

# Changes in Axonally Transported Proteins during Axon Regeneration in Toad Retinal Ganglion Cells

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**ABSTRACT** In an effort to understand the regulation of the transition of a mature neuron to the growth, or regenerating, state we have analyzed the composition of the axonally transported proteins in the retinal ganglion cells of the toad *Bufo marinus* after inducing axon regeneration by crushing the optic nerve. At increasing intervals after axotomy, we labeled the retinal ganglion cells with [<sup>35</sup>S]methionine and subsequently analyzed the labeled transported polypeptides in the crushed optic nerve by means of one- and two-dimensional electrophoretic techniques. The most significant conclusion from these experiments is that, while the transition from the mature to the regenerating state does not require a gross qualitative alteration in the composition of axonally transported proteins, the relative labeling of a small subset of rapidly transported proteins is altered dramatically (changes of more than 20-fold) and reproducibly (more than 30 animals) by axotomy. One of these growth-associated proteins (GAPs) was soluble in an aqueous buffer, while three were associated with a crude membrane fraction. The labeling of all three of the membrane-associated GAPs increased during the first 8 d after axotomy, and they continued to be labeled for at least 4 wk. The modulation of these proteins after axotomy is consistent with the possibility that they are involved in growth-specific functions and that the altered expression of a small number of genes is a crucial regulatory event in the transition of a mature neuron to a growth state.

In addition to these selective changes in rapidly transported proteins, we observed the following more general metabolic correlates of the regeneration process: The total radioactive label associated with the most rapidly transported proteins (groups I and II) increased three to fourfold during the first 8 d after the nerve was crushed, while the total label associated with more slowly moving proteins (group IV) increased about 10-fold during this same period. Among these more slowly transported polypeptides, five were observed whose labeling increased much more than the average. Three of these five polypeptides resemble actin and  $\alpha$ - and  $\beta$ -tubulin in their electrophoretic properties.

In the course of development of the nervous system, neurons extend axonal processes that form transient synapses, sprout collateral branches, and eventually form stable synapses. Some mature neurons (e.g., those of fishes and amphibia, and those of mammalian peripheral nervous systems) are able to reinitiate axon extension in response to axonal injury; in these neurons, axon regeneration can lead to functional recovery of synaptic connections. All of these events, which are critical in the formation of precise synaptic patterns during development and in recovery from axonal injury, involve transitions between the growing and mature states of axons. The molecular bases of

these transitions are thus of considerable importance but are currently unknown.

Two quite different mechanisms for the regulation of these transitions have been considered. According to the first, axon extension is regulated locally in the axon and involves only macromolecules that are functional components of the mature axon. For example, recent reports have emphasized the potential importance of the local control of an axonal protease in the regulation of axon extension (12). Alternatively, axon extension could require the products of genes that are expressed minimally, or not at all, in neurons with mature axons. To

distinguish between these two mechanisms, we have assessed the involvement of altered gene expression in axonal growth by comparing the polypeptides axonally transported by mature and regenerating retinal ganglion cells in adult toads (*Bufo marinus*). Because the axonally transported proteins are destined for the axon and terminals, it seemed likely that any proteins mediating axonal extension would be included among them. We have recently reported that at least five distinct groups of polypeptide are transported at different velocities down the axons of toad retinal ganglion cells (20). Here, we examine the consequences of injury and subsequent regeneration on the polypeptide composition of the four most rapidly transported groups. These experiments reveal four growth-associated polypeptides (GAPs) whose specific induction after axonal injury supports the idea that regenerative axon growth is regulated at the level of gene expression, i.e., the synthesis and transport of proteins.

## MATERIALS AND METHODS

### Optic Nerve Crushes

South American toads (*Bufo marinus*, obtained from Mogul-Ed, Oshkosh, WI.) were anesthetized by chilling them in ice; their left optic nerves were exposed by drilling through the skull, cutting a flap in the arachnoid, and gently displacing the forebrain. The optic nerves were crushed with jeweler's forceps at a point 1–3 mm from the optic chiasm; care was taken to minimize vascular damage. The arachnoid flap was sewn into place, Gelfoam (Upjohn Co., Agricultural Prods. MKT, Kalamazoo, Mich.) was placed in the wound, and the skull was sealed with dental acrylic. The animals were maintained at 30°C for 1 day to 16 wk after the operation.

### Labeling of Transported Proteins

To label axonally transported proteins, we immobilized toads by chilling them briefly in ice and injected [<sup>35</sup>S]methionine (usually 0.3 mCi, at 400 Ci/mmol, obtained from Amersham Corp., Arlington Heights, Ill., or New England Nuclear, Boston, Mass.) into the posterior eye chamber. The toads were warmed rapidly in water (10 min at 35°–40°C) and then maintained at 30°C for sufficient times to allow the transport of group I (4 h), groups I and II (12 h), or groups I–IV (8 d), into the cranial portion of the optic nerves. The animals were then chilled in ice or pithed and the optic nerves removed and frozen at –70°C.

### Electrophoretic Systems

We employed four analytical systems to analyze transported proteins in regenerating neurons.

**SYSTEM A:** One-dimensional SDS polyacrylamide gel electrophoresis (PAGE) on gradient (4–12% acrylamide) gels using the buffer system of Laemmli (10) as described elsewhere (20).

**SYSTEM B:** One-dimensional SDS PAGE on gels containing 12% acrylamide, 0.4% bis-acrylamide, 8 M urea (added to solutions immediately before use), and the buffers of system A.

**SYSTEM C:** Two-dimensional electrophoresis with isoelectric focusing (IEF) in the first dimension as described by O'Farrell (18). For IEF, gels (length 11.5 cm, diameter 1.4 mm) containing 4% acrylamide, 0.16% bis-acrylamide, 9 M urea, and 6% carrier ampholytes (3% pH 3.5–10, 3% pH 5–7 from LKB Instruments, Inc., Rockville, Md.) were prefocused for 1 h at 200 V. The electrolyte solutions were 10 mM phosphoric acid (lower, anode) and 20 mM NaOH (upper, cathode). The samples were loaded and electrophoresis was carried out at 450 V for 20–24 h. At the end of the run, the first-dimension gel was extruded and soaked for 20 min in interdimensional transfer buffer (2% SDS,  $2 \times 10^{-3}$  M dithiothreitol, 0.1 M Tris, pH 7.4, 8 M urea, and bromophenol blue tracking dye). The first-dimension gel was then laid over the stacking gel of SDS-urea PAGE gels of system A or B. The first-dimension gels were held in place by agarose (0.4%) in the buffer of the upper reservoir. For determination of the pH gradient established during the first-dimension run, a gel without protein sample was included in the run. This blank gel was cut into 1-cm segments, and each segment was soaked overnight in 0.2 ml of distilled water. The pH of each sample was then determined with a standard pH electrode.

**SYSTEM D:** Two-dimensional gel electrophoresis using nonequilibrium pH gradient electrophoresis in the first dimension as described by O'Farrell et al. (19). The first-dimension gels were identical to those of system C, except that the

only carrier ampholytes were pH 3.5–10 at a concentration of 6%. The electrolyte solutions were the same as in system C, except that the anodic reservoir (phosphoric acid) was on top and the cathodic reservoir (NaOH) was on the bottom. The samples were overlaid with 10–15  $\mu$ l of solubilizing buffer II (10% nonidet P-40,  $2 \times 10^{-3}$  M dithiothreitol, 4 M urea) and electrophoresed without prefocusing for 1,600–2,000 volt-hours, typically 4 h at 450 V. The subsequent treatment of the first gels was the same as for system C.

