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THE UNIVERSITY OF ALBERTA

PROPERTIES OF THE PARENT-DAUGHTER AXONAL JUNCTION IN REGENERATING NERVE

by

(C) HONEY CHAN

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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OF MASTER OF SCIENCE

IN

EXPERIMENTAL SURGERY

DEPARTMENT OF SURGERY

EDMONTON, ALBERTA

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled PROPERTIES OF THE DAUGHTER-PARENT JUNCTION IN REGENERATING NERVE submitted by HONEY CHAN in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE in EXPERIMENTAL SURGERY.

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Date: MARCH 22, 1989

ABSTRACT

The primary aim of this work was to investigate the properties of rapid axonal transport in regenerating myelinated axons in the sciatic nerve of Xenopus laevis, with particular attention to events at the junction between the proximal, intact axon (the *parent*) and the distal, newly formed axon (the *daughter*). Morphological studies indicated that all myelinated axons initiated regeneration and that at least 80% of these axons regenerated at a rate of 1 mm/day or greater (20°C). The ultrastructure of the junctional region was examined at regeneration times between 3 days and 20 weeks. The main qualitative change in the junctional axoplasm over this period was in its content of particulate organelles. At times up to 2 weeks regeneration the junction contained abnormal numbers of 50 nm diameter vesicles and 10 nm granules. Between 2 and 5 weeks the junction showed in addition a peripheral rim of large membrane-bounded organelles around a central core of microtubules and neurofilaments. At longer times the numbers of large membrane-bounded organelles diminished and all junctions contained prominent accumulations of 10 nm granules. The rate of rapid axonal transport of protein was similar in parent and daughter axons. Compared to the parent axons, a 2-5 times greater amount of protein was deposited to a stationary phase in daughter axons. Specimens of nerve that were subjected to mechanical stress during the removal of the perineurium showed a large accumulation of rapidly transported protein in the region of the crush at regeneration times up to 40 days; some of the accumulated protein was

subsequently transported retrogradely. Video microscopy of isolated axons supplied evidence that the transport deficit in mechanically stressed nerve was a partial block of anterograde vesicle transport, plus a reversal of anterograde transport, at the junction of parent and daughter axons. No structural changes were detected in mechanically stressed nerve. The results show that the junction between parent and daughter myelinated axons is a region with distinct morphology at which the dynamics of anterograde axonal transport may change dramatically.

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1 INTRODUCTION

The neuron is a cell of remarkable architecture. Its processes often reach far from the cell body and these contain cytoplasm with thousands of times the volume of the cell body. The cytoplasm of the axon, with few exceptions, does not contain ribosomes, hence the axon is essentially incapable of protein synthesis. Materials required for maintaining the function and structure of the axon are supplied by the neuronal cell body and are delivered to the axon by mechanisms known as fast and slow axonal transport.

The neuron also possesses the capability to regenerate. When an axon is crushed or severed, the segment of axon distal to the site of damage undergoes Wallerian degeneration. During regeneration, the distal segment of the axon is replaced by outgrowth from the proximal stump of the damaged axon. Thus, a regenerating axon consists of a proximal *parent* axon which is developmentally mature, and a distal *daughter* axon which is in a state of growth and maturation. Materials necessary to ensure successful regeneration are supplied by the neuronal cell body and are carried down the axon by axonal transport.

The junctional region between the parent and daughter axons is of interest in the study of the role of axonal transport in axonal regeneration. Processes that may be unique to this region are as follows:

a) At this region it is expected that there is an abrupt change in the requirements of the axon for transported material. The parent axon may retain many of its original properties and only require

materials to maintain its structure and function, while the daughter axon may be more actively involved in the incorporation of transported material.

b) The junctional region may be an important source of cytoskeletal elements at least during the initial development of axonal sprouts. Axonal sprouts are reported to occur as early as 4 hours after nerve crush (Zelená et al., 1968). These sprouts contain cytoskeletal components (microtubules and neurofilaments) that are normally transported by the axon at a rate no greater than a few millimeters per day. Unless the rate of transport of the structural materials from the cell body increases dramatically during regeneration, cytoskeletal elements of the new sprout can only be derived from existing sources in the immediate vicinity of the junction. The movement of cytoskeletal building blocks from the parent axon to the growing sprout may involve the reorganization of the cytoskeletal structures in the junctional region.

c) Studies of the transport of radiolabeled proteins and lipids in regenerating nerves show that while proteins and lipids are rapidly transported from the parent nerve into the daughter nerve, a significant fraction of these materials is retained in the region of the lesion (Carlsson et al., 1971; Bisby, 1978; Forman and Berenberg, 1978; Alberghina et al., 1983a,b; Danielsen et al., 1986). In some cases, the accumulation of transported materials at the lesion occurs during the phase of axonal elongation (2 to 4 weeks following injury) (Bisby, 1978; Forman and Berenberg, 1978), while in others, transported materials continue to

accumulate at the original site of injury even on functional recovery of end-organs. (Alberghina et al., 1983b; Danielsen et al., 1986). Several cellular bases have been suggested to explain this phenomenon. For example, transported materials may accumulate in the ends of axons which have not regenerated beyond the region of injury, or in the ends of aberrant or disoriented axons that fail to grow beyond the site of the lesion (Bisby, 1978; Forman and Berenberg, 1978). Other possible contributing factors which are not directly related to axonal growth processes but may cause an apparent accumulation of materials at the lesion include: blockade by local scarring and axonal constriction, increased local incorporation of blood-born radiolabels due to proliferation and hypertrophy of Schwann cells or epineurial sheath cells, and break-down of the diffusion barrier at the site of lesion (Bisby, 1978; Forman and Berenberg, 1978).

d) Recently, Tedeschi and Wilson (1987) reported that some species of rapidly transported polypeptides are selectively released by the daughter axons into the perineurial space. These polypeptides appeared to originate particularly from the site of damage. It was suggested that the released polypeptides may be involved in intercellular signalling. This would imply that the junctional region performs a specific signalling function which may be related to the process of regeneration.

This thesis describes studies of rapid (fast) axonal transport of radiolabeled proteins and of optically detectable organelles in regenerating sciatic nerves of the toad *Xenopus laevis*. In these

dynamic studies of intra-axonal movement of materials in the living nerve, particular attention was given to the events that occur at or close to the junction when regeneration was well under way. In addition, the structural features of the junction between parent and daughter axons were examined with both the electron microscope and video microscope. The results of this work have been accepted for publication (Chan et al., 1989).

The remainder of this chapter reviews current literature related to this work. It includes a historical perspective, an outline of some of the characteristics of axonal transport that are pertinent to this study, and a consideration of techniques that are used to detect axonal transport. The roles and characteristics of fast axonal transport in regeneration are also reviewed.

1.1 Historical perspective

As early as the middle of the 19th century, observations by Waller (1852) on the effect of injury in peripheral nerve led to the suggestion that the integrity of the axon depends on its structural continuity with the cell body. Several decades later, F. H. Scott (1906) postulated that the cell body of the neuron synthesizes substances which pass into the nerve fiber and eventually reach the nerve endings. He also suggested that these substances are transported as particles. Ramón y Cajal (1928) viewed the axon as a protoplasmic extension and the cell body as a "trophic center" which supports the axon. He suggested that the cell body sends substances to its axon through the cytoplasm. In his work with damaged axons, he saw fine regenerating fibers in and distal to partially constricted regions of

nerves and a series of bulges and constrictions in the nerve fibers just proximal to the constriction. He interpreted these observations as representing a process of nerve regeneration. In analogous experiments, Weiss and Hiscoe (1948) found similar irregular swellings in the axons above a partial constrictions. They viewed the swellings as a piling up or "damming" of the axoplasm and concluded from these experiments that the axoplasm advanced as a coherent column, continually flowing down within the fibers. They suggested that the movement of axoplasm "serves to replace catabolized protoplasmatic systems which cannot be synthesized in the peripheral cytoplasm". The rate of such flow was estimated at 1 to 2 mm per day. This supposition seemed to agree with the hypothetical deductions put forward by Waller a century earlier. However, subsequent re-examination of these results (Spencer, 1972) did not confirm the finding of Weiss and Hiscoe. Spencer suggested that Weiss described an effect that did not occur in normal axons, and that the swellings proximal to the constrictions represented the early onset of Wallerian degeneration.

When radioisotope-labeled amino acids became available in the '60s, they were recognized as useful tools for investigating axonal transport. An important advance was made by Droz and Leblond (1963) when they injected ^{3}H-leucine systemically in rats to label proteins. A wave of radioactive proteins was found to move down the sciatic nerve at a rate of about 1 mm/day, ironically the same value originally suggested by Weiss and Hiscoe (1948). Other studies such as that of Miani (1963) in which he examined movement of labeled phospholipid in axons suggested the possibility that some

components of the cytoplasm might move at a rate faster than 1 mm/day. More convincing evidence for a more rapid transport was presented by Lubinska and her colleagues (1964) and Dahlström (1965) in experiments in which they studied the accumulation of transmitter-related materials at a lesion. These studies revealed that some substances moved from the cell body to the nerve terminals at a fast rate of several hundred millimeters per day. Moreover, a movement of materials from axonal terminals to the cell bodies was demonstrated. The velocity of movement of these materials towards the cell body also occurred at a fast rate (Lubinska et al., 1964). As a consequence, it was gradually recognized that instead of a constant movement of the entire column of axoplasm down the nerve fiber, as proposed by Weiss and Hiscoe, there are different components of the axoplasm moving at different rates in the anterograde direction towards the nerve terminals. Also, there is a retrograde component of axonal transport which moves materials rapidly toward the cell body.

Interpretation of the results from early tracer experiments was hindered due to non-selective labeling caused by systemic injection. To increase the labeling of neurons and to decrease labeling of non-neuronal cells, radiolabeled amino acids as protein precursors were injected in the vicinity of the cell bodies (Grafstein, 1967) or directly into ganglia (Lasek, 1968a,b). This improvement in technique allowed a clear demonstration that two waves of radiolabeled proteins moved down the axon in the anterograde direction: a fast rate ranging from 100-500 mm per day and a slow rate at 1-2 mm per day.

