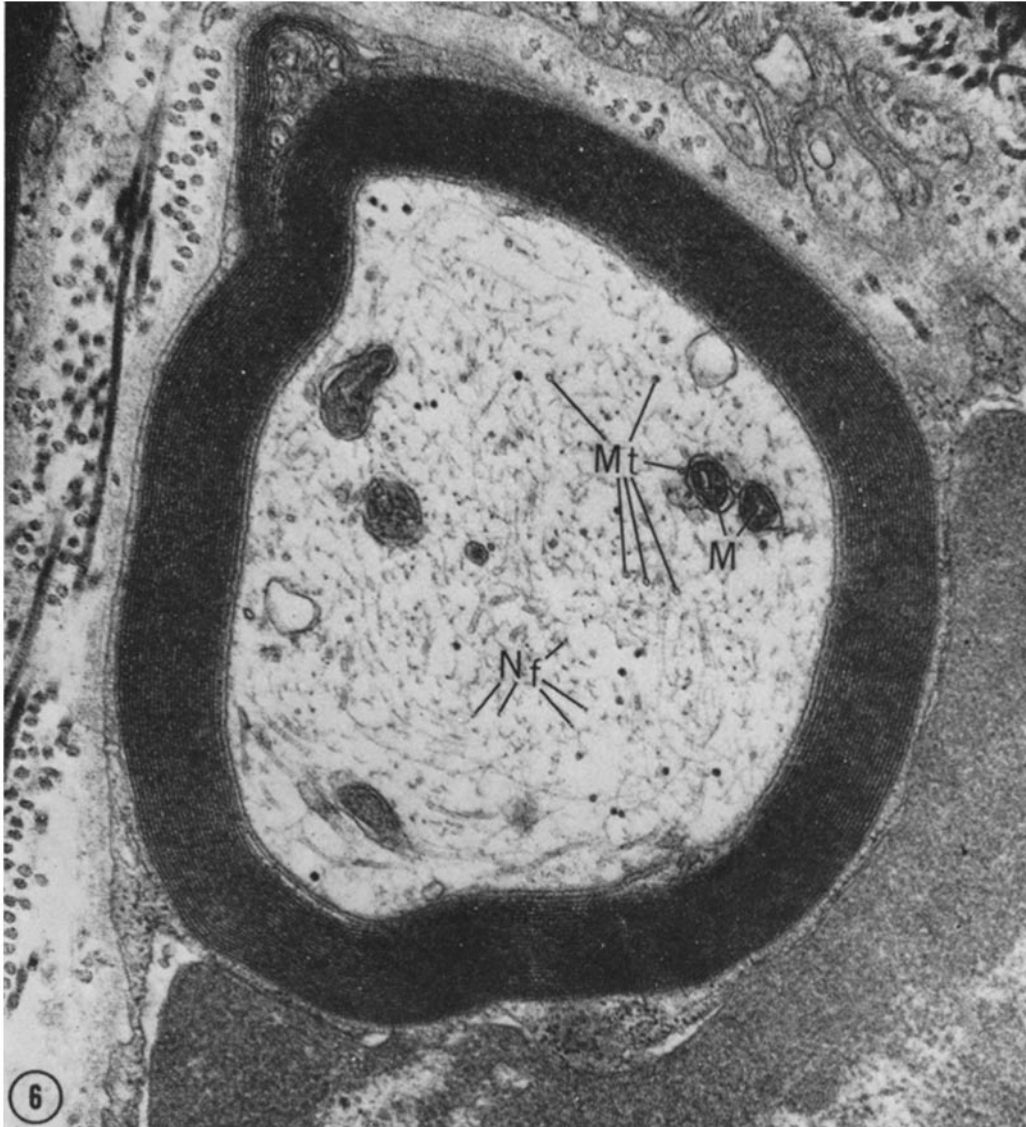


FIGURE 6 Myelinated axon in the brachial plexus of the regenerating newt. The axoplasm contains neurofilaments (*Nf*) and a few microtubules (*Mt*). Note that the organelles like mitochondria (*M*) tend to be distributed toward the periphery of the axon and that, except for the neurofilaments, the central zone of axoplasm contains fewer structures. $\times 43,000$.