The samples for systems A and B were prepared as described elsewhere (20). In the case of systems C and D, the samples were dissolved in solubilizing buffer I (0.5% SDS,  $2 \times 10^{-3}$  M dithiothreitol) and heated for 3–5 min at 90°–100°C. An equal volume of solubilizing buffer II (10% nonidet P-40,  $2 \times 10^{-3}$  M dithiothreitol, 4 M urea) was added and then urea, to a final concentration of 8 M (0.058 g solid urea/100  $\mu$ l). The gels were stained, impregnated with diphenyloxazole (3), dried, and put in contact with x-ray film that had been pre-exposed to an optical density of 0.1 (13). The intensity of the exposed bands or spots on the resulting fluorographs were quantitated by scanning them with a densitometer (Joyce, Loebel & Co., Gateshead-on-Tyne, England). We estimated the magnitudes of change in the relative labeling of individual polypeptides by comparing the optical density peak heights in samples from normal and regenerating nerves; these estimates agree with more recent estimates obtained by computer-directed densitometric scanning in two dimensions and comparison of peak volumes (J. H. P. Skene and J. A. Freeman, unpublished observations). In some instances, where significantly different amounts of total radioactivity were contained in samples that were being compared, we prepared multiple exposures of the gel, such that the products of the total radioactivity and the exposure time were comparable for each sample. Some of the figures of the next section are composite photographs of such multiple exposures; in these cases the exposure times are indicated in the legends.

## RESULTS

### Time-course of Regeneration and Enhanced Protein Synthesis

The axons of the toad retinal ganglion cells exit from the eye, pass through the optic nerve and contralateral optic tract, and form synaptic terminals in the optic tectum (Fig. 1). Although the retinal ganglion cells account for a minor fraction of the total retinal protein synthesis (7), their axonally transported gene products can be selectively analyzed in the optic nerve and optic tract, as the ganglion cell axons are the only centrally projecting fibers of the retina. To establish the time required for regenerating axons to traverse the optic tract and enter the optic tectum, we crushed the optic nerves of 20 toads at a point just in front of the optic chiasm and injected their eyes with [<sup>35</sup>S]methionine at various times later. In each case, the toad was killed 12 h after the injection (sufficient time for radioactive proteins to have reached the optic tectum in an unoperated toad; reference 20) and determined the amount of radioactivity in the optic tecta. Fig. 2 indicates that regenerating

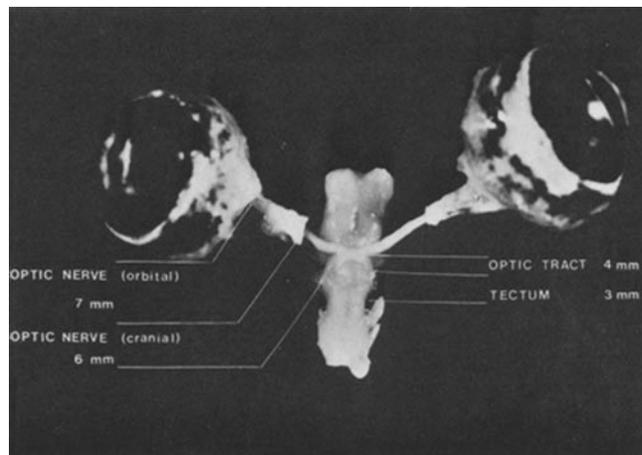


FIGURE 1 Ventral view of *Bufo marinus* brain, showing dimensions of the visual system.

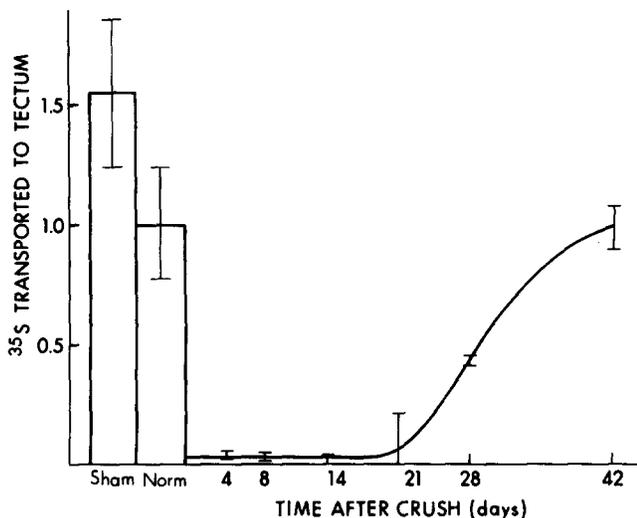


FIGURE 2 Return of regenerating retinal ganglion cell axons to the optic tectum. Toads' left optic nerves were crushed close to the optic chiasm, and after the indicated times at 30°C, [<sup>35</sup>S]methionine was injected into the left eyes; 12 h was allowed for transport of incorporated radioactivity to the optic tectum. TCA-precipitable radioactivity in the left and right tecta was determined as described in Materials and Methods. Blood-borne radioactivity (activity in the left tectum) was subtracted from the total radioactivity in the right tectum to determine the radioactivity transported in retinal ganglion cell axons. The activity in each sample was normalized to the value for unoperated toads (1.0 = 98,750 cpm). Surgical control (Sham) toads were injected 8 d after a sham operation, in which the optic nerve was exposed but not crushed.

axons reached the optic tectum ~3 wk after the nerve was crushed and that the amount of transported protein in the tectum increased for the next 3 wk. Because the optic tectum is ~6 mm from the site of the crush, the most rapidly regenerating axons must have grown with an average velocity of ~0.3 mm/day. If there is a lag of 5–10 d before the onset of axon elongation, as is the case with other regenerating neurons (1, 11, 14, 15), the average velocity during the period of growth would have been 0.4–0.5 mm/d.

The amount of label associated with the rapidly transported proteins increased substantially between 2 and 8 d after axotomy. When axonal transport was allowed to proceed for 12 h after an isotope injection, optic nerves that had been crushed 8 d previously contained  $3.4 \pm 0.34$  (particulate fraction) and  $4.4 \pm 1.6$  (soluble fraction) times more label than uncrushed nerves (6 experiments, 17 animals). The amount of labeled, rapidly transported proteins returned to the level of uncrushed control nerves between 4 and 6 wk after axotomy. The increased labeling of the rapidly transported proteins could not be explained simply by their accumulation at the crush site, since it did not commence until several days after axotomy and was apparent (Fig. 3) when the time allowed for transport (4 h) was insufficient to allow the most rapidly transported proteins to reach the crush (20). The amount of label associated with more slowly transported polypeptides also increased to 11 times the control level by 8 d after axotomy, and returned to control levels between 4 and 6 wk. These labeling increases most likely represent increased synthesis of the transported proteins, as it has been reported that protein synthesis in the retinal ganglion cells of the goldfish increased after the optic nerve was crushed (8, 16).