The discovery of the two rates of transport came at a time when time-lapse cinemicrography of the axoplasm of living axons was possible. Observations of intraaxonal organelles using bright field, phase contrast and differential interference contrast light microscopy showed that indeed there were anterograde and retrograde movement of optically detectable organelles in cultured neurons (Hughes, 1953; Nakai, 1956; Burdwood, 1965). A bidirectional transport of organelles was also demonstrated in mature axons (Smith, 1971, 1972; Kirkpatrick et al., 1972, 1973). The velocity of movement of these organelles is similar to the fast rate of transport obtained from radiolabeling studies (Smith, 1971, 1972). Moreover, the evidence also led to the suggestion that retrogradely transported organelles and anterogradely transported organelles are structurally different.

Analyses of the biochemical composition of the two different anterograde components of protein transport (fast and slow) revealed that they differ from each other not only in the rate of transport but also in the quantity, composition and intracellular localization of the materials transported (McEwen and Grafstein, 1968). About 20% of transported radiolabeled materials is carried by fast transport. The remaining 80% is transported slowly. Materials that are rapidly transported consist mostly of plasma membranes and membrane-associated materials. In contrast to fast transport, the slow component transports mostly fibrous cytoskeletal proteins. More recently, as a result of increasingly detailed analyses of the velocities and composition of transported materials, more components of axonal transport have been identified.

1.2 Characteristics of axonal transport

Although the present work is centered on axonal transport in regenerating axons, it is nevertheless necessary to introduce some properties of axonal transport in normal axons. This section reviews literature on some of the characteristics of axonal transport. These are:

a) the rates of different components of axonal transport;
b) the composition of materials transported at the different rates;
c) the movement of optically detectable organelles;
d) the mechanism of fast axonal transport;
e) the turnaround of rapidly transported materials; and
f) the deposition of materials along the axon by fast axonal transport.

The available information on axonal transport is vast and complex; characteristics of axonal transport not covered in this thesis are available in reviews of the literature (Schwartz, 1979; Grafstein and Forman, 1980; Ochs, 1982), and in the proceedings of international conferences (Weiss, 1982; Smith and Bisby, 1987).

1.2.1 Rates of transport

The original categorization (Lasek, 1968a,b) of axonal transport in terms of fast and slow components has been much elaborated. Current evidence from studies of axonal transport of proteins indicates at least five distinct rate components of anterograde transport and one rate component of retrograde transport (Lorenz and Willard, 1978).

Table 1 represents a summary of the work on the rate components of axonal transport of some commonly studied systems. Among these systems, the optic axons and the peripheral motor axons of the mammalian system are well characterized. Since the present work uses the sciatic axons of the amphibian Xenopus laevis, velocities of different components of transport in amphibian peripheral axons, if available in the literature, are also included. The rates cited in Table 1 are at the normal body temperature of the animals.

As stated earlier, fast axonal transport can also be studied by investigating the movement of optically detectable organelles. Table 2 presents some examples of the velocities of organelles as measured in various preparations.

Table 1 shows that the rates of different components are not the same in different nerves. In addition, although fast and slow transport cover wide range of velocities, the rates are so different that they do not overlap. Also, since axonal transport is a temperature sensitive process with a Q_{10} of 2-3 (Edström and Hanson, 1973; Takenaka et al., 1978; Smith and Cooper, 1981), velocities of axonal transport are generally faster in homeotherms than in poikilotherms.

Table 2 indicates that rapid retrograde transport is not necessarily slower than anterograde transport (Forman et al., 1987).

Table 1

Rate Components of Axonal Transport

		Max. velocities (mm/day)		
Transport group		Optic axons mammalian (38°C)	Peripheral motor axons mammalian (38°C)	Peripheral axons Amphibian (20-23°C)
Anterograde	Willard[1] Grouping			
Fast	I	>240[1]	410[3]	180[7]
	II	34-68[1]	-	-
Intermediate	III	4-8[1]	-	25[6]
Slow SCb	IV	2-4[1]	2-5[4]	-
SCa	V		0.7-1.1[1]	1.0-1.7[4]
Retrograde				
Fast		>72[2]	120-288[5]	158[7]

References

1. Lorenz and Willard (1978)
2. Halperin and LaVail (1975)
3. Ochs (1972)
4. Hoffman and Lasek (1980)
5. Kristensson and Olsson (1975)
6. Partlow et al. (1972)
7. Snyder (1986a)

Table 2

Velocities of Organelle Movement

Preparation	Types of organelles	Mean velocity µm/s (mm/day) Anterograde	Retrograde	Temp.
Lobster:giant axon of leg[1,2]	Mitochondria	0.72 (62)	1.33 (114)	21°C
	Particles*	0.63 (54)	1.73 (149)	21°C
Squid:giant axon[3]	Particles	2.5 (216)	2.5 (216)	21°C
Frog:sciatic axons (Rana catesbiana)[4]	Mitochondria	-	0.79 (68)	28°C
	Particles	1.08 (93)	-	28°C
Frog:sciatic axons (Xenopus laevis)[5]	Particles	0.93 (79)	0.91 (77)	22°C

* Particles refer to circular or elliptical images observed with light microscope, the dimensions of these particles are close to the wavelength of light.

References

1. Forman et al., 1987.
2. Smith and Forman, 1987
3. Allen et al., 1982a
4. Forman et al., 1977
5. Koles et al., 1982

1.2.2 Biochemical composition of transported materials

Different molecular types of materials are carried by different rate components of axonal transport. Recent work on the separation and identification of the composition of transported materials has revealed that many proteins are involved, many have yet to be identified and can only be distinguished by their apparent molecular weights and isoelectric points as determined by 2-dimensional SDS gel electrophoresis.

The type of proteins and cytoplasmic structures carried by the different components are very consistent among the various neuron types. Table 3 summarizes the major protein composition and cytoplasmic structures carried by the different anterograde and retrograde components. In addition to the materials given in Table 3, lipids (Abe et al., 1973; Sherbany et al., 1979) and nucleic acids (Lindquist et al., 1981; Ingoglia and Zanakis, 1982) are also transported rapidly.

1.2.3 Organelle transport

The cytoplasm of axon contains heterogeneous types of membrane-bounded organelles of various sizes and shapes (Smith, 1980; Tsukita and Ishikawa, 1980; Fahim et al., 1985). Typical dimensions range from 50 nm to 1 µm in diameter; mitochondria may be as long as 50 µm in some axons (Forman et al., 1987). The movement of these organelles, including those with dimensions below the resolution of the light microscope, can be detected with enhanced video microscopy (Allen et al., 1982a; Inoué, 1986).

Table 3

Composition of Transport Materials

Rate component	Biochemical composition	Cytological structure
Anterograde fast		
I	glycoprotein, phospholipids, transmitter-associated enzymes(eg. BDH,AChE), Na^+,K^+-ATPase, GAPs, serotonin, catecholamine	50-nm tubulovesicular structures
II	F1ATPase	mitochondria
III	myosinlike protein	
Retrograde fast	glycoproteins, phospholipids, lysosomal hydroxylase, NGF	Prelysosomal structures
Anterograde slow		
SCb	actin, clathrin, spectrin, calmodulin, some tubulin	microfilaments, clathrin complex,
SCa	neurofilament triplet, tubulin, tau proteins, spectrin	microtubules, neurofilaments

Current evidence strongly suggests that the movement of these organelles represents the basis of fast axonal transport. For example, the velocity of these organelles is similar to the fast anterograde and retrograde transport rates obtained from protein transport studies (Cooper and Smith, 1974; Allen et al., 1982b). Biochemical analysis of the composition of rapidly transported materials indicates that these molecules are associated with membranes (Lorenz and Willard, 1978). Conditions, such as changes in temperature and application of certain pharmacological agents, that cause an alteration in protein transport also induce a similar change in the movement of intraaxonal organelles (Hammond and Smith, 1977; Smith and Cooper, 1981; Smith, 1982). Autoradiographic studies using ^{3}H-glycerol as a precursor have demonstrated that labeled phospholipid is incorporated selectively into transported vesicular structures (Pfenninger, 1982). In addition, retrogradely transported horseradish peroxidase has been shown to be contained within discrete membrane-bounded organelles (LaVail et al., 1980).

The morphological character of optically detectable organelles has been examined in amphibian sciatic nerve (Smith, 1980), in mammalian peripheral axons (Tsukita and Ishikawa, 1980), in squid giant axons (Fahim et al., 1985), and motor axons from the walking legs of lobster (Forman et al., 1987). These ultrastructural studies demonstrated that although the type of organelles transported in either direction are similar among the different species, anterogradely transported organelles are structurally different from those that are retrogradely transported. Anterogradely transported organelles consist mostly of small

50 nm-diameter vesicular and tubular membranous structures, some dense-cored vesicles, and few mitochondria. By contrast, retrogradely transported organelles are generally larger, they include various lysosomes and pre-lysosomal bodies (multi-lamellar bodies and multi-vesicular bodies), mitochondria, as well as some small vesicles.

1.2.4 Mechanisms of fast axonal transport

Axonal transport requires at least 3 basic elements: a structure which exhibits the movement; a substrate with which the structure interacts to produce the movement; and a motor or translocator which generates the movement.

Anterograde and retrograde fast axonal transport consist of the movement of vesicles and membrane-bounded organelles (Smith, 1980; Tsukita and Ishikawa, 1980; Fahim et al., 1985). There is extensive evidence that microtubules are the cytoskeletal substrate with which the organelles interact to generate both anterograde and retrograde movement (Banks et al., 1971; Dahlström, 1971; Hammond and Smith, 1977; Smith, 1980; Allen et al., 1985b; Vale et al., 1985a; Schroer et al., 1985). Using preparations of extruded and dissociated squid giant axoplasm, it has been demonstrated that organelles move in both directions along the same microtubule (Allen et al., 1985b; Schnapp et al., 1985; Vale et al., 1985d; Langford et al., 1987).