Peripheral Nerve Bundles

After amputation of the limb, outgrowths from the cut axons invade the regeneration blastema (Lentz, 1967). The regenerating nerve fibers occur in small bundles loosely invested by Schwann cell cytoplasm. The individual fibers are unmyelinated and have the cytological characteristics of growing nerve fibers (Lentz, 1967). Some but not all of the nerve bundles in the blastema were labeled (Fig.

7). In those that were labeled, silver grains were sparsely distributed over the axons. More labeled nerves were found after cord injection than after injection into the ganglion, although the nerves labeled in both cases were structurally similar.

Peripheral Nerve Endings

Within the blastema, individual nerve fibers separate from the nerve bundles and terminate in

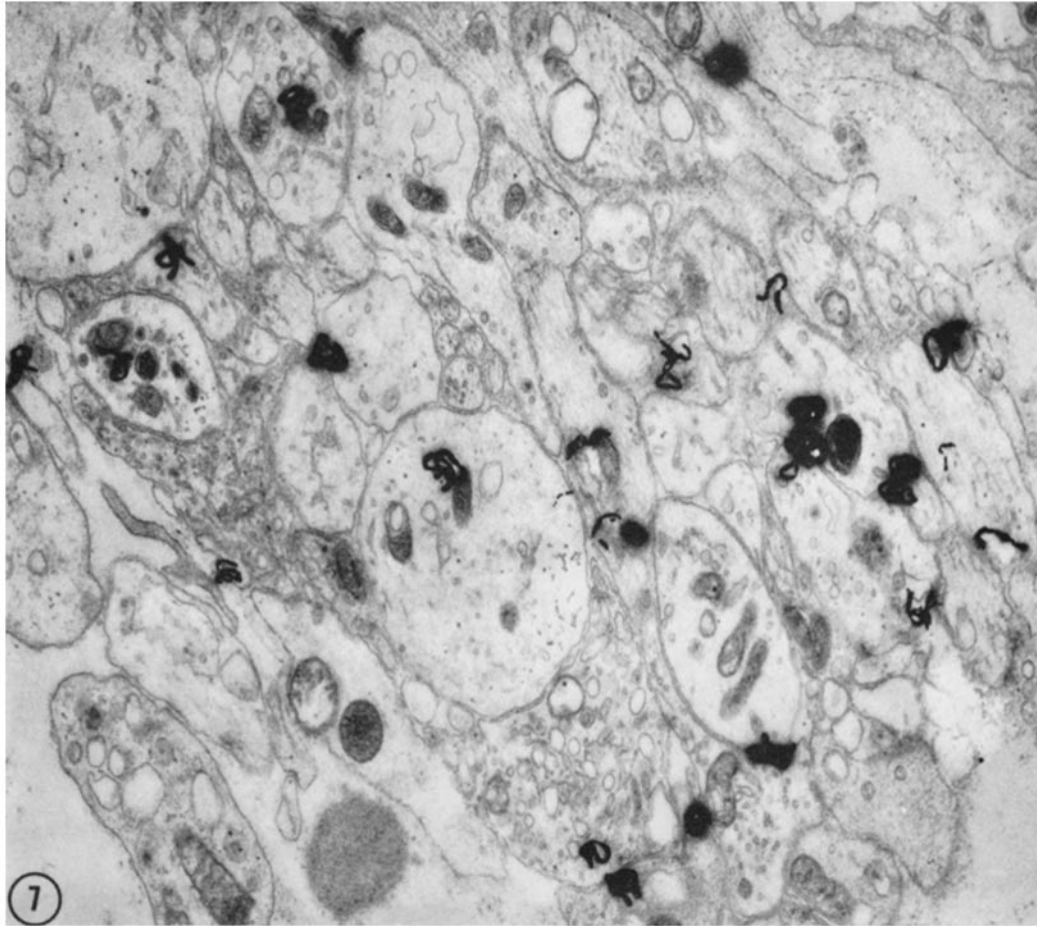


FIGURE 7 Bundle of regenerating nerves in the blastema of an 8 wk regenerate 3 days after infusion of leucine-³H into the spinal cord. A few silver grains are found over some of the nerve fibers. $\times 13,000$.

