# Proximodistal Degeneration of C-fibers Detached from Their Perikarya

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ABSTRACT Degeneration was followed in the garfish olfactory nerve after removal of the mucosa containing the cell bodies. Degeneration, as measured by a decrease in the weight of consecutive 3-mm nerve segments, spreads at constant velocity from the site of injury toward the synaptic area. The proximodistal degeneration is temperature dependent and progresses from 0.3 mm/d at 10°C to 13.0 mm/d at 35°C. Between 14 and 35°C, the velocity increases linearly with temperature. At all the temperatures investigated, these proximodistal degeneration velocities are identical to the rates of slow intraaxonal flow measured in axons detached from their cell bodies, or to the rates measured in regenerating fibers, and, except at 10°C, are 3.3 times faster than the rate of slow flow in intact nerves. These results were confirmed by light and electron microscopy. We hypothesize that the collapse and subsequent degeneration of the axons is the result of a proximodistal depletion of cytoskeletal elements no longer provided by the cell body to the axon by slow intraaxonal flow. A significant number of axons disappeared rapidly from the nerve before the arrival of the slow degenerative wave. From studies by other groups, this rapid degeneration may be the result of a lack of rapidly transported, mainly membranous components.

Axonal degeneration has been extensively studied in a large variety of preparations by anatomical and electrophysiological methods (for review, see references 1, 2, 3). The conflicting results have not answered the question of whether the degeneration process spreads centrifugally from the site of injury toward the periphery or simultaneously invades the entire nerve stump. Joseph and Whitlock (4) demonstrated a somatofugal degeneration in various regions of the toad CNS ranging from 0.07 to 2 mm/d at 20°C and 0.33 to 6 mm/d at 30°C with an average $Q_{10}$ of 3.5. In most other studies of proximodistal degeneration, much higher rates have been reported, usually on the order of several cm/d. Parker and Paine (5) measured a rate of 20–30 mm/d in the catfish lateral line. Lubinska (6) estimated that, in the rat phrenic nerve, degeneration spreads at 46 to 250 mm/d. More recently, Lubinska (7) has shown that, in the rat phrenic nerve, Wallerian degeneration of the distal stump progresses centrifugally by jumping from one internode to another and suggests that a trophic factor, transported in intact axons, keeps the Schwann cells in a quiescent state. Following axotomy, the factor being no longer provided, the Schwann cells hypertrophy and induce the destruction of the axon.

Lubinska (6) has postulated that the heterogeneity of these results is due to several causes: First, the studies have been conducted on nerves containing fibers of various types and diameters. Second, many nerves send or receive branches that influence degenerative processes. Third, the early degeneration of preterminal areas affects the results obtained when the injury occurs at different distances along the nerve.

In the present study, proximodistal degeneration was followed in the garfish olfactory nerve, which has the advantage of circumventing most of the causes of error mentioned above. The influence of nonneuronal elements on axonal degeneration is minimal because several hundred unmyelinated fibers are surrounded by a single Schwann cell and, therefore, the membrane-to-membrane contact between the two types of cells is limited to a very small number of axons. Furthermore, the nerve is composed of an extremely homogeneous population of unbranched C-fibers with an average diameter of 0.25 $\mu$m. Finally, the nerve is long (up to 30 cm) and the rapid degenerative process occurring in the preterminal area can be easily separated from the rest of the study.

## MATERIALS AND METHODS

### *Surgical Procedures*

The temperatures investigated ranged from 10 to 35°C. Garfish (*Lepisosteus osseus*), weighing 2–4 kg, were kept in 1,500-liter tanks maintained at constant



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temperatures (±0.1°C). The fish were acclimated to the selected temperature for 10 d before the beginning of the experiments.

NERVE CRUSH (DEGENERATION AFTER NERVE INJURY): We anesthetized the fish with 140 mg/liter of MS 222 (ethyl m-aminobenzonate methane sulfonate; Crescent Research Chemicals, Scottsdale, AZ). The right olfactory nerve was crushed as described by Cancalon and Elam (8). The entire nerve bundle, containing the olfactory and trigeminal nerves and the blood vessels, was exposed and lifted from its groove by means of a specially designed hook. The bundle was then crushed twice at a 45° angle with a fine pair of forceps at a distance of 1 cm from the center of the posterior naris.

MUCOSAL REMOVAL (DEGENERATION AFTER REMOVAL OF THE CELL BODIES): We split the tip of the upper jaw longitudinally along the line of cartilage separating the right and left parts of the rostrum. We then severed the right tip of the nose just behind the olfactory cavity in order to remove the entire olfactory mucosa and therefore the perikarya corresponding to the right nerve. The procedure leaves only the right distal stump in the animals. The left nerve is kept intact as a control (9). We performed all the experiments with this technique, except for a limited number in which the possible influence of the regenerating fibers was examined.

### Measurement of Weight Changes

Following the operation, the animals were kept at the selected temperature from 2 to >100 d. They were then killed, and the rostrum was removed and split longitudinally into two halves. By this procedure a distal segment representing about two-thirds of the nerve bundle is exposed in its groove. The rest of the nerve was then released by breaking off successive 1-cm bone segments. This method has the advantage of releasing the nerve bundle from the bone with minimal damage. The freed olfactory nerve was soaked in 5% trichloroacetic acid (TCA) for 4 h, then cut in 3-mm segments, and the weight of each segment was determined. We used a similar procedure in a limited number of cases on fresh nerves. The weight of the successive segments was plotted as a function of the distance from the site of injury. The distance traveled by the leading edge of the nerve shrinkage was measured from the site of injury and plotted as a function of time. The movement was linear and the rate of proximodistal degeneration was determined by calculating the slopes by linear regression analysis. The values of the X-intercepts were also measured in order to assess the lag period between the time of injury and the beginning of degeneration.

### Light and Electron Microscopy

Segments taken at various distances from the site of injury at different temperatures and at various times after surgery were prepared for light and electron microscopy as explained previously (8). They were fixed in 2.5% glutaraldehyde, 0.012 M $CaCl_2$ 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.

For light microscopy, thick sections of tissue embedded in Araldite were stained with a solution containing 1% toluidine blue and 1% sodium borate. Areas of nerve cross-sections were calculated with a computer-based digitizing system (Beehive Micro B).