There is good evidence for the existence of two different translocator proteins that control the direction of organelle transport (Vale et al., 1985d). One of these, kinesin, an ATPase that was first

isolated from the soluble fraction of the squid axoplasm (Vale et al., 1985b,c), supports organelle motion along microtubules in the anterograde direction (Vale et al., 1985c); the other translocator, probably a cytoplasmic dynein ATPase distinct from the dyneins of cilia and flagella (Gibbons, 1987; Paschal and Vallee, 1987; Vale, 1987), supports retrograde organelle motion.

The structural organization of the vesicle-microtubule complex has been examined by electron microscopy (Miller and Lasek, 1985; Langford et al., 1987; Weiss et al., 1987). Cross-bridges are seen preferentially anchored onto the surface of vesicles. However, in the presence of AMP-PNP (a non-hydrolyzable analogue of ATP), the cross-bridges become attached to microtubules, a behavior identical to that of kinesin (Brady, 1985; Vale et al., 1985c). Therefore, the workers proposed that these cross-bridges contain the translocator molecules which, via an ATP-dependent process, mediate vesicle and organelle transport (Miller and Lasek, 1985; Weiss et al., 1987).

1.2.5 Reversal of transport

Studies of rapid anterograde transport of endogenous materials indicate that some of the transported materials are returned towards the cell body via retrograde transport (Bisby and Bulger, 1977; Armstrong et al., 1985; Bisby, 1987). It is estimated that in rats as much as 50% of transported protein undergoes reversal of transport (Bisby and Bulger, 1977).

Current findings suggest that the reversal of endogenous materials occurs at the normal nerve endings (Bray et al., 1971; Abe

et al., 1974; Bisby and Bulger, 1977; Bisby, 1987), at an artificial ending caused by a lesion (Bisby and Bulger, 1977; Schmidt et al., 1980; O'Brien and Snyder, 1982), and at the tips of regenerating axons (Bulger and Bisby, 1978; Griffin et al., 1981).

Reversal of organelle transport on both sides of a lesion has been studied in vertebrate axons (Smith, 1987, 1988). The process of direction reversal includes a period of longitudinal oscillatory motion. Eventually, organelles move consistently in the direction away from the lesion. Hence, organelles originally moving in the anterograde direction become transported in the retrograde direction, and similarly, organelles originally moving in the retrograde direction become transported in the retrograde direction.

The results from organelle studies show that the reversal of organelle transport is not instantaneous (Smith, 1987, 1988). The delay is due to the time the organelle spends in its oscillatory movement before definitive direction reversal. This is in agreement with results obtained from protein studies which show that the reversal of transported proteins at a lesion is delayed for a similar period (Bisby and Bulger, 1977; Snyder, 1986a).

The mechanism of the reversal of transported proteins in nerve endings is not understood. However, some progress has been made towards an understanding of the mechanism underlying the reversal of organelle at a lesion. In vertebrate axons where two different molecular translocator motors appear to exist (Vale, 1987), it is possible that the two motors can bind to individual organelles that normally travel in either the anterograde or retrograde directions (Koles et al., 1982; Kendal et al., 1983). Reversal of the

direction of transport would then occur when the predominant type of motor binding to an organelle is altered (Smith, 1987, 1988).

While it is reasonable to assume that the purpose of reversal and retrograde transport of endogenous materials at nerve endings is to return exhausted materials to cell bodies for degradation (Nagatsu et al., 1976; Bisby, 1987), the physiological significance of reversal at a lesion is unclear. It is speculated that in axotomy, the reversal and retrograde transport of materials prematurely to the cell body serve as a signaling mechanism that initiates chromatolysis and other injury-related responses (Bisby, 1987).

1.2.6 Deposition of rapidly transported materials

Results from studies of fast anterograde transport of radiolabeled proteins, glycoproteins, and phospholipids (Gross and Beidler, 1975; Ochs, 1975; Bisby, 1976, 1978; Tessler et al., 1980; Alberghina et al., 1983a,b) showed that a residual amount of stationary radioactivity is detected in the segment of nerve behind the wave of rapidly transported material. The amount of radioactivity that remains in the nerve is significant. In the olfactory nerve of garfish, as much as 80% of the total radioactivity in the nerve is deposited into the axoplasm and never reaches the nerve terminals (Gross and Beidler, 1975).

Using a position-sensitive detector, Snyder (1986a,b) demonstrated that as an anterograde pulse of radiolabeled protein passes through an amphibian nerve, a portion of the transported protein is left behind. In the sciatic nerve of *Xenopus laevis*, the amount of radiolabeled protein that is deposited is estimated to be

6.8% of the anterograde pulse per each 3.18-mm segment of nerve (Snyder, 1989). The deposited protein exhibited no detectable movement during an observation period of 20-24 hours. A similar deposition of retrogradely transported protein has also been reported (Snyder, 1989).

Evidence suggests that deposited materials are possibly used for the local maintenance of the structure and function of the axon. For example, autoradiographic studies of transported labeled glycoproteins showed that the labels are selectively inserted into the axolemma along the entire length of the axon (Tessler et al., 1980). It is thought that this process may represent the replacement of old or exhausted membrane with new membrane. The deposition of rapidly transported material could represent a passive process in which some fixed fraction of the material in transit is dropped off at each position in the axon, or it could represent an active process that is controlled by local cellular demand.

1.3 Techniques used in investigation of axonal transport

It is clear from the description given above (Sections 1.1 and 1.2) that the view of the properties of axonal transport generated by an experiment depends to a large degree on the technical approach for the detection of transported material. In the work to be described in this thesis, more than one technical approach to the detection of axonal transport was used. In addition, the techniques used are not considered to be entirely conventional. Therefore, it is useful to consider briefly the techniques that may be used for the detection of axonal transport. These techniques are:

a) labeling endogenous materials with radioactive tracer elements, assayed by:
   i) autoradiography,
   ii) liquid scintillation spectrometry,
   iii) position sensitive detectors;

b) accumulation method,

c) optical detection of intra-axonal organelles,

d) labeling with exogenous markers.

1.3.1 Radiolabeling method

The transport of endogenous proteins, lipids, glycoproteins, phospholipids and transmitter-related substances can be detected by administering radiolabeled precursors, such as amino acids, fucose, glycerol and glucosamine, to the cell body and allowing these precursors to become incorporated into axonally transported molecules. Precursors are applied by injection directly into cell bodies, such as the giant neurons of Aplysia (Goldberg, 1987), or into the immediate region of the cell bodies such as the dorsal root ganglion and the ventral horn of spinal cord. This technique can be applied to in vivo preparations, or to in vitro preparations if the nerve is removed in continuity with its ganglion (Edström and Mattsson, 1972; Theiler and McClure, 1976; O'Brien and Snyder, 1982). At various intervals after the application of labeled precursors, the transport of macromolecules can be studied by examining the distribution profiles of radiolabels along the nerve with the time of transport as a parameter (Bisby, 1976). From the

profiles of radioactivity obtained at various times the velocity of transport can then be deduced.

The methods for assaying the amount and distribution of radiolabels in the nerve are:

a) autoradiography

In autoradiography, the nerve is sectioned at selected regions. These sections of nerve are placed closely apposed to photographic films or coated with a layer of photographic emulsion. After a suitable period of exposure, the films or emulsion-coated sections are developed. The latent images so formed consist of silver grains. The position of silver grains on the film or emulsion corresponds to the sites on the section of the nerve from which radiation is emitted. By counting the number of grains, the amount of radioactivity for each section can be determined. A radioactivity distribution profile at a given time can then be obtained by plotting grain counts against position along the nerve from which the sections are collected. The time-related behavior of axonal transport is then inferred from a number of estimates of the amount and position of the grains in different nerve preparations, with time as a parameter.

b) liquid scintillation spectrometry

In liquid scintillation spectrometry, the nerve is first divided into segments, dissolved in a suitable medium, then mixed with a fluor and counted in a liquid scintillation detector. The amount of radioactivity in each segment can then be evaluated. By plotting the amount of radioactivity in a segment against the position of the segment along the nerve, the distribution of radiolabels along

the nerve at a particular time is obtained. The velocity of axonal transport can then be determined in a manner similar to that of the autoradiographic method.

c) position-sensitive detectors

The development of position-sensitive detectors of ionizing radiation to assay the movement of radiolabeled materials in nerve (Widen et al., 1976; Snyder et al., 1976; O'Brien and Snyder, 1982; Takenaka et al., 1978; Takenaka and Ochs, 1980) has permitted the study of the dynamics of axonal transport in single preparations. One of these detectors used in this laboratory is the multiple proportional counter (MPC) (Snyder, 1984). It consists of a series of single proportional counters of ß-radiation. Each counter detects the emission of sufficiently energetic ß-radiation from a small segment of living nerve (Snyder and Smith, 1982). The activity distribution of ß-radiolabeled axonally transported material in a nerve can then be estimated from the amount of ß-radiation entering each counter.

There are certain limitations to the analysis of axonally transported radiolabels associated with the above techniques. For example, the techniques are only useful to the study of materials that are synthesized in the cell body prior to transport. When transport is assayed by conventional techniques of scintillation spectrometry and autoradiography, a number of preparations must be performed in order to determine the dynamics of the transport. For this reason, inter-preparation variability may be of concern in obtaining statistically reliable results. Also, it is difficult with

conventional techniques to obtain any measures of retrograde transport dynamics. Analyses using the MPC suffer other disadvantages: it is a non-standard technique; its application is restricted to the investigation of fast transport because of the limited viability of the in vitro preparation; suitable radiolabeled precursors are very limited, also the method cannot be used to determine the absolute amount of radioactivity in each nerve segment since the detection efficiency is greatly affected by the geometry of the nerve segments.