### Composition of Transported Proteins in Regenerating Neurons

The increase in transport of protein-associated label after the nerve was crushed might represent either an enhancement of the synthesis and transport of normally transported proteins or the induction of a completely new set of proteins associated with the regeneration process. When we analyzed the composition of rapidly transported (groups I and II) (20) proteins in toad optic nerves by means of one- and two-dimensional gel electrophoresis (systems A and C described in Materials and Methods), it was apparent that most of the transported polypeptides were identical to those transported in a normal optic nerve (Fig. 4). This extensive similarity indicates that the transition of a neuron from the mature state to the axon-extension state does not involve a global change in the composition of rapid axonal transport. On the other hand, the labeling of a small subset of the rapidly transported polypeptides was specifically increased after axotomy. For convenient reference we have designated these as GAPs, followed by their

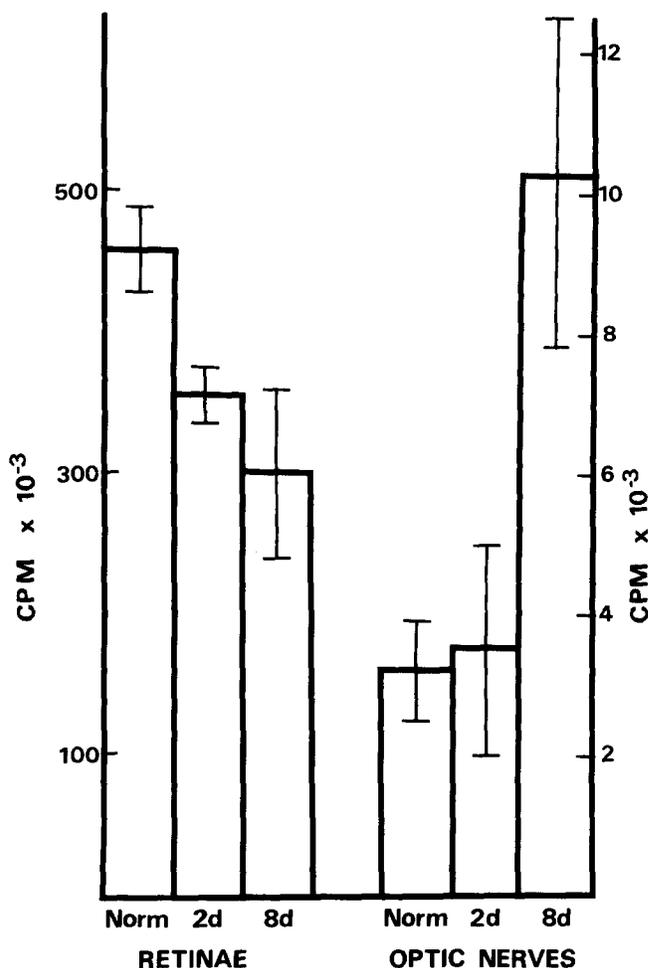


FIGURE 3 Increased labeling of group I axonally transported proteins in toad retinal ganglion cells after axotomy. [<sup>35</sup>S]methionine was injected into the posterior eye chambers of normal (unoperated) toads or toads whose optic nerves had been crushed 2 or 8 d before injection. 4 h after injection, the toads were killed and their retinae and optic nerves were removed. The TCA-precipitable radioactivity in the whole retinas and optic nerves is shown here. TCA-precipitable radioactivity was determined as in reference 20, Materials and Methods.

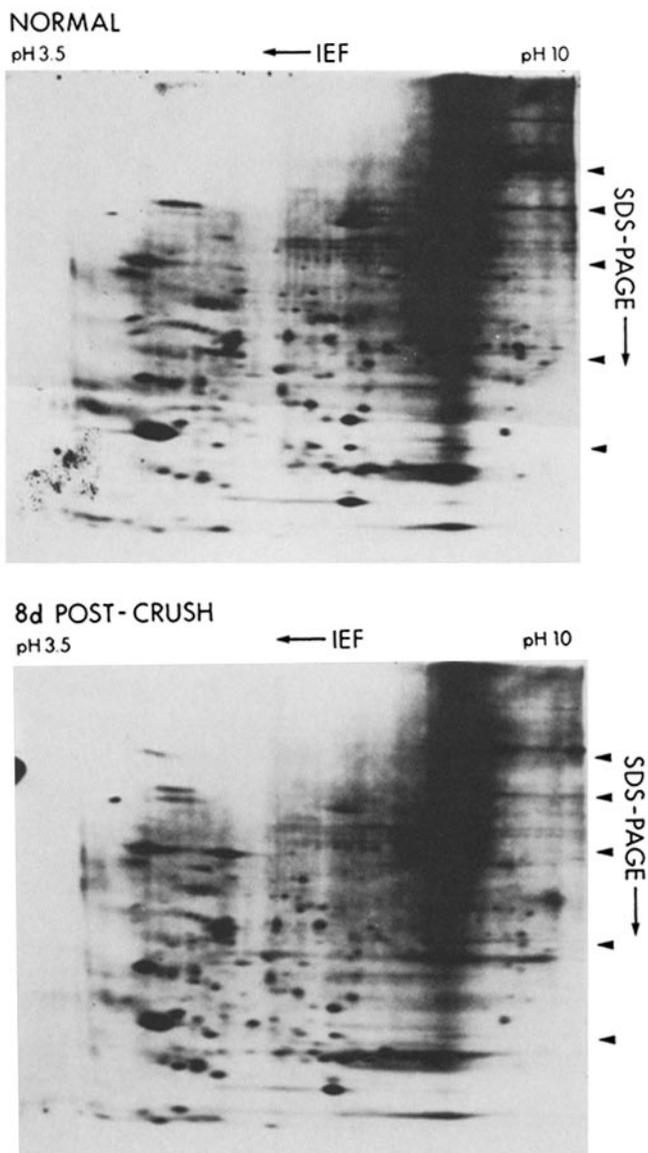


FIGURE 4 Lack of gross changes in rapidly transported proteins during regeneration of toad optic nerves (system C analysis). [<sup>35</sup>S]methionine was injected into the posterior eye chamber of a normal (unoperated) toad or a toad whose optic nerve had been crushed 8 d earlier. 12 h after injection, the optic nerves were removed, and a particulate fraction was prepared for electrophoresis and fluorography (system C, see Materials and Methods). The first dimension is IEF (pH 3.5–10); the origin is at right. The second dimension is SDS PAGE (4–12% polyacrylamide, 4–8 M urea; system A). This is a prolonged fluorographic exposure to reveal minor components. The arrowheads at right indicate molecular weights of 200,000; 150,000; 100,000; 50,000; and 25,000.

apparent molecular weight in daltons; these are described individually below.

### GAP-24

2 d after the optic nerve was crushed, we detected (by means of electrophoretic system B) the appearance of a 24,000-dalton polypeptide in the particulate fraction of the rapidly transported proteins (Fig. 5). GAP-24-associated label reached maximal levels by 8 d and remained labeled in the optic nerve for at least 5 wk after the nerve was crushed, then declined to the

background level by 10 wk (Fig. 6). The relative labeling of GAP-24 (GAP-24 label/total group I transported label) was at least 28 times the control (uncrushed) level 8 d after the crush, as determined by densitometry of the spots corresponding to GAP-24 on two-dimensional gels (system D [Fig. 6]). Because the total incorporation of [<sup>35</sup>S]methionine into group I polypeptides increases three- to fourfold when the nerve is crushed (Fig. 3), the absolute labeling of GAP-24 in regenerating nerves is more than 80 times the level in uncrushed nerves.

