

# STUDY OF REGENERATION IN THE GARFISH OLFACTORY NERVE

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## ABSTRACT

Previous studies of the olfactory nerve, mainly in higher vertebrates, have indicated that axonal injury causes total degeneration of the mature neurons, followed by replacement with new neuronal cells arising from undifferentiated mucosal cells. A similar regeneration process was confirmed in the garfish olfactory system. Regeneration of the nerve, crushed 1.5 cm from the cell bodies, is found to produce three distinct populations of regenerating fibers. The first traverses the crush site 1 wk postoperative and progresses along the nerve at a rate of  $5.8 \pm 0.3$  mm/d for the leading fibers of the group. The second group of fibers traverses the crush site after 2 wk postcrush and advances at a rate of  $2.1 \pm 0.1$  mm/d for the leading fibers. The rate of growth of this group of fibers remains constant for 60 d but subsequently falls to  $1.6 \pm 0.2$  for the leading population of fibers. The leading fibers in the third group of regenerating axons traverse the crush site after 4 wk and advance at a constant rate of  $0.8 \pm 0.2$  mm/d. The multiple populations of regenerating fibers with differing rates of growth are discussed in the context of precursor cell maturity at the time of nerve injury and possible conditioning effects of the lesion upon these cells.

Electron microscopy indicates that the number of axons decreases extensively after crush. The first two phases of regenerating axons represent a total of between 6 and 10% of the original axonal population and are typically characterized by small fascicles of axons surrounded by Schwann cells and large amounts of collagenous material. The third phase of fibers represents between 50 and 70% of the original axonal population.

**KEY WORDS** olfactory nerve · crush · degeneration · regeneration · growing phases · garfish

The succession of events taking place in a nerve cell following injury has been followed in detail in numerous preparations during both degeneration and regeneration. (For reviews, see 5, 11, 19, 27, 29, 32–34, 49.)

Most of these studies have been performed in myelinated and unmyelinated mammalian periph-

eral nerves and central nerves of lower vertebrates. These nerves, contrary to those in mammalian central nervous systems, are able to regenerate their outer dendritic and axonal segments. The olfactory neuron presents an apparently unique situation: a constant renewal of the unmyelinated olfactory neurons has been observed even in the intact nerves of adult animals (20). Furthermore, following axotomy both the axon and the cell body of mature cells degenerate and are subsequently replaced by nerve cells arising from un-

differentiated olfactory mucosal tissue (20–22, 40). This system should therefore provide the opportunity to study axonal transport during various stages of neuronal growth and maturation in an adult animal.

In our study, the regenerative properties of the olfactory neuron were investigated in the garfish olfactory nerve by biochemical and anatomical methods. The garfish olfactory nerve is up to 30 cm long, contains 12 million homogeneous C-fibers, and can be dissected from the cell bodies present in the mucosa to the synaptic area in the olfactory bulb. This therefore allows the study of the influence of injury on the various neuronal components as well as of the progression of changes induced by axotomy along the axon. Furthermore, extensive studies of the rate and composition of fast and slow axonal transport in the garfish olfactory nerve have been previously performed (9, 10, 16, 23–25). The rate of regeneration was followed by labeling the growing axons with rapidly transported radioactive proteins, a technique previously employed to demonstrate regeneration in peripheral fibers (6, 17, 18, 41).

## MATERIALS AND METHODS

### *Nerve Crush and Labeling*

Garfish (*Lepisosteus osseus*) weighing 2–5 kg were anesthetized with 140 mg/liter of water of MS-222 (ethyl m-aminobenzoate methane sulfonate, Crescent Research Chemicals, Scottsdale, Ariz.). About 3 mm of the right olfactory nerve was exposed by drilling away the bone covering the rostrum, at a distance of 1.5 cm from the center of the posterior naris. The entire nerve bundle, containing the olfactory and trigeminal nerves and the blood vessels, was lifted from its groove with a specially designed hook and crushed twice at a 45° angle with a fine pair of forceps. In a limited number of experiments, the olfactory nerve was cut, but the trigeminal and vascular elements of the bundle were left intact. Total olfactory nerve degeneration was also studied in fish from which the right half of the tip of the rostrum, containing the olfactory mucosa and olfactory cell bodies, was removed. The garfish were allowed to regain consciousness and were put in large tanks maintained at 21°C.

At various times ranging from 3 to 250 d, the animals were reanesthetized and the olfactory nerve was labeled as described previously (24). In most experiments, 100  $\mu$ Ci of L-[<sup>35</sup>S]methionine (400–900 Ci/mmol, Amersham Corporation, Arlington Heights, Ill.) were applied to the right olfactory cavity (crushed side). The proteins were allowed to be axonally transported for 24–48 h, a time sufficient to allow molecules to reach the most distal portion of an intact nerve (25) (Fig. 1). In double-labeling experiments, the regenerating nerves were labeled with 100  $\mu$ Ci of [<sup>35</sup>S]methionine and 8 h later with 150  $\mu$ Ci of L-[4,5-<sup>3</sup>H]leucine (30–50 Ci/mmol, New England Nuclear, Boston, Mass.). An hour after the second labeling, the nerve was crushed again by the procedures described above.

Normal rapid axonal transport of proteins was found to occur

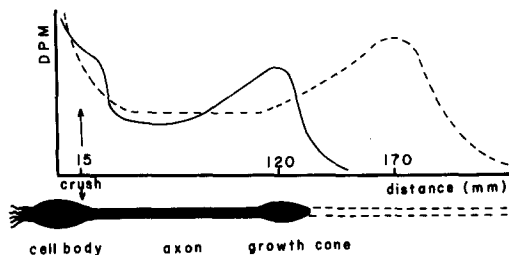


FIGURE 1 Schematic representation of the determination of regeneration velocity by intraaxonal transport. In intact nerves (dotted line), the radioactive amino acids ([<sup>35</sup>S]methionine or [<sup>3</sup>H]leucine) were taken up by the cell bodies and incorporated into proteins, which were transported along the axons at ~220 mm/d (23°C). The distribution shown was characteristic of nerves isolated 24 h postlabeling (see Gross and Beidler [22]). In regenerating nerves (solid line; in this example, 30 d after injury), labeled proteins accumulated at the growing axon tips and formed a peak of radioactivity corresponding to the position of the regenerating fibers. In all experiments, the nerve was crushed at 1.5 cm from the center of the posterior naris, and the proteins were allowed to be axonally transported for 24–48 h (depending on the position of the regenerating fibers) before the animals were killed.

in nerves after a sham operation in which the nerve bundle was lifted from its groove but neither cut nor crushed. To assess the distribution of systemic labeling in crushed nerves, experiments were conducted in which the mucosa of the contralateral intact side was labeled. The amount of systemic labeling remained constant along the entire length of the experimental nerve except in the area proximal to the crush, where a relatively large amount of radioactivity was found (data not shown).

### *Processing of the Tissue*

After transport of the proteins, the garfish were killed, the rostrum was sawed off and cooled in ice, and both olfactory nerves were removed according to the method of Easton (13). The wet weight of nerves, mucosae, and bulbs was recorded. Possible variations of the weight of intact nerves in response to the contralateral nerve's injury were determined by comparing the nerve's weight per unit length to the size of the fish. A similar study was done previously by Gross (23) with unoperated animals and no significant difference could be found between the two groups of animals. The same conclusion was drawn from measurement of the mucosae and the bulbs.

The distribution of radioactivity along both nerves was determined according to the method of Gross and Beidler (24). The nerves were washed in 5% TCA and cut into 3-mm segments, and each segment was counted for radioactivity. In a limited number of cases the weight of each segment was also recorded. The distances traveled by the leading edge of the front of radioactivity and of the peak apex were measured from the center of the posterior naris and plotted as a function of time (Fig. 1). The rates of wave front and peak displacements were determined by calculating the slopes by linear regression analysis. The values of the x-intercepts were measured for regeneration experiments in order to assess the somal reaction time. The radioactivity

profiles were normalized according to the method of Gross and Beidler (25).

