Changes in the Amounts of Cytoskeletal Proteins within the Perikarya and Axons of Regenerating Frog Motoneurons

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ABSTRACT Changes in the amounts of tubulin, actin, and neurofilament polypeptides were found in regenerating motoneurons of grass frogs during the period of axonal elongation. Ventral roots 9 and 10 were transected unilaterally about 7 mm from the spinal cord. 35 d later, [^{3}H]colchicine binding had decreased in the proximal stumps to approximately one-half of contralateral control values, well before the regenerating motor axons had reinnervated skeletal muscles of the hind limb. [^{3}H]colchicine binding did not change significantly in the operated halves of the 9th and 10th spinal cord segments over a 75-d period. The relative amounts of actin, tubulin, and neurofilament polypeptides in the operated ventral roots were measured by quantitative densitometry of stained two-dimensional electrophoretic gels. Alpha-tubulin, beta-tubulin, and the 68,000 molecular weight subunit of neurofilaments (NF68) decreased within the transected ventral roots to 78%, 57%, and <15% of control values, respectively. The amount of actin increased to 132% of control values within the operated ventral roots, although this change was not statistically significant. Opposite changes were found within motoneuronal cell bodies isolated from the spinal cord. The relative amounts of alpha-tubulin, beta-tubulin and NF68 within axotomized perikarya increased, respectively, to 191%, 146%, and 144% of that in control perikarya isolated from the contralateral side of the spinal cord. Thus, the changes in NF68 and tubulin did not occur uniformly throughout the injured cells. The possible structural and functional consequences of these changes are discussed.

Cytoskeletal proteins, including actin, tubulin, and neurofilament polypeptides, are particularly abundant within axons and play important roles in axonal structure and function (35, 36, 67). Axons receive a continuous supply of cytoskeletal proteins from their nerve cell bodies by slow axonal transport (20). When an axon in the peripheral nervous system is transected (axotomy), the isolated distal segment degenerates and is regenerated from the proximal segment (23). Whether or not the normal continuous supply of cytoskeletal proteins from the perikaryon is sufficient for axonal regeneration is unclear at present (21, 30). A prevailing hypothesis states that the synthetic priorities of axotomized neurons become reordered to favor the increased production and delivery of structural proteins to the regenerating axons (19, 49).

Altered axonal amounts of cytoskeletal proteins can arise from changes in their synthesis, degradation, and/or transport. Previous studies of cytoskeletal proteins within regenerating neurons have focused on changes in their synthesis and transport. Several laboratories have reported increased rates of tubulin and actin synthesis in the region of axotomized retinal ganglion cell bodies (6, 17, 26), although such increases were not statistically significant in autonomic and sensory ganglia (24, 46). Increased amounts of radioactivity associated with newly synthesized tubulin and actin are transported within regenerating optic (17, 56) and motor axons (30, 34) following administration of labeled amino acids to their cell bodies. In contrast, decreased amounts of radiolabeled neurofilament polypeptides enter regenerating motor axons (30).

In the present study, we looked for changes in the *total amounts* of individual cytoskeletal proteins within the perikarya and axons of axotomized spinal motoneurons of the frog. The cell bodies of axotomized motoneurons, obtained by a new procedure for isolating perikarya from small neuronal populations, and the proximal stumps of transected ventral roots

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were analyzed by quantitative two-dimensional electrophoresis. This study shows that proteins can change in opposite directions in different regions of the axotomized motoneurons, and that individual cytoskeletal proteins respond differently to injury.

Portions of this work have been published in abstract form (54).

MATERIALS AND METHODS

Tissue Preparation: The ninth and tenth ventral roots of grass frogs (*Rana pipiens*; Carolina Biological Supply Co., Burlington, NC) maintained at 18–23°C were transected unilaterally on the left side 6–9 mm from the spinal cord as described previously (55). 2–75 d later, the proximal stumps of operated ventral roots were dissected from the spinal cord up to and including the site of transection; similar lengths of the contralateral unoperated ventral roots were used as controls (Fig. 1). Meninges were removed from the spinal cord, and the ninth and tenth spinal segments were hemisected at the midline into control and experimental halves. These halves of spinal segments 9 and 10 either were homogenized for biochemical analyses or were used for the isolation of motoneuronal cell bodies. Homogenates were prepared at a final concentration of 40 mg wet weight per ml in 20 mM sodium phosphate buffer, pH 7.2.

Isolation of Motoneuronal Cell Bodies: The left or right halves of spinal segments 9 and 10 from four to seven frogs were minced with a razor blade into ~1-mm cubes and were stored for up to 3 months at −70°C in a medium consisting of 3 parts 0.9 M sucrose in 15 mM glucose, 1.7 mM citrate-NaOH, pH 5.0 and 7 parts ethylene glycol. The use of ethylene glycol at subzero temperatures, first employed by Giller and Schwartz (16) to aid in the microdissection of invertebrate neurons, greatly enhanced the yield and light microscopic appearance of the isolated frog motoneurons. The effects of ethylene glycol have been tested in preliminary experiments, and it does not appear to reduce the total protein content of the isolated perikarya. In previous studies, we have described the absence of synaptic boutons and glial processes on isolated bovine spinal motoneurons (27) and the retention of most soluble protein within the isolated perikarya when buffers of relatively low pH and ionic strength are used (64).

Motoneuronal perikarya were isolated from the minced spinal tissue by bulk fractionation procedures followed by the collection of individual cell bodies with a micropipette (55). Individual motoneuronal perikarya were identified for collection by their characteristic morphology (Fig. 2*a*) and large size (average dimensions: 38 × 74 μm) using a Leitz Diavert microscope equipped with phase contrast optics. The scarcity of other cells of such large size (50, 58, 59) and the lack of gamma-motoneurons in the frog spinal cord (22) support our belief that the isolated cells were alpha-motoneurons.

[^{3}H]Colchicine-binding Assay: The procedure of Castle and Crawford (8) was modified for the assay of small amounts of frog tissue at saturating concentrations of [^{3}H]colchicine. Homogenates were prepared at 4°C to a final concentration of 20 mg wet weight/ml in 25 mM sodium phosphate, pH 6.8, containing 0.375 M sucrose, 0.125 M sodium glutamate, 1.25 mM EDTA, and 1.25 mM GTP. 4-μl aliquots were assayed immediately in 400-μl microcentrifuge tubes. 1 μl of [^{3}H]colchicine (5–20 Ci/mmol; New England Nuclear, Boston, MA) in water was added to a final concentration of 20 μM, and the tubes were incubated for 30 min at 37°C. At this concentration of [^{3}H]colchicine, binding equilibrium is reached within 20 min. Unbound radioactivity was removed by vacuum filtration of each sample through a single 2.5-cm DE81 filter disc (Whatman Inc., Clifton, NJ) wetted with 10 mM sodium phosphate, pH 6.8, followed by six washes with 10 ml of the same buffer. Binding activity did not decay during the incubation period, and increasing the number of filters did not increase the amount of bound radioactivity. The filters were counted after overnight elution of bound radioactivity in 5 ml of scintillation cocktail consisting of (per liter) 330 ml of Triton X-100, 666 ml of toluene, 5 g of 2,5-diphenoxazole, and 100 mg of 2,2′-p-phenylbis(4-methyl-5-phenyl) oxazole. Counting efficiency was ~35% and was monitored in each sample by a channels-ratio method. Nonspecific binding was estimated by boiling aliquots of each homogenate for 5 min prior to the addition of [^{3}H]colchicine. Specific binding activity was saturable with a K_d of 0.99 μM, was inhibited by unlabeled colchicine, and was eliminated by preincubation of samples for 15 min with 20 μM podophyllotoxin. The nonspecific binding measured in boiled blanks was equal to that measured in the presence of podophyllotoxin and was <10% of the total bound radioactivity in all cases. As little as 0.03 pmol of bound [^{3}H]colchicine could be measured reliably, and the assay was linear with increasing tissue concentration up to at least 10 pmol.

