

Incorporation of Axonally Transported Glycoproteins into Axolemma during Nerve Regeneration

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ABSTRACT The insertion of axonally transported fucosyl glycoproteins into the axolemma of regenerating nerve sprouts was examined in rat sciatic motor axons at intervals after nerve crush. [³H]Fucose was injected into the lumbar ventral horns and the nerves were removed at intervals between 1 and 14 d after labeling. To follow the fate of the "pulse-labeled" glycoproteins, we examined the nerves by correlative radiometric and EM radioautographic approaches.

The results showed, first, that rapidly transported [³H]fucosyl glycoproteins were inserted into the axolemma of regenerating sprouts as well as parent axons. At 1 d after delivery, in addition to the substantial mobile fraction of radioactivity still undergoing bidirectional transport within the axon, a fraction of label was already associated with the axolemma. Insertion of labeled glycoproteins into the sprout axolemma appeared to occur all along the length of the regenerating sprouts, not just in sprout terminals. Once inserted, labeled glycoproteins did not undergo extensive redistribution, nor did they appear in sprout regions that formed (as a result of continued outgrowth) after their insertion. The amount of radioactivity in the regenerating nerves decreased with time, in part as a result of removal of transported label by retrograde transport. By 7–14 d after labeling, radioautography showed that almost all the remaining radioactivity was associated with axolemma. The regenerating sprouts retained increased amounts of labeled glycoproteins; 7 or 14 d after labeling, the regenerating sprouts had over twice as much of radioactivity as comparable lengths of control nerves or parent axons.

One role of fast axonal transport in nerve regeneration is the contribution to the regenerating sprout of glycoproteins inserted into the axolemma; these membrane elements are added both during longitudinal outgrowth and during lateral growth and maturation of the sprout.

Regeneration of a transected axon requires both the restoration of a substantial volume of axoplasm and the addition of new axolemma to the regenerating sprouts. Because the axon itself is unable to synthesize significant amounts of protein, transfer of new protein from the cell body to both axoplasm and axolemma must be mediated by the axonal transport systems. Slow axonal transport provides the bulk of the axoplasmic and cytoskeletal proteins (15, 18, 24, 25), whereas several lines of evidence suggest that fast transport contributes elements to the axolemma (4, 12, 13, 19). During regeneration, fast axonal transport continues at the normal rates (2, 13, 19, 30, 32), passing unimpeded through the site of axotomy into regenerating sprouts (2, 17, 19, 31). Rapidly transported materials

accumulate in the distal regions of the regenerating sprouts soon after delivery (17, 19, 28, 31, 33, 38). The subsequent disposition of rapidly transported materials, however, is poorly understood.

The present study was designed to examine the fate of rapidly transported glycoproteins in regenerating rat sciatic nerves. We were interested in several related problems: first, whether rapidly transported glycoproteins were inserted into sprout axolemma; second, at what stages of sprout outgrowth and maturation insertion occurred; third, how the proportion of transported glycoproteins associated with axolemma (rather than intraaxonal constituents) changed with time after delivery; and fourth, how insertion into sprout axolemma compared in

quantity to insertion into the axolemma of parent axons or unoperated controls.

From 1 to 12 wk after one sciatic nerve had been crushed, [³H]fucose was injected into the lumbar ventral horns (the contralateral nerve was used as an unoperated control). At intervals after labeling, the nerves were removed. The amount of transported radioactivity in different segments of the nerves was determined by liquid scintillation spectrometry. Quantitative EM radioautographic studies measured the associated of label with axolemma and with intraaxonal structures. In addition, the proportion of radioactivity still being transported was determined by ligating the nerves at intervals after labeling (3, 5, 14, 21). The results were used to construct a model of glycoprotein addition to sprout axolemma.

MATERIALS AND METHODS

Nerve Crush

All studies were performed on 250-g Sprague-Dawley female rats. In rats under chloral hydrate anesthesia, the right sciatic nerve was crushed for 10 s with watchmaker's forceps just distal to the branching of the nerve to the biceps

femoris or, in some groups, at the merging of lumbar roots 4 and 5. In all animals, the contralateral side was used as a control.

Radiometric Studies

These studies were done to analyze the distribution and relative amount of transported radioactivity along regenerating and control nerves at different times after labeling (i.e., after delivery of a "pulse" of transported glycoproteins to the axon). Groups of animals were subjected to unilateral crush at various times from 7 to 90 d before labeling. [³H]Fucose (sp act, 42 or 60 Ci/mM; New England Nuclear, Boston, Mass.) was injected into the lumbar ventral horns as previously described (19). In the present studies, three injections—each of 0.8 μl and separated by 0.75 mm—were made symmetrically in each side. Animals were returned to their cages after labeling. Groups of four animals each were killed 1, 3, 7, or 14 d after labeling. Table I summarizes the timing of the various experimental groups.

The nerves were dissected, solubilized, and counted as previously described (19). In preliminary studies, the amount of nontransported radioactivity present along the sciatic nerves was assessed by measuring disintegration per minute per 3-mm segment in regions distal to nerve ligations. The highest values, found 24 h after labeling, were 70 dpm in control nerves and 240 dpm in regenerating nerves. The values (for each interval after labeling) were subtracted from the measured values to obtain values for transported radioactivity. Results were plotted as disintegrations per minute per 3-mm nerve segment against position of each segment along the nerve.

An additional experiment was designed to determine the extent to which inserted glycoproteins might be carried distally as the sprouts continue to elongate. We compared the distribution of glycoproteins in the regenerating nerves 7 d after labeling with the position of the growing tips of the regenerating nerves at the time of nerve removal. Two animals were subjected to a crush at the merging of the L-4 and L-5 lumbar roots in the pelvis, [³H]fucose was injected into the lumbar ventral horns 7 d later, and on day 13 after crush, [³⁵S]methionine (sp act, 400 Ci/mM) was injected into the lumbar ventral horns. On day 14 after crush, the nerves were removed and counted by double-label techniques.

Ligation Studies

These experiments, done in parallel with the radiometric studies, were designed to estimate the proportion of radiolabel that continued to be transported at various times after labeling. Animals were labeled 7 d after crush; on the day on which the animals had been killed in the studies described above, the animals in these studies were anesthetized and the regenerating nerves were ligated at a level 15 mm proximal to the crush (see Table I). These animals were killed 24 h after ligation, and the nerves were removed, divided into segments, and counted.