## RESULTS

### Decrease in the Total Nerve Weight as a Function of Temperature

The decrease in the weight of the entire nerve was followed at various temperatures after removal of the olfactory mucosa (Fig. 1). The changes in nerve weight as a function of time for each temperature can be characterized by two linear functions: a rapid fall of the weight followed by a much slower decrease (8). The initial fall of the total nerve weight has been plotted at 10, 14, 21, 25, and 35°C (Fig. 2). These changes are highly temperature dependent. At 10°C the nerve weight still represents 80% of the control value after 100 d. At 35°C, on the contrary, a value of only 20% of control is reached within 10 d.

### Somatofugal Degeneration

WEIGHT CHANGES: Proximodistal degeneration was followed at temperatures ranging from 10 to 35°C after nerve crush or mucosal removal (Fig. 3). At each temperature, degeneration progresses from the site of injury toward the olfactory bulb at constant velocity. The degeneration rates increase linearly with temperature from 1.6 mm/d at 14°C to 13 mm/d at 35°C (Fig. 4). The increase in degeneration velocity as a function of temperature can be expressed as a rate increase of 0.56 ± 0.06 mm/d/°C (Fig. 5).

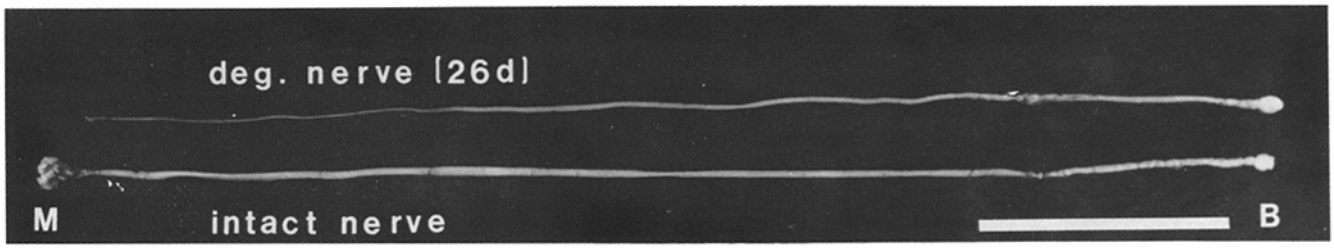


FIGURE 1 Degenerating and intact nerves 26 d after removal of the right mucosa. *M*, mucosa. *b*, olfactory bulb. Bar, 5 cm. × 0.7.

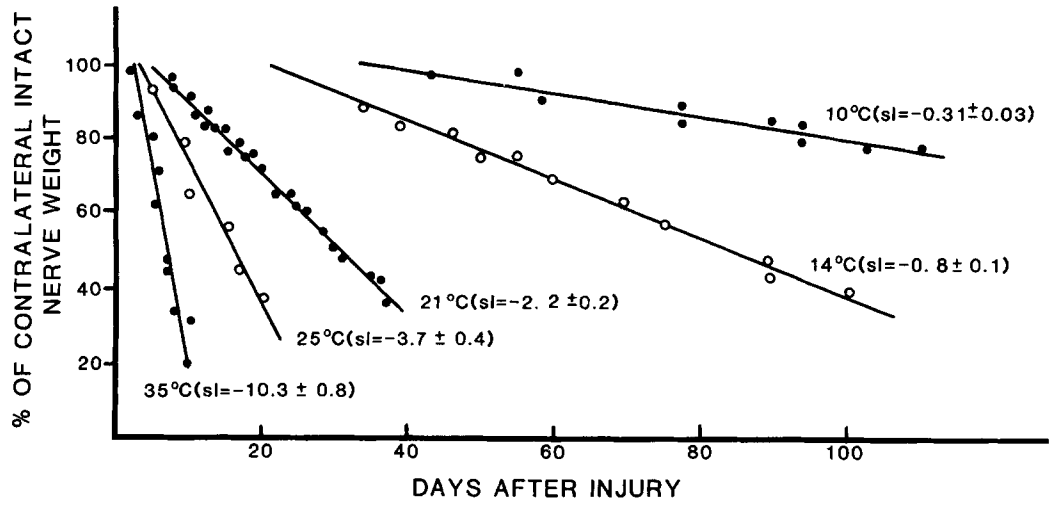


FIGURE 2 Decrease in the total weight of the nerve (as percent of the contralateral nerve) during the initial phase of degeneration.

Extrapolation of the degeneration velocity vs. temperature function to zero velocity indicates that degeneration in the somatofugal direction should cease at 10.4°C. However, a degeneration velocity of 0.3 ± 0.1 mm/d was measured in injured nerves maintained at 10°C.

The lag period for the beginning of the somatofugal degeneration decreases exponentially from >40 at 10°C to <5 h at 35°C. The variations can be expressed by the equation: $\log_{10} t(\text{days}) = -0.090T\ (°C) + \log_{10} 454$.

CHANGES IN NERVE DIAMETER: The proximodistal degeneration determined by the weight changes can be confirmed by light microscopy by measuring the area of nerve sections taken at various distances along the nerve (Table I). At the different temperatures investigated, the area of a nerve section in the degenerated zone is approximately half the area of a section taken in the distal zone.

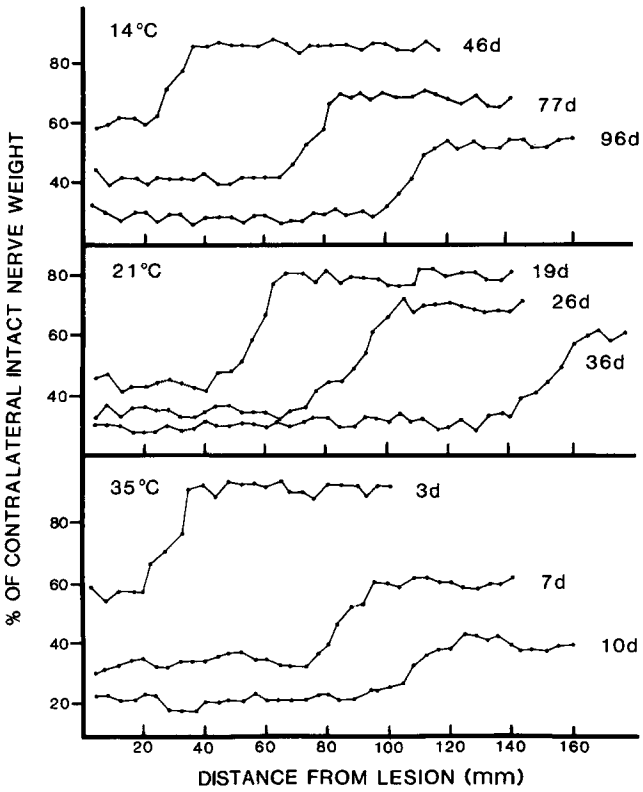


FIGURE 3 Proximodistal degeneration, as measured by the weight decrease of successive 3-mm nerve segments at 14, 21, and 35°C.