### Light and Transmission Electron Microscopy

Various segments of nerve were analyzed by transmission electron microscopy. Pieces of the entire nerve bundle were prepared according to Graziadei and DeHan (21). They were fixed in 2.5% glutaraldehyde, 2% paraformaldehyde, 0.012 M  $\text{CaCl}_2$  in 0.07 M sodium cacodylate buffer, pH 7.4, and postfixed in 2% osmium tetroxide in the same buffer. Small segments of tissue were embedded in Araldite 506, and the sections were stained with both lead citrate and uranyl acetate. The sections were examined with a Philips 300 electron microscope. The number of individual axons in the various types of nerve was estimated by counting the number of fibers in areas of electron micrographs of similar magnifications and extrapolating the results for the total surface of the nerve cross section. The number of axons in the first two regenerating phases was estimated at 100 d by comparing distal cross sections of nerves (where the 1st phase was present and where the 2nd phase had not penetrated) with more proximal sections where both phases were present. Degenerating axons remaining at this time were estimated from nerves where the entire mucosa was removed.

For light microscopy, thick sections of tissue (1–1.5  $\mu\text{m}$ ) embedded in Araldite were stained with a solution containing 1% toluidine blue and 1% sodium borate.

## RESULTS

### Rates of Regeneration

Regeneration of the olfactory fibers was followed up to 250 d after surgery by monitoring the accumulation of radioactive intraaxonally transported proteins in the terminals of the growing fibers (Fig. 1). The efficacy of the crush in totally blocking axonal transport was confirmed by labeling the olfactory mucosa during the 1st wk after nerve crush. This produced a large accumulation of radioactivity proximal to the injury while the labeling distal to the crushed area remained at the level of the systemic radioactivity recovered in the unlabeled contralateral nerve (Fig. 2). At later times after crush, two successive peaks of radioactivity were observed to move along the nerve, corresponding to two different populations of regenerating axons (Fig. 3). For each population, the front edge of the peak should correspond to the position of the most rapidly growing fibers and the apex to the position of the largest number of fibers (6, 17). At very late times, a large amount of additional radioactivity moved into the nerve (Fig. 4). It decreased in the proximodistal direction and did not produce a peak of radioactivity. This phase of radioactive material was associated with an increase in nerve weight and with a large increase in the number of axons present in the nerve (see below).

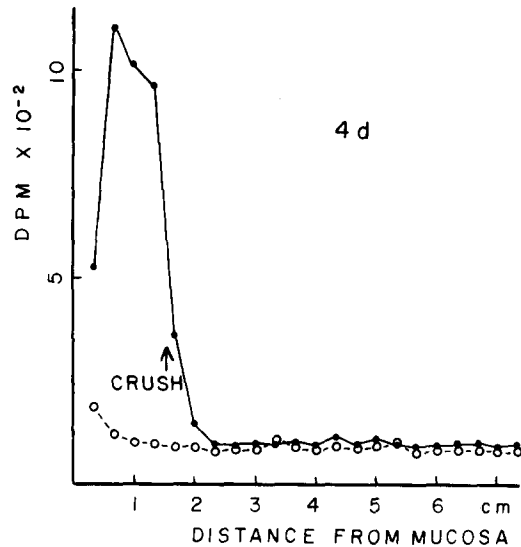


FIGURE 2 Distribution of radioactivity along the nerve 4 d after crush. (●) crushed nerve; (○) contralateral intact nerve.

To confirm that the radioactivity along the nerve was due to intraaxonally transported proteins accumulated in the tips of the growing axons, control experiments were performed for the three phases of regenerating fibers. The regenerating nerves were first labeled with [ $^{35}\text{S}$ ]methionine, and when the labeled intraaxonally transported protein had entered the axon, the nerve was relabeled with [ $^3\text{H}$ ]leucine and crushed 1 h later. This prevented the second isotope from being intraaxonally transported. With the three populations of presumed regenerating fibers, only the first isotope, which was intraaxonally transported, accumulated in the growing tips (Fig. 5).

The first population of fibers (1st phase) was experimentally observed in the nerve distal to the crush 9 d p.o. (Fig. 3). The leading fibers grow at a constant rate of  $5.8 \pm 0.3$  mm/d (linear regression analysis coefficient, 0.995), and by calculating the  $x$ -intercept value it was estimated that fibers would start to grow from the mucosa after 3.4 d and cross the crush area after 6 d (Fig. 6a). The largest number of fibers in this 1st phase, defined as the apex of the peak of radioactivity (see Materials and Methods), appeared in the axon after 4.7 d, traversed the crush area after 7.8 d, and progressed at a constant rate of  $4.9 \pm 0.2$  mm/d ( $r = 0.947$ ) (Fig. 6b).

The second peak of radioactivity (2nd phase) was observed in the nerve distal to the crush by 20

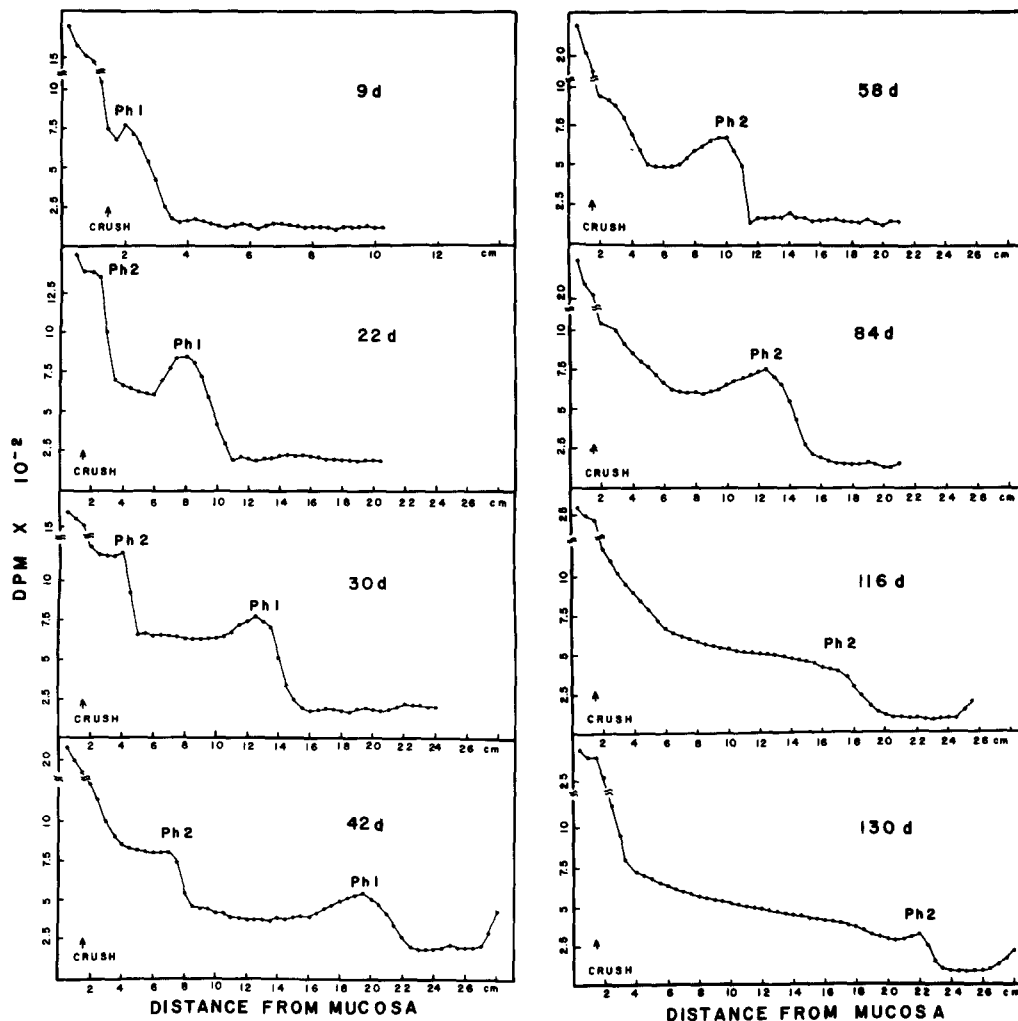


FIGURE 3 Profiles of radioactivity determined by the accumulation of [ $^{35}\text{S}$ ]methionine-labeled proteins. Left, the rapidly growing fibers (phase 1) at 9, 22, 30, and 42 d p.o. At 22, 30, and 42 d, the intermediate phase of growing fibers was visible in the nerve (phase 2). Right, the intermediate phase of growing fibers at 58, 84, 116, and 130 d p.o. (phase 2).

d (Fig. 3), and between 20 and 50 d both 1st and 2nd phase fibers were growing simultaneously. The regeneration rate of the leading fibers in the 2nd phase was only  $2.1 \pm 0.1$  mm/d ( $r = 0.976$ ) (Fig. 6a). This velocity was maintained for 60 d and then declined to  $1.6 \pm 0.2$  mm/d ( $r = 0.936$ ). It was extrapolated that the leading fibers would start growing from the cell body 6.5 d after crush and cross the crush area after 13.4 d. The main population of fibers in the 2nd phase started to grow after 8.3 d (Fig. 6b) and crossed the crush area after 16 d at a velocity of  $1.9 \pm 0.1$  mm/d ( $r = 0.862$ ) and after 60 d declined to  $0.8 \pm 0.1$

mm/d ( $r = 0.843$ ). Data in Fig. 6b were confined to 130 d because the peak apex became poorly defined at later times.