6-Phosphogluconate Dehydrogenase (6PGDH) Assays: 6PGDH activity was measured in homogenates of ventral roots and spinal cord by the fluorometric method of Luine and Kauffman (39) in 1 ml of reagent. For the assay of 10–20 isolated motoneuronal cell bodies, sensitivity was increased by reducing the assay volume to 100 μl and by increasing both the intensity of excitation illumination and the gain of a Farrand ratio fluorometer.

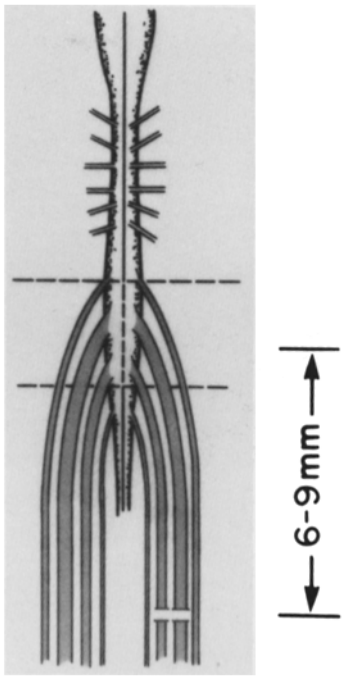

FIGURE 1 Diagram of frog spinal cord as seen from the ventral side. Illustrated are the location of intravertebral transections of the ninth and tenth ventral roots 6–9 mm from the spinal cord and of the spinal segments that were dissected for biochemical analyses (dashed lines).

Protein Assays: The protein concentration of ventral root and spinal cord homogenates was measured by the method of Lowry et al. (38) in a final volume of 1 ml, using bovine serum albumin as the standard. For analyses of motoneuronal cell bodies isolated in the sucrose-containing medium described above, proteins were first precipitated by a modification of the procedure described by Bensadoun and Weinstein (4). 50 μl of 0.025% (wt/vol) sodium deoxycholate was added to 400-μl microcentrifuge tubes containing 30–50 isolated cell bodies in a volume of 1–5 μl. The tubes were mixed and allowed to stand for 15 min at ice temperature prior to the addition of 50 μl of 2 M perchloric acid. Following thorough mixing, the tubes were kept at ice temperature for an additional 15 min and were then centrifuged at 9,500 *g* for 5 min to precipitate the protein. The protein pellet was resuspended and precipitated twice more in fresh deoxycholate-perchloric acid as described above, omitting incubation of the samples on ice. The final pellet was dissolved in 12 μl of 1 N NaOH and was assayed for protein in a final volume of 100 μl. As little as 0.05 μg of protein could be precipitated quantitatively and measured reliably by this method.

Two-dimensional Electrophoresis: Two-dimensional electrophoresis of the total proteins extracted from ventral roots or from isolated motoneuronal cell bodies was performed as described previously (65) with the following modifications. 5–10-μl aliquots of ventral root homogenates in phosphate buffer, or 100–200 isolated motoneuronal cell bodies in 1–3 μl of the sucrose-containing medium described above, were evaporated to dryness in a vacuum centrifuge. The dried samples were dissolved in 15 μl of lysis buffer modified to contain 5 mM dithiothreitol instead of mercaptoethanol, 0.5% (wt/vol) SDS, and 2% (wt/vol) LKB Ampholines mixed in the following proportion: 3 parts of pH range 3.5–10, 1 part of pH range 5–7, and 1 part of pH range 6–8. Isoelectric focusing gels were prepared using the same Ampholine mixture, and 10% polyacrylamide gels were employed for the second dimension. Ventral root proteins were stained with Coomassie Blue as previously described (65), or with silver by the method of Oakley et al. (45). ~100 protein spots could be detected in silver-stained gels containing extracts of 200 isolated cell bodies.

Actin, tubulin, and neurofilament polypeptides were identified in electrophoretic gels containing extracts of frog ventral roots by co-electrophoresis with purified samples of these proteins. *Alpha*-actin, purified from rabbit skeletal muscle, was purchased from Worthington Biochemical Corp. (Freehold, NJ). Tubulin polypeptides, purified by two cycles of assembly and disassembly from bovine brain, were a gift from Dr. George Langford (University of North Carolina at Chapel Hill). Neurofilaments were isolated from frog and bovine spinal cord by the method of Chiu et al. (9). Although the three polypeptides that have been identified in mammals as neurofilament subunits (36) were present in bovine neurofilaments, only the 68,000 molecular weight subunit (NF68) was visible in gels containing frog neurofilament polypeptides.