The resolution of GAP-24 depended strongly on the electrophoretic conditions. Although it was poorly resolved by gradient SDS polyacrylamide gels (system A), GAP-24 was reproducibly detected on the 12% SDS polyacrylamide gels containing 8 M urea with a pH of 9.15 (system B); using this system, we detected GAP-24 in more than 30 comparisons of regenerating and normal nerves. Its mobility was affected both by the urea (it was not resolved without urea) and by the pH (its mobility increased with decreasing pH in such a manner that it was not resolved on gels with a pH of <9.1). The resolution of GAP-24 by two-dimensional electrophoresis was dependent upon these same factors; we detected it when either IEF (systems C or D) as the first dimension was combined with system B as the second dimension (Fig. 5) but not reproducibly when

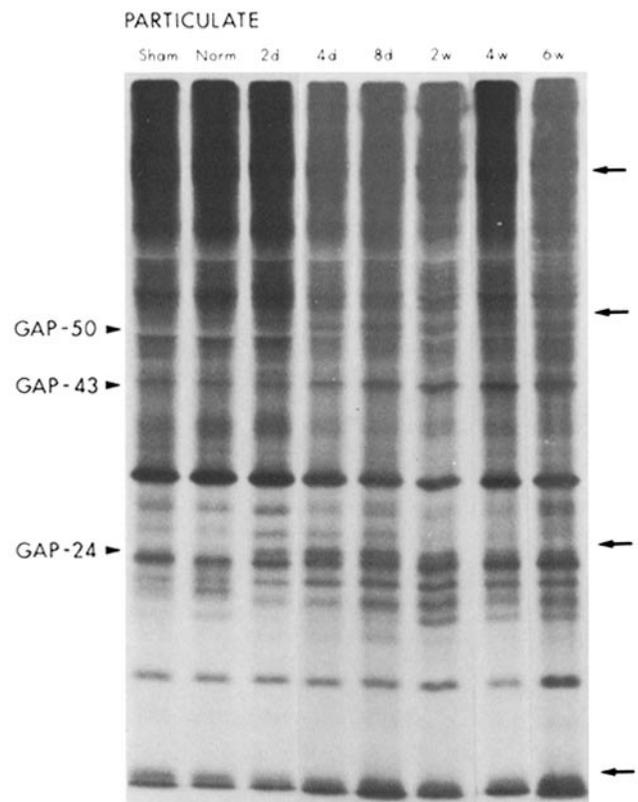


FIGURE 5 Time-course of induction of individual rapidly transported polypeptides during optic nerve regeneration in toads (system B analysis). At the indicated times after optic nerve crush, the rapidly transported proteins (groups I and II) were labeled with [<sup>35</sup>S]methionine. Particulate fractions of optic nerves were analyzed on SDS PAGE gels containing 12% polyacrylamide and 8 M urea, pH 9.1; system B). This is a composite fluorograph. The sham-operated, normal, and 4-wk samples are from a 12-d exposure; the remaining samples are from a 2-d exposure. Arrows at right indicate molecular weights of 100,000; 50,000; 25,000; and 15,000.

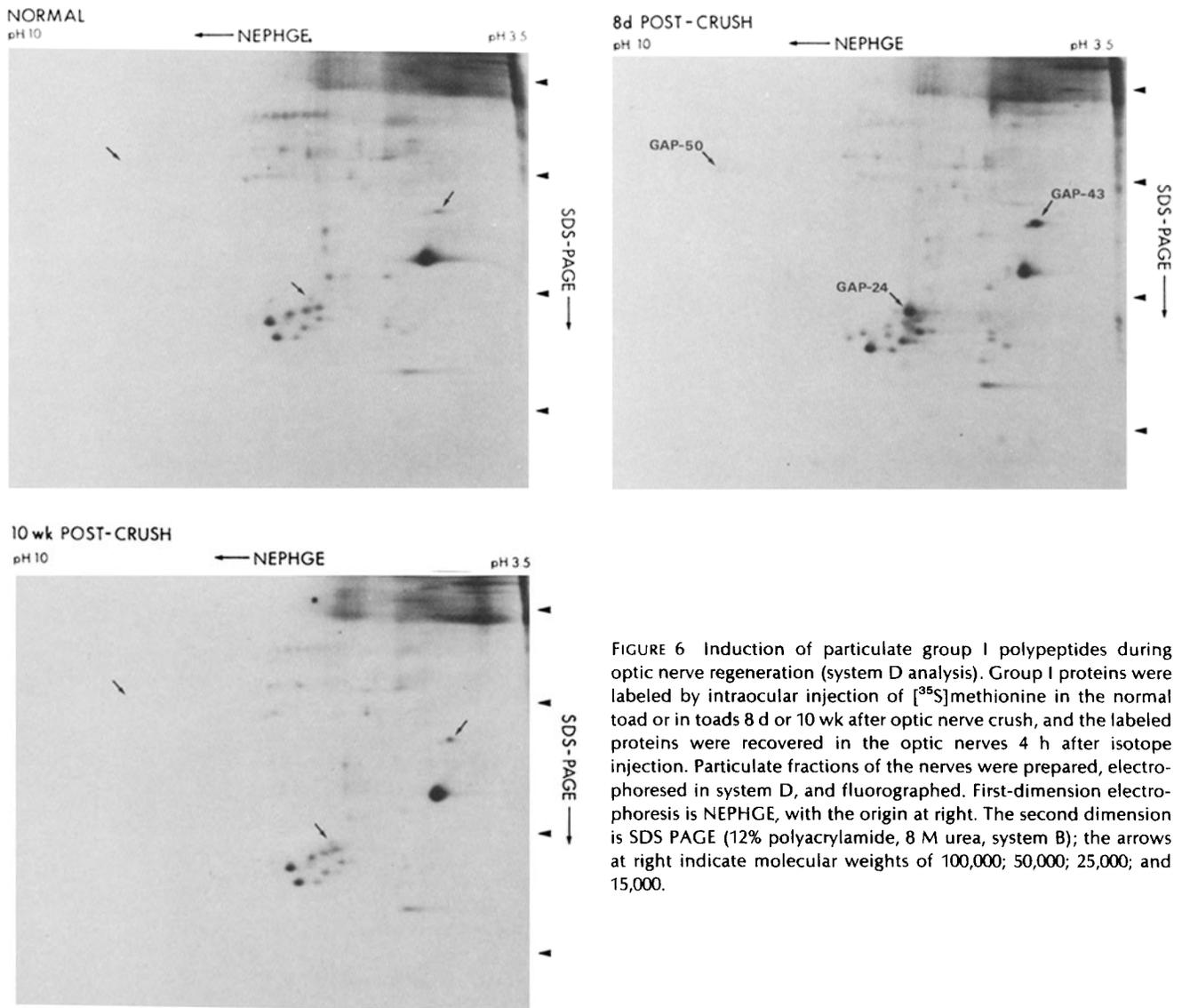


FIGURE 6 Induction of particulate group I polypeptides during optic nerve regeneration (system D analysis). Group I proteins were labeled by intraocular injection of [ $^{35}$ S]methionine in the normal toad or in toads 8 d or 10 wk after optic nerve crush, and the labeled proteins were recovered in the optic nerves 4 h after isotope injection. Particulate fractions of the nerves were prepared, electrophoresed in system D, and fluorographed. First-dimension electrophoresis is NEPHGE, with the origin at right. The second dimension is SDS PAGE (12% polyacrylamide, 8 M urea, system B); the arrows at right indicate molecular weights of 100,000; 50,000; 25,000; and 15,000.

system A was the second dimension. GAP-24 has an isoelectric point (pI) of 6.7.

#### GAP-50

A second polypeptide associated with the particulate fraction of the group I transported proteins became labeled 4 d after the optic nerve was crushed. This polypeptide was reproducibly resolved only by means of two-dimensional analysis using NEPHGE (system D) in the first dimension (Fig. 6). Its migration in this system indicated that it has a molecular weight of 50,000 and is extremely basic with a pI of >8.9. GAP-50 was resolved into two polypeptides of indistinguishable molecular weight and similar charge when the NEPHGE in the first dimension was limited to <1,600 volt-hours. As was the case with GAP-24, the labeling of GAP-50 reached maximum levels (its relative labeling was 15 times the highest level in control animals) by 8 d after axotomy.

#### GAP-43

The relative labeling of a third polypeptide (GAP-43) associated with the particulate fraction of group I began to increase between 4 and 8 d after axotomy, reached a maximum level

between 2 and 4 wk, and retained this level until 5 wk (Fig. 7A). By 10 wk, the relative labeling of this polypeptide had decreased to control levels (Fig. 6). GAP-43 was resolved by two-dimensional analysis with either IEF (system C) or NEPHGE (system D) in the first dimension and either system A or B in the second dimension. Its electrophoretic properties indicated that it has a molecular weight of 43,000 and a pI of 4.3. Although GAP-43 was readily detected as a minor component of group I in control animals, its relative labeling increased eightfold by 8 d, and 15- to 20-fold by 4 wk after axotomy.