## Electron Micrographs

The results indicate that during degeneration four different areas of the nerve can be distinguished (Fig. 6). (*a*) The degenerated zone: In this area, next to the degenerative wave, no intact axons can be seen. The appearance of this segment varies greatly with temperature. At low and ambient temperatures the domains remain filled with axonal debris for extended periods of time (Figs. 6*a* and 7*a*). At high temperatures, the debris is removed rapidly and only collagen and Schwann cells can be seen (Fig. 8*a*). (*b*) The intermediate zone: In this area of the nerve, corresponding to the drop in nerve weight, degenerating axons can be seen (Fig. 6, *b*). Their diameter is one-third to one-fourth that of a normal axon. The inside is electron-dense and few internal structures can be recognized. (*c*) The distal zone: In this area (Figs. 6*c*, 7*b*, and 8*b*) the axons appear morphologically intact although a large proportion of them seems to contain neurofilaments associated with a decrease in the number of microtubules (Figs. 6*c* and 9, *b* and *c*). Intact axons are characterized by the presence of four to six microtubules and some endoplasmic reticulum (8) but neurofilaments are rarely seen (Fig. 9*a*). The number of axons in the distal area varies greatly with temperature: Preliminary data have shown a decrease from >70% of the original axonal population after 78 d at 10°C (Fig. 6*c*) to <20% after 10 d at 35°C (Fig. 8*b*). Various stages of axonal degeneration in the distal zone are shown in Fig. 9. The preterminal zone corresponding to the last few mm of nerve near the olfactory bulb (Fig. 6*d*): In this area, degeneration is much more advanced than in the distal zone, the number of intact axons is greatly reduced, and large amounts of debris are present. A more complete analysis of the changes in axonal populations is presently being performed.

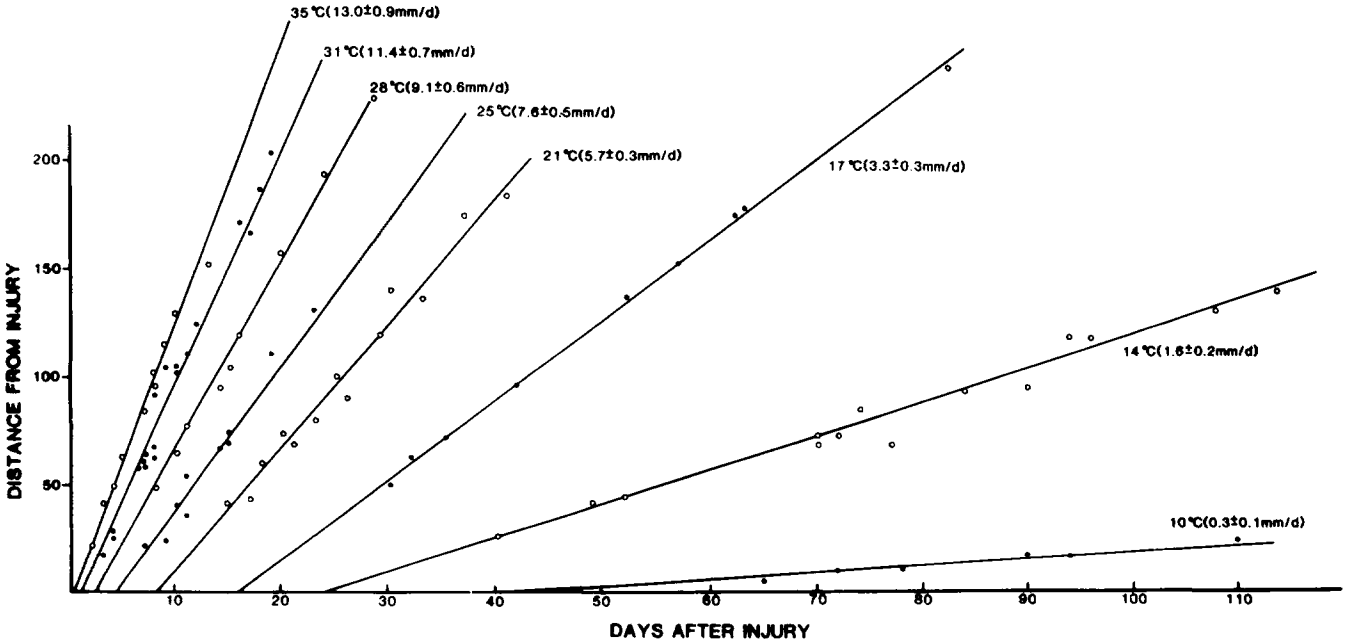


FIGURE 4 Degeneration velocities at temperatures ranging from 10 to 35°C. "Distance from injury" measured in millimeters.