intercellular swellings known as growth cones or end bulbs. Some of the nerve terminals were heavily labeled, especially after spinal cord injection (Fig. 8). Examination of the micrographs clearly gives the impression that the majority of the grains are situated at the periphery of the terminal. The distribution of grain density relative to the terminals confirms this impression and shows that the greatest density of grains occurs 1500 A (1 HD unit) inside the limiting membrane of nerves with an average radius of 9000 A (6 HD) (Fig. 9). The experimental distribution of grains corresponded most closely with the theoretical distribution for a circle with a radius of 7500 A (5 HD units) and labeled at its periphery (Fig. 9).

The end bulbs (Figs. 8, 10, 11) of the regenerating nerves in newts contain many small vesicles, larger dense vesicles or granules, and mito-

chondria (Hay, 1960; Salpeter, 1965; Lentz, 1967). These structures fill most of the axoplasm, but usually are separated by a narrow space (~ 500 A) from the axolemma (Figs. 10, 11). This space was frequently occupied by fine filamentous material (Fig. 11). Occasionally, a vesicle or granule was immediately adjacent to or fused with the membrane (Fig. 8, inset).

DISCUSSION

Both spinal cord neurons and sensory ganglion neurons were heavily labeled 3 days after infusion of leucine-³H. Several investigators have shown that radioactively labeled leucine injected into nervous tissue is incorporated into newly formed proteins (Droz and Warshawsky, 1963; Ochs et al., 1967; Lasek, 1968). Free, unincorporated leucine is apparently washed out during fixation