#### Changes in the Soluble Fraction

The relative intensity of labeling of several polypeptides associated primarily with the soluble fraction of groups I and II changed after the optic nerve was crushed. These soluble polypeptides fell into two classes: those whose labeling decreased after the nerve was crushed, and those whose labeling increased. The former class included a polypeptide (mol wt = 190,000) whose relative labeling decreased within 1 d after the nerve was crushed, as well as two polypeptides (mol wt = 68,000 and 115,000) whose labeling did not decrease until 3-4

d after axotomy (Fig. 7B). Only one polypeptide (mol wt = 33,000) in the soluble fraction showed a reproducible selective increase in its labeling in response to the crush (Fig. 7B). The

increased labeling of GAP-33 was apparent by 2 d and persisted for at least 16 wk. Because these polypeptides were not found in optic nerves when only 4 h was allowed for transport of labeled proteins, they appear to be transported in group II (20).

### Changes in Slowly Transported Proteins during Regeneration

In mature toad retinal ganglion cells, the third and fourth groups of axonally transported proteins reach the optic nerve by 8 d after intraocular labeling. Figs. 8 and 9 show one- and two-dimensional (systems A and C) electrophoretic analysis of polypeptides labeled by intraocular injection at various times after axotomy and recovered in the optic nerve 8 d after the injection. At all times after axotomy, the electrophoretic profile of the particulate fractions are similar to those of unoperated or sham-operated controls; the relative labeling of only two polypeptides (molecular weights 210,000 and 185,000) decreased 4–8 d after axotomy (Fig. 7A). These increased again 4–6 wk postcrush, although they were still below control levels.

The labeling of several slowly transported polypeptides in the soluble fraction increased after axotomy: Two basic polypeptides with molecular weights of ~42,000 (Figs. 8 and 9) increased 15-fold in labeling intensity (relative to the total protein-associated label) by 4 d after axotomy; they maintained this increased level throughout the period of axon elongation, returning to control levels 4–6 wk after axotomy. A comparable time-course of increased labeling was displayed by a 43,000 mol wt polypeptide that was resolved only by two-dimensional electrophoresis (Fig. 9). Finally, several polypeptides that produced a 50,000–55,000 mol wt smear on one-dimensional gels and complex spots of 51,000 and 55,000 mol wt on two-dimensional gels increased in relative labeling at least 10-fold 4–8 d after axotomy and returned to control levels in the period between 2 and 4 wk. These polypeptides were easily detected in control animals (Fig. 9). All of these slowly transported crush-sensitive polypeptides co-electrophoresed with major Coomassie-stained polypeptides, suggesting that they are major structural elements of the axon. It is consistent with this interpretation that certain of these polypeptides had molecular weights (51,000–55,000 and 44,000) and isoelectric points (~4.9 and 5.1, respectively) corresponding to those of tubulin and actin (4, 17), which are major structural components of the neuron; however, we did not unequivocally identify these polypeptides. Because these slowly transported polypeptides appear to be major components of mature axons as well as regenerating axons, we have reserved the designation "GAP" for the rapidly transported proteins described previously, because they seem more likely to be involved in specifically growth-related processes.

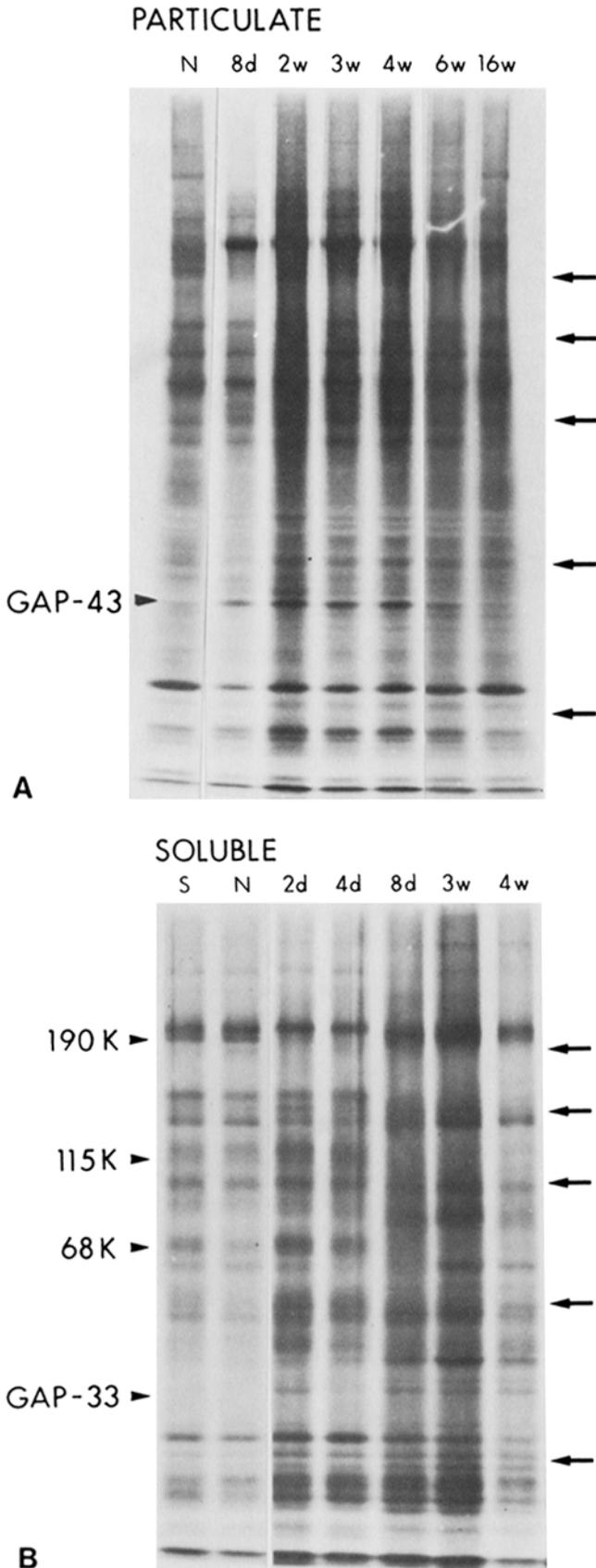
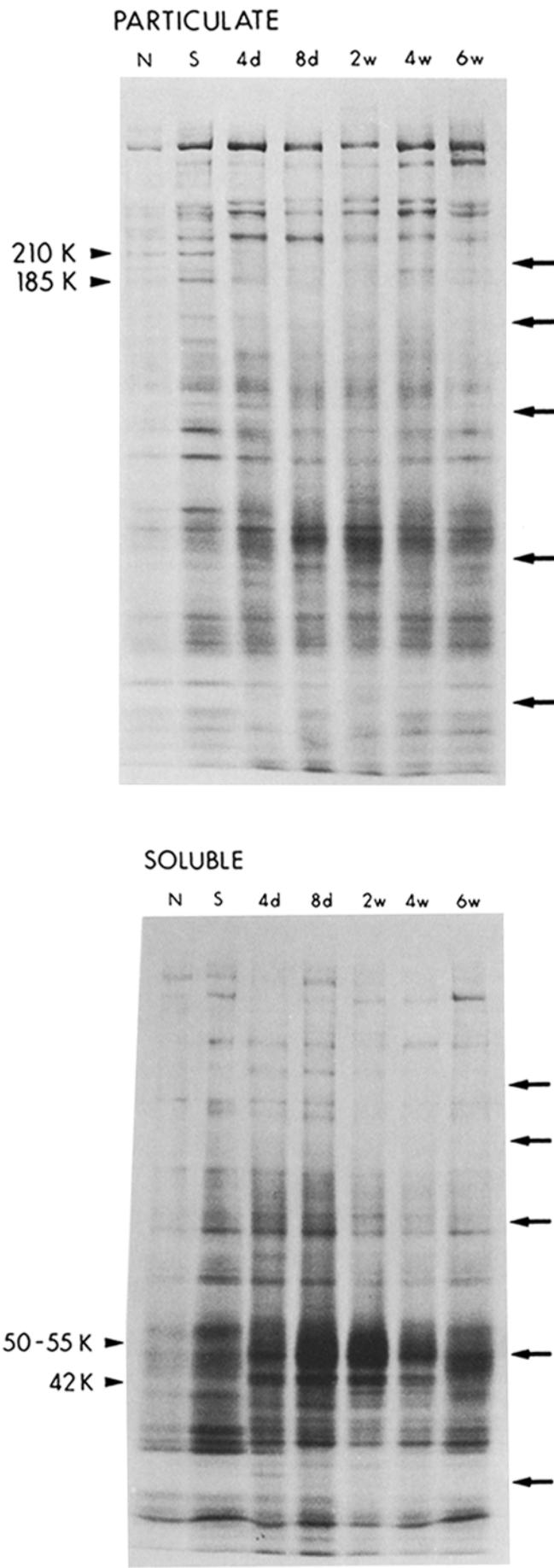