The leading fibers of the 3rd population of axons (3rd phase) started growing after 12.3 d (Fig. 7), crossed the crush area after 30 d, and progressed at a constant rate of  $0.8 \pm 0.2$  mm/d ( $r = 0.851$ ). A similar study was conducted on a limited number of animals whose olfactory nerve was totally severed but the cell bodies were retained (see Materials and Methods). Rates of regeneration and growth-starting times for the first two phases were similar to those found in crushed

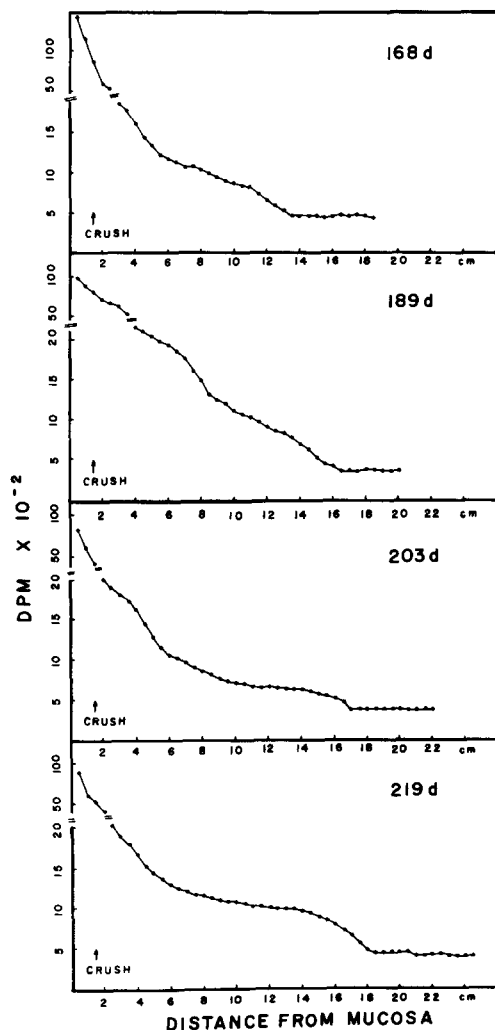


FIGURE 4 Profiles of radioactivity determined by the accumulation of [ $^{35}$ S]methionine-labeled proteins in the slowly growing fibers at 168, 189, 203, and 219 d p.o. (phase 3).

nerve experiments (Fig. 6, triangles); however, the amount of radioactivity accumulating in the axon tips was approximately half the amount measured in nerves regenerating after crush.

#### *Tissue Weight Changes After Olfactory Nerve Crush*

The weights of the injured nerve, the corresponding mucosa (containing the cell bodies), and the olfactory bulb (containing the olfactory glomeruli) were monitored at various times after nerve crush. The weight of the injured nerve re-

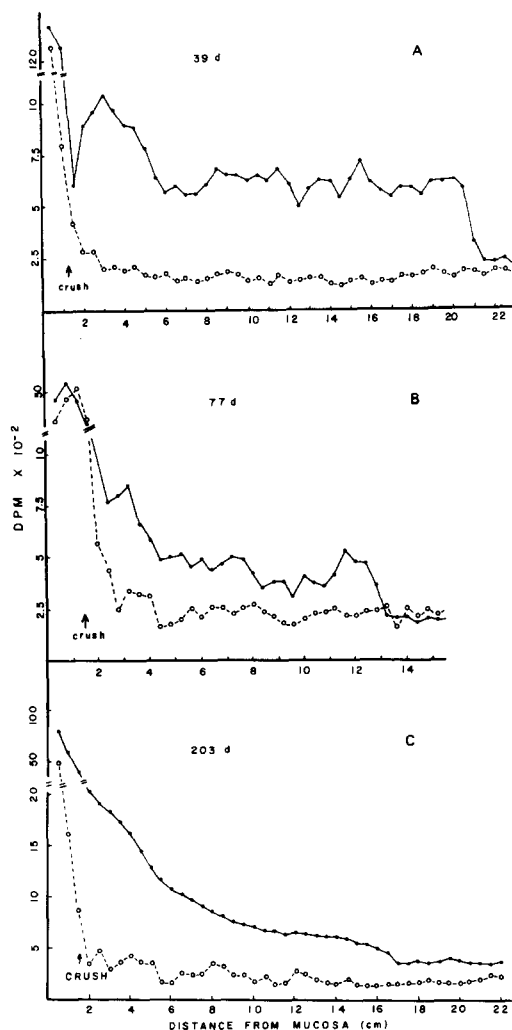


FIGURE 5 Profiles of radioactivity determined by [ $^{35}$ S]methionine-labeled proteins (●) in the regenerating axons at (A) 39 d, (B) 77 d, and (C) 203 d p.o. and by [ $^3$ H]leucine-labeled proteins (○), deposited in the same nerve 8 h after the first isotope deposition and 1 h before the nerve was crushed a second time.

mained constant for 6 d (Fig. 8) and then decreased linearly for the next 30 d according to the equation weight ( $w$ ) =  $-2.17$  (days [d]) + 112.63 ( $r = 0.876$ , as determined by linear regression analysis). After this rapid decrease, the nerve weight reached a plateau at 33% of the weight of the intact contralateral nerve. At 100 d after surgery, the nerve weight started to increase linearly according to the equation  $w = 0.53$  d - 20.73 ( $r = 0.847$ ) and reached a plateau of 75% of the original value 180 d p.o. In a few animals, the