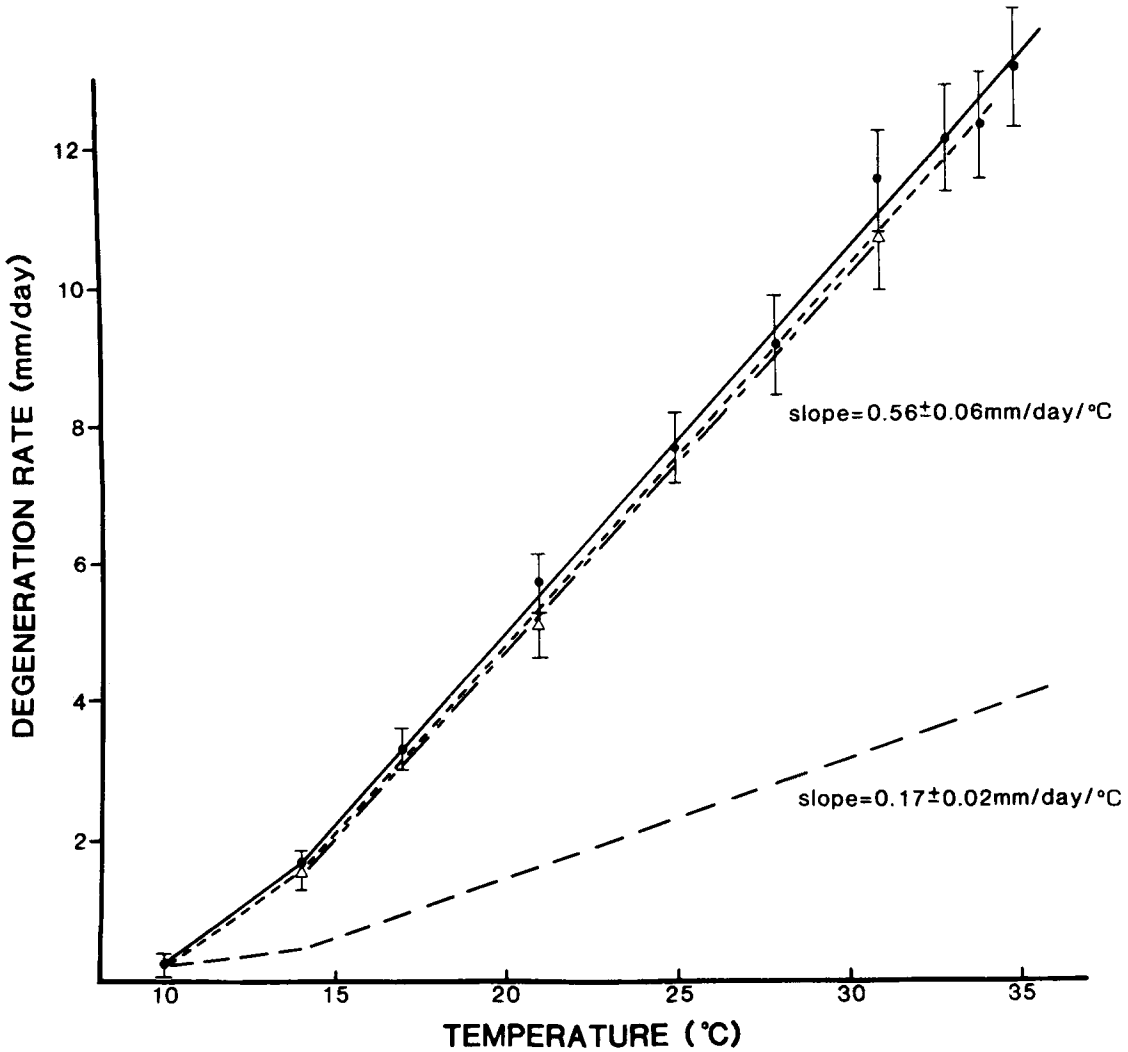


FIGURE 5 Velocity-temperature functions: (●) proximodistal degeneration, (△) slow flow in axons detached from their cell bodies, (---) slow flow in regenerating nerves, (——) slow flow in intact nerves. The velocity of proximodistal degeneration is identical to the rate of slow flow in degenerating and regenerating nerves and 3.3 times faster than the rate of slow flow measured in intact nerves.

TABLE I

*Area of Nerve Cross-sections**

| | | Distance from injury | | | |
|---|---|---|---|---|---|
| | | 20 mm | | 200 mm | |
| Temperature | Days after mucosa removal | % of contralateral nerve | $mm^2$ | % of contralateral nerve | $mm^2$ |
| 14°C | 115 | 37.9 ± 1.7 | 0.36 ± 0.02 | 52.9 ± 2.5 | 0.52 ± 0.02 |
| 21°C | 19 | 43.7 ± 2.1 | 0.56 ± 0.03 | 78.9 ± 1.4 | 1.01 ± 0.02 |
| 31°C | 10 | 28.1 ± 1.1 | 0.26 ± 0.01 | 67.9 ± 1.3 | 0.64 ± 0.01 |
| 35°C | 5 | 34.6 ± 1.5 | 0.23 ± 0.01 | 63.9 ± 3.2 | 0.42 ± 0.02 |

* Average of 4 different sections ± standard deviation.

## DISCUSSION

### *Proximodistal Degeneration of Unmyelinated C-fibers*

Degeneration can easily be followed since it results in a major weight decrease (Fig. 1). This shrinkage is not artifactual and can be recorded in fresh nerves or by measuring changes in the area of nerve cross-sections by light microscopy (Table I). Whether injury occurs by nerve crush or removal of the mucosa, degeneration progresses at the same velocity from the lesion toward the olfactory bulb. This observation precludes the possibility that degeneration of the severed axons is triggered by the arrival of new regenerating fibers.

Degeneration progresses linearly at all the temperatures investigated (10–35°C) at velocities ranging from 0.3 to 13 mm/d (Fig. 4). It represents a more than 40-fold increase in degeneration rate over a 25°C range. A large increase has also been reported by Joseph and Whitlock (4) after section of the