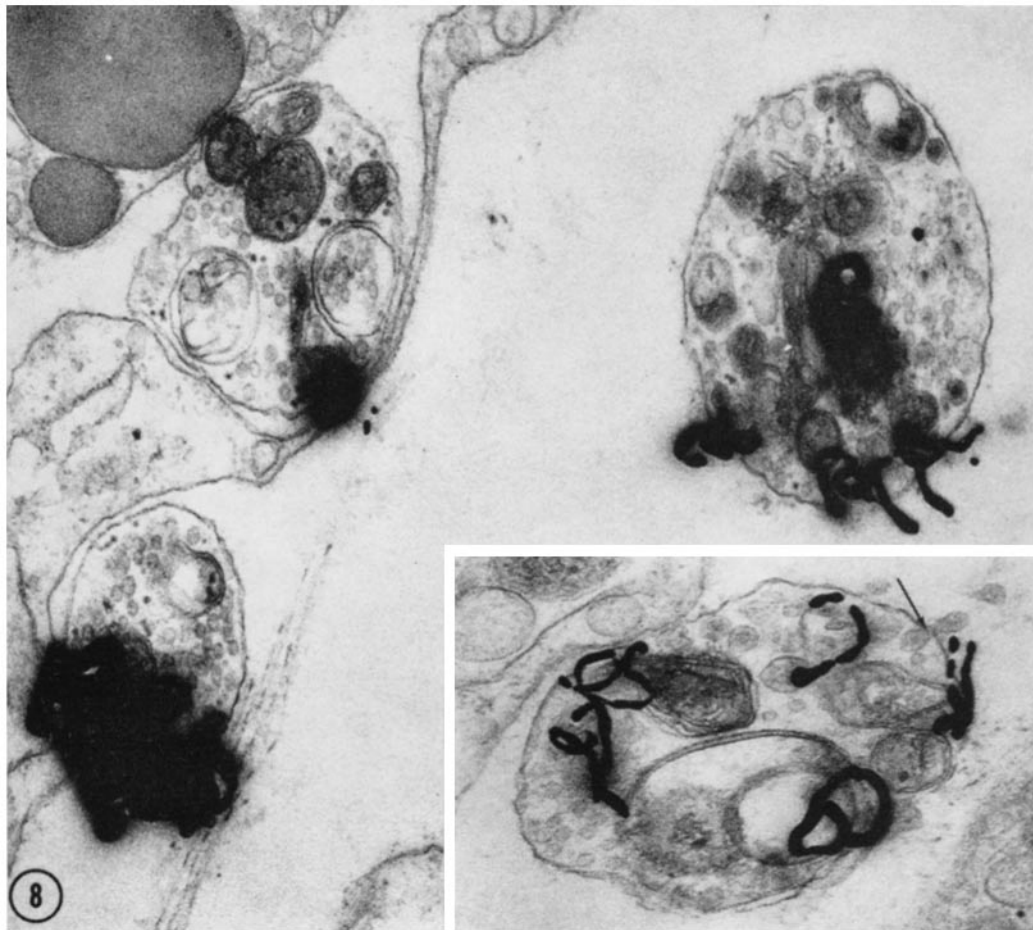


FIGURE 8 Nerve terminations in the blastema of an 8 wk regenerate 3 days after infusion of leucine- ^3H into the spinal cord. The terminals or end bulbs appear as circular profiles containing mitochondria, small clear vesicles, and larger dense vesicles or granules. The label is concentrated at the periphery of the terminals. An occasional vesicle (arrow) is closely associated with the axolemma. $\times 39,000$; *inset*, $\times 45,000$.

and dehydration of tissues. In the present study, all perikaryal constituents were labeled to some extent, indicating incorporation of leucine- ^3H into cellular enzymes and structural proteins. The highest concentration of label per unit area occurred over the rough-surfaced endoplasmic reticulum which is considered to be the site of synthesis of the major portion of the axoplasmic proteins.

Radioautographic grains were found over the large brachial nerves, bundles of regenerating nerves in the blastema, and the terminals of these nerves. Control experiments rule out the possibility of background radiation, chemography,

extracellular diffusion from the injection site, or transport by the blood stream as significant sources of the peripheral nerve radioactivity. Thus, the label observed within these nerves is attributed to axoplasmic transport of leucine- ^3H within proteins synthesized in the neuronal perikarya.¹

¹ Detailed studies were not made of rates of transport. The portions of brachial nerves removed for fixation were 2 mm from the site of infusion and the peripheral endings were 8–12 mm. While most of the observations were made 3 days after infusion, label has been detected in a similar pattern within the terminals 1 day after infusion, indicating a rate of transport of at least 10 mm per day. Thus, it is assumed

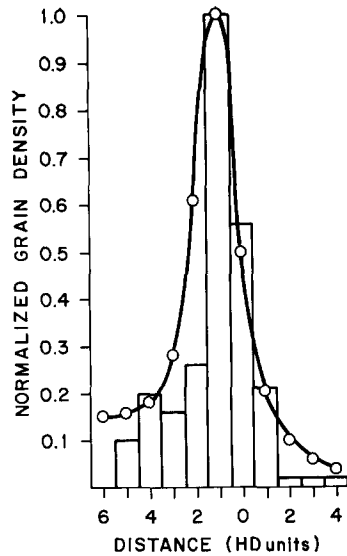


FIGURE 9 Grain density around nerve terminals in the blastemata of 8-wk regenerates 3 days after the infusion of leucine- ^3H into the spinal cord. The left side of the histogram from point 0 (axon membrane) is inside the terminal; the right side is exterior to the terminal. Distance is measured in units of HD (1500 Å). Grain density is greatest 1 HD unit inside the axolemma. The solid curve is the theoretical distribution for a circular source labeled at its edge and with a radius of 5 HD units. The observed distribution and theoretical curve are normalized to unity at 1 HD inside the membrane. Histogram based on 14 terminals with an average radius of $0.6\ \mu$, 54 grains, 707 random points.