FIGURE 7 Changes in rapidly transported proteins during toad optic nerve regeneration (system A analysis). At the indicated times after optic nerve crush, rapidly transported proteins in toad optic nerves were labeled with [<sup>35</sup>S]methionine. Fractions of the optic nerves were analyzed by one-dimensional electrophoresis on gradient gels (4–12% polyacrylamide, 4–8 M urea; system A) and the gels were fluorographed. In A, the normal sample (N, unoperated toad) was from a 5-d fluorographic exposure; the remaining samples were from a 12-h exposure. In B, the sham-operated (S) and normal (N) samples were exposed for 45 d, and the remaining samples were exposed for 17d. The arrows at right indicate molecular weights of 200,000; 150,000; 100,000; 50,000; and 25,000.



### Control Experiments

The selective enhancement of labeling of a few axonally transported polypeptides (the GAPs) after axotomy suggests the possibility that their synthesis is selectively induced by the injury. The following experiments argue against less interesting alternatives. First, to rule out the possibility that the GAPs were generated artifactually during the preparation for electrophoresis by the action of an injury-related protease, we co-homogenized normal optic nerves containing labeled groups I and II with unlabeled regenerating nerves (8 d post axotomy); this procedure failed to generate GAPs-24 or -43. Furthermore, these GAPs were not generated when labeled group I polypeptides were prepared in the absence of protease inhibitors, nor was the enhancement of GAP labeling in regenerating nerves decreased when the nerves were homogenized directly in 2% SDS and immediately heated to 100°C to destroy proteases. GAP-50 was not resolved on the one-dimensional gels used in these experiments.

Second, a comparison of the GAPs with polypeptides locally synthesized in the optic nerve (e.g., by glia, mesodermal, and meningeal elements) shows that the locally synthesized polypeptides do not include GAPs-24, -43, or -50 (Figs. 10 and 11); therefore, the GAPs must be neuronal polypeptides that are synthesized in the cell bodies of the retinal ganglion cells.

Third, the enhancement of GAP labeling in axotomized nerves is not the result of the selective accumulation of normally transported polypeptides at the crush site, because the GAPs were augmented in crushed optic nerve (8 d after axotomy) when insufficient time (4 h) was allowed for the transported proteins to have reached the site of the crush (20) (Fig. 6). This result also argues against the possibility that the GAPs might be generated by the modification of normally transported proteins when they arrive at the site of the crush.

Fourth, the enhanced labeling of the GAPs cannot be explained by a crush-induced increase in transport velocity of proteins that are normally transported slowly; when the GAPs were compared to normal slowly transported proteins by means of one- (Fig. 12) and two-dimensional electrophoresis, no slowly transported polypeptide corresponded to GAP-24 (Fig. 12) or GAP-43. GAP-50 also appeared to differ slightly from slowly transported polypeptides of similar molecular weights (Fig. 12); we did not compare GAP-50 to these polypeptides in the relevant two-dimensional electrophoretic system (D).

Finally, the axotomy-induced increase in GAP-24 labeling appears to be initiated in the ganglion cell bodies rather than in the axon; the kinetics of labeling GAP-24 (it appears maximally labeled by 4 h after the retina is labeled) are more rapid than would be expected if it were produced by a modification of some other group I polypeptide (many of which don't become maximally labeled until 6-12 h) in the axon. Further-

FIGURE 8 Changes in slowly transported polypeptides (group IV) during toad optic nerve regeneration (system A analysis). At the indicated times after optic nerve crush, [<sup>35</sup>S]methionine was injected into the posterior eye chambers; labeled group IV axonally transported proteins were recovered in the optic nerves 8 d after isotope injection. The nerves were separated into particulate and soluble fractions and prepared for electrophoresis as described in Materials and Methods. Equal amounts of total radioactivity from each sample were analyzed by electrophoresis/autoradiography (4-12% polyacrylamide, 0-30% glycerol, system A). N, unoperated control; sham-operated 8 d before isotope injection. The arrows at right indicate molecular weights of 200,000; 150,000; 100,000; 50,000; and 25,000.

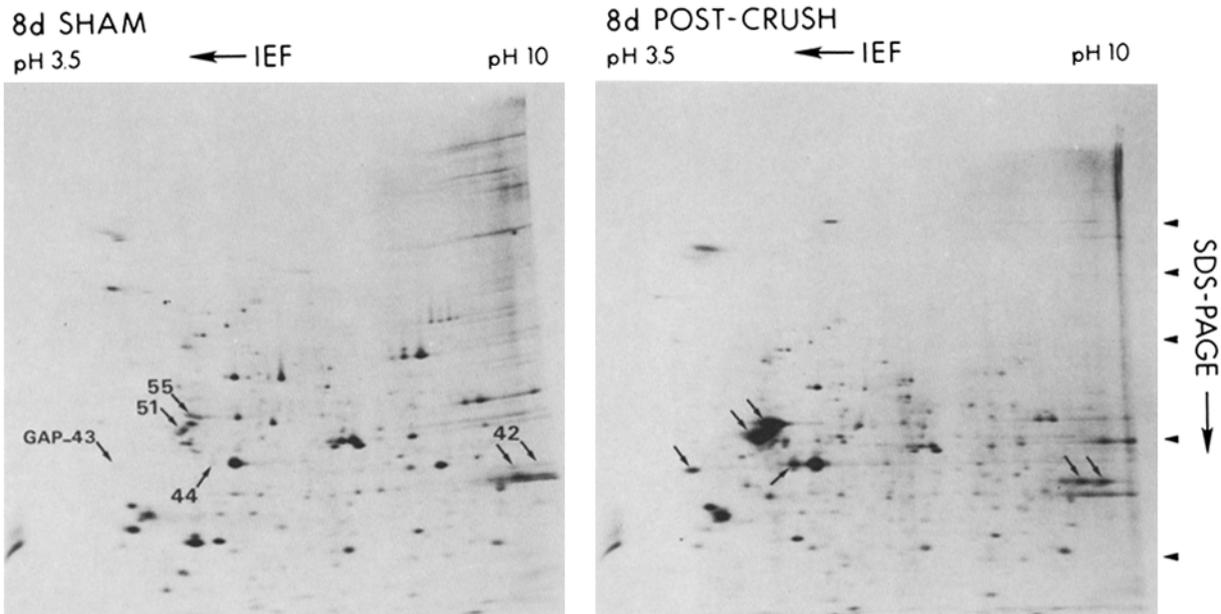


FIGURE 9 Changes in slowly transported polypeptides (group IV) during toad optic nerve regeneration (system C analysis). Toads were subjected either to optic nerve crush or to a sham operation; 8 d later, [<sup>35</sup>S]methionine was injected intraocularly; after an additional 8 d, labeled group IV axonally transported proteins were recovered in the optic nerves. The optic nerves were homogenized in H buffer (see Materials and Methods), and the labeled proteins were precipitated with TCA and washed with ether. The TCA-precipitated samples were analyzed by two-dimensional electrophoresis (system C) and autoradiography; the first dimension is IEF (pH 3.5–10), with the origin at right; the second dimension is SDS PAGE (4–12% polyacrylamide, 0–30% glycerol, system A). The arrowheads at right indicate molecular weights of 200,000; 150,000; 100,000; 50,000; and 25,000.