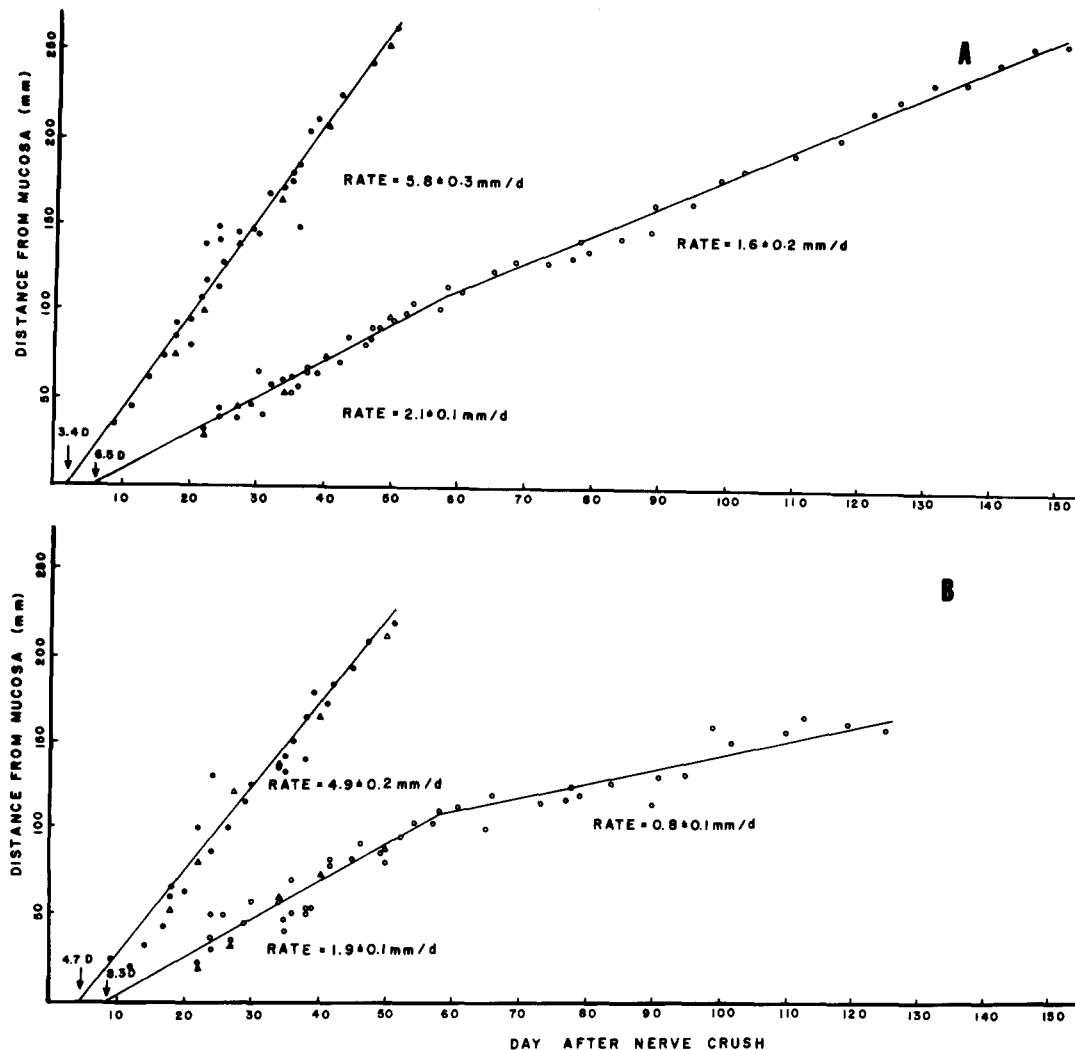


FIGURE 6 Distance traveled as a function of time, (A) by the leading fibers (wave front of the peak of radioactivity) of the rapidly growing population of axons (●), and of the intermediate population of axons (○) following nerve crush; (B) by the bulk of fibers (apex of the peak of radioactivity) of the rapidly growing population of axons (●), and of the intermediate population of axons (○) following nerve crush. Similar experiments in the regenerating nerves after section of the entire olfactory nerve (Δ).

right mucosa containing the olfactory perikarya was removed and the corresponding nerve was allowed to degenerate (Fig. 8a). The initial decrease in weight during the first 36 d was similar to that described for crushed nerve. After this initial period, the weight of degenerating nerves kept decreasing but at a much slower rate (Fig. 8a). After 224 d, the weight of the degenerating nerve represented 15% of the intact nerve value. A more detailed analysis of the nerve weight changes during degeneration and regeneration was

achieved by measuring the weight of consecutive 3-mm segments of nerves following TCA treatment at 22–219 days (Fig. 9). During degeneration, a proximodistal decrease in nerve weight was seen to progress along the nerve at a rate of ~5 mm/d. This centrifugal degeneration was confirmed by comparing the diameters of proximal and distal nerve segments during early degeneration by light microscopy; preliminary studies of such segments by electron microscopy also revealed that degeneration is more advanced in the proximal region

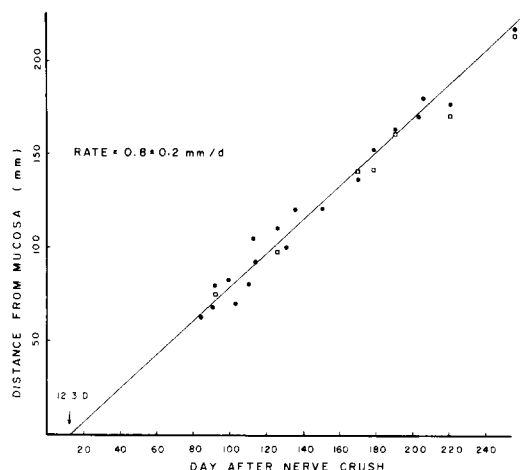


FIGURE 7 Distance traveled as a function of time by the leading fibers of the slowly growing population of axons (●) and by the leading edge of the proximodistal increase in regenerating nerve weight (□); see Fig. 9.

of the nerve. The arrival of the 3rd phase of regenerating fibers was characterized by a proximodistal increase in nerve weight. The rate of the nerve weight increase was shown to parallel closely the velocity of regeneration of the more slowly regenerating fibers as determined by intraaxonal transport.

Similar but quantitatively smaller decrease and increase in weight were recorded for the mucosa (Fig. 8b). The weight decrease started after 4–5 d, continued for 60 d, and can be best described by the power function  $w = 114.5 d^{-0.091}$  ( $r = 0.846$ ). A subsequent increase began after 88 d and reached 100% of the weight of the intact mucosa at 150 d p.o. The bulb weight decreased linearly for 150 d according to the equation  $w = -0.30 d + 91.72$  ( $r = 0.811$ ) to a plateau of 45% of the original weight (Fig. 8c). It was only when the second wave of regenerating axons arrived in the bulb that a rapid linear increase in the bulb weight occurred. The increase can be described by the equation  $w = 0.87 d - 85.07$  ( $r = 0.916$ ). A plateau of 85% of the original bulb weight is reached 190 d p.o.

#### *Anatomical Changes in Degenerating and Regenerating Olfactory System*

Degenerative and regenerative changes in the mucosa and bulb were monitored by light microscopy at 31 d, when the degeneration of the nerve was prominent (Fig. 8a) and at 203 d, when the

nerve, mucosa, and bulb had regenerated to a plateau weight (Fig. 8a–c). The appearances of the normal and regenerated mucosae (Fig. 10a, c) were very similar and were characterized by the presence of elongated sustentacula and olfactory cells. At 31 d p.o. (Fig. 10b), the mucosa was characterized by a short superficial layer of mainly sustentacular cells and a thick layer of small round cells which were probably differentiating basal cells. A similar succession of events can be followed in the bulb. In the intact bulb, as described previously by Andres (2) in various fish, a well-characterized glomerular layer was present (Fig. 10d). At 31 d p.o., the ventricle had enormously increased and the glomerular layer had almost completely disappeared (Fig. 10e). After the arrival of the first two phases of axons, the glomerular layer was not as well characterized as in the intact bulb but was clearly visible (Fig. 10f).

The distribution pattern of the axons varied greatly during degeneration and regeneration. In intact nerves (Fig. 11a), sections taken at various distances from the mucosa revealed domains of several hundred densely packed fibers. The nerves had an average diameter of  $0.25 \mu\text{m}$  (Table I) and were surrounded by a single Schwann cell (14). During degeneration, the number of axons in each domain decreased rapidly, and eventually entire domains became empty. Degeneration in the absence of possible regeneration was examined in nerves where the entire mucosa was removed. At 96 d after cell body removal (Fig. 11b), examination of nerve sections at various distances from the mucosa revealed that 1–3% of the original axonal population was still present. Most of these remaining axons did not appear to be as well enclosed in small fascicles as was seen in regenerating nerves.

In regenerating nerves, it was estimated that 102 d after crush, each of the first two phases of regenerating axons contained 3–5% of the original axonal population. Typically, small domains containing 10–50 small axons with an average diameter of  $0.15 \mu\text{m}$  were present in both phases (Table I). Each of these fascicles of fibers was surrounded by a Schwann cell and a large amount of collagenous tissue, as demonstrated by sections taken at various distances from the mucosa (Fig. 11c). With the arrival of the 3rd population of fibers, a major reorganization of the nerve took place, as demonstrated by nerve sections taken 5 cm from the mucosa at 189 and 203 d p.o. (Figs. 11d and 12a). The amount of collagenous material decreased drastically and the appearance of the nerve was