We related the distribution of radioactivity in these ligated nerves to that of the corresponding unligated nerves (Table II). The amount of radioactivity accumulating distal to the ligature was used to calculate a "mobile fraction", whereas the radioactivity remaining in the regenerating sprouts of the ligated nerves was used to calculate a "stationary fraction."

Radioautographic Studies

Regenerating nerves from animals 1 and 7 d after labeling were examined by radioautography (8 d and 14 d after crush). The animals were perfused with 50 ml of 4% paraformaldehyde followed by 1 liter of 5% glutaraldehyde in sodium phosphate buffer, pH 7.3, and samples were taken from the regenerating nerve proximal to the crush, as well as from the first eight 3-mm segments distal to the

TABLE I
Summary of Time Relationships between Experiments

Intraspinal injection of [³ H]fucose	Nerve ligation	Nerve removal	Number of regenerating nerves	Control nerves
d after crush	d after crush	d after crush		
7	—	8*	4	4
	—	10	4	4
	—	14*	4	0
	—	14‡	2	0
	—	21	4	4
7	8	9	4	4
	10	11	4	4
	14	15	4	4
	21	22	4	4
14	—	15	4	0
	—	21	4	0
21	—	28	5	5
90	—	97	4	3

* Animals from these intervals were also perfused with fixatives and the nerves were studied by EM radioautography.

‡ The nerves of these animals were crushed proximally; [³H]fucose was injected 7 d after crush, and [³⁵S]methionine injected on day 13 (see text).

TABLE II
Changes in the Mobile and Stationary Fractions of Transported Radioactivity with Time after Labeling

Day after injection*	dpm in Regenerating sprouts—unligated group‡ (A)	dpm in Regenerating sprouts—ligated group‡ (B)	Retrogradely transported dpm (distal to ligature)‡§ (C)	Stationary fraction (B/A)	Mobile fraction (C/A)
1	159,766 ± 17,445	57,958 ± 3,561	39,296 ± 9,075	.36	.25
3	120,120 ± 15,549	87,238 ± 15,780	18,783 ± 2,517	.73	.16
7	58,758 ± 7,646	48,607 ± 6,422	6,818 ± 1,520	.83	.12
14	43,821 ± 9,653	39,253 ± 9,530	2,818 ± 1,172	.90	.06

Changes in the mobile and stationary fractions with time after labeling. In all groups, animals were injected with [³H]fucose 7 d after nerve crush. At the times indicated, nerves were either removed, or ligated 15 mm proximal to the crush and removed 1 d later. The amounts of radioactivity in the regenerating sprouts (columns A and B) were obtained by adding the dpm in the first eight 3-mm segments distal to the crush.

* The day after injection designates the day experiments were terminated in the unligated group, and the day ligatures were applied in the ligated group.

‡ Mean ± SD.

§ To correct for intrinsic stationary radioactivity in the segment distal to the ligature, the dpm in the second segment from the ligature was subtracted from that in the first. The resulting value is a better measure of the radioactivity arriving by retrograde transport.