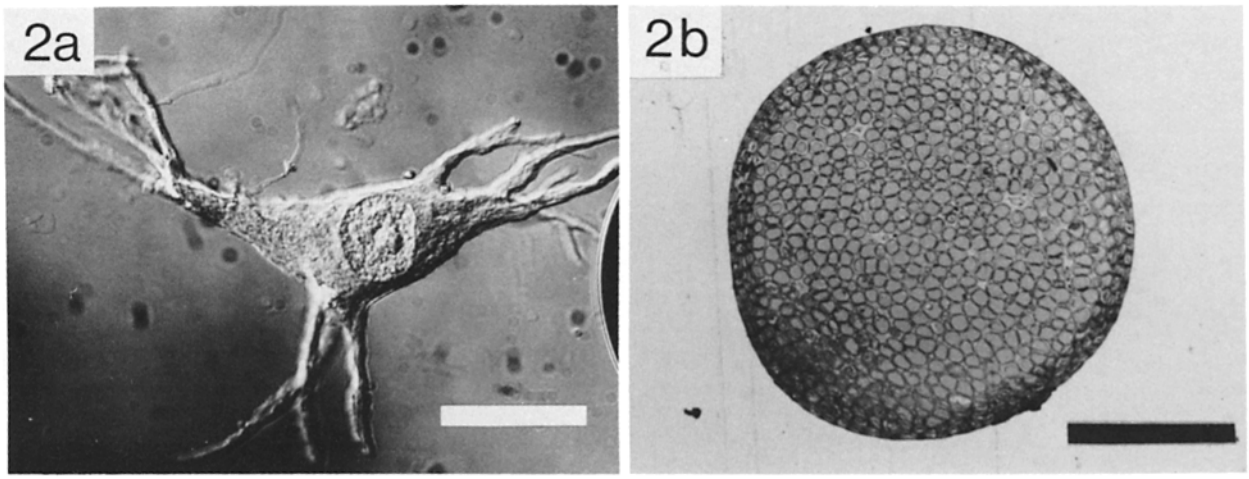

FIGURE 2 (*a*) Unfixed motoneuron isolated from a normal frog. Nomarski optics. Unlike mammalian *alpha*-motoneurons, most *alpha*-motoneurons in the ninth and tenth spinal segments of the frog are arcuate; rather than stellate (59). Bar, 50 μm. (*b*) Toluidine-blue stained cross-section of the 9th ventral root of a normal frog. Large myelinated axons are especially predominant features of the ninth and tenth ventral roots in the frog (61). Bar, 100 μm.

Densitometry of Two-dimensional Electrophoretic Gels: The integrated (total) optical density (IOD) of individual stained proteins in two-dimensional gels was measured with the television-based system described previously (2). The IOD of each spot was divided by the IOD of ovalbumin, a fixed amount of which was added as an internal standard to each sample prior to electrophoresis, to normalize for differences in the degree of staining or in the amount of protein that entered each gel. These IOD ratios are a measure of the relative mass of each spot. The IOD of ovalbumin stained with either Coomassie Blue or silver was linearly related to the amount that was applied to the gels over the range of densities observed for the cytoskeletal proteins. The smallest amount of bovine serum albumin that could be quantified was ~100 ng when gels were stained with Coomassie Blue and 10 ng when stained with silver.

Histology and Morphometry: Frog ventral roots were fixed in 75 mM sodium phosphate buffer, pH 7.4, containing 0.8% (vol/vol) glutaraldehyde for 24 h at 4°C. After fixation, the tissue was washed for 30 min in two changes of 50 mM sodium phosphate, pH 7.4, dehydrated and embedded in Spurr plastic (Polysciences, Inc., Warrington, PA). 1-μm sections of the ventral roots were stained with toluidine blue (Fig. 2*b*) and their image was projected onto a data tablet interfaced to a digital laboratory computer. The perimeters of individual fibers were traced with a manually guided cursor and the enclosed areas were calculated by a numerical integrative procedure.

RESULTS

Time Course of Changes in [^{3}H]Colchicine Binding

The binding of [^{3}H]colchicine by tubulin dimers was assayed in homogenates of spinal cord and ventral roots between 1 and 75 d after ventral root transection (Fig. 3). No changes were found in homogenates of the operated halves of spinal segments 9 and 10 throughout the period examined. In contrast, binding activity decreased significantly in the combined ninth and tenth ventral roots about the tenth postoperative day, reaching a minimum of ~52% of control values by days 20–35. Absolute binding activity (pmol [^{3}H]colchicine bound · mg protein^{-1}) in control ventral roots was comparable to that in ventral roots from unoperated animals. The concentration of total protein per unit tissue wet weight did not change significantly in homogenates of ventral roots and spinal cord throughout the 75-d postoperative period.

Electrophoresis of Ventral Root Proteins

The thirty-fifth postoperative day was chosen for electrophoretic analysis of the cytoskeletal proteins in ventral roots because at this time [^{3}H]colchicine binding, as well as the activities of several other proteins, showed maximal changes. By day 35, acetylcholinesterase activity had decreased to 66% (55) and choline acetyltransferase activity had decreased to 23% of control values (Sinicropi, D. V., and D. L. McIlwain, unpublished data). The activity of 6PGDH, an enzyme that increases in axotomized superior cervical ganglia (25, 52, 53), had increased ($P < 0.05$) to 138 ± 14 [10] percent of control values (mean ± SEM [number of experiments]) within operated ventral roots by days 20–35 (absolute activity within control ventral roots was 12.2 ± 1.5 [10] pmol 6-phosphogluconate oxidized · min^{-1} · μg protein^{-1}). Motor axons were elongating when these

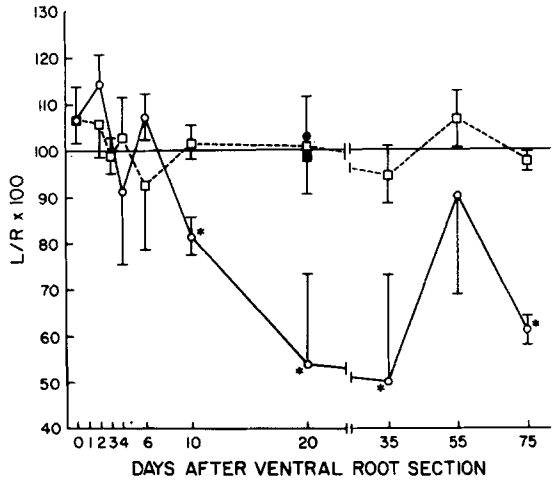

FIGURE 3 Time course of changes in [^{3}H]colchicine binding activity in homogenates of ventral roots (open circles) and spinal segments (open squares). Ordinate: ratio of specific binding activity on the left (operated) side to that on the right (control) side multiplied by 100. Each symbol represents the mean value from at least three animals and the vertical bars represent the SEM. Filled symbols represent data obtained in sham-operated animals. Normal values (mean ± SEM; *n*) in unoperated animals were 31.4 ± 2.6 (6) pmol [^{3}H]colchicine bound per mg ventral root protein and 122.6 ± 5.8 (10) pmol per mg spinal cord protein. The logarithms of ratios at each time point were compared to the 0 day ratios by analyses of variance. *, $P < 0.05$.

protein changes were maximal; the fastest regenerating motor axons reinnervate hind limb muscles about day 50–55 (14).

Regions of a pair of two-dimensional electrophoretic gels containing cytoskeletal polypeptides extracted from experimental and control ventral roots are illustrated in Fig. 4. Slight differences in the staining or amount of protein that entered each pair of gels can be judged by the density of ovalbumin, which was added to each sample as an internal standard. The cluster of spots to the left of NF68 are artifacts that result from the presence of thiol reducing agents in the isoelectric focusing step of the electrophoretic procedure; the density of these spots is variable, and they are present in gels run without protein. NF68 and the tubulin polypeptides appeared to be decreased and actin appeared to be increased in the proximal stumps of ventral roots 35 d after transection (Fig. 4).