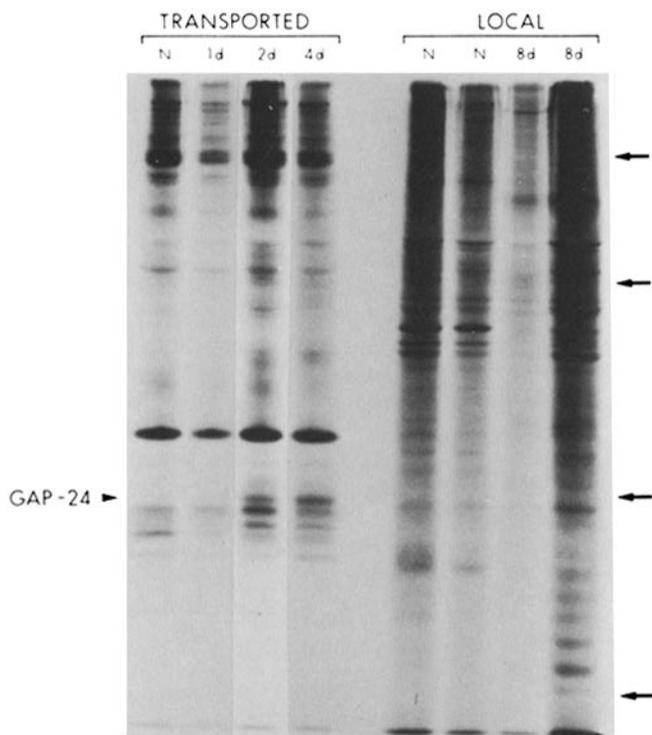


FIGURE 10 Comparison of group I axonally transported polypeptides and locally synthesized polypeptides in normal (N) and regenerating toad optic nerves. At the indicated times after optic nerve crush, proteins were labeled with [<sup>35</sup>S]methionine by intraocular isotope injection (0.5 mCi) and group I polypeptides were recovered in optic nerves 4 h later; proteins synthesized locally in the optic nerves were labeled by direct application of [<sup>35</sup>S]methionine (0.5 mCi) to the nerves. Particulate fractions were prepared and analyzed by one-dimensional SDS PAGE/fluorography (system B). This is a

more, two-dimensional electrophoresis (system D) of retinal polypeptides has indicated that GAP-24 labeling is increased in the retinas of toads whose optic nerves have been crushed, although the level of labeling is near the limits of detection. Thus, the axotomy-induced enhancement of at least GAP-24 appears to occur in the cell body, most likely by increased synthesis, or alternatively by post-translational modification of a pre-existing polypeptide.

#### DISCUSSION

We undertook these experiments to evaluate the question of whether axon growth requires the introduction into the axon of gene products normally present in negligible amounts. The changes we have observed in the labeling of axonally transported proteins after axotomy of toad retinal ganglion cells are summarized in Fig. 13. They fall into three general classes: First, most proteins that are normally transported are labeled to a greater degree after axotomy. Second, a few proteins that are normally transported are less intensely labeled after axotomy. Third, and most relevant to the question of gene regulation in axon growth, a few proteins that are normally labeled only minimally are increased in their labeling intensity far more than the average. The possibility that these changes reflect metabolic alterations directly related to (and perhaps prerequisite for) axon regeneration is suggested by the temporal correlation between these changes and events in the regeneration process. In particular, the period (3–8 d) required to establish a “regenerating composition” of transported proteins

composite fluorograph; all lanes are from a 14-d fluorographic exposure, except the 2-d postaxotomy lane, which is from a 4-d exposure. Arrows indicate molecular weights of 100,000; 50,000; 25,000; and 15,000.

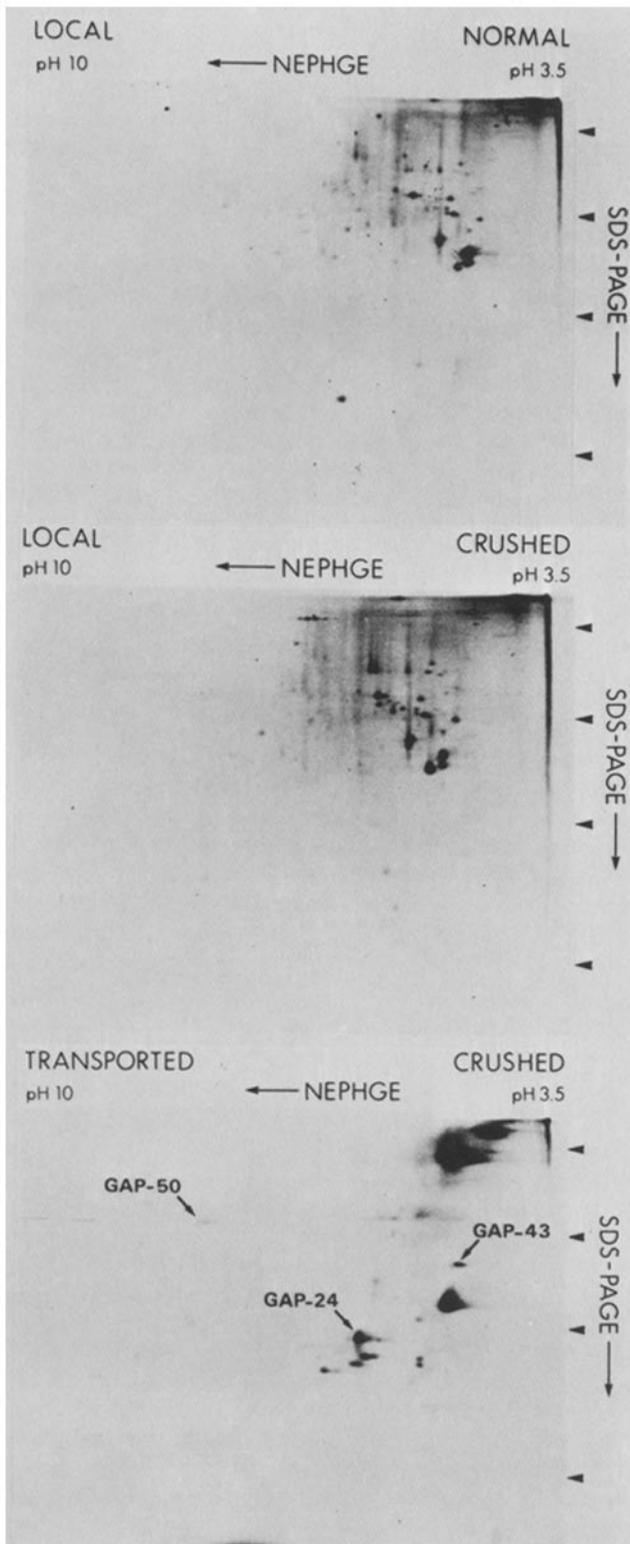


FIGURE 11 Two-dimensional electrophoretic analysis (system D) of locally synthesized proteins in normal and regenerating (crushed) toad optic nerves. Proteins were labeled by direct application of [<sup>35</sup>S]methionine to normal optic nerves or to nerves crushed 8 d before. Particulate fractions were prepared, electrophoresed (system D), and fluorographed as described in Materials and Methods. First-dimension electrophoresis was on NEPHGE, with the origin at right; the second dimension was SDS PAGE (system B). A comparable gel of [<sup>35</sup>S]methionine-labeled group I axonally transported proteins from a regenerating optic nerve is included for comparison. Arrowheads at right indicate molecular weights of 100,000; 50,000; 25,000; and 15,000.