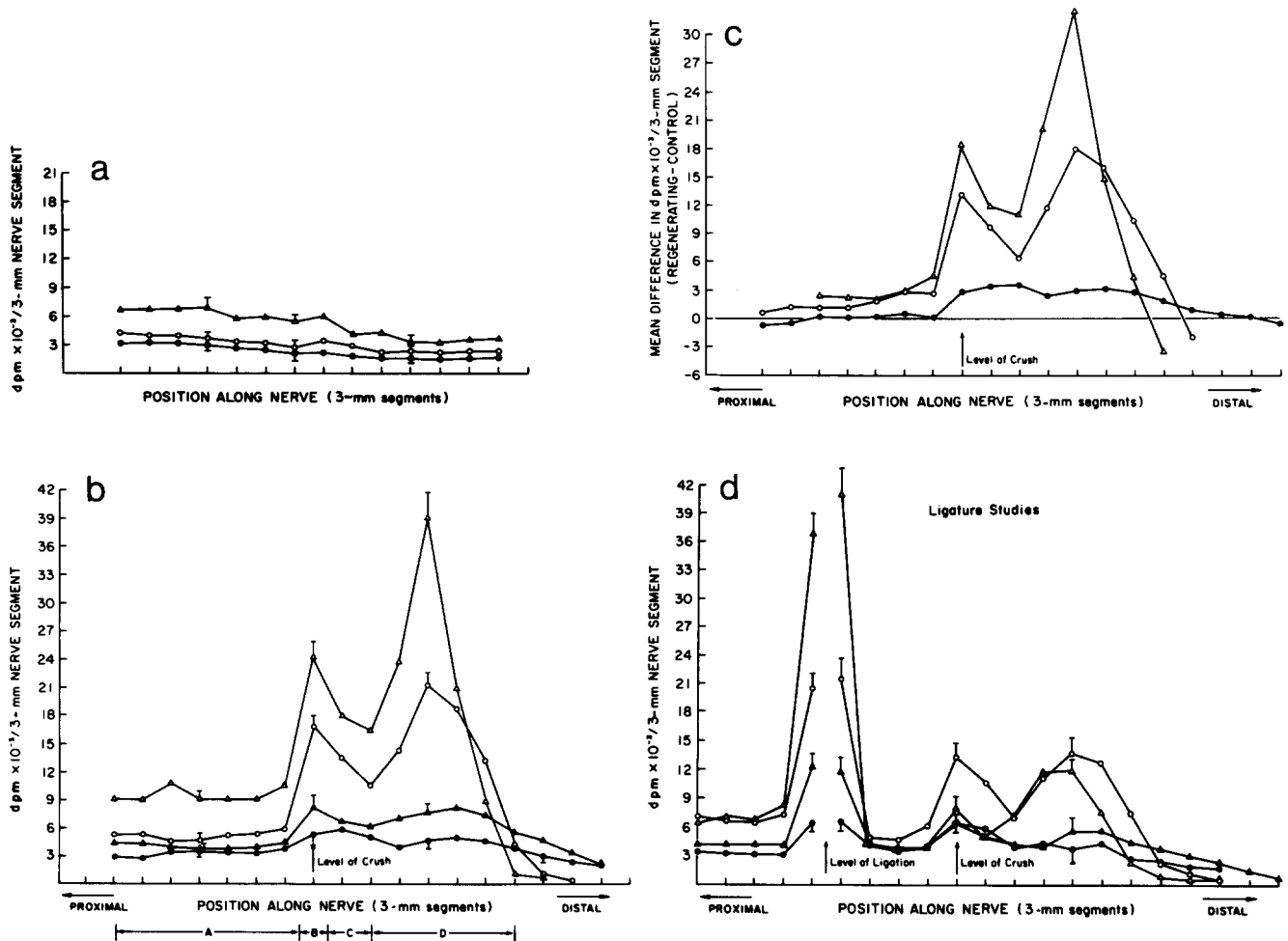


FIGURE 1 Plots of the distribution of transported radioactivity along rat sciatic nerves at intervals after injection of [^3H]fucose into the lumbar ventral horns, showing retention of [^3H]fucosyl glycoproteins in (a) control nerves and (b–d) regenerating sprouts. Each curve is the mean of four nerves (\pm SD). (a) Control nerves (contralateral to the regenerating nerves shown in b). The slightly lower levels of radioactivity in the distal segments of the nerve are the result of nerve branching (particularly the nerve to the biceps femoris). Δ , 1 d postinjection; \circ , 3 d postinjection; \bullet , 14 d postinjection. (b) Regenerating nerves, labeled 7 d after crush and removed at times shown. Regions A–D are as defined in the text. Note that the general conformation of the curves is maintained with time although the relative prominence of the peak in region D decreases. Note also that relatively little transported radioactivity is found distal to region D at late times after labeling, even though the regenerating sprouts are growing out >4 mm/d (cf. Fig. 7). Δ , 8 d postcrush, 1 d postinjection; \circ , 10 d postcrush, 3 d postinjection; \blacktriangle , 14 d postcrush, 7 d postinjection; \bullet , 21 d postcrush, 14 d postinjection. (c) Mean differences in disintegrations per minute between regenerating and contralateral control nerve at intervals after labeling. These curves represent the differences between the curves in b and those in a, with the mean values of the control nerves in each segment plotted as 0 on the y-axis. Note that 1 and 3 d after labeling, more radioactivity was present in region A on the regenerating side. However, by 14 d after labeling, the amount of radioactivity was essentially identical on the two sides. Note also that the amount of radioactivity in the regenerating sprouts (regions B, C, and D) was greater than comparable segments of the control nerves at all times after labeling (see also Table III). Δ , 8 d postcrush, 1 d postinjection; \circ , 10 d postcrush, 3 d postinjection; \bullet , 21 d postcrush, 14 d postinjection. (d) Ligature studies. Plots of the distribution of radioactivity in regenerating nerves labeled 7 d after crush and subjected to nerve ligation at intervals after labeling. The animals in each group were sacrificed 1 d after ligation. Note that the amount of radioactivity accumulating distal and proximal to the ligation decreases dramatically with time. Note also that, 1 d after labeling in the group ligated, the amount of radioactivity in region D is much less than in unligated nerves (b). In contrast, nerves ligated 14 d after labeling have a mean curve similar to that of the 14-d group in b. As described in the text and Table II, these changes reflect the decrease in the mobile fraction and relative increase in the stationary fraction with time after labeling. Δ , 9 d postcrush, 2 d postinjection, 1 d postligation; \circ , 11 d postcrush, 4 d postinjection, 1 d postligation; \blacktriangle , 15 d postcrush, 8 d postinjection, 1 d postligation; \bullet , 22 d postcrush, 15 d postinjection, 1 d postligation.

crush. All samples were osmicated (1% OsO_4 for 2 h), dehydrated, and embedded in Epon 812. Transverse sections ($1\ \mu\text{m}$) were used for light radioautography. For electron microscope radioautography, pale gold sections were placed on carbon-coated grids, dipped in Ilford L4 emulsion, exposed for 50 and 80 d, and developed in Kodak D19 developer (35). The specimens were photographed with an AEI 801 electron microscope, and the distribution of grains was obtained from prints (final magnification, $\times 15,000$) by the method of Salpeter et al. (36). The grains were circumscribed by the smallest possible circle, and the distance of the

center of each circle on the perpendicular line from the axolemma was determined in half-distance (HD) units. 1 HD unit = $1,600\ \text{\AA}$ (36). Next, the perimeter of each axon was measured and the radius (r) of a comparable circle was calculated. Then the areas of circular bands of $(r + 0.5\ \text{HD}) - (r - 0.5\ \text{HD})$, $(r - 0.5\ \text{HD}) - (r - 1.5\ \text{HD})$, etc., were calculated, and the same procedure was repeated for circles outside the axolemma. For each resulting band inside and outside the axolemma, grain density ($\text{grains}/\mu\text{m}^2$) was calculated and mean grain densities for each band were normalized. For three regions along the regenerating nerve

(i.e., 6–9 mm proximal to the crush, 3–6 mm distal to the crush, and 15–18 mm distal to the crush), plots of the normalized mean grain densities were compared with universal curves for hollow circular sources (36).

RESULTS

For clarity, the results from groups of animals killed 1 d after labeling are presented first, and contrasted with those from groups killed at later times.

1 d after Labeling

RADIOMETRIC STUDIES: The unoperated control nerves showed nearly constant amounts of radioactivity all along the sciatic nerve (Fig. 1 *a*). In regenerating nerves labeled 7 d (Fig. 1 *b*) or 14 d (Fig. 2) after crush, four distinct regions were identified. Proximal to the crush (region *A*), there was a flat plateau of radioactivity. At the level of the crush (region *B*), there was a small peak of radioactivity, resulting from accumulation of rapidly transported label within transected axons that had not grown beyond the crush (see below). Distal to the crush, there was a “valley” (region *C*) that preceded a larger peak of accumulated radioactivity (region *D*). This peak of radioactivity represented in large part [³H]fucosyl glycoproteins transported to, and accumulated within, sprout endings; as discussed below, radioautographic studies showed that growth cones were heavily labeled and were most numerous in this region.

SUBCELLULAR DISTRIBUTION OF TRANSPORTED GLYCOPROTEINS: Quantitative radioautography was used to assess the association of label with axolemma, with the intraaxonal compartment, and with extraaxonal structures, and also to compare the patterns in sprouts of different degrees of maturity. Sections from the crush site showed a small proportion of axons that were markedly enlarged and contained dense collections of membranous organelles. These axonal swellings, which were heavily labeled, presumably represented axons that had not begun to grow out effectively (8, 20). They explain the small peak of radioactivity in region *B*.