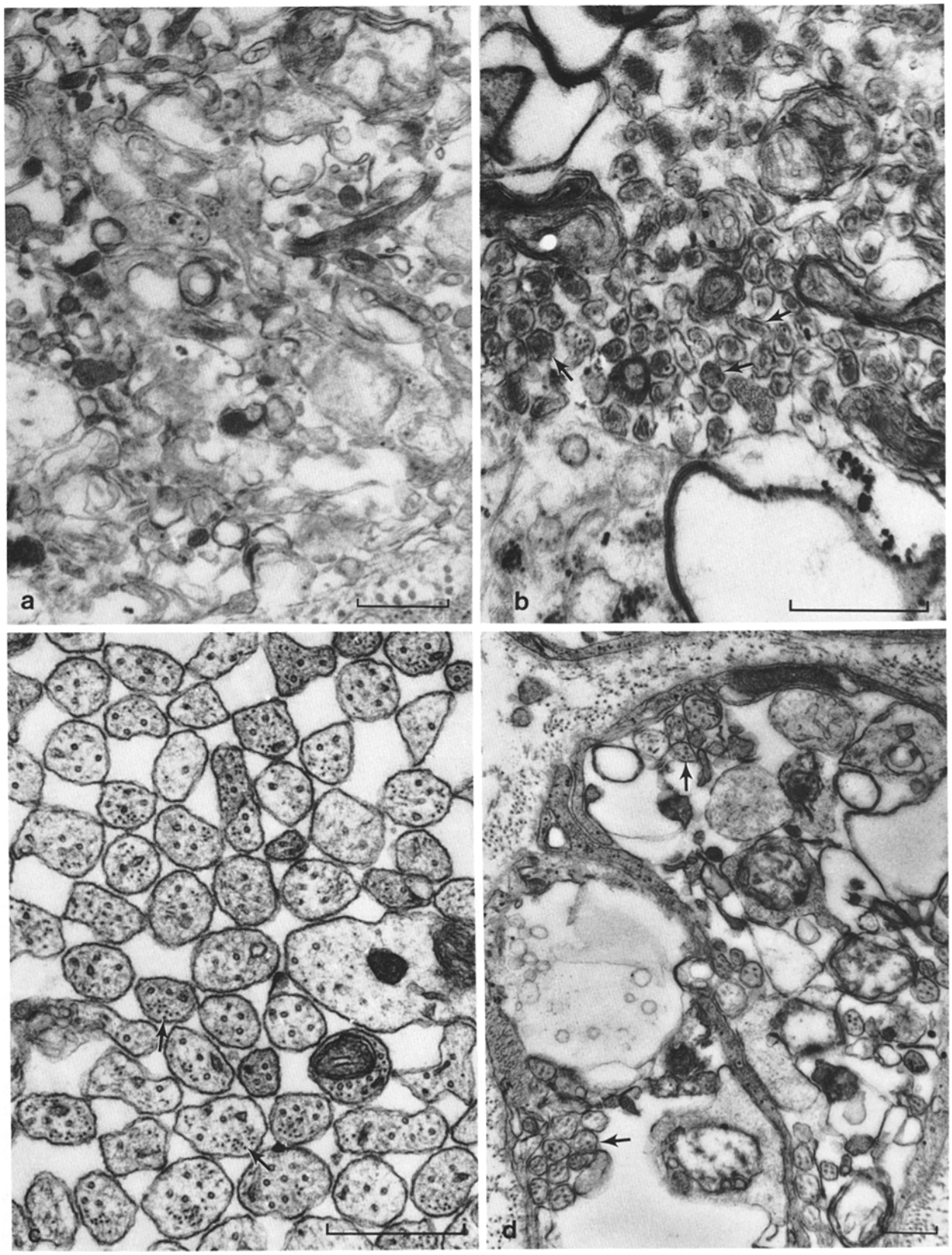


FIGURE 6 Electron micrographs of degenerating olfactory nerves at 10°C, 78 d post-operative (P.O.), (*a*) 5 mm from the site of injury: degenerated zone (× 35,000). This area does not contain any intact axons but is filled with membranous material. (*b*) At 15 mm: intermediate zone (× 45,400). Only shrunken axons (arrows) about a one-third to one-fourth of their normal size can be found. (*c*) At 120 mm: distal zone (× 45,400). The area contains numerous loosely packed axons. A large number of them appears to be filled with neurofilaments (arrows). (*d*) At 185 mm: preterminal area. The nerve segment close to the olfactory bulb contains fewer axons (arrows) (× 21,500). Bar, 0.5 μm.

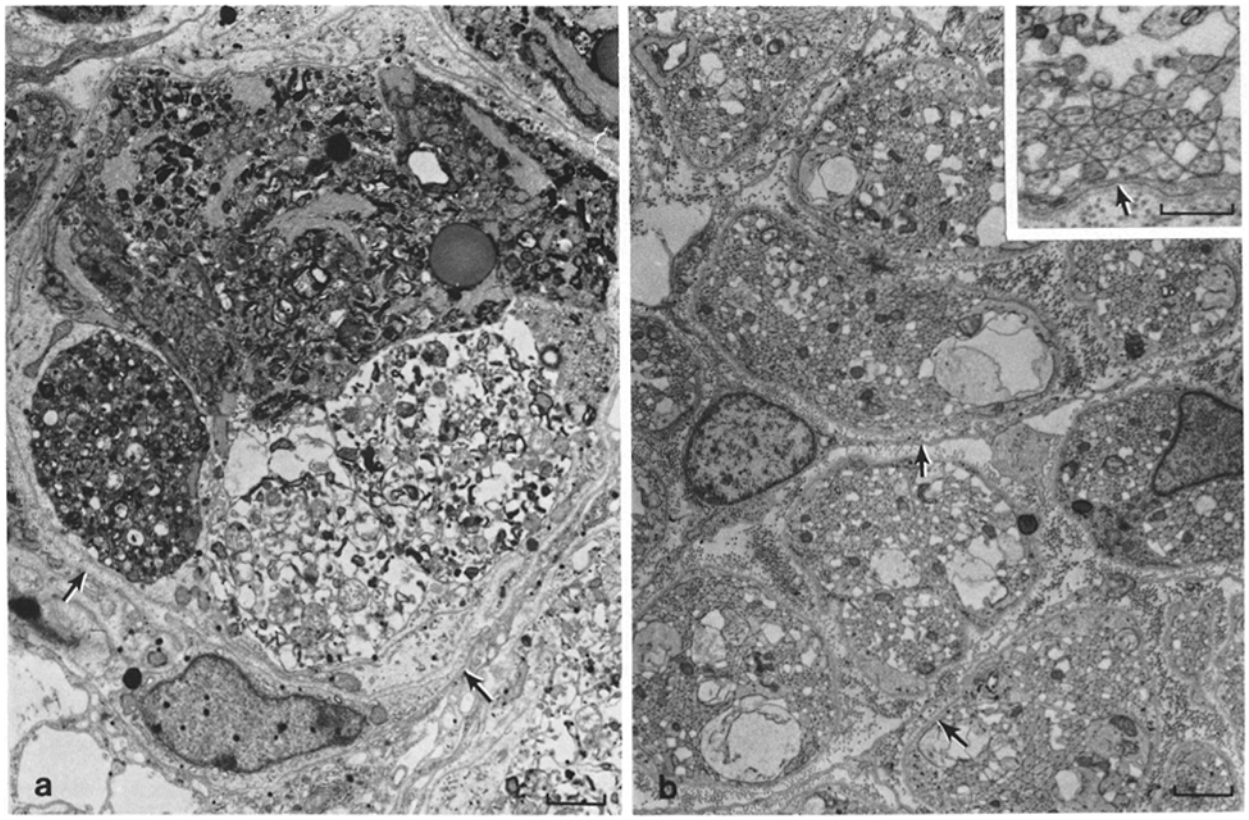


FIGURE 7 Electron micrographs of degenerating olfactory nerves 19 d P.O. at 21°C. (*a*) At 20 mm from the site of injury: degenerated zone. This area does not contain any intact axons. A Schwann cell (arrows) still surrounds a domain filled with membranous debris. Bar, 1 μm. × 6,200. (*b*) At 200 mm: distal zone. Numerous domains, surrounded by a Schwann cell and collagen (arrows), are filled with a large number of axons. Bar, 1 μm. × 6,200. *Inset*: Bar, 0.5 μm. × 18,600.

ninth spinal dorsal root of the toad: centrifugal degeneration progresses at an average rate of 1.15 mm/d at 20°C and 3.74 mm/d at 30°C.