Considerably more labeled nerve fibers were observed after injection of the spinal cord than after infusion of the ganglia. This observation might seem somewhat surprising since the ratio of sensory to motor fibers in the newt limb is about 3.5:1 (Singer, 1946). However, only one of the three ganglia supplying the limb was infused. Furthermore, the small size of the ganglion probably results in a greater proportion of its cells being damaged by the infusion procedure and greater spillage of the labeled solution into the surrounding tissues. Cord infusion, on the other hand, is accompanied by less cell damage and leakage, and appears to represent a more efficient way of labeling the peripheral nerves, even though the cord supplies a smaller number of nerves.

The most significant finding of the present study is that at least some of the labeled material is transported by the rapid component of axoplasmic flow (see Barondes, 1967).

study was obtained by comparing the density distributions of silver grains in the peripheral nerves with the theoretical curves (Salpeter et al., 1969) for labeled sources. These comparisons clearly show that the labeled materials are not distributed uniformly in the axoplasm during transport, but are restricted in their movements to certain regions of the axon. Thus, in myelinated axons with a diameter of about $3\ \mu$, almost all of the radioautographic grains can be accounted for by the presence of a uniformly labeled band occupying the area 1500–9000 Å inside the axon membrane. The central core of the axon is largely unlabeled.

In the nerve fibers of the newt (Lentz, 1967), as well as other species (Yamada et al., 1971), the core of the axon contains a greater number of neurofilaments and is encircled by a peripheral zone in which microtubules and organelles predominate. The peripheral band to which label is localized after infusion of leucine- ^3H corresponds in position to the region containing microtubules whereas the central unlabeled zone is occupied by neurofilaments. This correlation supports the hypothesis that microtubules are related to axonal transport (Dahlström, 1969; Kreutzberg, 1969; see also Schmitt, 1969) but is not consistent with the notion that the central core containing neurofilaments is involved in transport (see Yamada et al., 1971), at least during the time course of these experiments. Martinez and Friede (1970) have suggested that convection of axoplasm and organelles may occur in "streets" or clefts between bundles of neurofilaments. The present study indicates that transport occurs within a wide peripheral band, but within the band smaller corridors may exist between neurofilaments.

Because of the limitations of resolution in electron-microscope radioautography, the precise site of localization of label in axons (microtubules, neurofilaments, vesicles, mitochondria, endoplasmic reticulum, axoplasm, etc.) is not known. Ochs et al. (1967) and Barondes (1968) have presented evidence that labeled amino acids are incorporated into soluble protein and small particulate components including vesicles which are free to move in the fluid part of the axoplasm. Droz and Koenig (1969) suggest that labeled proteins are also closely associated with structural components of the axon such as neurofilaments and microtubules.