1.3.2 Accumulation method

A local accumulation of transported material occurs when transport is disrupted at some point along the axon. The dynamics of axonal transport can then be inferred by examining the temporal pattern of accumulation (or depletion) of transported material. Transport can be irreversibly arrested by a ligature or by locally freezing the axon, or reversibly arrested by cooling a segment of the axon to about 5°C. Unlike the radiolabeling technique, the transport of specific materials such as acetylcholinesterase can be examined by this method. In addition, the accumulation of transported organelles can be studied using ultrastructural or cytochemical techniques.

Since the principle of the technique is to study the pattern of accumulation of material, it can be equally useful in the study of anterograde or retrograde transport. This is well demonstrated in studies of organelle transport in which anterogradely transported vesicles accumulated proximal to a local blockage of transport while

retrogradely transported lysosomal bodies accumulated distal to the block (Smith, 1980; Tsukita and Ishikawa, 1980).

The accumulation technique in its simplest form involves the disruption of transport at a single location on the axon. More sophisticated techniques involve the disruption of transport at two locations, in which case an isolated segment of axon is produced. The dynamic of transport can be determined by studying the redistribution of material within the isolated segment, as well as the material that accumulates at the interruption.

Although the technique is basically simple, the results must be interpreted with caution. The pattern and quantity of accumulation of materials can be altered by transport reversal, local synthesis, or differential routing of rapidly transported substances to branches of the axonal tree (Bisby and Bulger, 1977; Wooten et al., 1977; Goldberg, 1987; Smith, 1987, 1988). In addition, the interpretation of the results obtained with this technique is complicated by the fact that axonal transport occurs at a range of velocities rather than one particular rate. Also, the physical disruption of the axon may render the axon abnormal, and therefore the properties of axonal transport observed may not be representative of a normal phenomenon.

1.3.3 Optical detection of intra-axonal organelles

Another approach to study axonal transport is to observe the behavior of optically detectable organelles by light microscopy. The use of optical systems to study the transport of individual subcellular organelles provides information with a much greater temporal and

spatial resolution than is obtainable by the methods described above. Conventional optical systems were used for early work (Kirkpatrick et al, 1972, 1973; Smith, 1971, 1972). The development of differential interference contrast optics (DIC) with greatly improved detection capabilities, combined with video detection of the image and computer enhancement routines, has greatly increased the sensitivity of light microscopy (Allen et al, 1981; Inoué, 1986). Under suitable conditions, video-based systems are capable of detecting sub-resolution particles that are not detectable with conventional light microscopes.

One of the limitations of the use of optical system to the study of axonal transport is the need to isolate single cells for optimal imaging. Also, the viability of such preparations is a major concern.

1.3.4 Labeling with exogenous markers

The study of axonal transport is not limited to the investigation of movement of endogenous materials. Exogenous markers can be employed in the study of retrograde transport. This method relies on the uptake of markers at the nerve endings. Exogenous materials, such as nerve growth factor or horseradish peroxidase, can be taken up by pinocytosis or receptor mediated endocytosis, and retrogradely transported to the cell body (Stockel et al., 1974). Various methods are used to detect the movement of these markers. The markers may be radiolabeled, or fluorescent dyes may be attached to the marker, or histochemical methods may be available for the detection of the marker. However, the usefulness of

the technique in the study of the dynamics of axonal transport is limited by the low quantity of markers that are taken up by the nerve endings.

1.4 Axonal regeneration

A basic principle in understanding the significance of axonal transport in regeneration is that the axon itself is not able to synthesize proteins. Virtually all axonal proteins are synthesized in the cell body and transported into the axon. Axonal regeneration requires new materials to replace the portion of the axon lost due to axotomy. Replacement of the damaged axon can only be achieved if proteins synthesized in the cell body are delivered to the axon by the processes of fast and slow axonal transport.

Slow transport is mainly concerned with the transport of cytoskeletal components which include the neurofilament proteins, tubulin and actin. This thesis is primarily concerned with rapid axonal transport in regeneration; the process of slow transport is beyond the scope of this review. This section discusses:

a) current ideas and findings on the roles of fast axonal transport in regeneration;

b) alterations in fast axonal transport during axonal regeneration; and

c) morphological observations of the structure of the region of the axon between the parent and daughter axons.

1.4.1 Role of fast axonal transport in regeneration

Rapid anterograde transport conveys mostly membranous materials. There is little doubt that these materials are components of the plasma membrane. During regeneration, these materials are thought to be required for the expansion of the surface membrane of the axon and for some undefined growth promoting functions.

Autoradiographic studies (Pfenninger, 1982) demonstrated that labeled phospholipid is incorporated selectively into anterogradely transported vesicles. These labels are then inserted into the growth cone at the tip of the regenerating axon as well as along the axolemma of the newly formed axon for axonal elongation, radial growth and maturation. Studies using radiolabeled glycoproteins lead to similar conclusions (Tessler et al., 1980; Griffin et al., 1981).

Another important role of fast anterograde transport in regeneration is the delivery of a class of substances known as the growth-associated proteins, or GAPs (Skene and Willard, 1981a,c). It is possible that many of the GAPs are constituents of the membrane of the growth cone (Willard et al., 1987; Benowitz, 1987) and therefore their function may be related to the behavior of the growth cone. Although the functions of GAPs in regeneration are yet to be delineated, elevated levels of GAPs in regenerating axons are believed to be necessary to induce the axon to change from a normal physiological equilibrium to a competent growth state (Skene and Willard, 1981b). The findings that GAPs are also transported at an elevated level during fetal or neonatal development (Kalil and Skene, 1986; Jacobson et al., 1986), but are not reinduced in non-regenerative mammalian CNS neurons after axotomy (Skene and

Willard, 1981b), and that mammalian CNS axons are capable of regeneration when injury occurs during neuronal development (Kalil and Reh, 1982) further reinforce the hypothesis.

Retrograde transport conveys both endogenous and exogenous materials to the cell body. In regeneration, the transport of these materials is speculated to serve as a signalling mechanism to the cell body of the neuron.

The occurrence of chromatolysis in the cell body when its axon is injured some distance away indicates that the cell body receives information about the status of the axon. There is evidence to suggest the involvement of retrograde transport in this informative role. For example, the appearance of chromatolysis in the cell body after injury is time-correlated with the appearance of retrogradely transported materials from the site of injury (Kristensson and Olsson, 1975). In addition, when colchicine is applied proximal to the site of injury to block axonal transport, the onset of cell body reaction is delayed (Singer et al., 1982).

There is evidence to suggest that the retrograde transport of specific factors is necessary to induce a regenerative response in the cell body. For example, after axotomy, the administration of nerve growth factor (NGF) stimulates the regenerative process in NGF-sensitive neurons (Bjerre et al., 1973; Hendry, 1975); and when a peripheral nerve segment is transplanted into non-regenerating CNS (Kao et al., 1977; Aguayo et al., 1982), a regenerative response is induced in the CNS neurons. Recently an increase in retrograde transport of NGF produced by Schwann cells has been demonstrated in transected sensory nerves (Abrahamson et al., 1986; 1987). It has

been suggested that extrinsic trophic factors, such as NGF, undergo retrograde transport in regenerating axons (Bisby, 1987); the introduction of the trophic factors to the cell body activates biological pathways that lead to the regenerative response (Grafstein and McQuarrie, 1978).

It has also been proposed (Bisby, 1987) that in axotomy, transport reversal at the lesion causes a premature return of endogenous materials to the cell body. The retrograde transport of all or some selected endogenous proteins in abnormal amounts, ratios or forms, and the elimination of proteins normally returning from the axon distal to the injury, may constitute a signal to the cell body that axotomy has occurred. Furthermore, during regeneration, the progressive change in timing, amount, and perhaps composition, of endogenous and exogenous materials returned to the cell body, may enable the cell body to monitor and respond to the progress of regeneration (Bulger and Bisby, 1978).

1.4.2 Alteration of fast axonal transport during regeneration

Four aspects of the alteration of rapid axonal transport during nerve regeneration are reviewed here. These are:

a) alteration in the rate of transport;

b) alteration in the amount of transport;

c) changes in the composition of materials transported; and

d) changes in the deposition of transported materials along the axon.

Whenever possible, distinction is made between changes in transport in the *parent* segment of the axon and changes in transport

in the *daughter* segment of the axon. It is presumed that alterations in axonal transport in the parent segment of the axon represent changes in supply of materials necessary to sustain regeneration, as well as changes induced in the parent axon due to the physiological status of the neuron, while alterations observed in the daughter axon reflect, in addition, the incorporation of materials to increase the mass of the regenerating axon.

1.4.2.1 Rate of materials transported

In mammalian peripheral systems, axonal transport has been studied in great variety of species and nerve types (Frizell and Sjostrand, 1974a,b; Ochs, 1976; Bisby, 1978; Griffin et al., 1981). The maximum velocity of fast anterograde transport during period of axonal outgrowth, as measured by the advance of labeled protein or glycoprotein, shows little change from normal values in the parent axons. The velocity of transport in the daughter portion of the regenerating axons also appears to be similar to that of the parent axons (Griffin et al., 1976; Bisby, 1978).

1.4.2.2 Amount of materials transported

Possible changes in the amount of materials rapidly transported in the anterograde direction in regenerating mammalian peripheral systems have been investigated extensively.

Conflicting reports indicate detectable increases, decreases, or no obvious change in the quantity of anterogradely transported materials. Table 4 summarizes the results from these studies. The diversity of these results may be attributed to the many different

types of nerves used, the type of trauma induced, the distance from the lesion to cell body, the type of labeling technique as well as the times or phases of regeneration at which axonal transport was investigated. In general, it appears that the transport of glycoprotein increases during the phase of axonal elongation. A comparable increase in the transport of labeled membrane lipids has also been observed. It has been suggested that the increase in the amounts of glycoprotein and lipid transported represent an enhanced cotransport of membrane constituents necessary for the expansion of the axolemma of the regenerating axons (Elam, 1984). A dramatic and consistent increase in quantity of materials transported is reported only in the retinal ganglion cells of the goldfish and amphibians.