In region *C*, sprouts in varying degrees of maturity were seen, ranging from clusters of fine sprouts within single Schwann cells to larger sprouts already beginning myelination (Fig. 3 *a*). Growth cones were rare. The grain distribution (Fig. 4 *b*) showed that a fraction of transported label was already associated with axolemma, whereas another fraction was intraaxonal. The axolemma was labeled even in the larger, myelinating axons.

Region *D* contained fine sprouts with relatively numerous growth cones (Fig. 3 *b* and *c*). The growth cones, containing loosely packed membranous organelles including branched

tubulovesicular profiles (7), were often intensely labeled. The grain distribution in the fine sprouts (excluding growth cones) was similar to that seen in region *C* (cf. Fig. 4 *b* and *d*).

Later Disposition of Transported Glycoproteins

RADIOMETRIC AND LIGATION STUDIES: Between 1 and 14 d after labeling, the amount of radioactivity in the regenerating sprouts decreased by >70% (Fig. 1 *b* and Table II). In addition, the relative distribution of radioactivity between regions *C* and *D* changed. Region *D* became progressively less prominent (Figs. 1 *b* and *c* and 2), although a small peak corresponding to region *D* could still be distinguished 14 d after labeling (Fig. 5 *a*).

The ligation studies showed a progressive increase in the proportion of radioactivity in the stationary fraction. 36% of the total radioactivity in the regenerating sprouts was in the stationary fraction on day 1; by day 14 the stationary fraction was 90% of the total (i.e., the amount of radioactivity within the regenerating sprouts was nearly the same in ligated and unligated nerves) (Table II). There was a corresponding reduction in the mobile fraction from 25 to 6% between days 1 and 14 (Table II).

SUBCELLULAR DISTRIBUTION OF TRANSPORTED GLYCOPROTEINS: By 7 d after labeling (14 d after crush), region *C* contained sprouts of various calibers, including a substantial proportion of sprouts over 4 mm in diameter that were in advanced stages of myelination (Fig. 6 *a*). In region *D*, sprouts were mostly smaller and had not begun to myelinate (Fig. 6 *b*), or were in early stages of myelination.

A large proportion of the [³H]fucosyl glycoproteins were associated with axolemma; the distributions of grains in both the parent axons and the regenerating sprouts in regions *C* and *D* were nearly identical, and closely approximated those predicted for a hollow circle (Fig. 4 *A*, *C*, and *E*). In the sprouts, the same association of grains with axolemma was found in myelinating axons as well as in axons that had not yet begun to myelinate.

These grain distributions were assessed from normalized grain densities. When raw grain densities in different regions of the same nerve were compared, there was a greater density in the small density sprouts than in the more proximal regions. For example, 7 d after labeling, the grain density was 2.6-fold greater in region *D* (the former growth cone region) than in region *C* (just distal to the crush).

Extent of Redistribution of Transported Glycoproteins

We next asked to what extent labeled glycoproteins might be redistributed after delivery. For example, we wondered whether labeled constituents in the growing tips would be carried more distally as the sprouts continued to grow out, and whether substantial intramembranous migration might occur. At 7 and 14 d after labeling, transported radioactivity extended only slightly more distally than at 1 d after labeling (Figs. 1 *c*, 2, and 5). In groups labeled 7 d after crush, radioactivity in the distal stump reached baseline levels at 21 mm beyond the crush on day 8 (1 d after labeling) and 25 mm on day 10; even on day 21 (14 d after labeling), most of the radioactivity was within 25 mm of the crush, although low levels of transported radioactivity were found 30–36 mm distal to the crush (Figs. 1 *b* and 5 *a*).

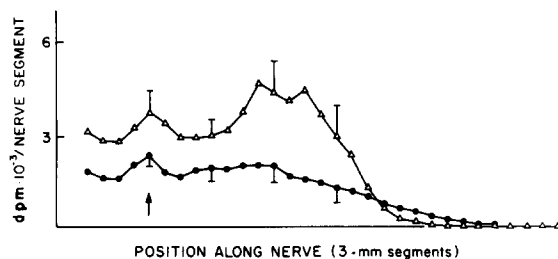


FIGURE 2 Regenerating nerves labeled 14 d after crush at the merging of the L4 and L5 spinal roots. Note the decrease in prominence of region *D* compared with region *C* between 1 and 7 d after labeling. Δ , 15 d postcrush, 1 d postinjection; \bullet , 21 d postcrush, 7 d postinjection.