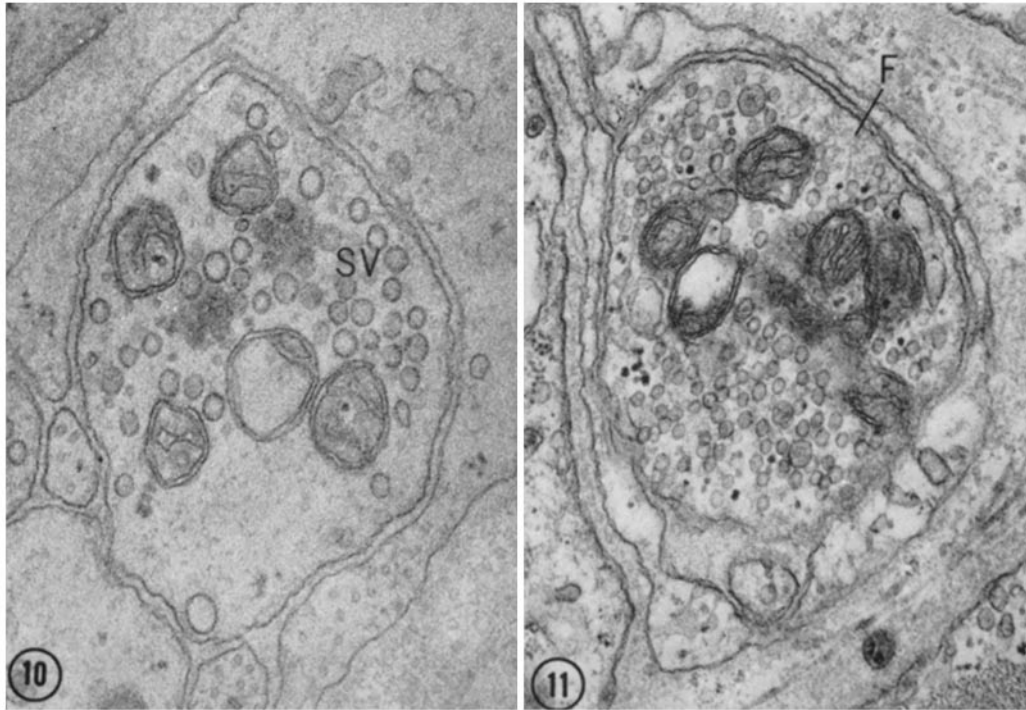


FIGURE 10 Nerve terminal in the blastema of a limb processed for radioautography but opposite the side of infusion of the sensory ganglion. Note absence of silver grains. The ending contains mitochondria and synaptic vesicles (*SV*). Most of the vesicles are not immediately adjacent to the axolemma but are separated from it by a short distance. $\times 51,000$.

FIGURE 11 Nerve terminal in a 22 wk regenerate. Mitochondria and synaptic vesicles fill the ending. Note the presence of fine filamentous material (*F*) in the narrow zone beneath the axolemma from which vesicles are excluded. $\times 38,000$.

In the nerve terminals, the observed density distribution of silver grains fit the theoretical distribution of a circle labeled at its edge and situated 1500 Å inside the axon membrane. Thus, the labeled material is restricted to a thin zone beneath the plasma membrane while the central region of the terminal is largely unlabeled. It should be noted that the greatest density of silver grains was not at the plasma membrane but a short distance (1 HD unit, 1500 Å) inside the membrane. Similarly, the organelles within the nerve terminal were separated from the membrane by a narrow space except for an occasional vesicle or dense granule. Yamada et al. (1971) have observed a network of fine filaments within the zone immediately beneath the membrane.

The peripheral pattern of labeling in the endings is consistent with two possibilities. First, if growth of the terminal end bulb is accompanied by

successive addition of newly synthesized proteins near or at its periphery, labeling would be expected in this region. Bray (1970) has suggested that new surface materials formed during axonal growth are added in the region of the growing tip. Yamada et al. (1971) have proposed a model for axon elongation in which membranous elements originating in the perikaryon are transported down the axon, accumulate in the growth cone, and fuse with the plasma membrane. Thus, the growth cone serves as the site of deposition of new surface material for the elongating axon. The observation of labeled material near the periphery of the growth cone is consistent with this hypothesis. Replacement of the total mass of axoplasm should produce a more widespread distribution of label.

Secondly, if the labeled proteins delivered to the nerve terminals are subsequently released, a

greater concentration of label at the periphery should be observed. Some release of protein might occur through leakage or as a result of normal turnover of axoplasmic constituents, although these events would not account for all of the radioactivity being located at the periphery of the fiber. Another possibility is the transport and release of trophic substances, if these are proteins. It is well-known that nerves exert a trophic effect during limb regeneration of the newt (Singer, 1952), and most evidence indicates that these effects are mediated by a substance released by nerve cells (see Singer, 1960; Lentz, 1971). Thus, some of the peripheral labeling of nerve terminals could represent transport of trophic materials to their site of release.

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