There are only a few studies on changes in the amount of material undergoing retrograde axonal transport during regeneration (Table 5). Some of these studies investigated changes of endogenous proteins while others used exogenous tracers as markers of material transported towards the cell body. The results seem to indicate an increase in materials returning to the cell body immediately after axotomy (Frizell et al., 1976; Bisby and Bulger, 1977). When regeneration is under way, there are conflicting reports on changes in the amount of materials returning to the cell body. Again, the diversity in results may be caused by the different types of experimental protocol involved. The results of these studies must be interpreted with caution. The increase in retrograde transport of labeled endogenous materials reported may at least partly caused by an increase in anterograde transport and a premature reversal of the

Table 4

Changes In Overall Amount Of Anterogradely Transported Materials During Regeneration

	Type of nerve	Quantity	Ref.
protein	rat sciatic motoneuron	↑	1
	rat sciatic sensory	no change	2,3
	cat sensory	no change	4
	Amphibian sciatic sensory	no change	5
	guinea pig hypoglossal	↑	6
	rabbit hypoglossal	↑	7
	goldfish RGC	↑↑↑	8,9
	Amphibian RGC	↑↑↑	10,11
glycoprotein	rabbit hypoglossal	↑↑	12a,b, 13
	rabbit vagus	↓	12b
	rat sciatic motoneuron	↑↑	14
phospholipid	rat sciatic nerve	↑↑	15a
	rabbit hypoglossal	↑↑	15b

References
1. Griffin et al., 1976.
2. Bulger and Bisby, 1978.
3. Bisby, 1978.
4. Ochs, S. 1976.
5. Perry and Wilson, 1981
6. Kreutzberg and Scherbert, 1971.
7. Danielsen et al., 1986.
8. McQuarrie and Grafstein, 1982.
9. Grafstein and Murray, 1969.
10. Skene and Willard, 1981.
11. Szaro et al., 1985.
12. Frizell and Sjostrand, 1974a,b
13. Tessler et al., 1980.
14. Griffin et al., 1981.
15. Alberghina et al., 1983a,b.

Table 5

Changes In Amount Of Retrogradely Transported Material During Regeneration

	Type of nerve	Quantity	Ref.
Endogenous protein or glycoprotein	rat sciatic motoneuron	no change	1
	rabbit hypoglossal	↑	2,3
	rabbit vagus	↑	3
	goldfish optic	↑	4
Tracer-horseradish peroxidase	mouse facial nerve	↑	5
	rabbit hypoglossal	↓	6
	chick isthmo-optic nerve	↓	7

References
1. Bulger and Bisby, 1978.
2. Frizell et al., 1974a.
3. Frizell et al., 1976.
4. Whitnall et. al., 1982.
5. Olsson et al., 1978.
6. Kristensson and Sjostrand, 1972.
7. Halperin and LaVail, 1975.

transported materials (Frizell et al., 1976). Also, it is not known whether the dynamics of uptake of exogenous materials is altered during regeneration.

1.4.2.3 Composition of transported materials

The general finding of various studies of anterograde axonal transport during regeneration indicates that changes in the pattern of major protein composition, as analyzed by polyacrylamide gel electrophoresis, are generally small and involve very few proteins. It appears that no new protein is transported and none of the existing proteins are removed from the transport system (Bisby, 1978, 1980; Perry and Wilson, 1981; Skene and Willard, 1981a,b; Szaro et al., 1985).

While successful regeneration does not require a gross qualitative alteration in the composition of rapid axonal transport, changes have been observed in the relative abundance of a small number of components that are quantitatively minor. It is generally agreed that during regeneration, there is a decrease of transport of enzymes related to transmitter metabolism. For example, in cholinergic neurons, the transport of cholinesterase is reduced (Frizell and Sjöstrand, 1974a; Heiwall et al., 1979); in adrenergic neurons, the transport of catecholamine storage granules containing dopamine-ß-hydroxylase and norepinephrine is similarly reduced (Karlström and Dahlström, 1973; Ohshiro et al., 1978). A decrease in transport of these materials is commonly interpreted as an indication that the neuron alters its protein synthesis from a pattern which

maintains normal neuronal functions to a pattern which is directed to axonal growth (Forman, 1983).

In regenerating toad optic axons, Skene and Willard (1981a,b) showed that the relative amount of labeling in a small number of rapidly transported proteins, GAPs, is elevated as much as 100-fold their basal level in the control nerve. Increased transport of specific neuronal proteins similar to that in toad optic nerve has been reported during successful axonal regeneration in a variety of other nerves (Theiler and McClure, 1978; Skene and Willard, 1981b; Redshaw and Bisby, 1984; Benowitz and Lewis, 1983; Zwiers et al., 1987). All of these proteins are identified as components of group I axonally transported proteins (Lorenz and Willard, 1978).

Large increases in the axonal transport of RNA have been demonstrated during regeneration (Ingoglia, 1982). Electron microscopic autoradiograms show that this RNA is concentrated at the growth cones (Gambetti et al., 1978). These observations led to the suggestion that the function of increased level of RNA is growth-related.

1.4.2.4 Deposition of materials along the axon

During regeneration, an increased deposition of radiolabels occurs along the entire length of the regenerating portion of the axon (Griffin et al., 1976, 1981; Bisby, 1978; Forman and Berenberg, 1978; Tessler et al., 1980; Alberghina et al., 1983a,b; Danielsen et al., 1986). Some studies show a further accumulation of radiolabels at the tip of the regenerating axon (Griffin et al., 1976; Frizell et al., 1976; Forman and Berenberg, 1978; Tessler et al., 1980) and in the region of the

original lesion (Carlsson et al., 1971; Forman and Berenberg, 1978; Bisby, 1978; Griffin et al., 1981; Alberghina et al., 1983a,b; Danielsen et al., 1986).

Studies using the autoradiographic technique to investigate the intra-axonal distribution of rapidly transported proteins and glycoproteins in the regenerating segment of the axon indicate that the radiolabels are preferentially incorporated into the axolemma (Griffin et al., 1976, 1981; Tessler et al., 1980). In addition, transported radiolabels are continuously inserted into the plasma membrane of the growth cone (Tessler et al., 1980). Therefore, the increase in the deposition of proteins and glycoproteins in the daughter axon and the growth cone seems to reflect the increase in the requirement for axonally delivered membranous materials as the regenerating axon increases its surface area.

1.4.3 Morphology of the axon in the junctional region between the parent and daughter axons

During regeneration, the first growth of axonal sprouts occurs mostly at the retracted first node of Ranvier, occasionally at the second node proximal to the lesion, and sometimes at the terminal club of the proximal stump (Lubinska, 1959, Zelená et al., 1968; Morris et al., 1972; Friede and Bischhausen, 1980; McQuarrie, 1985). Retrograde degeneration occurs ascending up to the node where axonal sprouts originate (Morris, 1972; Lanners and Grafstein, 1980; McQuarrie, 1985). The transition to the daughter axon, therefore, is marked by an abrupt end of myelination of the proximal stump, beyond which the non-myelinated daughter axons extend distally

(Morris, 1972). As regeneration proceeds, axonal sprouts gradually become myelinated. At the interface between the surviving parent axon and the regenerating axon which has become myelinated, a new node is formed. This node represents a junctional region between the parent axon and the daughter axon.

Although the junction between parent and daughter axons has not been extensively investigated, a morphological study of regenerating axons by Morris et al. (1972) shows that various kinds of intraaxonal organelles may accumulate in the axoplasm of the junctional region. These organelles were identified as multi-lamellar bodies, multi-vesicular bodies, dense bodies, dense-cored vesicles, branched tubules, vesicles and granular/filamentous materials.

2 GENERAL METHODS

This section describes the animals used, the procedures for crushing the nerve, and the techniques of desheathing the nerve. Methods used that are specific for either ultrastructural, or radiolabeled protein transport, or organelle transport studies are described in subsequent chapters.

2.1 Animals

Adult specimens of female Xenopus laevis weighing 110-150g were obtained from South African Snake Farms, Fish Hoek, S.A. These animals were maintained in water tanks kept at room temperature (20°C). Operated animals with regenerating nerves and normal animals were housed in separate tanks. They were fed once a week with a diet of lean ground beef; the water was changed on the day after feeding.

At various times of regeneration up to 20 weeks, animals used for ultrastructural and organelle transport studies were pithed, while animals used for radiolabeled protein transport studies were anesthetized by submersion in a 2% solution of urethane (ethyl carbamate, Sigma Chemical). The regenerating nerve was removed for the experiment.

2.2 Nerve crush

Normal animals conditioned to the laboratory environment were anesthetized by immersion in 2% aqueous urethane solution (normally for 20 to 30 minutes until the animal lost the pinch reflex).

An incision was made at the left or right mid-thigh region to expose the sciatic nerve. With a 6-0 thread, a transverse crush was made by ligating the nerve against a glass rod for 60 seconds. This lesion was normally made on the main trunk of the sciatic just proximal to its bifurcation into the tibial and peroneal branches. This location was about 40 to 50 mm from the 9th dorsal root ganglion. The procedure produced a narrow compressed region across the nerve. After crushing the nerve, the ligature was removed, the skin incision was closed and the animal was returned to the water tank. At various time of regeneration, the animals were sacrificed and the regenerating nerve was removed for study.

Histological examination showed that crushing the nerve with the above procedure resulted in Wallerian degeneration in all fibers of the nerve distal to the lesion.

2.3 Identifying the original site of lesion

The position of the lesion in the regenerating nerve was identified by microscopic examination. At short periods of regeneration, the position of the lesion was determined by locating the most proximal extent of myelin interruption. At long periods of regeneration, the junction was marked by abrupt change in the refractile characteristic of the axon due to a change in the thickness of the myelin sheaths between the parent and the daughter axons.

2.4 Removal of the nerve sheath

The perineural sheath was removed from most of the nerves used for morphological work, from some of the nerves in the studies

of labeled protein transport, and from all of the nerves used for studies of organelle transport.