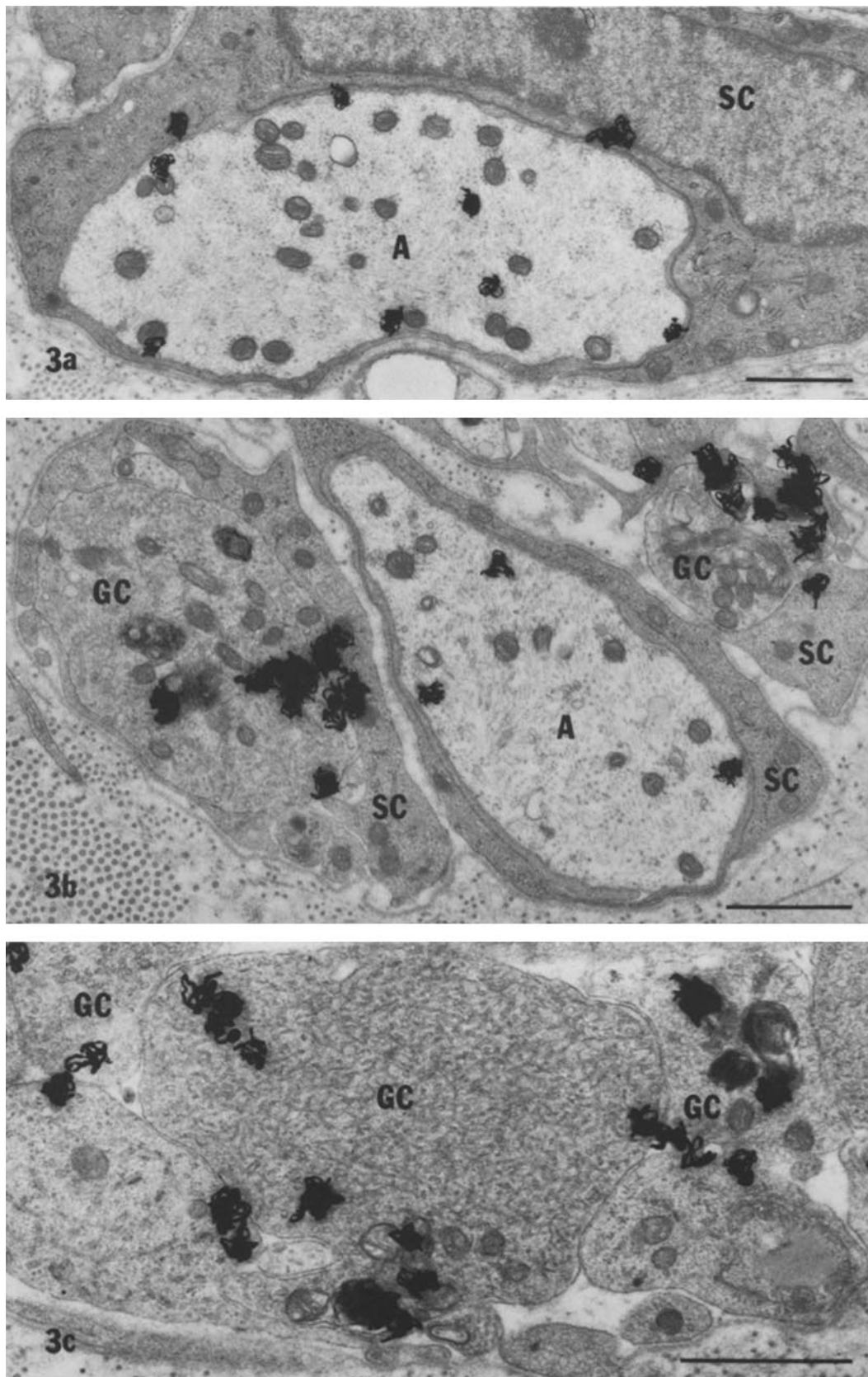


FIGURE 3 Electron microscope radioautograms from regenerating sprouts 1 d after injection of [3 H]fucose into the lumbar ventral horns. (a) region C, early myelinating axon (A), with silver grains present both near the axolemma and within the axon. SC, Schwann cell. Bar, 1 μ m. \times 17,000. (b) Region D, an early myelinating axon (A) and two growth cones (GC) containing particulate organelles. Bar, 1 μ m. \times 20,100. (c) Region D, several densely labeled growth cones (GC) containing tubulovesicular profiles. Bar, 1 μ m. \times 27,700.