Results of densitometric quantification of all four of the electrophoretic gel pairs are summarized in Table I. In agreement with the results of [^{3}H]colchicine binding assays, the amount of beta-tubulin decreased to 57% of control levels in the transected ventral roots; the smaller percent change in alpha-tubulin was seen in all four experiments. NF68 decreased below quantifiable levels and actin increased to an average of 132% of control within the transected ventral roots. The increase in actin was not observed in one of four experiments. Because the relative masses of the cytoskeletal proteins in ventral roots were normalized for the total amount of protein loaded on each gel and the total protein per mm did not change significantly in the transected ventral roots (Table I), the tabulated values reflect changes in the total amounts of cytoskeletal proteins per unit ventral root length.

Morphometry of Ventral Roots

Cross-sections of the ninth and tenth ventral roots 3–4 mm from the spinal cord (e.g., Fig. 2 *b*) were examined to determine the probable origins of the cytoskeletal proteins extracted from this tissue. Data obtained from equal numbers of the ninth and tenth ventral roots were combined since the results were similar for both roots. Large myelinated axons are a major constituent of normal ventral roots, comprising 83.6 ± 2.1 [8] % of the total cross-sectional area (mean ± SEM [number of ventral roots]). This major portion of the ventral roots was distributed approximately equally between axons (39.9 ± 2.3 [8] %) and myelin (43.7 ± 1.9 [8] %). Unmyelinated axons, Schwann cells, capillaries, as well as other cellular and extracellular constituents, occupy the remaining 16.4 ± 2.1 [8] % of the cross-sectional area. 35 d after transection, the percentage of cross-sectional area comprised by axons decreased ($P < 0.05$) in proximal

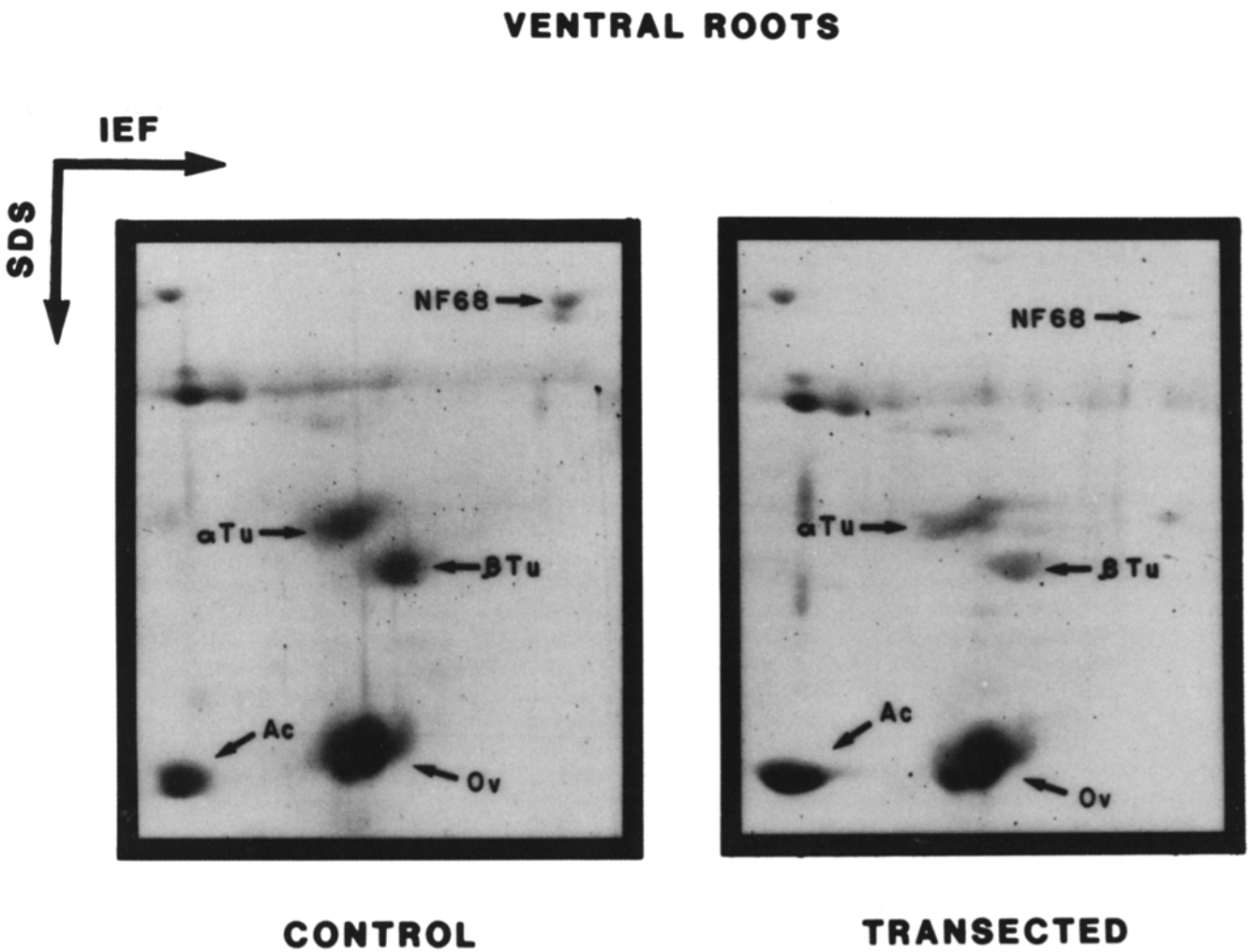

FIGURE 4 Photographs of the regions of two-dimensional electrophoretic gels containing actin (*Ac*), alpha-tubulin (*α-Tu*), beta-tubulin (*β-Tu*), the 68,000-mol wt subunit of neurofilaments (*NF68*), and ovalbumin (*Ov*). Proteins were extracted from the proximal stumps of ventral roots 35 d after transection (*right*) and from contralateral controls (*left*). Coomassie Blue stain.

TABLE I

Changes of Cytoskeletal Proteins in Axotomized Motoneurons

Protein	Cell bodies	Ventral roots
	mean percent of control ± SEM [n] *	
Actin	ND	132 ± 18 [4]‡
		NS
Alpha-tubulin	191 ± 27 [4]	78 ± 3 [4]
	P < 0.05	P < 0.01
Beta-tubulin	146 ± 16 [4]	57 ± 6 [4]
	NS	P < 0.05
NF68	144 ± 10 [4]	< 15 [4]§
	P < 0.05	
Total protein‖	171	112 ± 8 [10]
	P < 0.05	NS

* The relative masses (see methods) of stained protein spots were normalized either for the number of perikarya or for the amount of ventral root protein applied to the gels. Perikarya isolated from four to seven different animals were analyzed in each experiment. Percent of control equals the ratio of the normalized relative mass measured in the experimental side to that measured in the control side of each animal multiplied by 100. The logarithms of ratios obtained from each animal were used for paired, two-tailed t-tests. *ND*, not detectable, spot densities were below quantifiable levels; *NS*, not significant.

‡ Although variable, increases were found in three of the four experiments.

§ Spot densities were decreased below the threshold of detection (~15% of control) in three of the four experimental gels.