Two different techniques were used to remove the perineural sheath completely. The initial steps of both techniques were essentially the same. The sciatic nerve without the ganglion was placed on a wet piece of filter paper in a petri dish. The lower blade of a pair of iris scissors was inserted between the nerve bundle and the sheath of the stump of the 8th ventral ramus, and the sheath was slit to a point approximately 5 mm distal to the fusion of the 8th and 9th rami. The sheath was then cut around the circumference of the nerve. While gripping the 8th ramus with a pair of forceps, the distal portion of the sheath was removed by pulling it so that the sheath everted over the distal nerve bundle. Then, the nerve was gripped near its distal end and the proximal portion of the sheath was similarly removed by eversion. This method will be called *desheathing by eversion.*

In the second method, after the sheath had been cut around its circumference, both the distal and proximal portions of the sheath were slit longitudinally along the entire length of the nerve with iris scissors. The sheath was then peeled from the nerve in the same manner as before. This method will be called *slit-desheathing*.

Both of these techniques of desheathing, when performed carefully, caused no visible damage to the fibers.

3 THE SUCCESS OF REGENERATION AND THE RATE OF REGENERATION IN CRUSHED SCIATIC NERVES

A preliminary survey was conducted to establish both the approximate rate and success of regeneration of axons in the crushed sciatic nerve of *Xenopus laevis*. The approach adopted was mainly morphological, consisting of an examination of fixed axons by electron microscopy and of living axons by video microscopy. A verification of the results of the morphological method for determining regeneration rate was carried out by a radiolabel method.

3.1 Methods

3.1.1 Electron microscopy and the estimation of the regeneration rate

Primary fixation of nerves was carried out in a solution of 3% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4) for 2 hours or more. The fixed tissues were rinsed in 0.1 M cacodylate buffer, post-fixed in 2% osmium tetroxide in phosphate buffer (pH 7.4), rinsed with distilled water, dehydrated in graded dilutions of ethanol, transferred to propylene oxide to complete dehydration, and left overnight in a mixture of 1:1 propylene oxide and Poly/bed 812 medium (Polysciences Inc.). After infiltration overnight in the Poly/bed mixture, the tissues were embedded in 100% Poly/bed and were oriented for cross or longitudinal sectioning as necessary. All the above procedures were performed at room temperature. Unpolymerized Poly/bed was cured at 60°C for 48 hours.

Sectioning was performed using a Reichert Ultracut microtome and a diamond knife. Thin sections of thickness approximately 600 Å to 800 Å were collected on 200 mesh copper grids, stained with saturated uranyl acetate in 50% ethanol for 10 minutes and saturated aqueous lead citrate for 5 minutes. The sections were examined with a Siemens 102 electron microscope.

The rate of regeneration was evaluated by examining the extent of regrowth of crushed myelinated axons at two regeneration periods. Nerves that had regenerated for 21 days and 56 days were desheathed and processed for electron microscopy. The location of the most proximal extent of myelin interruption was determined by light microscopic examination. Cross-sections were obtained at 5 mm intervals distal to this site. Areas of the sections were selected in a random manner for quantitative evaluation. Each such area measured at least $2X10^4$ μm^2.

3.1.2 Radiolabel method for the estimation of the regeneration rate

The farthest point of regrowth was also examined with a radiolabeling method. The transport of radiolabels in nerves that had regenerated for 21 days were examined.

A regenerating nerve in continuity to its 8th and 9th dorsal root ganglia was transferred to a three-compartment tray. The ganglia was in the first compartment, the rami and the proximal portion of the sciatic nerve were in the second compartment and the sciatic nerve containing the region of the crush was in the third compartment. The connections between the compartments where the

nerve threaded through were then sealed with silicon grease. Twenty-five microliters of saline solution containing 0.15-0.25 mCi of L-[^{35}S] methionine at a specific activity of 1000-1400 Ci/mmol was added to the ganglia compartment. The saline solution had the composition (mM): NaCl, 112; KCl, 3.0; $MgSO_4$, 1.6; $CaCl_2$, 3.0; glucose, 5.0; N-2-hydroxyethlypiperazine-N'-2-ethanesulfonic acid (HEPES), 3.0; the pH of the solution was adjusted to 7.40±0.02 with NaOH. Saline solution not containing any radioactive methionine was added to the other two compartments. The nerve preparation was allowed to incubate at room temperature (20-22°C) for 20 to 24 hours.

At the end of the incubation period, the nerve was removed from the three compartment tray and was divided into 3-mm segments. Each segment was treated with 0.5 ml of 5% trichloroacetic acid (TCA) solution for 1-2 hours at 80-85°C. The TCA solution precipitated proteins and extracted unincorporated free amino acids. The nerve segments were then dissolved in 0.6 ml Protosol (New England Nuclear) overnight at 40-45°C. Ten milliliters of Scinti-Verse II (Fisher Scientific) was added to each of the TCA-soluble samples and 10 ml of Econofluor (New England Nuclear) was added to TCA-insoluble samples. The amount of radioactivity in the samples was assayed with a liquid scintillation spectrometer (Nuclear Chicago Mark I). Quenching was uniform among the samples and counts were not corrected. The results were expressed as counts of TCA-insoluble activity per hour per segment.

3.1.3 Video microscopy and the assessment of the success of regeneration

A segment of five to six centimeters of regenerating nerve containing the junctions was removed from the animal. The original site of lesion was generally about two centimeters from the distal end of the nerve bundle. The nerve was transferred to either a physiological saline solution (for composition, see Section 3.1.2) or a glutamate solution of composition (mmoles/l): potassium glutamate, 120; HEPES, 3.0; buffered to pH 7.2. Behavior of bidirectional intraaxonal organelles transport are essentially identical using either one of these bathing media (Kendal et al., 1983). The nerve was then desheathed by either one of the methods described above (see Section 2.4). Since the regenerating nerve was fragile, the nerve bundle was treated with a 1% solution of trypsin (Sigma Chemical) in saline or glutamate for approximately 10 minutes. The use of trypsin solution causes no detectable adverse effect on the transport of organelles in these axons but the treatment enables the regenerating axons to be isolated without damage (Smith et al., 1985). The nerve bundle was rinsed briefly in a medium without trypsin and transferred onto a microscope slide which had been ringed with a thin layer of silicone grease to contain sufficient bathing medium for the nerve preparation. The nerve bundle was teased apart with fine needles to expose a number of single axons over a 5 to 7 mm length in the region of the crush. The region of the nerve bundle containing the isolated axons was supported from below by a drop of Dow Corning 710 silicone fluid. When a cover glass (number 0) was placed over the entire preparation the silicone fluid gently pressed the

isolated axons against the underside of the cover glass. Optimum optical conditions were achieved with the axon just beneath the cover glass.

Single myelinated axons were viewed with differential interference-contrast optics (Zeiss photomicroscope; objective, X63, na, 1.4). Illumination was provided by a 100 W quartz-halogen lamp with a band-pass green filter (60 nm bandpass centered at 550 nm). Heat filters were used in the light path to prevent heating of the nerve preparation. Some analogue enhancement of the microscope image was obtained with a television camera (Dage-MTI, chalnicon target tube) as described by Allen et al. (1981) and Inoué (1986). Further enhancement of the image was achieved by real-time digital manipulation of the television signal using Quantex Corp. processors. The image was high pass spatial filtered to remove low frequency shadows and highlights originated in the myelin. Most of the material carried by rapid anterograde transport consists of 50 nm diameter vesicles and vesiculo-tubular organelles (Smith, 1980; Tsukita and Ishikawa, 1980). To detect the diffraction images caused by these small organelles within the intact axons it was necessary to eliminate the image of the stationary cellular background. To do this, a running exponential average of the output of the high pass spatial filter was subtracted from the spatially filtered but unaveraged image of the current television frame. Finally, noise in the difference image was reduced by exponential averaging. The method was described in detail by Smith (1989).

Individual preparations were viewed for a maximum of two hours. During this time organelle transport in terms of the velocities

of the organelles and the number of organelles moving in the region of interest were stable.

All procedures were performed at room temperature (20-22°C).