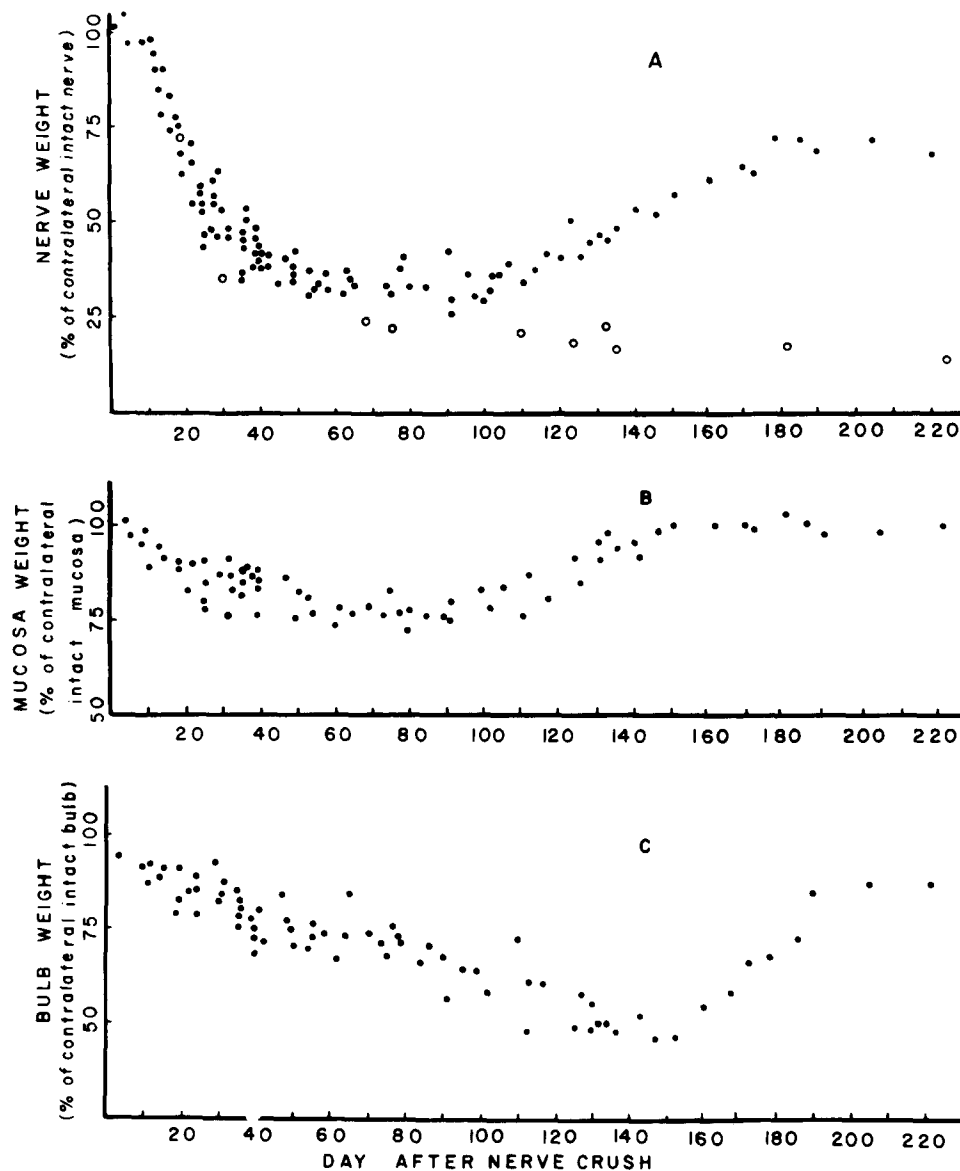


FIGURE 8 (A) (●) Variations of the weight of the olfactory nerve after nerve crush. (○) Totally degenerating nerves, after removal of the mucosa containing the cell bodies. (B) Variations of the weight of the olfactory mucosa after nerve crush. (C) Variations of the weight of the olfactory bulb after nerve crush.

reminiscent of intact nerve. Large domains of several hundred fibers were surrounded by a Schwann cell and, exterior to it, a perineural cell. At 5 cm from the mucosa, it was estimated that 50–70% of the original axonal population was present (Figs. 11*d* and 12*a*). The nerve at this stage of regeneration was characterized by the presence of large numbers of small and very small

fibers (Table I) and by large bodies which in longitudinal sections (not shown) appeared to represent a combination of growth cones and glial cells (43). It should be noted, however, that in the distal part (20 cm from the mucosa) of the late regenerating nerves (189 d), where the 3rd axonal population had not penetrated (Fig. 12*b*), most of the collagenous material had disappeared and was



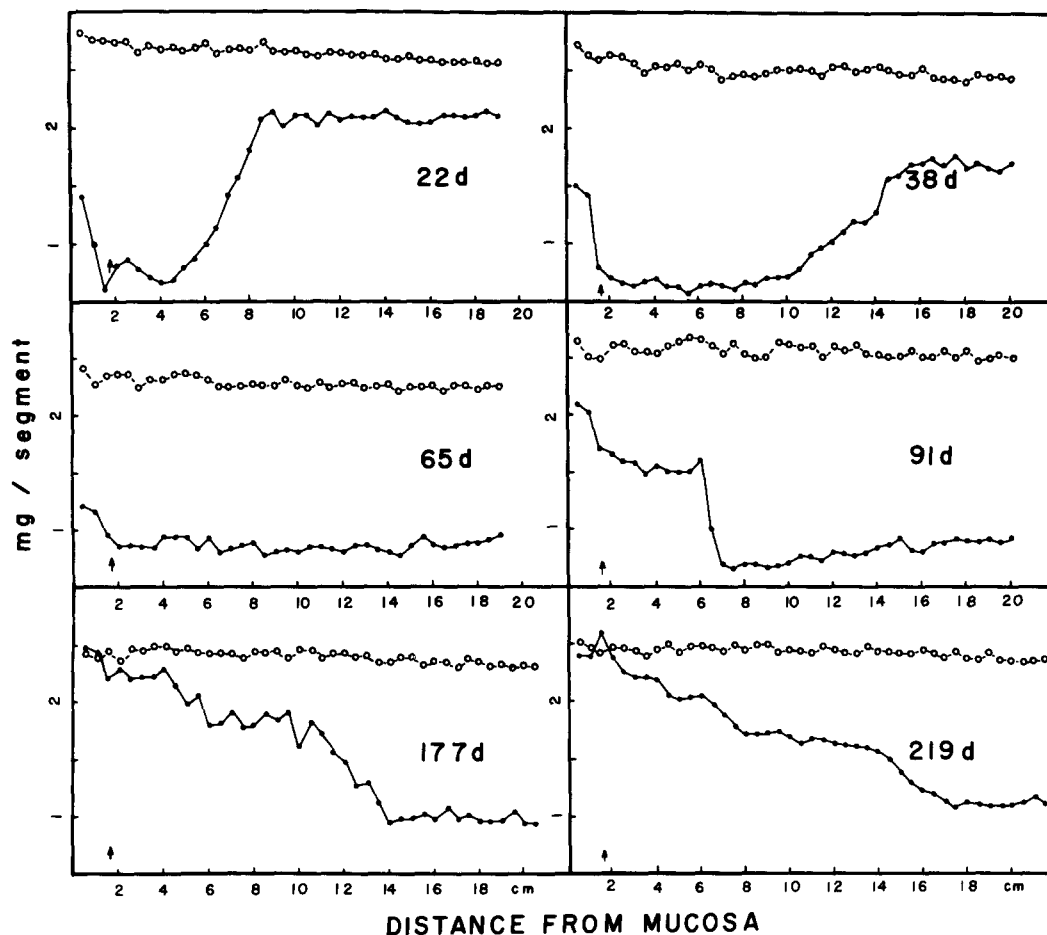


FIGURE 9 Weight of consecutive segments of degenerating and regenerating nerves 22, 38, 65, 91, 177, and 219 d after nerve crush. Arrow, crushed area; (●) crushed nerve, (○) intact contralateral nerve.

replaced by large domains constituted by a Schwann cell, a perineural cell, and a number of axons similar to that found at 100 d p.o.

## DISCUSSION

### *Changes in Nerve Weight*

A latent period of 6 d for the onset of postoperative degeneration is much longer than the latency measured in the case of the rabbit olfactory nerve, where the first signs of degeneration can be detected as early as 8 h after injury (4), but is similar to the latent periods determined in cold-blooded animals. Parker and Paine (42) showed that, at 18°C, the catfish lateral line nerve appears to remain intact for 11 d. At 24°C, no degeneration of the olfactory nerve of the frog is noticed before the 4th d p.o. (20).

It is of interest to note that in the very long olfactory nerve of the garfish, degeneration did not occur simultaneously along the entire nerve but progressed in the proximodistal direction. Numerous studies on spatiotemporal Wallerian degeneration have shown variations among species (for review, see Lubinska [34] and Joseph [27]). However, the proximodistal spreading of degeneration has been observed in various nerves such as the catfish lateral line nerve (42), the toad dorsal roots (28), and the rat phrenic nerve (35).