‖ In some cases, experimental and control perikarya were isolated from different groups of animals. The cell body value is the ratio of the mean absolute data (see text) multiplied by 100. The mean μg protein per mm combined ventral roots 9 and 10 was 15.2 ± 1.1 [10] on the control side.

ventral root segments to 28.6 ± 2.1 [4], whereas the remaining area increased ($P < 0.01$) to 28.9 ± 2.4 [4] and that occupied by myelin did not change (42.5 ± 2.6 [4]). The decreased percentage of axonal cross-sectional area was probably associated with a decrease in the diameter of the regenerating axons, as has been reported by others (11, 28, 32).

Isolated Cell Bodies

Perikarya isolated from the experimental sides of the spinal cords displayed several of the morphological alterations that have been noted frequently after axotomy (37), including a swollen appearance and an eccentrically located nucleus. In addition, the yield of motoneuronal perikarya obtained at the isopycnic sedimentation step of the isolation procedure was invariably greater ($P < 0.05$) from the experimental side than from the control side of the spinal cord, a finding consistent with that of Watson (63). A mean of 270 ± 36 [4] perikarya (± SEM; [number of experiments]) was obtained from each left half, while 136 ± 37 [5] perikarya were obtained from each right half of spinal segments 9 and 10. Since each side of the ninth and tenth frog spinal segments contains ~1,100 motoneurons (55), the numbers of recovered perikarya correspond to yields of 25% and 12% on the experimental and control sides, respectively.

Protein Changes within Isolated Cell Bodies

The absence of a change in [^{3}H]colchicine binding in homogenates of the ninth and tenth spinal segments (Fig. 3) did not rule out the possibility that a change had occurred within the axotomized motoneuronal cell bodies, since a large amount of tubulin probably resides in structures surrounding the motoneurons. Therefore, motoneuronal cell bodies were isolated from the operated and unoperated sides of the spinal cord and were analyzed electrophoretically. Based on the time course of protein changes within transected ventral roots, the twentieth postoperative day was selected for electrophoretic analysis of isolated cell bodies. Because tubulin and neurofilament polypeptides are transported anterogradely within axons at a rate of ~0.5 mm/d (33, 57), we estimated that changes within axotomized cell bodies would probably be most pronounced about 14 d earlier than they were within the proximal 7 mm of ventral roots on day 35.

In contrast to the decreases of NF68 and tubulin in ventral roots, the amounts of these polypeptides increased within axotomized perikarya (Fig. 5 and Table I). Similar changes were visible in gels from a single experiment in which perikarya were isolated 35 d after ventral root transection. Actin was seen, but could not be quantified in gels containing 200 or fewer perikarya. 6PGDH activity increased ($P < 0.05$) from 0.99 + 0.24 [3] in control to 1.89 ± 0.35 [3] pmol 6-phosphogluconate oxidized · h^{-1} · $cell^{-1}$ (mean ± SEM [number of experiments]) in axotomized cell bodies. The majority of other perikaryal proteins in the gels also appeared to increase, while some decreased after axotomy. These changes were not quantified. A net increase in the total protein content to 171% of control values was found within the axotomized frog perikarya (Table I), in close agreement with the value obtained in axotomized rabbit motoneurons (5). The protein content (mean ng protein per cell ± SEM; [number of experiements]) was 4.99 ± 0.88 [3] in normal perikarya, 4.56 ± 0.21 [4] in control (contralateral unoperated), and 7.82 ± 0.59 [5] in axotomized perikarya.

DISCUSSION

Each of the cytoskeletal proteins examined in this study responded differently to axonal transection and did not change uniformly throughout the injured frog motoneurons. Specifically, tubulin and NF68 increased within the axotomized cell bodies and decreased within the proximal stumps of the transected ventral roots, whereas actin increased within the roots. These changes were more complex than predicted by the hypothesis cited in the Introduction and, as discussed further below, cannot be explained solely by alterations in protein synthesis. These conclusions are drawn from direct analyses of the mass of cytoskeletal proteins within the cell body and axonal regions of the injured motoneurons.

Ventral Root Analyses

Most of the proteins extracted from the ninth and tenth ventral roots, including actin, tubulin, and NF68, were probably derived from alpha-motor axons, which occupy most of the volume in these roots (Fig. 2). Gamma-motor axons are not present in the frog (22) and unmyelinated fibers constitute only a small fraction of the total axonal volume in ventral roots 9 and 10 (61; designated roots 8 and 9 in that study). Virtually all of the NF68 was undoubtedly derived from axoplasm, since neurofilament subunits are neuron-specific proteins (1, 36, 68). It is unlikely that myelin was a major source of the actin and tubulin in ventral root extracts for two reasons. First, the protein concentration of myelin is approximately one-half that of axoplasm (44). Second, although myelin may contain some actin and tubulin, the concentrations of these proteins within isolated myelin preparations are low (12, 18, 62) compared to our extracts of whole ventral roots. Finally, the concentrations of actin and tubulin within other constituents of the ventral roots, such as Schwann cells, capillaries, and unmyelinated axons, would have to be much higher than their concentrations