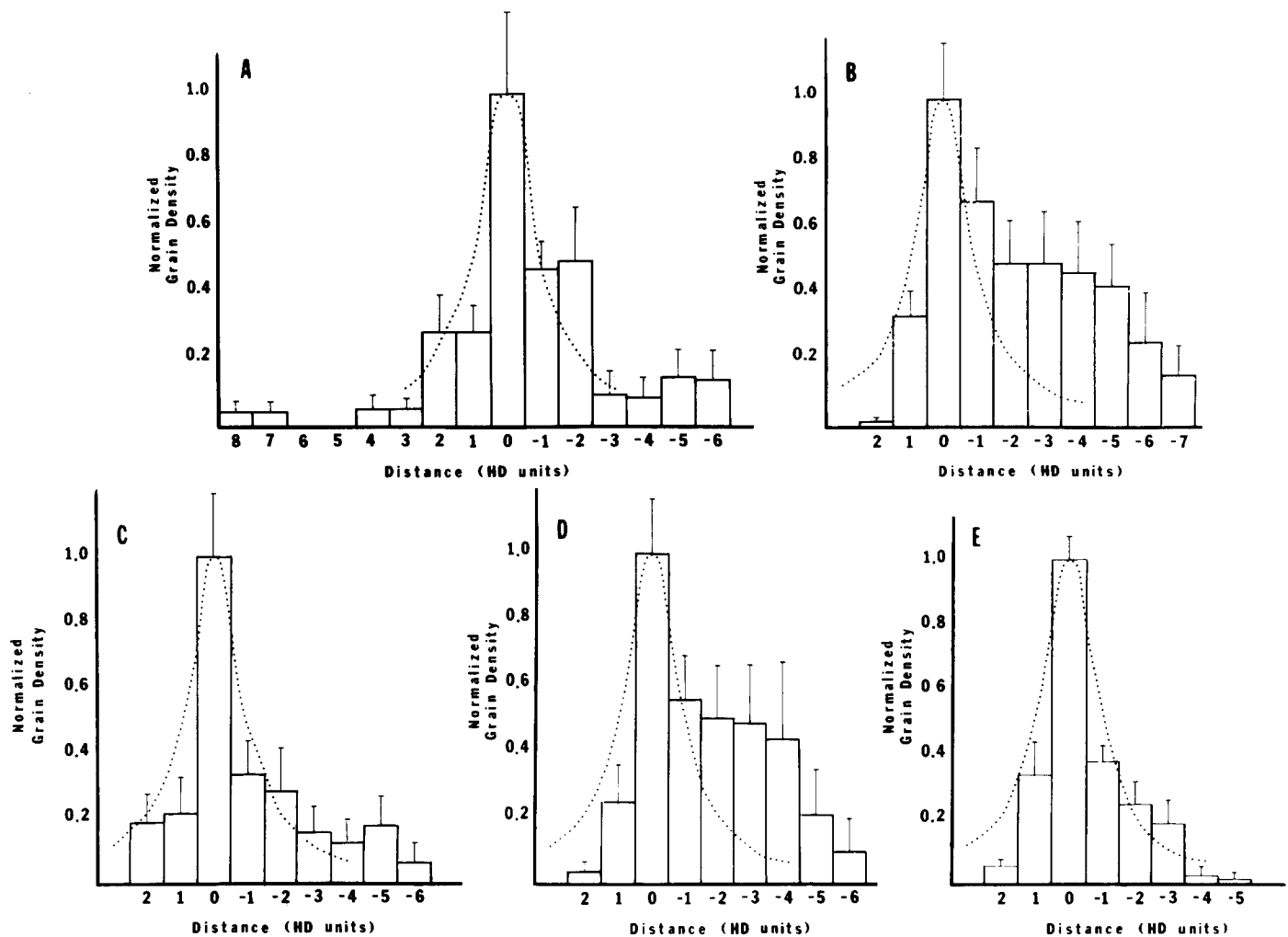


FIGURE 4 Histograms showing the distribution of silver grains in EM radioautograms of regenerating sprouts. All radioautograms were developed after 50 d of exposure and were photographed at $\times 15,000$ total magnification. Each HD unit is 1,600 Å. The axolemma is at 0 with + HD units outside and - HD units inside. In all histograms, grain density is normalized to 1.0 at the axolemma. The universal curve for axons of infinite radius is superimposed (no axons of <6 HD units in radius were counted). (A) Grain distribution in parent axons proximal to the crush (7 d after intraspinal injection of [^3H]fucose). (B) Grain distribution in regenerating sprouts 3-mm distal to the nerve crush (8 d postcrush, 1 d after intraspinal injection of [^3H]fucose). (C) Grain distribution in regenerating sprouts 3-mm distal to the crush (14 d postcrush; 7 d after intraspinal injection of [^3H]fucose). (D) Grain distribution in regenerating sprouts 15-mm distal to the crush (8 d postcrush, 1 d after intraspinal injection of [^3H]fucose). (E) Grain distribution in regenerating sprouts 15-mm distal to the crush (14 d postcrush; 7 d after intraspinal injection of [^3H]fucose).

The double-labeling experiment allowed direct comparison within individual nerves of the distribution of radioactivity 7 d after labeling (14 d after crush) with the position of the sprout terminals at that time. In this experiment (Fig. 7), the sciatic nerve was crushed; on day 7 after crush, [^3H]fucose was injected; on day 13 after crush, [^{35}S]methionine was injected; and on day 14 after crush, the animal was killed. The results showed that proteins labeled by [^{35}S]methionine extended 20 mm beyond the segments labeled by [^3H]fucose. Thus, these results indicated that inserted glycoproteins were not carried distally with the growing tip, nor was there any substantial redistribution of transported glycoproteins to regions distal to region D in spite of continued outgrowth.

Amount of Transported Radiolabel in Regenerating Nerves

The regenerating sprouts retained considerably more radioactivity than either parent axons or contralateral control nerves;

even 14 d after labeling, there was more than twice the amount of radioactivity in the regenerating sprouts as in the control nerve (Fig. 5A and Table III). Increased retention was not restricted to region D, although a small peak persisted in this region (Fig. 5A). Region C also showed more radioactivity than control nerves, as is best seen in animals labeled 21 d after crush and sacrificed 7 d later (Fig. 5B and Table III). In these animals, the growing tips had grown beyond the available length of nerve. The regenerating sprouts contained 2.22 times the amount of radioactivity in comparable control nerves (Table III). However, in animals labeled 90 d after crush and killed 2 wk later, the same amounts of radioactivity were found all along the regenerating and control nerves (not shown).

It is of interest that the amount of radioactivity in the parent axons (region A) was increased at early times after labeling, presumably reflecting an increased amount of fucosyl glycoproteins undergoing fast transport (13). However, by 14 d after labeling, the number of counts in this region was identical on the two sides (Fig. 1c).

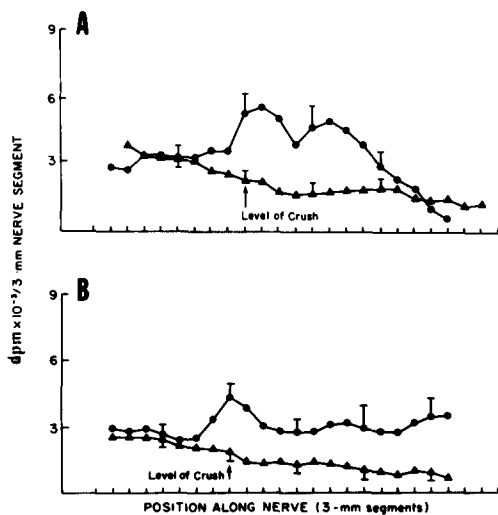


FIGURE 5 Plots allowing direct comparison of amounts of radioactivity in regenerating (●) and contralateral controls (▲) nerves. Each curve is the mean of four nerves. (A) [³H]fucose was injected into the lumbar ventral horns 7 d after nerve crush, and the nerves were removed 14 d later. The level of radioactivity on the two sides is similar proximal to the crush, but increased above control levels for 28-mm distal to the crush. Note a small peak persists in region D. (B) [³H]Fucose was injected 21 d after nerve crush, and the nerves were removed 7 d later. The amount of radioactivity is increased on the regenerating side throughout the available length of nerve distal to the crush, demonstrating increased retention during sprout maturation.

DISCUSSION

This study was designed to construct a spatial and temporal picture of aspects of the insertion of glycoproteins into the axolemma of regenerating sprouts. Interpretation of these correlative radiometric and radioautographic studies is based on the fact that labeled fucosyl glycoproteins are synthesized in the cell body and delivered to regenerating axon in relatively "pulsed" fashion, with fast axonal transport providing the means of delivery (1, 11, 12, 16, 26, 39, 40). Because, in young rats, a large proportion of sciatic motor axons grow out in a coherent fashion (17, 19, 31, 33), it was possible to examine regions of the regenerating nerve rich in growth cones, in mature sprouts, or in maturing sprouts, and to follow the fate of a "pulse" of transported glycoprotein over time.

The autoradiographic results showed that some axonally transported [³H]fucosyl glycoproteins were incorporated into the axolemma of the regenerating sprouts within the first day after synthesis. 1 d after labeling, in addition to the silver grains located within the axon, another fraction was clearly associated with axolemma (Fig. 4B and D). It is likely that these labeled glycoproteins within the axolemma correlate in large part with the stationary fraction as determined by ligature studies. 1 d after labeling, a definite stationary fraction was present within the sprout (Fig. 1d and Table II). In addition, there was a relatively large mobile fraction, presumably corresponding to the intraaxonal glycoproteins still undergoing bidirectional transport (Fig. 1d and Table II). In contrast, by 7 d after labeling, the grains remaining in the regenerating sprouts were associated almost exclusively with axolemma, and the grain distribution closely approximated that expected for a hollow circular source (Fig. 4C and E). In the ligature studies, most of the radioactivity was stationary, with a much reduced mobile phase (Fig. 1d and Table II). Thus, at late times after labeling,

the radioactivity remaining in the sprouts can be equated with the stationary axolemmal radioactivity.