3.2 Results

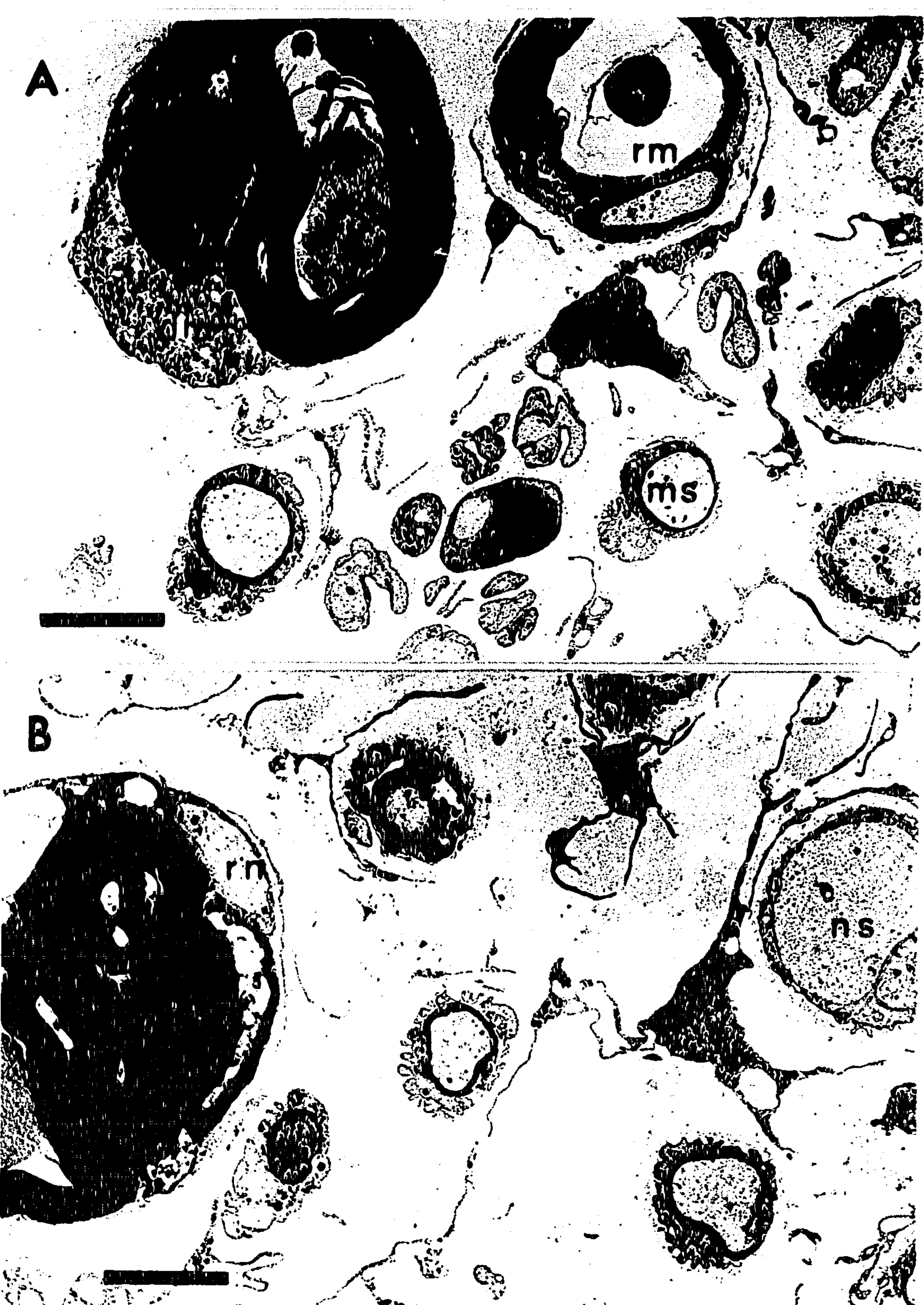
3.2.1 The rate of regeneration

The rate of regeneration was assessed by a morphological method and a radiolabeling method.

At each level of the nerve that was examined by electron microscopy, the number of regenerating myelinated axons was assessed by summing 1) the number of thinly myelinated axons, 2) the number of degenerating myelinated fibers with regenerating sprouts within the basal lamina, and also 3) the number of "regenerating units" (Morris et al., 1972) consisting of at least i) one myelinated sprout or ii) non-myelinated sprout with a diameter greater than 5 μm (Fig. 1A, B).

In some of the segments of regenerating nerve examined, the regenerating units were rarely associated with degenerating myelinated axons. Regenerating units containing myelinated fibers clearly had developed from myelinated axons, but the origin of the units containing only non-myelinated axons was not easy to determine. Earlier work in this laboratory has shown that the mean (±SD) diameter of non-myelinated axons in this nerve is 1.1 (±0.38) μm (Smith et al., 1985). Therefore, regenerating units consisting of non-myelinated axons with a diameter of 5 μm or more were considered to have originated from myelinated axons.

Figure 1.
Electron micrographs of cross-sections of a 56 days regenerating nerve. The nerve consists of both regenerating and degenerating myelinated axons. **A**, 45 mm distal to crush; dm, degenerating axon with no sprout within its basal lamina; rm, regenerating axon with myelinated sprout; ms, thinly myelinated regenerating sprout not associated with degenerating axon. **B**, 40 mm distal to crush; rn, regenerating axon with non-myelinated sprout within its basal lamina; ns, non-myelinated sprout with a diameter greater than 5 µm. Scale bars, 5 µm.


A
rm
ms
B
rm
ns

This approach represents a conservative evaluation of the number of regenerating myelinated axons since regenerating units from myelinated axons that failed to meet either one of the two criteria were excluded from this estimate.

The numbers of regenerating axons and degenerating axons at each level of the nerves are given in Table 6. These results were expressed by taking the percentage of regenerating fibers to the total number of myelinated axons in each area. The total number of myelinated fibers was estimated as the sum of the regenerating myelinated axons and the degenerating myelinated axons that contained no sprout within their basal lamina. Figure 2A is a plot of the percentages of regenerating fibers versus the distances from the site where sprouts emerged.

The results were also expressed in terms of a "minimum rate of regeneration". A plot of "minimum rate of regeneration" versus percentage of regenerating fibers (Fig. 2B) allows an estimate of the proportion of axons that grew at a rate equal or greater than the value shown on the ordinate. Figure 2A shows that at least 90% of the sciatic myelinated fibers successfully regrew their axons at these two regeneration times. Figure 2B shows that 80% of the myelinated fibers regenerated at a minimum rate of 1 mm/day at 20°C.

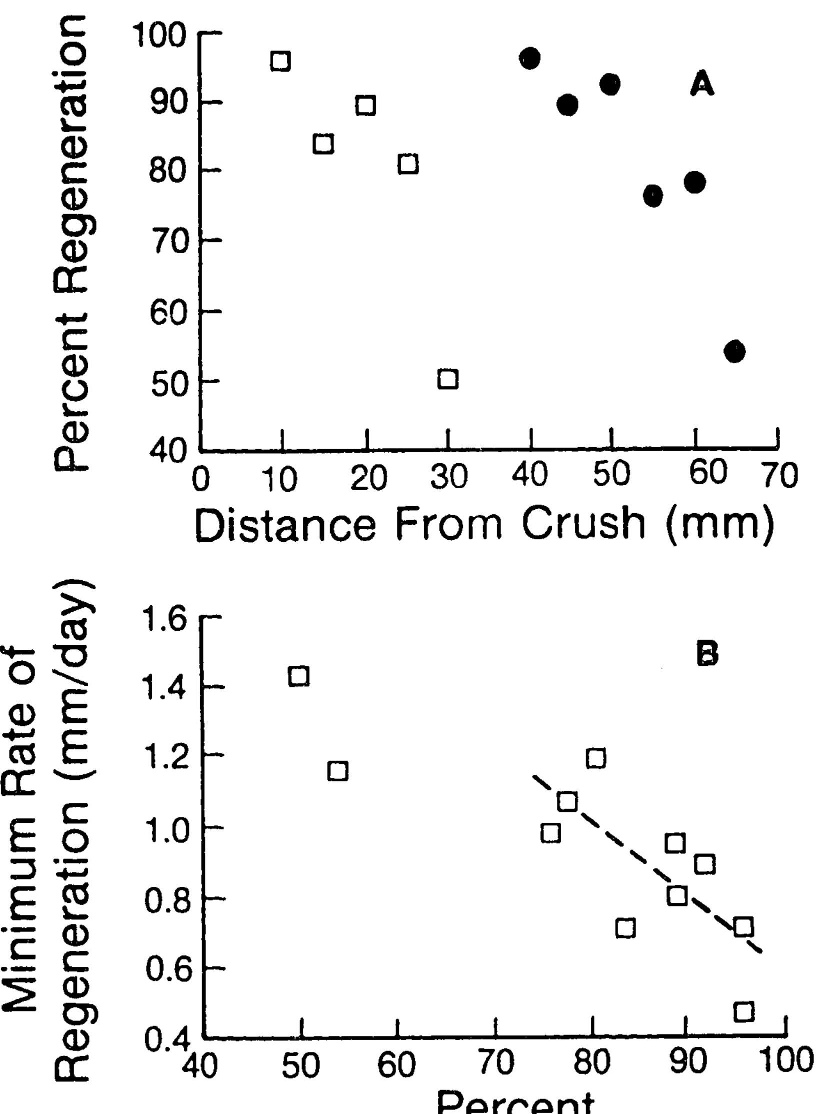
The regeneration rate estimated by locating the most distant position of the rapidly transported radiolabeled proteins (Bisby, 1978; Forman and Berenberg, 1978) was compared to that obtained by the morphological method described above. The most distal location along the nerve where radioactivity at above background levels could be detected was taken as the position of the tips of the

Table 6

Numbers of regenerating and degenerating axons at measured distances from the lesion in 21 days and 56 days regenerating nerves

Regeneration Time (days)	Distance from the crush (mm)	Number of axons regenerating	Number of axons degenerating	Total
21	10	127	5	132
	15	62	10	72
	20	72	8	80
	25	72	14	86
	30	109	54	163
56	40	98	4	102
	45	111	12	123
	50	62	5	67
	55	29	7	36
	60	18	4	22
	65	13	6	19

Figure 2.
Regeneration of myelinated axons in the sciatic nerve following a crush lesion. **A**, the percentage of myelinated axons that had regenerated measured distances from the proximal side of the crush at 21 days (open squares) and 56 days (filled circels) after the crush. **B**, the data of part **A** plotted to show the percentage of myelinated fibers that grew at least at the rate shown on the ordinate. The cluster of 9 points at the right of the plot was fitted with a least squares line. From the line, 80% of the myelinated fibers grew at 1.0 mm/day or greater.



A
Percent Regeneration
100
90
80
70
60
50
40
0
10
20
30
40
50
60
70
Distance From Crush (mm)
B
Minimum Rate of Regeneration (mm/day)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
40
50
60
70
80
90
100
Percent

most rapidly growing axons. An average regeneration rate of the most rapidly growing axons was calculated by dividing the distance between the site of lesion and the tips of regenerating axons by the number of days elapsed since the crush. A regeneration rate of approximately 1.2 mm/day was obtained with these analyses (n=2).

The results obtained by both the methods described above represent a minimum estimate of the regeneration rate since the lag period between nerve crush and initiation of axonal sprouts was not determined and hence not included in this estimation.

3.2.2 The success of regeneration

The results described above indicated that most, if not all, axons successfully regenerated under the experimental conditions. With video microscopy (at magnification X2,000 to X10,000), over 1000 freshly teased living fibers from nerves of regenerating times varying between 5 days and 20 weeks were examined for continuity in the region of the lesion. All fibers examined had regenerated axonal sprouts that could be followed for a considerable distance. This observation indicates that the sciatic myelinated neurons efficiently regenerate and that there is no evidence of axonal "blind-ending" in the region of the crush.