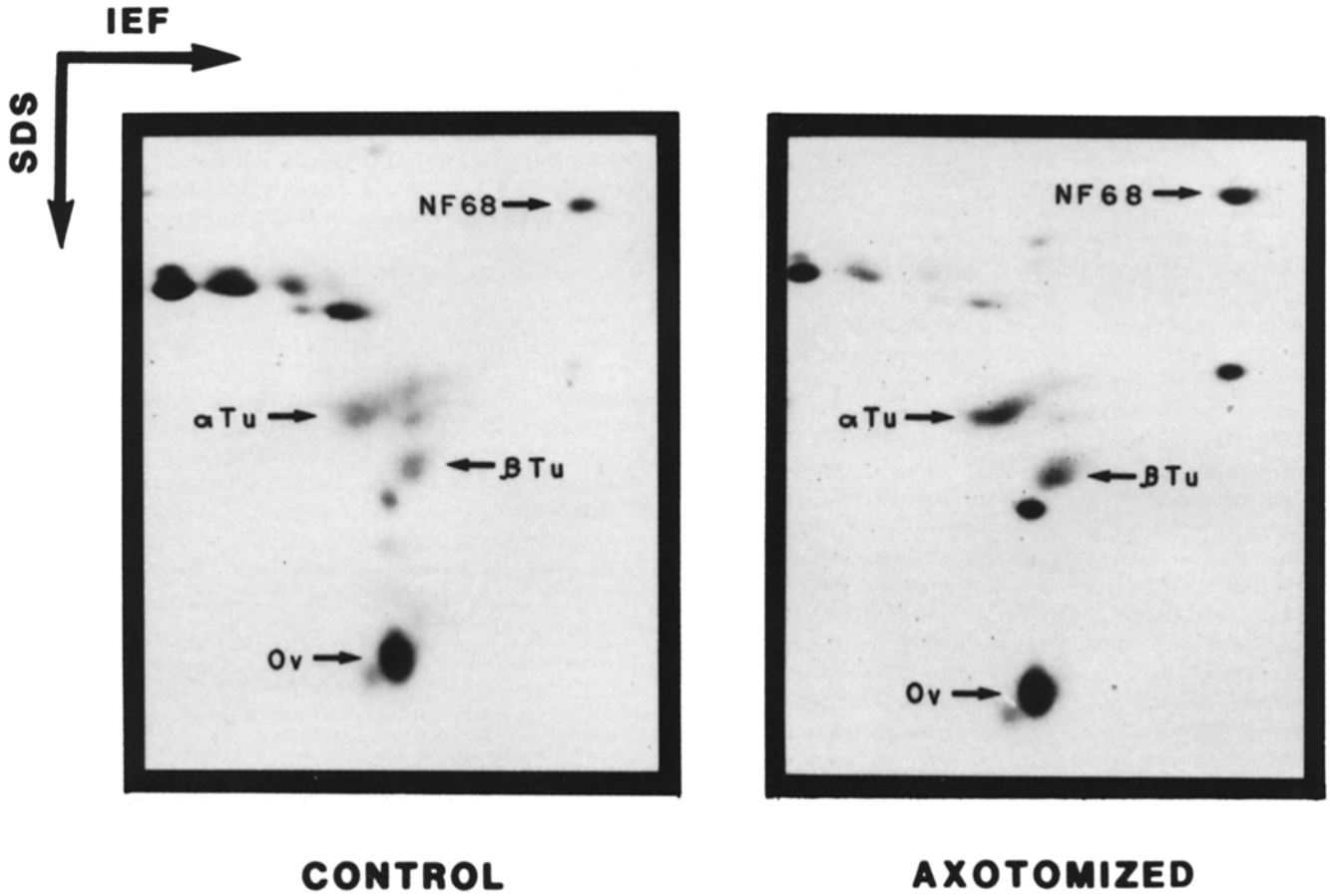

FIGURE 5 The regions of electrophoretic gels containing cytoskeletal proteins extracted from isolated motoneurons axotomized 20 d earlier (*right*) and from contralateral controls (*left*). Abbreviations as in Fig. 4. Silver stain.

within axoplasm in order to account for much of the total extracted amounts. We cannot discount extra-axonal sources of 6PGDH because this enzyme activity is relatively abundant in glia of the central nervous system (51).

Structural Correlates of Axonal and Cell Body Changes

The decreases in axonal NF68 and tubulin which we observed by biochemical methods are consistent with the electron microscopic observations of others. Murray (41) noted the virtual absence of neurofilaments within regenerating goldfish retinal axons, and found that their numbers increased following reinnervation. Similar increases in numbers of neurofilaments and microtubules have been described within developing axons after they establish synaptic connections and stop elongating (15, 47). Reduced amounts of tubulin and neurofilament polypeptides might be responsible for the decreased diameters of regenerating axons (28), since axonal diameter is directly proportional to the number of neurofilaments and microtubules they contain (15). Although the proximal stumps of the regenerating axons contained reduced amounts of NF68 and tubulin at day 35, increased amounts may be needed later when the axons stop elongating and regain their normal diameter. Microfilaments have not been quantified in regenerating axons by electron microscopy.

In agreement with the perikaryal increases in neurofilament and tubulin polypeptides reported here, increased numbers of neurofilaments and microtubules have been noted in most electron microscopic studies of axotomized nerve cell bodies (3, 42, 43, 60) including frog motoneurons (48), although decreases have been described in some cases (13, 40). Moreover, there were indications from our electrophoretic gels and total protein analyses that many other perikaryal proteins also increased within the axotomized cell bodies. These increases could produce the swelling of the neuronal perikaryon and the displacement of the nucleus that occur after axotomy (48).

Functional Implications

Regenerating motoneurons may require increased amounts of some, but not all structural proteins. The increased amounts of actin in the proximal stumps of transected ventral roots and of 6PGDH in the cell bodies and ventral roots may directly support axonal regeneration. Actin is believed to play a central role in the motility of growth cones (67) and 6PGDH in the production of biosynthetic precursors of lipids (25, 31). On the other hand, it is not clear whether the perikaryal increases in neurofilament and tubulin polypeptides facilitate or impede the process of neuronal regeneration. Increased numbers of neurofilaments have been noted in some central neurons that degenerate after axotomy (3). Moreover, chromatolysis and

increased numbers of neurofilaments in motoneuronal perikarya and proximal axons have been reported for several primary motoneuron diseases (7, 10, 66).

Possible Mechanisms Underlying the Altered Mass of Cytoskeletal Proteins

Changes in the mass of individual proteins within different regions of nerve cells can arise by at least three mechanisms: alterations in protein synthesis, degradation, and/or transport. Increased rates of synthesis of actin and tubulin, similar to those observed in goldfish retinal ganglion cells (6, 17, 26), could contribute to the increased amounts of alpha- and beta-tubulin we find within axotomized motoneuronal perikarya and to the increase in actin within the proximal stumps of the transected ventral roots. Despite possible neuronal and species differences, it is clear that a change in the rate of synthesis of tubulin or NF68 cannot account for the reciprocal alterations in their amounts within the cell bodies and axons of regenerating frog motoneurons. Additional or alternative mechanisms for the reciprocal changes in tubulin and NF68 include: (*a*) selective degradation of these proteins in the regenerating axons with increased synthesis and/or decreased degradation in the cell bodies; (*b*) the return of increased amounts to the cell body from its axon by retrograde transport, with or without a change in their synthesis or degradation; (*c*) increased synthesis or decreased degradation in the perikaryon accompanied by an increased rate of anterograde transport of these polypeptides, resulting in the delivery of increased amounts to the elongating portions of the axons and decreased amounts within the proximal stumps; or (*d*) a reduction in the amounts of tubulin and neurofilament polypeptides that are transported into the axons from the perikarya, leading to a decrease in these polypeptides within regenerating motor axons and to their accumulation within the axotomized cell bodies. The possibility of altered transport is noteworthy, since tubulin and NF68 appear to be transported by the same process, termed "slow component a" by Lasek and coworkers (33). However, a single change in slow component a would not explain why NF68 decreased more than tubulin within the regenerating axons (Table I).

A number of laboratories have reported alterations in the transport of radiolabeled cytoskeletal proteins within regenerating axons. Rates of slow transport have been reported to be unchanged (30) or increased (29) in the proximal stumps of transected motor axons in rats, and variable results have been obtained in other neuronal populations (reviewed in reference 21). Increased amounts of radioactive tubulin are transported anterogradely within regenerating optic (17, 56) and spinal motor (30, 29, 34) axons. Although these reports appear to conflict with the decreased amounts of tubulin we find in the proximal stumps of regenerating motor axons, these data are not necessarily inconsistent with our own. For instance, it is possible that tubulin is labeled to higher specific activity after axotomy as a result of an increased rate of synthesis, but still enters the axon in reduced total amounts. Hoffman and Lasek (30) have observed a decrease in radiolabeled neurofilament polypeptides in regenerating rat motor axons, that together with our data suggests a decreased delivery of neurofilament protein to the injured axon. However, as noted above, other explanations are conceivable.