## Slow Flow and Degeneration

We have seen previously (9) that, after removal of the mucosa containing the olfactory perikarya, slow flow in the isolated stump does not stop but continues at an accelerated velocity. At 14, 21, and 31°C, slow flow in axons detached from their cell bodies is 3.3 times faster than the rate measured in intact nerves (10). It increases linearly with temperature at the rate of 0.53 ± 0.06 mm/d/°C. The labeled material slowly transported in detached nerve stumps has a polypeptide composition (as determined by PAGE) similar to that determined previously for slow flow in intact nerves (9). Furthermore, these slow flow rates are identical to the velocities of slow transport in regenerating fibers in which the accelerated rate increases linearly with temperature (0.52 ± 0.04 mm/d/°C) between 14 and 35°C (11). Slow flow in both degenerating and regenerating fibers exhibits similar $Q_{10}{}^{15}_{25}$ (3.3 and 3.1, respectively). This $Q_{10}$ is also similar to that measured for the centrifugal degeneration process in the garfish olfactory nerve ($Q_{10}{}^{15}_{25}$ = 3.2). A similar value was determined in the toad CNS (3.5) by Joseph and Whitlock (4). Therefore, identical velocities were measured at various temperatures for slow flow in degenerating and regenerating axons and for the spreading of the degenerative process. We suggested previously (9) that the accelerated slow flow rate might be characteristic of axons lacking continuity from the perikarya to the synapses. A factor maintaining slow flow at an accelerated velocity might be repressed in intact neurons and released after injury. The acceleration of slow flow is triggered by the injury, not by the needs of the neurones. This link between degeneration and slow transport is corroborated by in vitro studies performed on neurite segments cut from their cell bodies: Shaw and Bray (12) have shown that, following nerve transection, the isolated neurite segment retracts and forms a bead but eventually grows again, from the distal end, by a volume equivalent to the volume lost by the proximal collapse. They found the rate of regrowth of the transected neurite to be equivalent to the velocity measured in intact growing neurites and they estimated it to be between 1 and 2.4 mm/d. Shaw and Bray also demonstrated that neurite retraction and regrowth are active phenomena that can be stopped by low temperature and lack of glucose. Regrowth was shown to be stopped by colchicine, an inhibitor of axonal transport. It is interesting to note that Shaw and Bray (12) prevented the retraction of the transected neurite with cytocholasin B, a fungal metabolite which has been shown to disrupt various mechanisms associated with microfilaments. These results strongly indicate that slow transport is still occurring in isolated neurites and that the movement of the transported material is similar in intact growing neurites or isolated segments.

ANATOMICAL EVIDENCE OF PROXIMODISTAL DEGENERATION: Evidence for somatofugal degeneration is also provided by electron micrographs (Fig. 6). A large number of apparently intact axons can always be seen in the distal region of a degenerating nerve, while no fibers are present in the proximal segment. A clue regarding the mechanism of proximodistal degeneration was provided by micrographs taken in

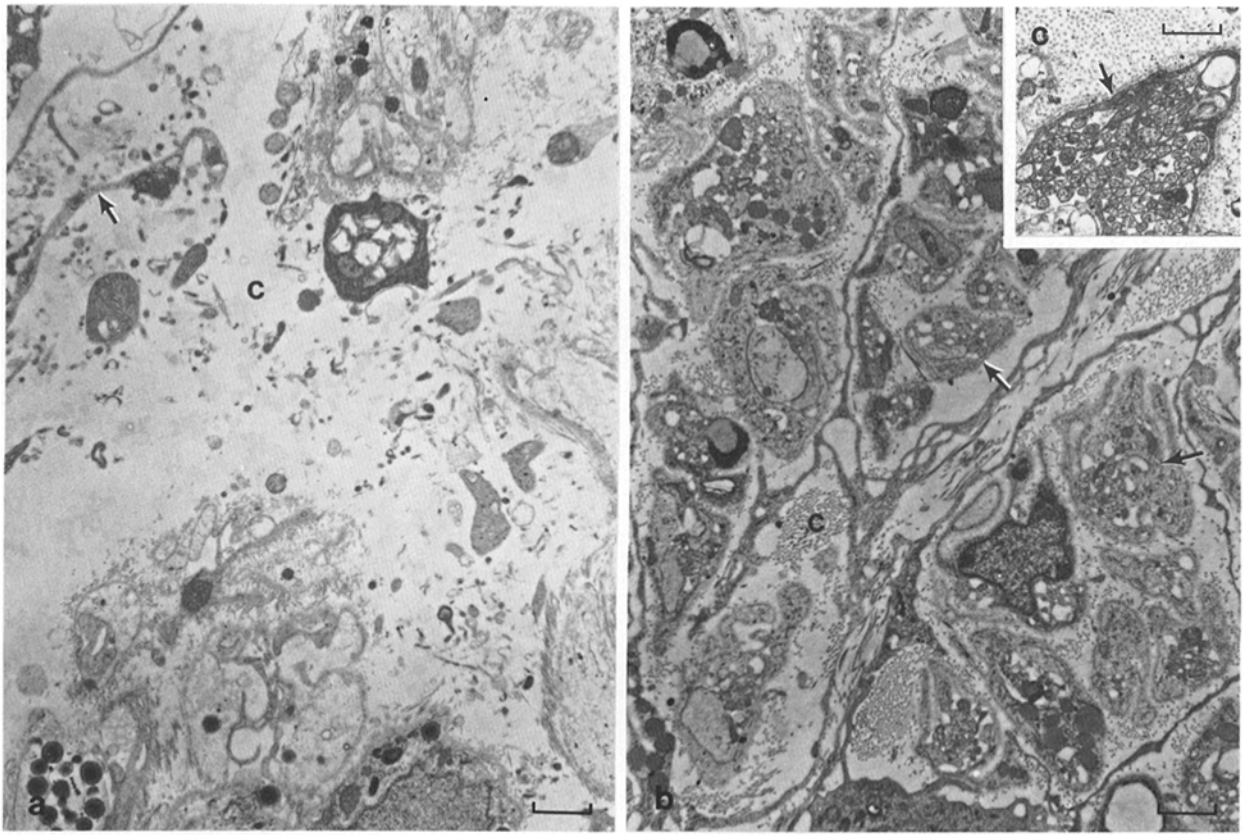


FIGURE 8 Electron micrographs of degenerating olfactory nerve 10 d P.O. at 35°C. (a) At 55 mm from the site of injury: degenerated zone. All the axons have degenerated, and most of the debris has been removed. The nerve is filled with collagen (C). Bar, 1 μm. × 6,200. Schwann cells can still be found (arrow). (*b*) At 210 mm: distal zone. The nerve contains large amounts of collagen (C). The axonal domains have been reduced to small fascicles of axons (arrows). (*Inset*) fascicle of axons. Bar, 0.5 μm. × 13,900.

the transition area between the degenerated and distal zones (Fig. 6*b*). In this area the axons are shrunken to a fraction of their normal size and do not appear to contain identifiable structures. These abnormal fibers might represent the last form of the degenerating axons once most of their components provided by slow flow have disappeared. The shrunken axons, which have been seen at all the temperatures under investigation, can be found only in the short intermediate segment between the distal and degenerated zones. They disappear completely from the degenerated zones where fragments of membranous appearance fill identifiable domains (Fig. 7*a*).