We next asked at what stage or stages of sprout development axolemmal insertion of transported glycoprotein occurred. In particular, we tested whether insertion of transported glycoproteins might occur only at the growing tip (4), or whether a significant proportion might be inserted locally all along the regenerating sprout (lateral insertion). Our data suggests that both terminal and lateral insertion contribute transported glycoproteins to the axolemma. Indirect evidence suggesting that terminal insertion occurred was found in the peak of stationary radioactivity in the growth-cone-rich region D in the ligation studies (e.g., in nerves ligated 1 and 3 d after labeling [Fig. 1d]). A similar persistent peak in region D was found in the radiometric studies, even 14 d after labeling (Fig. 5A). These patterns are consistent with the possibility of terminal insertion.

On the other hand, several related observations indicated that the growth cone was not the sole site of addition of transported glycoproteins. Radioautography showed that even large myelinating sprouts, whose growth cones must be many millimeters distal to the level of the histologic section, contained label within axolemma, even 1 d after labeling (Fig. 3a). In addition, local insertion all along the regenerating sprouts was suggested by the relatively large amounts of radioactivity found in region C 7 d after labeling. This retained radioactivity is best seen in groups labeled 14 or 21 d after crush (Figs. 2 and 5B, respectively), because of the greater length of region C available for examination. Taken together, these radioautographic and radiometric observations are best explained by local axolemmal insertion of glycoproteins all along the regenerating sprouts.

An alternative interpretation might be that terminal insertion was followed by retrograde intramembranous movement of glycoproteins resulting in general axolemmal labeling. Although such a process (27, 29) cannot be excluded by present data, intramembranous redistribution (27, 29) would have to be rapid (to account for widespread axolemmal labeling within 1 d), extensive, selective (the abrupt increase in the amount of radioactivity at the level of the crush never ascended into the parent axon [Figs. 1c and 5]), and unidirectional (see below). Local insertion seems a more likely mechanism for addition of the bulk of axolemmal glycoproteins.

We next asked whether labeled glycoproteins, once delivered to the sprouts, might subsequently be carried along as the sprouts continued outgrowth. The radiometric studies showed no evidence of a major redistribution of transported glycoproteins into sprout regions that develop after delivery. In particular, the double-labeling experiment demonstrated that most of the new sprout growth that developed after delivery of the pulse of fucosyl glycoprotein remained unlabeled (Fig. 7). There is a small difference in the most distal segment containing transported radioactivity 14 d after labeling (compared with 1 d) (Figs. 1b, 2, 5A, and 7); this difference is best accounted for by the continued delivery of small amounts of material still moving distally 3 or 7 d after intraspinal injection of isotope (i.e., the radioactivity proximal to the ligation in Fig. 1d). Thus, we conclude that the sprout axolemma with the greatest proportion of recently synthesized glycoproteins is always the most distal (the most heterogeneous sprout axolemma with regard to the day of synthesis of axolemmal constituents would be the segment just distal to the crush).

There was a progressive reduction of the prominence of region D relative to region C with time after labeling. This change in conformation of the radiometric curves suggests that

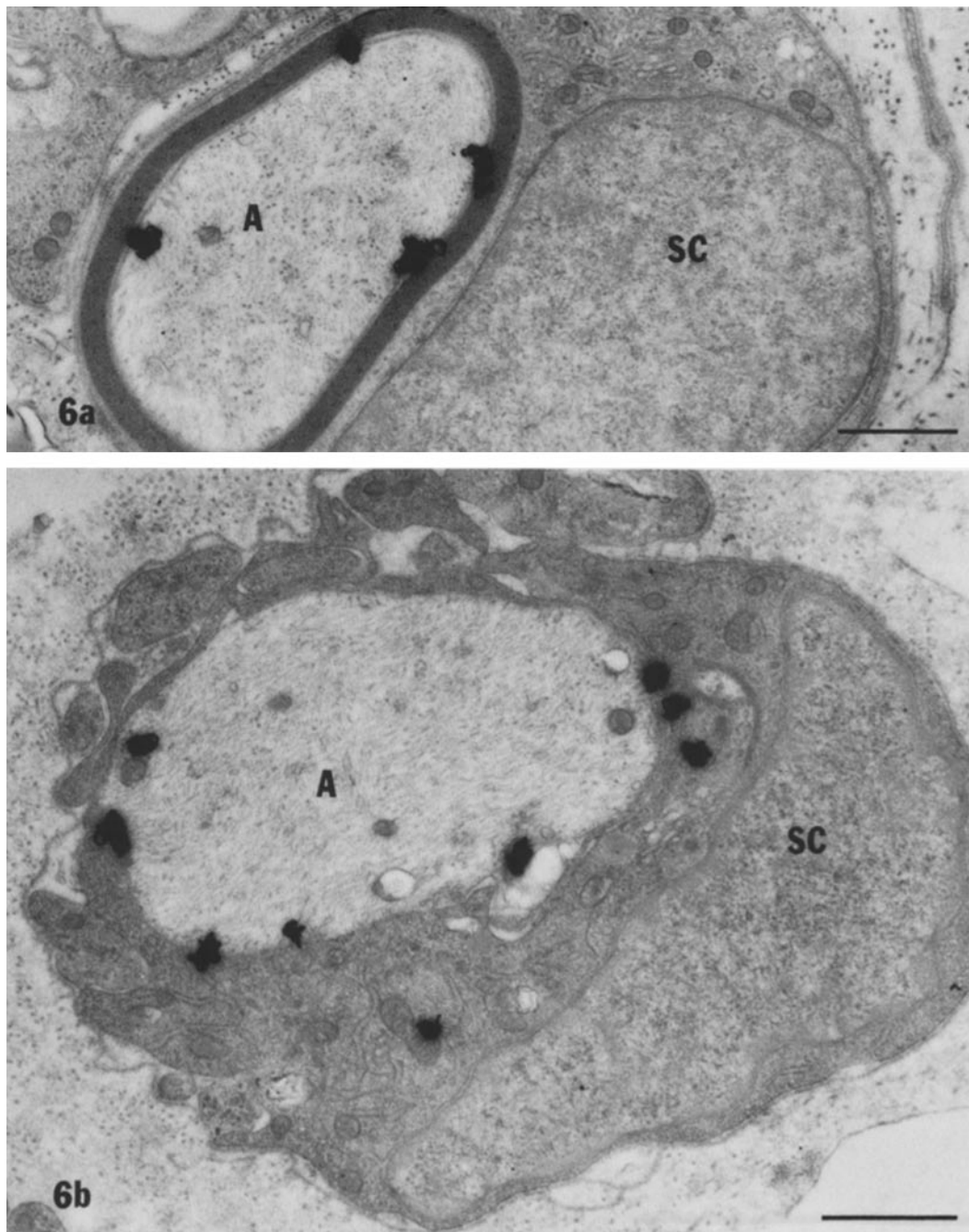


FIGURE 6 Electron microscope radioautograms from regenerating nerves removed 7 d after intraspinal injection of [^3H]fucose, showing association of silver grains with axolemma. (a) Region C, sprout in advanced stage of myelination (cf. Fig. 4 C). Bar, 1 μm . $\times 17,000$. (b) Region D, large axon that has not yet begun to myelinate (cf. Fig. 4 E) Bar, 1 μm . $\times 19,500$.