The weight of the crushed nerve remained at a minimum up to 100 d after the injury. Such a long plateau might reflect the combined influence on the nerve of degeneration, regeneration, and changes in the glial content of the nerve. A moderate Schwann cell multiplication after crush of the unmyelinated fibers of the cervical sympa-

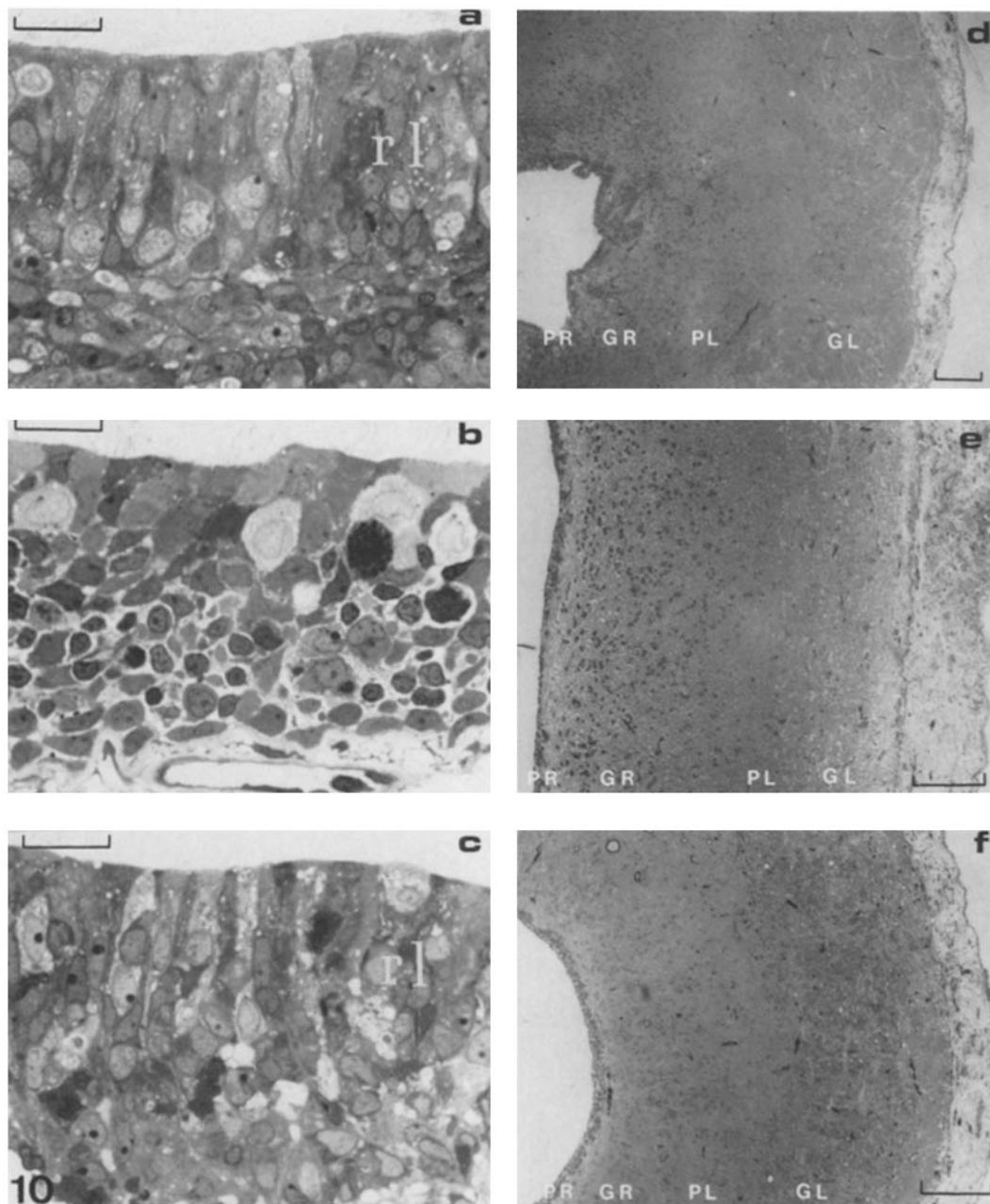


FIGURE 10 Light micrographs. (a) Normal olfactory mucosa.  $\times 1,053$ . (b) Degenerated mucosa, 31 d p.o.  $\times 1,053$ . (c) Regenerated mucosa, 203 d p.o.  $\times 1,053$ . (d) Normal olfactory bulb.  $\times 66$ . (e) Degenerated olfactory bulb, 31 d p.o.  $\times 105$ . (f) Regenerated olfactory bulb, 203 d p.o.  $\times 105$ . Bars in a, b, c, 10  $\mu\text{m}$ ; bars in d, e, f, 100  $\mu\text{m}$ . rl, receptor layer; GL, glomerular layer, PL, plexiform layer; GR, granular layer; PR, periventricular layer.