LAG PERIOD: A last piece of information regarding the mechanism of proximodistal degeneration was provided by the distance/time functions. These functions indicate that degeneration does not start before the end of a lag period. We have, however, previously seen that the rate of slow flow does not stop in the distal stump after axotomy (9). This lag period may represent the time necessary for the exhaustion of slowly transported molecules from the proximal nerve segment. It is not clear at the moment whether degeneration is triggered by the lack of one or a few molecules or by a massive dissappearance of proteins.

## Mechanism of Proximodistal Degeneration

In this study the slow proximodistal degeneration of the detached fibers is unequivocally linked to the depletion of the slow-moving components (Fig. 10). This may be best explained by Lasek's central theory of axonal transport (13). Preliminary experiments (Cancalon, P., S. T. Brady, and R. J. Lasek, unpublished observations) have demonstrated that in intact garfish olfactory nerve the two components of slow flow (Sca and Scb) are not distinct peaks but move as a single wave. Following cell body removal, new axonal elements are no longer provided. Since slow flow keeps moving in the detached stump (9), the proximal area of the severed axons becomes depleted of its matrix elements and, finally, the disappearance of the cytoskeleton brings about the collapse of the neuronal architecture, leaving only the axolemmal ghost as seen in the intermediate zone. The collapse of the axons progresses toward the nerve endings at a rate corresponding to the velocity of withdrawal of the cytoskeleton and therefore at the rate of slow flow in detached nerves. This proximodistal degeneration resembles the retraction of an isolated neurite after transection (12). Since the retraction can be stopped by cytochalasin B, it is likely that the neurite cytoskeletal elements are involved in the process.

Finally, the axolemmal ghosts are decomposed very rapidly into membranous components. At high temperature the axonal fragments are quickly disposed of by macrophages and Schwann cells (Fig. 8*a*). At low temperatures, on the contrary, they remain in the nerve for extended periods of time (Fig. 7*a*).

## Rapid Degeneration of C-fibers

Although a well defined somatofugal slow degeneration has been characterized at all the temperatures investigated, a significant degeneration also occurs in the distal zone before

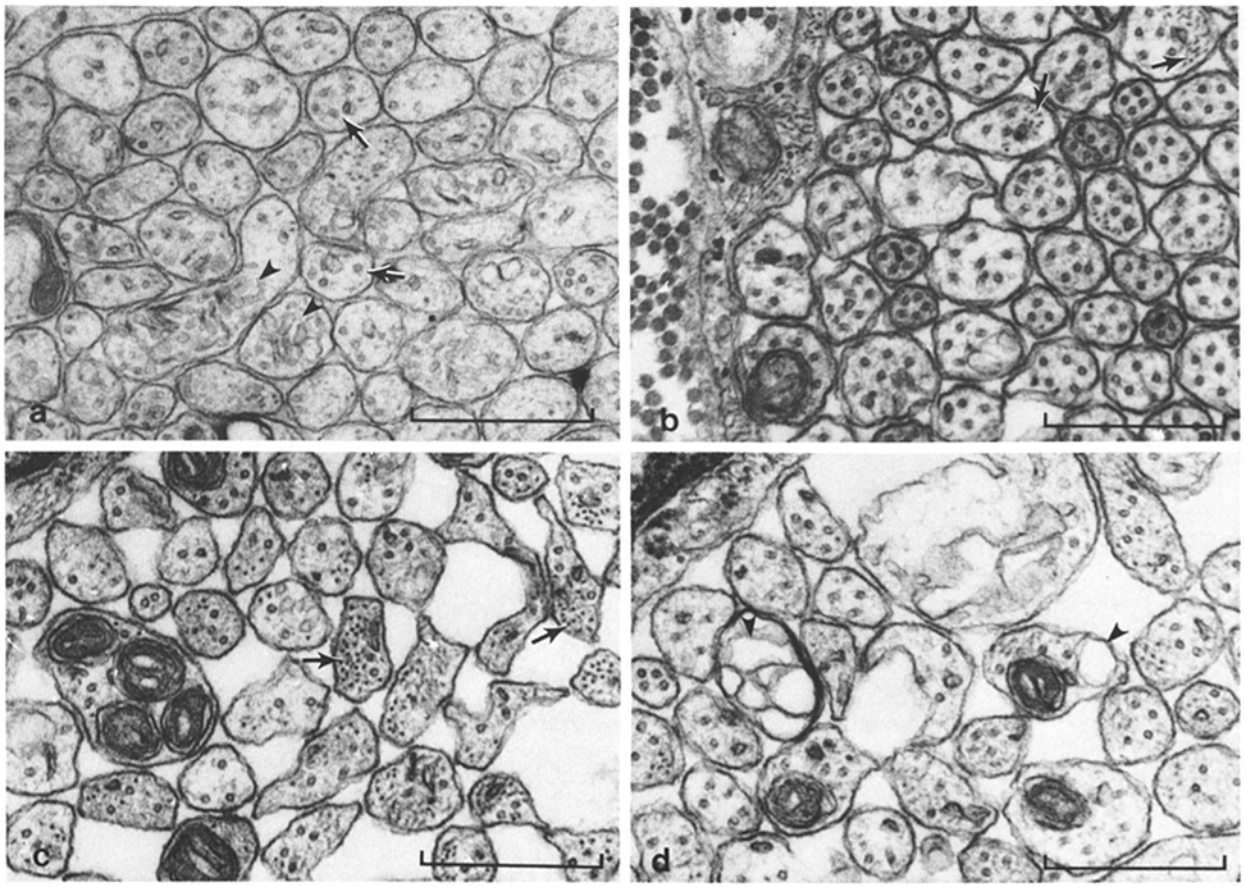


FIGURE 9 Since in the distal zone of the nerve some axons degenerate and others remain intact (see Discussion), various stages of axonal degeneration can be seen. (*a*) Intact nerve: the axons contain microtubules (arrows) and some endoplasmic reticulum (arrowheads) but few neurofilaments. Increasingly degenerated areas of the distal zone, 2–6 d P.O. at 21°C. (*b*) Densely packed axons. (*c*) Loosely packed axons. During degeneration, numerous axons seem to be filled with neurofilaments (arrows). (*d*) Axons becoming filled with vacuoles (black arrows). Bar, 0.5 μm. × 45,400.

the arrival of the slow wave. This distal degeneration can be demonstrated by a decrease in nerve weight and diameter. These changes most probably represent the compounded effect of numerous events occurring in the isolated stump, but they are mainly due to a decrease in the number of axons, although a slight decrease in axonal diameter was also noted. The decrease in the axonal population is highly temperature dependent (Figs. 7*b* and 8*b*).