a portion of the radioactivity that accumulates in the growth cones at early times turns over more quickly after delivery than that in region C (Figs. 1 a, 2, and 7). As demonstrated by the ligation studies, this turnover occurs in part by retrograde transport back towards the cell body (Fig. 1 d) and also, presumably, by local degradation. It is possible that some of the transported glycoproteins reaching the growth cone undergo a cycle of insertion, endocytosis, and subsequent retrograde transport (7), similar to that suggested at the neuromuscular junction (23).

Retention of [^3H]fucosyl glycoproteins within regenerating sprouts occurred in amounts considerably greater than either parent axons or control nerves (in regions comparable to the

regenerating sprouts). For example, 2 wk after labeling, the 24 mm distal to the crush contained 2.3 times the amount of radioactivity present in the same region of the contralateral nerve (Figs. 1 c and 5 A; Table III). That this increase was not simply the result of the increased fast transport of fucosyl glycoprotein that occurs during regeneration (13) was shown by the fact that no greater retention was seen in parent axons than in the control nerves 7 or 14 d after labeling (Figs. 1 c and 5). Increased retention continued in sprout axolemma at least 3 wk old (the region just distal to the crush in Fig. 5 b), but, by 3 mo after crush, counts in control and regenerating nerves were comparable.

The following model relates previous models of membrane

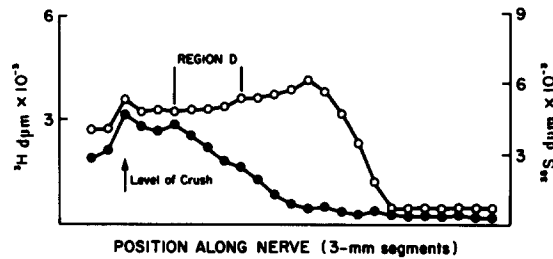


FIGURE 7 Double-label experiment comparing, in the same regenerating nerve, the distribution of [^3H]fucosyl glycoproteins labeled 7 d before removal and the distribution of [^{35}S]methionine proteins labeled the day before nerve removal. This nerve was crushed proximally at the merging of the L4 and L5 roots, and, 7 d later, [^3H]fucose was injected into the lumbar spinal cord. 6 d later, [^{35}S]methionine was injected into the same sites of the spinal cord. 1 d later, the nerve was removed and counted by double-label techniques. Transported [^{35}S]methionine-labeled proteins are present >45-mm distal to the crush; this is the extent of the outgrowth in the 14 d since the crush. In contrast, [^3H]fucosyl glycoproteins are present in significant amounts only in the first 25-mm distal to the crush. For comparison, region D (for 8 d after crush) is identified. This experiment shows that the labeled glycoproteins, for the most part, remain in the segments in which they are initially inserted; they are not redistributed or carried more distally with subsequent outgrowth. ●, 14 d postcrush, 7 d after injection of [^3H]fucose; ○, 14 d postcrush, 1 d after injection of [^{35}S]methionine.

TABLE III

Comparison of Amounts of Radioactivity in Regenerating Sprouts and Control Nerves

[^3H]- Fucose injection	Nerve re- moval	Total dpm in sprouts (A)	Total dpm in control (B)	Regener- ating/ Control (A/B)
d after crush	d after crush			
7	8	159,766 \pm 17,445	39,716 \pm 4,458	4.02
	10	120,120 \pm 15,549	25,664 \pm 5,564	4.68
	21	43,821 \pm 9,653	18,807 \pm 4,719	2.33
21	28	48,318 \pm 11,115	21,732 \pm 7,107	2.22

Comparison of amounts of radioactivity in the regenerating sprouts (24 mm distal to nerve crush) with the comparable 24 mm of contralateral control nerves. Note that by 21 d after crush (14 d after injection), whereas there is over twice as much radioactivity in the sprouts as in control nerves, the amount of radioactivity in the parent axons (proximal to the crush) is nearly identical to that in comparable segments of control nerves (see Fig. 1 c).

biogenesis in nonneural and neural systems with the results of the present study.

(a) Fucosyl glycoproteins are synthesized in the cell body and carried by fast transport into the axon in association with smooth endoplasmic reticulum (1, 4, 6, 9–12, 30).

(b) At early times after delivery, there is a preferential accumulation of newly synthesized glycoproteins in the growth cones where they may undergo insertion and “recycling,” with a substantial fraction returning back toward the cell body by retrograde transport. Such recycling has been suggested to serve “sampling” of the extracellular milieu by the sprout terminal (7).

(c) In addition to providing a flux of membrane for subsequent retrograde transport, rapidly transported glycoproteins are added to axolemma. In the growth cone, new axolemma presumably subserves longitudinal outgrowth. Elsewhere along the axon, the laterally inserted glycoproteins may contribute to

the lateral growth or enlargement of the axolemma. Calculations indicate that the total area of axolemma added must be considerably greater during lateral growth (e.g., from 1 to 10 μm) than during longitudinal outgrowth of a 1- μm sprout terminal. In addition, the added glycoproteins could contribute to an increase in particle density within the sprout. Freeze-fracture studies (34) have suggested a low particle density within growth cone axolemma and intraaxonal vesicles, with more proximal regions of the sprouts showing a progressive increase in particle density toward that of mature axolemma. Finally, a speculative possibility might include a role for inserted glycoproteins in “signaling” Schwann cells by axon–Schwann cell contact. There is evidence that axonal membrane-associated elements can influence Schwann cell division (22, 37) and they possibly could be involved in signaling the initiation of myelination.

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