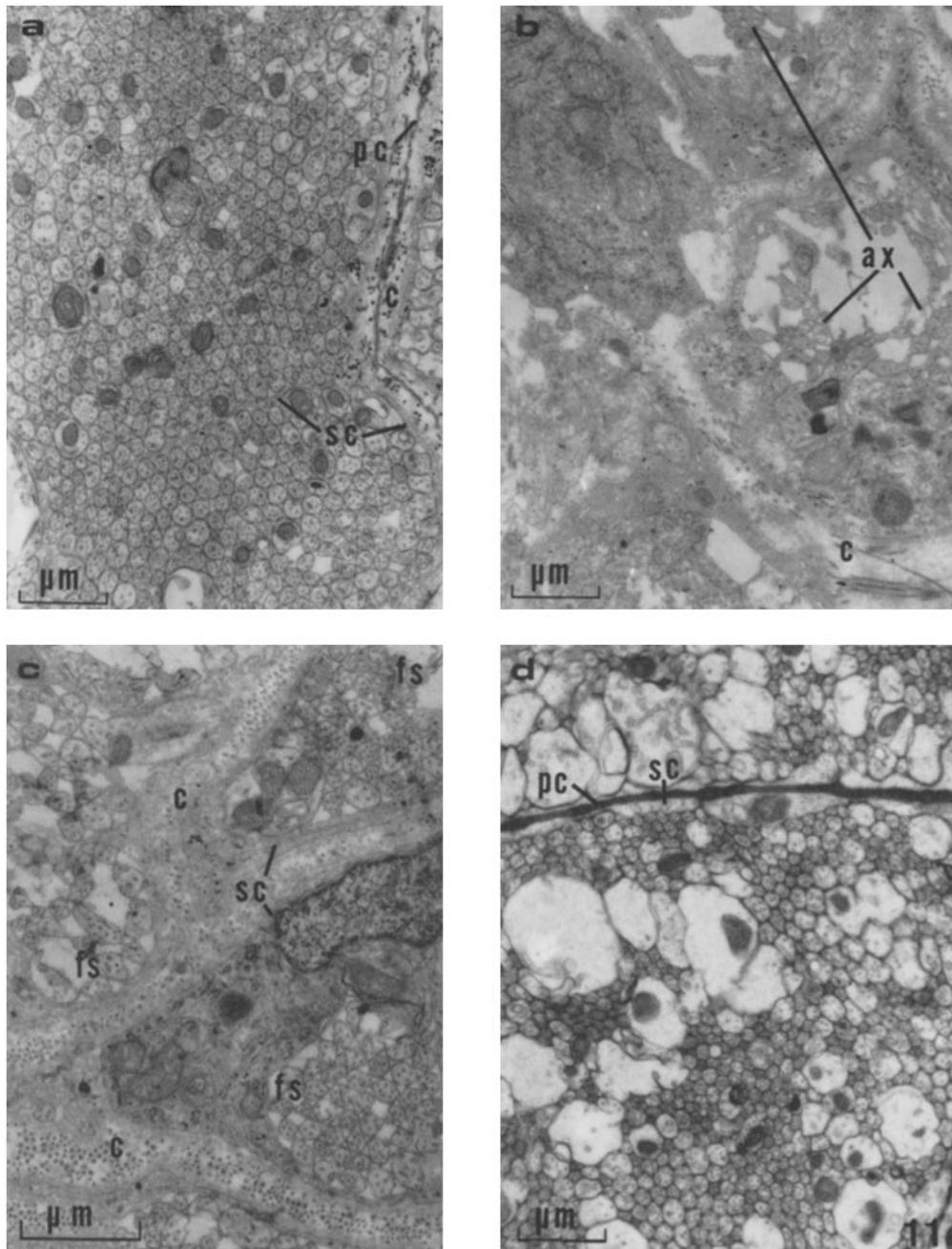


FIGURE 11 Transmission electron micrographs. (a) Intact olfactory nerve: each domain is constituted by a large number of fibers surrounded by a Schwann cell. Section taken 12 cm from the mucosa.  $\times 12,000$ . (b) Degenerating olfactory nerve, 96 d after removal of the olfactory mucosa, section taken 12 cm from the mucosa.  $\times 12,000$ . (c) Regenerating olfactory nerve, 102 d after crush: small fascicles of axons were surrounded by a perineural cell and large amounts of collagenous fibers. Section taken 12 cm from the mucosa.  $\times 16,700$ . (d) Regenerating olfactory nerve, 203 d after crush; a massive invasion of new axons was occurring. Section taken 5 cm from the mucosa.  $\times 12,000$ . Ax, axons; c, connective tissue; fs, fascicle of axons; pc, perineural cell; sc, Schwann cell. Bars in a, b, c, and d, 1  $\mu\text{m}$ .

TABLE I  
Distribution of Axon Sizes in Normal and Regenerating Nerves

Nerve diameter $\mu\text{m}$	Percent of total axonal population		
	Normal nerve	Regenerating nerve	
		1st and 2nd phase	3rd phase
0.07–0.1	3	10	40
0.10–0.15		85	52
		(Mean = 0.25 $\mu\text{m}$ )	(Mean = 0.15 $\mu\text{m}$ )
0.15–0.20	88		
	(Mean = 0.25 $\mu\text{m}$ )		
0.20–0.30		5	8
0.30–0.40	9		

thetic trunks has been observed by Romine et al. (45).

The increase in the weight of the regenerating nerve began 100 d after crush, and the rate of increase was approximately four times slower than the rate of initial nerve weight decrease. This late nerve weight increase was concomitant with the arrival of the third population of regenerating fibers in the proximal part of the nerve and was most likely due to the increase in the number of growing axons (see below). The weight reached a plateau of only 75% of the original weight, despite the continuing appearance and growth of 3rd phase fibers. Graziadei and DeHan (21) have shown that in the frog, regenerating olfactory nerves are also unable to regain their original size. However, in garfish, the complete arrival of the third population of fibers in the synaptic area should take at least a year, and therefore it is not known from the present study whether the nerve ultimately regains its original weight. It is also not known whether the new axons eventually regain the diameter of undamaged fibers.

The postcrush decrease in mucosa weight can be explained by the disappearance of the cell bodies of the injured neurons (Fig. 10). Graziadei and Monti-Graziadei (22) could not find any mature olfactory perikarya in the rat mucosa 8 d after cutting the nerve. Mucosal degeneration in the frog (21) as well as in the goldfish (26) shows a latency of 2–4 d, similar to the latent period measured in the garfish. These studies, however, estimated that mucosal degeneration is then completed in ~2 wk. Because in garfish, mucosal weight decreased for 60 d and did not start to increase before almost 90 d, the changes in mucosal weight do not seem to be entirely correlated with the corresponding degeneration and regen-

eration of the olfactory cells. However, the olfactory perikarya represent only a small percentage of the total number of cells present in the mucosa (8); the weight variations might reflect other changes occurring in the mucosal epithelium.

The analysis of the olfactory bulb showed a constant decrease of the bulb weight for 150 d p.o., a period during which the first wave of regenerating fibers should have made synaptic connections. The increase in bulb weight occurred only when the second population of fibers reached the bulb. A similar effect has recently been demonstrated in the goldfish optic system (47) where the reinnervation of the optic tectum by regenerating optic nerve fibers enhances cell proliferation in the tectum. In the present case, a minimum number of fibers might be necessary to mediate this trophic effect, and the 1st phase axons may not be numerous enough to provide it. Although an increase of bulb weight to 85% of normal might suggest extensive functional recovery, light microscopy indicates that after 200 d the glomerular layer is only partially reconstituted (Fig. 10f). The study was not continued until the arrival of the 3rd phase of regenerating fibers in the bulb to determine whether total reconstitution occurs at later times.

#### Anatomical Analysis

Light microscopy analysis of the olfactory mucosa at various times after axotomy (Fig. 10a–c) confirmed a massive injury-induced degeneration, during which most of the olfactory perikarya disappeared, and a return to an almost normal situation during regeneration. A similar succession of events occurred in the bulb (Fig. 10d–f). These results show that the mechanism of olfactory nerve regeneration described in detail for higher vertebrates (36) is also operative in fish.

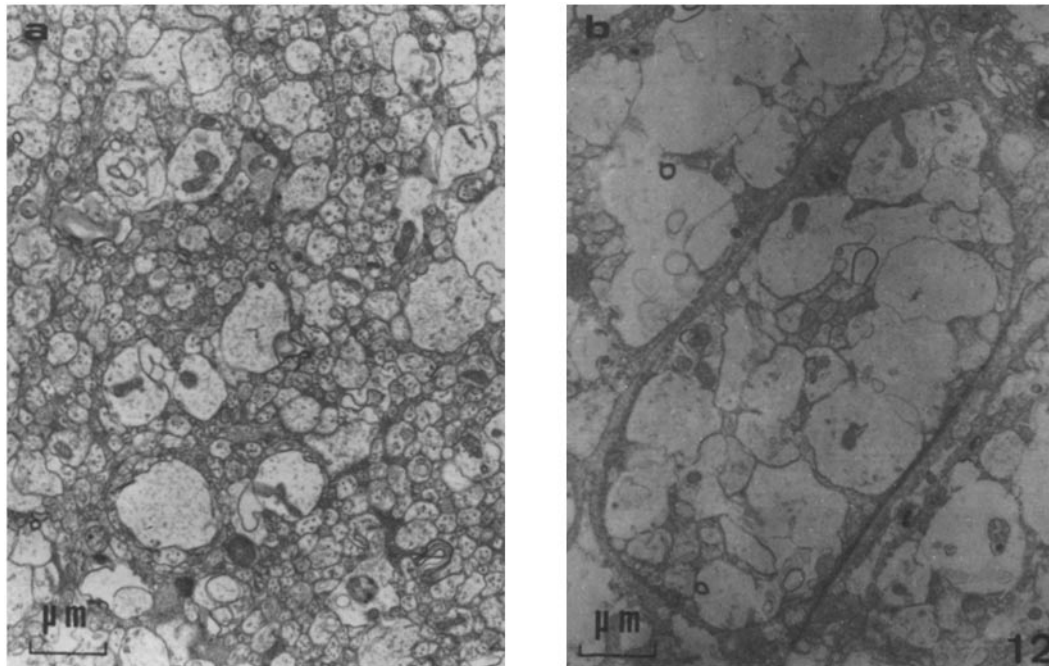


FIGURE 12 Transmission electron micrographs of regenerating olfactory nerve 189 d after crush. (a) Section taken 5 cm from the mucosa; (b) section taken 20 cm from the mucosa. Bars in *a* and *b*, 1  $\mu$ m.  $\times$  10,200.

Electron microscopy demonstration that degeneration following injury was characterized by a large decrease in the number of axons and the presence of large empty perineural domains can be compared to a similar, although faster, succession of events (2–7 d) occurring in the rabbit olfactory nerve (4). Shantha and Bourne (46) have demonstrated that, after nerve section, the perineural epithelium remains intact in the proximal and distal stumps and plays an active role in regeneration by guiding the growing fibers. That 1–3% of the original axonal population remains in the degenerating nerve 100 d after total removal of the olfactory mucosa indicates that, after the initial massive degeneration, a few axons can survive for several months. It was reported previously by Berger (4) for the rabbit olfactory nerve that a limited number of axons can remain apparently intact after being disconnected from the cell body for extended periods of time.

Judged by electron micrographs at 102 d after crush, it appears that each of the first two phases of regenerating fibers represented only a very small percentage of the original population of axons, with fibers mainly enclosed in small fascicles of 10–50 axons. Turner and Singer (48) have

characterized early regeneration in the newt optic nerve by the presence of small fascicles of fibers. In the regenerating garfish nerve, the fibers were of a significantly smaller diameter than in the intact nerve (0.15  $\mu$ m compared with 0.25  $\mu$ m, Table I). A similar difference (0.25  $\mu$ m compared with 0.4  $\mu$ m) was reported for the regenerating unmyelinated nerve of the rat cervical sympathetic trunk (12).