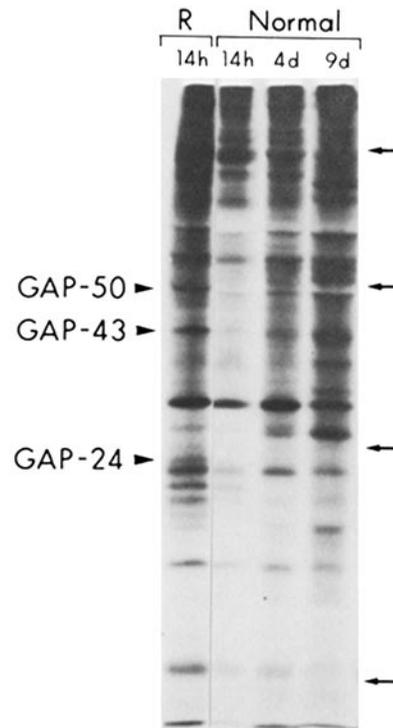


FIGURE 12 Comparison of GAPs in regenerating toad optic nerves with slowly transported polypeptides in normal nerves. [<sup>35</sup>S]methionine was injected into the posterior eye chambers of normal toads and a toad whose optic nerve was crushed 8 d previously (R). At the indicated times after isotope injection, the optic nerves were removed, particulate fractions were prepared, and the labeled proteins were analyzed by electrophoresis/fluorography (system B). This is a composite fluorograph; the regenerating nerve sample (R) is taken from a 7-h exposure; the remaining samples are from a 48-h exposure. The arrows at right indicate molecular weights of 100,000; 50,000; 25,000; and 15,000.

corresponds to the “lag phase” (estimated as 8–10 d in *Bufo* [14] and 2–7 d in other systems [1, 9, 8]) between axotomy and the beginning of axon growth. The lag phase appears to represent the time required to accomplish certain rate-limiting steps in the initiation of axon growth, because it is reduced in growing (1, 2) and previously axotomized axons (1, 15). The establishment of the “regenerating pattern” of transported proteins could thus be the basis of the lag phase. Similarly, the maintenance of the regenerating pattern of axonally transported proteins through the period of axonal elongation and its reversion to the normal pattern at about the time that new synaptic connections are established suggest that the altered metabolism serves to provide specialized requirements of the growing axon. We consider here the nature of requirements that each class of changes may supply and the implication of the third class of changes for the mechanism of control of axon growth.

The first class of changes appears to represent increases in transport of normal components of the axon and can most readily be interpreted as providing the structural materials necessary for the elaboration of the new axon. The majority of both rapidly transported proteins (which includes material destined for the plasma membrane) and slowly transported proteins (which includes cytoskeletal elements [e.g., tubulin and actin]) fall into this class. The second class of changes is represented by three rapidly transported polypeptides whose relative labeling decreases during regeneration. These polypeptides are candidates for functions that are necessary only when

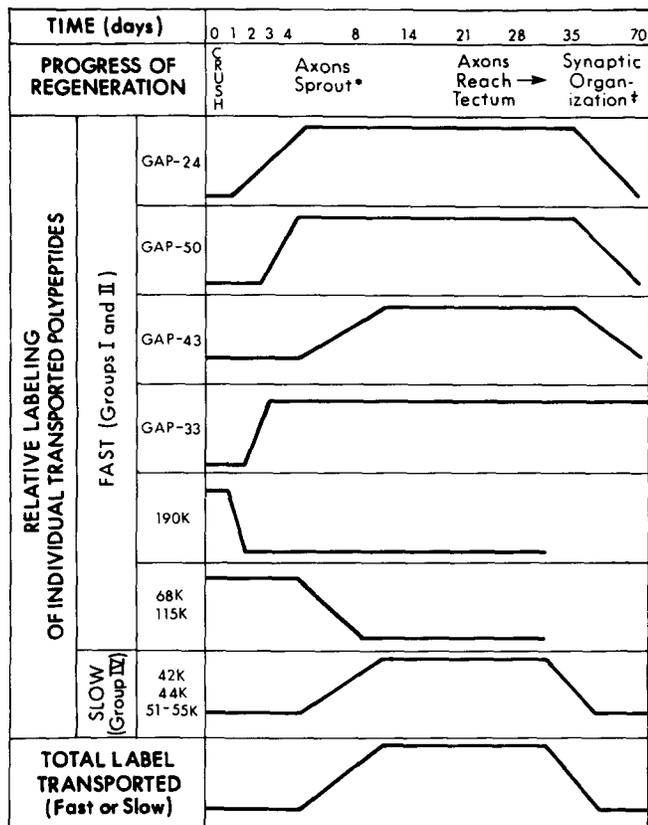


FIGURE 13 Time-course of changes in axonally transported proteins during regeneration of toad optic nerves. In each panel, the heavy line indicates the total radioactivity transported (lower panel) or the labeling of a particular polypeptide relative to the total radioactivity in its transport group. The lower line in each panel represents the lowest labeling observed, and the upper line represents the maximal labeling; the difference in the heights of these lines is not proportional to the magnitude of the change in labeling.

\* Initial outgrowth of axons after optic nerve crush is assumed to occur after a lag of 4–8 d, as in *Xenopus* (1) and goldfish (9, 11), and as suggested for *Bufo* (14).

† See references 5 and 6.

retinotectal connections are intact (e.g., synaptic transmission or synaptic maintenance) or for functions that normally prevent the entry of the axon into a growth state (i.e., the negative regulation of growth-specific functions).

The third class of changes, most interesting from a mechanistic point of view, involves rapidly transported polypeptides (the GAPs) whose degree of labeling was near the lower limits of detection in normal nerve and increased 50- to 100-fold after axotomy. Although such behavior might be expected of a structural protein that turned over very slowly in the mature axon, the GAPs are clearly not major components of the axonal structure, because (a) they are not present in sufficient abundance to be stained by Coomassie Brilliant Blue; (b) they, in fact, turn over rapidly with a half-time of hours to days (21); and (c) onset of their increased labeling precedes the onset of increased labeling of proteins presumed to serve structural functions. We propose, therefore, that the GAPs include pro-

teins that mediate functions peculiar to and essential for axon growth; the low level of GAP labeling in normal nerve could represent the depressed basal level in all neurons or, alternatively, fully induced levels in a few neurons involved in some form of axon growth. This proposal, which forms the basis of the "GAP hypothesis," has three immediate consequences for the mechanism of regulation of axon extension. First, it implies that the transition of a neuron from the mature state to the growth state is regulated at the level of gene expression (i.e., the synthesis and transport of proteins from the cell body) and cannot be accomplished solely by local regulation of axonal constituents. Second, the GAP hypothesis requires that signals initiating and terminating the expression of GAP genes are conveyed from the axon terminals to the cell body during the course of regeneration. Third, the GAP hypothesis suggests that the failure of some neurons to regenerate after injury could result from their failure to induce GAPs.

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