Rapid changes in detached unmyelinated axons have been reported by various groups. A reaction of the distal segment of the rabbit olfactory nerve characterized by the swelling and vacuolization of numerous axons has been observed as early as 8 h after lesion of the nerve (14). Matsumoto and Scalia (15) estimated that, in frogs maintained at 20°C, more than half of the unmyelinated optic axons have disappeared in the first 10 d after injury but that 12% remains after 10 w.

An explanation for the rapid degeneration of isolated axons has been provided by Schlaepfer (16) who demonstrated that this type of degeneration is induced by calcium-dependent proteases affecting neurofilaments. The author postulated that the increase in proteolytic activity might be triggered by an influx of calcium inside the axon due to a higher permeability of the axolemma. The changes in the membranous properties in turn seem to be due to a lack of membranous components delivered by fast axonal transport. Indeed, Schlaepfer and Bunge (17) characterized a similar type of degeneration after interruption of fast transport with vincristine.

It remains to determine why certain fibers are rapidly affected while others remain intact for extended periods of time (Fig. 9). A constant turnover of the olfactory neurones has been demonstrated (18) and several studies (19, 20) have indicated that young axons might lack axolemmal protein components. Such fibers which already have an immature membrane might be less resistant to an influx of calcium and eventually to degeneration than older axons having a fully developed membrane.

The degree of degeneration of the distal zone is not equivalent over the entire nerve segment. Degeneration was much more extensive in the last few millimeter of the nerve. It has indeed been shown in various studies that in individual fibers the nerve endings and the preterminal branches degenerate earlier than the part of the axon running in the nerve trunk (21, 22). Lasek and Black (23) have postulated that in intact axons the neurofilaments and microtubules are degraded by proteases when they reach the synaptic area. Considering the mechanism of degeneration put forward by Schlaepfer (16), a higher concentration or the presence of more active proteases might be responsible for a faster decay of the nerve segment close to the synapses. Alternatively, this distal degeneration can be triggered by a rapid decomposition of the synaptic area due to a lack of synaptic components no longer provided by fast transport.

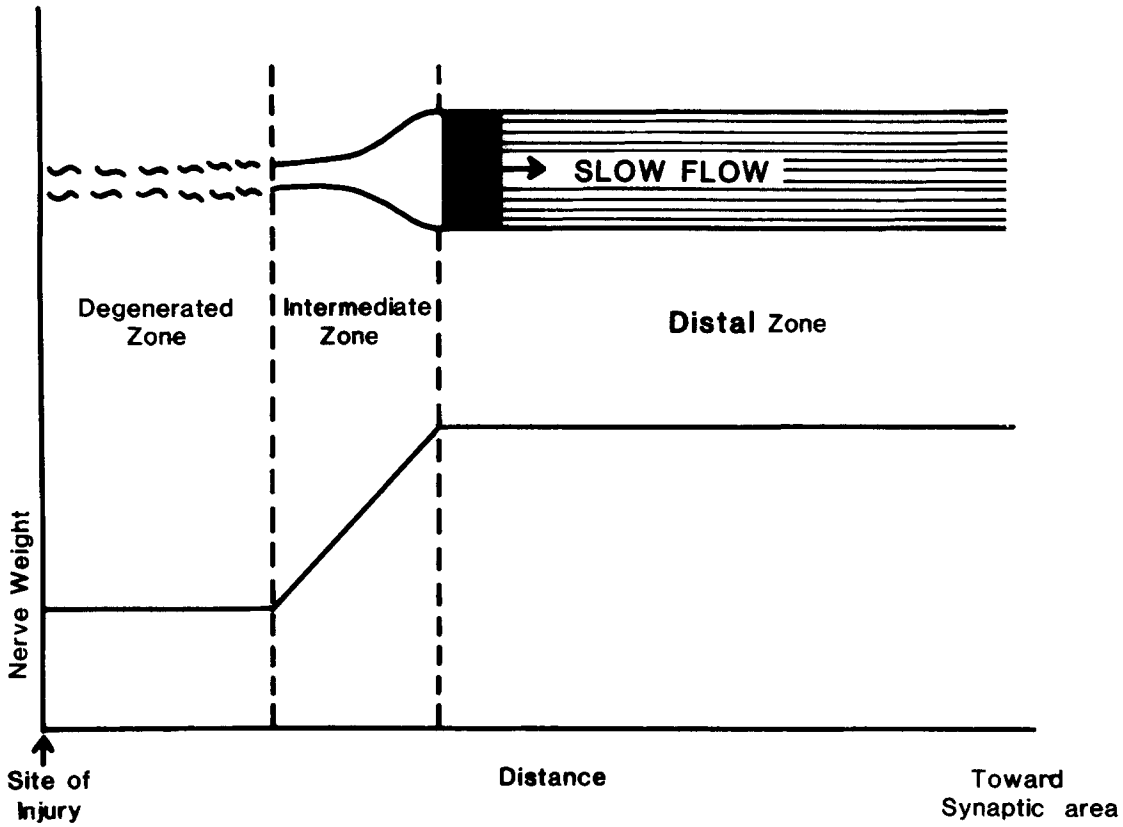


FIGURE 10 Schematic of slow proximo-distal degeneration: The proximal area of the axon becomes depleted of cytoskeletal elements, no longer provided by the cell body, as slow flow moves centrifugally. As a result the axon collapses and is rapidly decomposed.

## Conclusion

We conclude that a lack of both rapidly and slowly transported components triggers axonal degeneration. Wallerian degeneration per se has been more specifically associated with the rapid phase of degeneration (6, 7) and might be the only degenerative process occurring in myelinated fibers where the association between the axolemma and the myelin is essential for axonal survival. A large number of unmyelinated fibers on the contrary is able to survive the initial lack of rapidly transported elements and collapse only with the withdrawal of the slowly moving cytoskeleton.

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