The organization of the nerve changed drastically with the arrival of the third population of fibers. The appearance of cross sections from a proximal nerve segment (189 and 203 d p.o., Figs. 11*d* and 12*a*) was very similar to that of a normal nerve, although most of the axons were of a very small diameter (Table I). Similarly, in the optic nerve of the newt (48), the olfactory nerve of the pigeon (3), and the unmyelinated cervical sympathetic trunk of the rat (12), early regeneration is characterized by the appearance of a few axons, and the arrival of a large number of axons is restricted to the late phase of regeneration. That electron micrographs showed that cross sections of distal nerve segment at 189d (Fig. 12*b*), where the 3rd phase fibers had not penetrated, were very different from cross sections of the same segment

(Fig. 11c) at 100 d suggests that the nerve reorganization occurring during late regeneration was not induced by the massive arrival of nerve fibers but took place earlier under the influence of the pioneer fibers.

### *Rates of Regeneration*

Rates of nerve regeneration have been studied in a large variety of animals and fibers (For review, see Lubinska [34]). In cold-blooded animals, rates of only a fraction of a millimeter to 1 mm/d are usually measured at temperatures ranging from 8° to 25°C. Very different results were obtained in the garfish olfactory nerve where three distinct populations of growing fibers were detected. The most rapid rate of regeneration is clearly extremely high for cold-blooded animals. The velocity of regeneration of the second population of fibers is still significantly high, although a velocity of 1.2 mm/d was determined by Lubinska and Olekiewicz (36) for the regeneration of the frog sciatic nerve at 21.9°C.

The two slower rates of regeneration can be compared with the rates of slow axonal flow measured previously in intact garfish (9). The rate of regeneration of the leading fibers of the 2nd phase of regeneration (2.1 mm/d) is very similar to the velocity of a small peak of slowly transported radioactivity moving along intact axons at 2.38 mm/d at 21°C (9). A similar small peak of slowly transported material has been described in the rat sciatic nerve (31). The authors showed that during regeneration the amount of material present in this peak increases due to an increase in the slowly transported tubulin. A similar process could occur in the garfish although the influence of regeneration on the labeling of this small peak has not been analyzed.

A correlation also exists between the velocity of the main peak of slow flow ( $0.92 \pm 0.02$  mm/d at 21°C) and the rate of growth of the 3rd phase of regenerating fibers ( $0.8 \pm 0.2$  mm/d) after the first 60 d p.o. Previous subcellular and polypeptide analysis of the material transported by slow intraaxonal flow in intact nerves has revealed that its composition is very similar to the composition of the axons themselves (10). Therefore a rate of axonal growth similar or slightly lower than the rate of slow flow could reflect a limitation of axonal growth by the rate of arrival of the molecules necessary to build the new axon. However, the rate and composition of the slow flow in regenerating nerves have not yet been studied. It

should also be conceded that a nonnegligible number of fibers grew at a velocity greater by far than the rate of slow flow. The material necessary for the elongation of these fibers must have come from rapidly transported molecules, yet we have recently found that fast transport in the rapidly regenerating fibers contained mainly high molecular weight polypeptides unrepresentative of the nerve as a whole.<sup>1</sup> It is not clear at this time how fast transport was able to provide the total material for the rapid growth of new axons. Alternatively, an increase in the rate of slow flow might have occurred.

The presence of three distinct populations of regenerating fibers might be explained by the unusual nature of the olfactory nerve. Following axotomy or nerve crush, the injured mature cells degenerated and disappeared. Graziadei and Monti-Graziadei (22) showed that in the rat olfactory mucosa all the mature olfactory perikarya have been eliminated 8 d p.o.; however, they also showed that a small percentage of immature neurons arising from normal turnover remain present at all times. This small population of neurons metabolically in its initial growth stage might be ready to grow at a high velocity after the signal produced by the lesion. Such fibers should also be able to grow through the crush with minimum latency. Initial delays between nerve crush and the beginning of axonal growth, varying from 1 to 5 d, have been determined for both cold- and warm-blooded animals (for review, see Grafstein and McQuarrie [19]).

The latter two populations of regenerating fibers might represent newly differentiated neurons. In the rat olfactory mucosa Monti-Graziadei and Graziadei (40) have shown that, after section of the nerve, a mitotic activity takes place in the layer of basal cells underlying the degenerating olfactory perikarya. These dividing cells differentiate into typical olfactory neurons, capable of growing an axon and eventually making synaptic contact. The 2nd phase of fibers might originate from basal cells undergoing final differentiation at the time of the crush. This would explain the longer latency (6.5 d) measured for this population. The 3rd phase of fibers might represent the more massive and protracted response of the mucosa following the injury. Basal cells, originally in premitotic phase, must divide and differentiate after the in-

<sup>1</sup> Cancalon, P., and J. S. Elam. Manuscript in preparation.

jury, producing a latency of 12 d for the fastest growing fibers of this group. A latency of 18–20 d was determined by Graziadei and DeHan (21) for the appearance of the majority of the new neurons in the frog olfactory system.

The different rates of regeneration among the three axonal populations might be due to the presence of various types of olfactory neurons. However, Ichikawa and Ueda (26) have identified only two types of cells as olfactory nerve perikarya. The rate decrease could also be the result of the postoperative proliferation of collagenous material (37, 44). However, the effect of collagen on regeneration has not been completely clarified, and recent experiments (7) have shown that for normal development of nerve fibers the Schwann cells must be in contact with the connective tissue as well as with the axons themselves. In addition, microscopy indicated that the collagen largely disappeared at the time of entry of the third and most slowly growing phase (Fig. 12*b*). As an alternative to the connective tissue hypothesis, it is possible that a conditioning effect was responsible for the changes in velocities. It has been demonstrated that the rate of regeneration of the rat sciatic nerve that was crushed in its proximal segment, 2 wk after an initial crush of the nerve in its distal part, is 23% faster than the regeneration velocity in a nerve that did not have a prior conditioning lesion (39). A similar study was performed by Agranoff et al. (1) in *Xenopus* retinal cells. Recent experiments have indicated that this effect might be the result of a transient increase in the amount of material rapidly transported intraaxonally (38).

In our experiments, a conditioning effect was expected to act most strongly on the first population of fibers, which were presumed to be differentiated growing neurons at the time of injury and therefore in a situation similar to that of neurons conditioned by a first distal crush. The effect of the injury might have been less prominent on the second population of fibers, because they were not fully differentiated neurons at the time of surgery. Finally, cells giving rise to the last population of fibers were still quiescent basal cells at the time of crush and might not have been conditioned at all by the injury. Variations in the intensity of the conditioning effect as a function of time have recently been demonstrated in the goldfish optic system (15). The growth enhancement is strongest 14 d after the conditioning lesion. A smaller effect is observed after 7 and 21 d.

Remaining to be explained is the fall in the rate

of regeneration of the second population. It can be noted that the velocity dropped to 0.8 mm/d only a few days after arrival of the 1st phase fibers in the bulb. This slower rate was identical to the velocity of the slowest growing population of axons and similar to the rate of slow intraaxonal flow. This raises the possibility that the 1st phase synaptic connections have a “deconditioning” effect on the fibers still in the process of growth and reduces the growth rate to a nonconditioned value. The mechanism of such transaxonal effect is not known but the activation of proteases upon interaction between the axon terminal and the postsynaptic cell has been postulated by Lasek and Black (30) as a mechanism for intraaxonally stopping axonal growth.

## CONCLUSION

The study has revealed a unique pattern of regrowth of the axons corresponding to the complex cellular dynamics occurring in the olfactory nerve after injury. Some neurons, possibly beginning growth at the time of nerve crush, were able to grow at very high velocities while a second population of cells presumed to be undergoing final differentiation during surgery appeared in the nerve later and grew at a much slower rate. These two phases represent only 6–10% of the original axonal population. At later times, axons that most likely originated from premitotic basal cells invaded the nerve in large numbers. Changes in axonal growth rates and in bulb weight and structure suggest that the regeneration mechanism was controlled by both positive and negative factors.

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