Our data and that of other laboratories make it obvious that a number of variables may act in concert to produce changes in the mass of some proteins in different regions of the regenerating neuron. If, as the hypothesis predicts, frog spinal motoneurons respond to axotomy by increasing the synthesis and delivery of structural proteins to the elongating axons, they must also undergo other changes that lead to reductions in the amounts of axonal tubulin and NF68 proximal to the site of injury. The growing body of information regarding the synthesis and axonal transport of proteins in regenerating neurons, augmented by fresh approaches to protein degradation throughout the injured cell, should lead to definitive explanations of the changes in many proteins during the axon reaction.

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REFERENCES

1. Autilio-Gambetti, L., M. E. Velasco, J. Sipple, and P. Gambetti. 1981. Immunochemical characterization of antisera to rat neurofilament subunits. *J. Neurochem.* 37:1260–1265.
2. Aycock, B. F., D. E. Weil, D. V. Sinicropi, and D. L. McIlwain. 1981. Television-based densitometric analysis of proteins separated by two-dimensional gel electrophoresis. *Comput. Biomed. Res.* 14:314–326.
3. Barron, K. D., M. P. Dentinger, L. R. Nelson, and J. E. Mincy. 1975. Ultrastructure of axonal reaction in red nucleus of cat. *J. Neuropathol. Exp. Neurol.* 34:222–243.
4. Bensadoun, A., and D. Weinstein. 1976. Assay of proteins in the presence of interfering materials. *Anal. Biochem.* 70:241–250.
5. Brattgård, S.-O., J.-E. Edström, and H. Hydén. 1957. The chemical changes in regenerating neurons. *J. Neurochem.* 1:316–325.
6. Burrell, H. R., A. M. Heacock, R. D. Water, and B. W. Agranoff. 1979. Increased tubulin messenger RNA in the goldfish retina during optic nerve regeneration. *Brain Res.* 168:628–632.
7. Carpenter, S. 1968. Proximal axonal enlargement in motor neuron disease. *Neurology.* 18:841–851.
8. Castle, A. G., and N. Crawford. 1978. The [^{3}H]colchicine binding properties of platelet tubulin. *Int. J. Biochem.* 9:439–447.
9. Chiu, F.-C., B. Korey, and W. T. Norton. 1980. Intermediate filaments from bovine, rat, and human CNS: mapping analysis of the major proteins. *J. Neurochem.* 34:1149–1159.
10. Cork, L. C., J. W. Griffin, J. F. Munnell, M. D. Lorenz, R. J. Adams, and D. L. Price. 1979. Hereditary canine spinal muscular atrophy. *J. Neuropathol. Exp. Neurol.* 38:209–221.
11. Cragg, B. G., and P. K. Thomas. 1961. Changes in conduction velocity and fiber size proximal to peripheral nerve lesions. *J. Physiol.* 157:315–327.
12. DeNechaud, B., C. Jeantet, and J. M. Bourre. 1982. Occurrence of tubulin in myelin from CNS. *Trans. Am. Soc. Neurochem.* 13:191.
13. Dixon, J. S. 1968. Changes in the fine structure of neurons after axon section. *J. Anat.* 103:396–397.
14. Farel, P. B. 1978. Reflex activity of regenerating frog spinal motoneurons. *Brain Res.* 158:331–341.
15. Friede, R. L., and T. Samorajski. 1970. Axon caliber related to neurofilaments and microtubules in sciatic nerve fibers of rats and mice. *Anat. Rec.* 167:379–388.
16. Giller, E., Jr., and J. H. Schwartz. 1971. Choline acetyltransferase in identified neurons of abdominal ganglion of Aplysia californica. *J. Neurophysiol. (Bethesda).* 34:93–107.
17. Giulian, D., H. Des Ruisseaux, and D. Cowburn. 1980. Biosynthesis and intra-axonal transport of proteins during neuronal regeneration. *J. Biol. Chem.* 255:6494–6501.
18. Gozes, I., and C. Richter-Landsberg. 1978. Identification of tubulin associated with rat brain myelin. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 95:169–172.
19. Grafstein, B. 1975. The nerve cell body response to axotomy. *Exp. Neurol.* 48:32–51.
20. Grafstein, B., and D. S. Forman. 1980. Intracellular transport in neurons. *Physiol. Rev.* 60:1167–1283.
21. Grafstein, B., and I. G. McQuarrie. 1978. Role of the nerve cell body in axonal regeneration. *In* Neuronal Plasticity. C. W. Cotman, editor. Raven Press, New York. 151–195.
22. Gray, E. G. 1957. The spindle and extrafusal innervation of a frog muscle. *Proc. R. Soc. Lond. B. Biol. Sci.* 146:416–430.
23. Guth, L. 1956. Regeneration in the mammalian peripheral nervous system. *Physiol. Rev.* 36:441–478.
24. Hall, M. E., D. L. Wilson, and G. C. Stone. 1978. Changes in synthesis of specific proteins following axotomy: Detection with two-dimensional gel electrophoresis. *J. Neurobiol.* 9:353–366.
25. Härkönen, M. H. A., and F. C. Kauffman. 1974. Metabolic alterations in the axotomized superior cervical ganglion of the rat. II. The pentose phosphate pathway. *Brain Res.* 65:141–157.
26. Heacock, A. M., and B. W. Agranoff. 1976. Enhanced labeling of a retinal protein during regeneration of optic nerve in goldfish. *Proc. Natl. Acad. Sci. U. S. A.* 73:828–832.
27. Hester, S., P. Capps-Covey, and D. L. McIlwain. 1978. The plasma membrane of bulk-isolated mature spinal neurons. *Brain Res.* 159:41–54.
28. Hoffman, P. N., J. W. Griffin, and D. L. Price. 1980. The role of slow component a (SCa) in determining axon caliber: changes during regeneration. *Soc. Neurosci. Abstr.* 6:93.
29. Hoffman, P. N., J. W. Griffin, and D. L. Price. 1981. Changes in the axonal transport of the cytoskeleton during development, aging, and regeneration. *Soc. Neurosci. Abstr.* 7:743.
30. Hoffman, P. N., and R. J. Lasek. 1980. Axonal transport of the cytoskeleton in regenerating motor neurons: Constancy and change. *Brain Res.* 202:317–333.

31. Krebs, H. A., and L. V. Eggleston. 1974. The regulation of the pentose phosphate cycle in rat liver. *Adv. Enzyme Regul.* 12:421–434.
32. Kreutzberg, G. W., and P. Schubert. 1971. Volume changes in the axon during regeneration. *Acta Neuropathol.* 17:220–226.
33. Lasek, R. J. 1980. Axonal transport: a dynamic view of neuronal structures. *Trends. Neurosci.* 3:87–91.
34. Lasek, R. J., and P. N. Hoffman. 1976. The neuronal cytoskeleton, axonal transport, and axonal growth. *In* Cell Motility. R. Goldman, T. Pollard, and J. Rosenbaum, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 3:1021–1049.
35. Lasek, R. J., and M. L. Shelanski, editors. 1981. Cytoskeletons and the Architecture of Nervous Systems. *In* The Neurosciences Research Program Bulletin. Massachusetts Institute of Technology Press. Boston, MA. Vol. 19.
36. Lazarides, E. 1980. Intermediate filaments as mechanical integrators of cellular space. *Nature (Lond.).* 283:249–256.
37. Lieberman, A. R. 1971. A review of the principal features of perikaryal responses to axon injury. *Int. Rev. Neurobiol.* 14:49–124.
38. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.
39. Luine, V. N., and F. C. Kauffman. 1971. Triphosphopyridine nucleotide-dependent enzymes in the developing spinal cord of the rabbit. *J. Neurochem.* 18:1113–1124.
40. Mackey, E. A., D. Spiro, and J. Wiener. 1964. A study of chromatolysis in dorsal root ganglia at the cellular level. *J. Neuropathol. Exp. Neurol.* 23:508–526.
41. Murray, M. 1976. Regeneration of retinal axons into goldfish optic tectum. *J. Comp. Neurol.* 168:175–196.
42. Murray, M., and D. S. Forman. 1971. Fine structural changes in goldfish retinal ganglion cells during axonal regeneration. *Brain Res.* 32:287–297.
43. Nathaniel, E. J. H., and D. R. Nathaniel. 1973. Electron microscopic studies of spinal ganglion cells following crushing of dorsal roots in adult rat. *J. Ultrastruct. Res.* 45:168–182.
44. Norton, W. T., and L. A. Autilio. 1966. The lipid composition of purified bovine myelin. *J. Neurochem.* 13:213–222.
45. Oakley, B. R., D. R. Kirsch, and N. R. Morris. 1980. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Anal. Biochem.* 105:361–363.
46. Perry, G. W., and D. L. Wilson. 1981. Protein synthesis and axonal transport during nerve regeneration. *J. Neurochem.* 37:1203–1217.
47. Peters, A., and J. E. Vaughn. 1967. Microtubules and filaments in the axons and astrocytes of early postnatal rat optic nerves. *J. Cell Biol.* 32:113–119.
48. Price, D. L., and K. R. Porter. 1972. The response of ventral horn neurons to axonal transection. *J. Cell Biol.* 53:24–37.
49. Reis, D. J., R. A. Ross, G. Gilad, and T. H. Joh. 1978. Reaction of central catecholaminergic neurons to injury: model systems for studying the neurobiology of central regeneration and sprouting. *In* Neuronal Plasticity. C. W. Cotman, editor. Raven Press, New York. 197–226.
50. Silver, M. L. 1942. The motoneurons of the spinal cord of the frog. *J. Comp. Neurol.* 77:1–39.
51. Sims, K. L., F. C. Kauffman, E. C. Johnson, and V. M. Pickel. 1974. Cytochemical localization of brain nicotinamide adenine dinucleotide phosphate (oxidized)-dependent dehydrogenases. Qualitative and quantitative distributions. *J. Histochem. Cytochem.* 22:7–19.
52. Sinicropi, D. V., and F. C. Kauffman. 1979. Retrograde alteration of 6-phosphogluconate dehydrogenase in axotomized superior cervical ganglia of the rat. *J. Biol. Chem.* 254:3011–3017.
53. Sinicropi, D. V., F. C. Kauffman, and D. R. Burt. 1979. Axotomy in rat sympathetic ganglia: reciprocal effects on muscarinic binding and 6-phosphogluconate dehydrogenase activity. *Brain Res.* 161:560–565.
54. Sinicropi, D. V., and D. L. McIlwain. 1981. Changes of cytoskeletal proteins in the perikarya and axons of regenerating frog motoneurons. *Soc. Neurosci. Abstr.* 7:907.
55. Sinicropi, D. V., K. Michels, and D. L. McIlwain. 1982. Acetylcholinesterase distribution in axotomized frog motoneurons. *J. Neurochem.* 38:1099–1105.
56. Skene, J. H. P., and M. Willard. 1981. Changes in axonally transported proteins during axon regeneration in toad retinal ganglion cells. *J. Cell Biol.* 89:86–95.
57. Skene, J. H. P., and M. Willard. 1981. Electrophoretic analysis of axonally transported proteins in toad retinal ganglion cells. *J. Neurochem.* 37:79–87.
58. Sotelo, C., and I. Grofova. 1976. Ultrastructural features of the spinal cord. *In* Frog Neurobiology. R. Llinás, and W. Precht, editors. Springer-Verlag, NY. 707–727.
59. Székely, G. 1976. The morphology of motoneurons and dorsal root fibers in the frog's spinal cord. *Brain Res.* 103:275–290.
60. Turner, J. E., R. K. Delaney, and R. E. Powell. 1978. Retinal ganglion cell response to axotomy in the regenerating visual system of the newt (Triturus viridescens): an ultrastructural morphometric analysis. *Exp. Neurol.* 62:444–462.
61. Vance, W. H., G. L. Clifton, M. L. Applebaum, W. D. Willis, Jr., and R. E. Coggeshall. 1975. Unmyelinated preganglionic fibers in frog ventral roots. *J. Comp. Neurol.* 164:117–126.
62. Waehneldt, T. V., and J. Malotka. 1980. Comparative electrophoretic study of the wolfgram proteins in myelin from several mammalia. *Brain Res.* 189:582–587.
63. Watson, W. E. 1966. Alteration of the adherence of glia to neurons following nerve injury. *J. Neurochem.* 13:536–537.
64. Weil, D. E., W. H. Busby, and D. L. McIlwain. 1977. Choline acetyltransferase activity in large ventral spinal neurons. *J. Neurochem.* 29:847–852.
65. Weil, D. E., and D. L. McIlwain. 1981. Distribution of soluble proteins within spinal motoneurons: a quantitative two-dimensional electrophoretic analysis. *J. Neurochem.* 36:242–250.
66. Wohlfart, G., and R. L. Swank. 1941. Pathology of amyotrophic lateral sclerosis: fiber analysis of the ventral roots and pyramidal tracts of the spinal cord. *Arch. Neurol. Psychiatry.* 46:783–799.
67. Yamada, K. M., B. S. Spooner, and N. K. Wessells. 1971. Ultrastructure and function of growth cones and axons of cultured nerve cells. *J. Cell Biol.* 49:614–635.
68. Yen, S.-H., and K. L. Fields. 1981. Antibodies to neurofilament, glial filament, and fibroblast intermediate filament proteins bind to different cell types of the nervous system. *J. Cell Biol.* 88:115–126.