Some fibers were further examined at a magnification of X10,000. In all fibers examined, there was bidirectional traffic of intraaxonal organelles through the junctions. It can be concluded that there was both morphological and functional continuity at the junctional region.

3.3 Conclusion

Crush sciatic nerves of Xenopus laevis regenerated at 20°C at a rate such that 80% of the myelinated axons grew at 1 mm/day or faster. All myelinated axons appeared to initiate growth successfully. No failure to regenerate was observed.

4 Morphological characteristics of the junction between parent and daughter axons in regenerating myelinated axons

The structure of the junction between the parent axon and the regenerating daughter axon was examined at regeneration times up to 20 weeks. The continuity of microtubules across the junction was also investigated. This work was expected to provide information on those structural properties that are characteristic of the junction, and hence of possible importance to the transport of materials across the junction. In addition, this work allowed an assessment of the structural status of the junction in nerves that had been desheathed by different methods.

4.1 Methods

4.1.1 Structure of axons at the region of the junction

The ultrastructure of the axons at the region of the junction were studied mainly by electron microscopic examination. For electron microscopy studies, axons were processed as described in Section 3.1.1. Some of the features of regenerating axons were also studied in living axons with the video microscope. The method for video microscopy was described in Section 3.1.3. In these studies, nerves that had regenerated from 3 days to 20 weeks were used.

As will become clear in Chapter 5 (Protein transport studies), the properties of the junction between the parent and daughter axon may be altered depending on the type of desheathing techniques used, therefore, for electron microscopic work, the structure of the

axons at the junction was studied from axons prepared by one of the following procedures.

a) The regenerating nerve was exposed in the animal and fixed in situ by flooding the area with glutaraldehyde fixative. The perineurial sheath was slit open longitudinally prior to fixation to facilitate fixation. After 30 minutes of in situ fixation, the nerve was removed from the animal, processed, and embedded as a whole nerve without removing the sheath. This method presumably produced little or no stress to the junctions.

b) The nerve was desheathed by eversion, fixed, processed and transferred to unpolymerized Poly/bed mixture. Individual myelinated axons or small bundles of axons containing the region of the junction were isolated and embedded.

In most cases the morphology of the junctions was studied in single, isolated axons or small bundles of axons (using the method b above). This approach facilitated the process of locating the junctions for sectioning. To ensure this procedure did not create artifacts in the junction between parent and daughter axons, junctions in whole nerve bundles prepared by the first method were examined for comparison. Nodes of Ranvier proximal and distal to the junction were also examined. All axons were sectioned longitudinally.

4.1.2 Continuity of microtubules across the junction

A small diameter sensory nerve branch emerging from the sciatic trunk at its bifurcation into the peroneal and tibial branches was selected for a detailed morphological study by electron

microscopy. The branch of nerve was identified in the anesthetized animal, crushed, and allowed to regenerate for a period of 21 days. The nerve was thick and thin cross-sectioned at regular intervals through the region of the crush. Thick sections (1.0 μm) were cut, collected on glass slides and stained with a 1:1 mixture of 1% toluidine blue and 1% Azure II in 1% borax, and then examined with a light microscope. These thick sections were used for survey purposes. Thin sections were examined with an electron microscope and photographed at a magnification of X12,500. Final electron micrographs were printed at a magnification of X37,500. The number of microtubules on both sides of the junction was assessed from micrographs obtained at the region of the junction.

4.2 Results

4.2.1 Morphology of axons

The morphology of the axon in the region of the junction was examined by electron microscopy and video microscopy. In addition, nodes of Ranvier proximal and distal to the junction were examined with the electron microscope.

In all fibers that were examined by electron microscopy, regenerating sprouts were seen distal to the site of the lesion. The appearance of the junctional region depended on the time allowed for regeneration.

An examination of the axons at the junction revealed no detectable morphological differences between axons obtained from nerves in which the sheath was merely slit and nerves which were desheathed by eversion. The junctions of axons from nerves of same

regeneration period were similar in appearance and cytoplasmic content.

The process of regeneration could be divided into 3 phases according to the cytoplasmic content in the region of the junction: i) a short period 3 days to 2 weeks, ii) an intermediate period 2 to 5 weeks, and iii) a long period 5 to 20 weeks.

At 3 days after the crush injury, the earliest period examined, sprouting had already occurred. Figures 3A and 4A shows the structure of axons at the junction which had regenerated for 3 and 7 days respectively. At this short period of regeneration, myelin retraction from the axolemma of the paranode was a common feature of the axons in the region of lesion. Thin unmyelinated axonal sprouts typically originated at the first retracted node of Ranvier proximal to the crush and extended distally. They adhered closely to the outside surface of the Schwann cell but remained within the basement membrane of the degenerating segment of the axon. Cytoskeletal components in the region of the junction appeared normal; typically, microtubules occurred in bundles within a field of neurofilaments. The microtubules appeared to be continuous through the junction at the earliest stages examined. The organelle content of the axoplasm was, however, different at the two sides of the junction. In addition to the organelles normally found in the axoplasm, diffusely scattered granular materials of approximately 10 nm diameter were common on the proximal side of the junction. Clusters of 50 nm vesicles were seen occasionally. These profiles were not observed distal to the junction.

Figure 3.
Electron micrographs of longitudinal sections through the junction between parent (to the left) and daughter axons (to the right) at three regeneration times. **A**, at three days, the parent axon with thick myelin extends a non-myelinated sprout (s). The sprout runs alongside degenerating myelin (dm) that surrounds degenerating axoplasm (ax). Within the parent axon there is an accumulation of small vesicles (v); the same region of axoplasm (g) also contains fine granules that cause an overall grayness in the electronmicrograph at low magnification. **B**, at 28 days the daughter axon has a thin myelin sheath (my) and is connected to the parent axon by a long node of Ranvier. The node contains a rim of large membranous organelles, many of them dense bodies (d). **C**, at 141 days the myelin sheath of the daughter axon is thicker but is still distinguishable from the myelin sheath of the parent axon. Dense bodies (d) are present in the junctional region of this specimen. Fine granular material at the proximal side of the junction gives an overall grayness to the image. Scale bars: **A**, 10 µm; **B**, 2 µm; **C**, 5 µm.

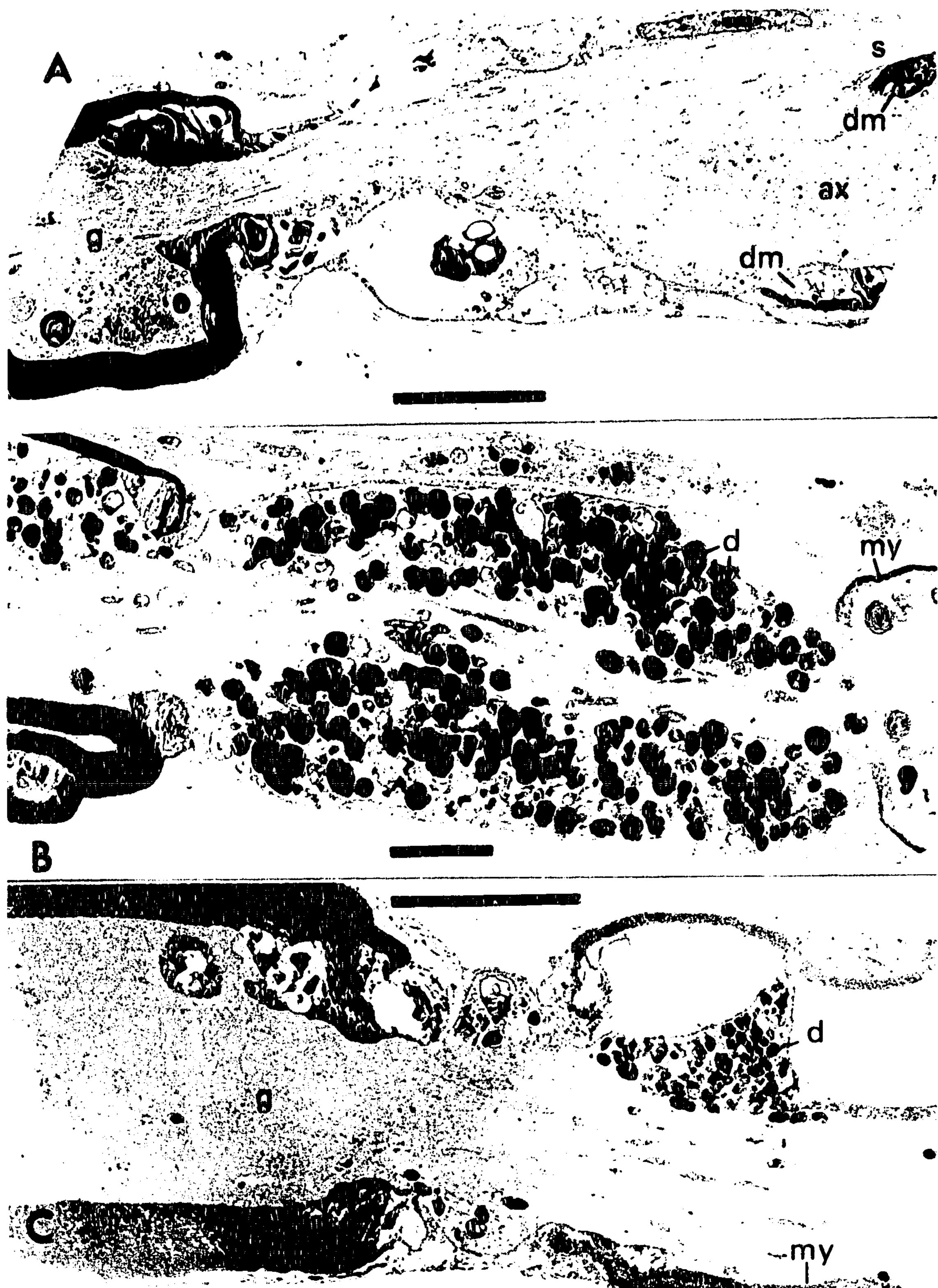
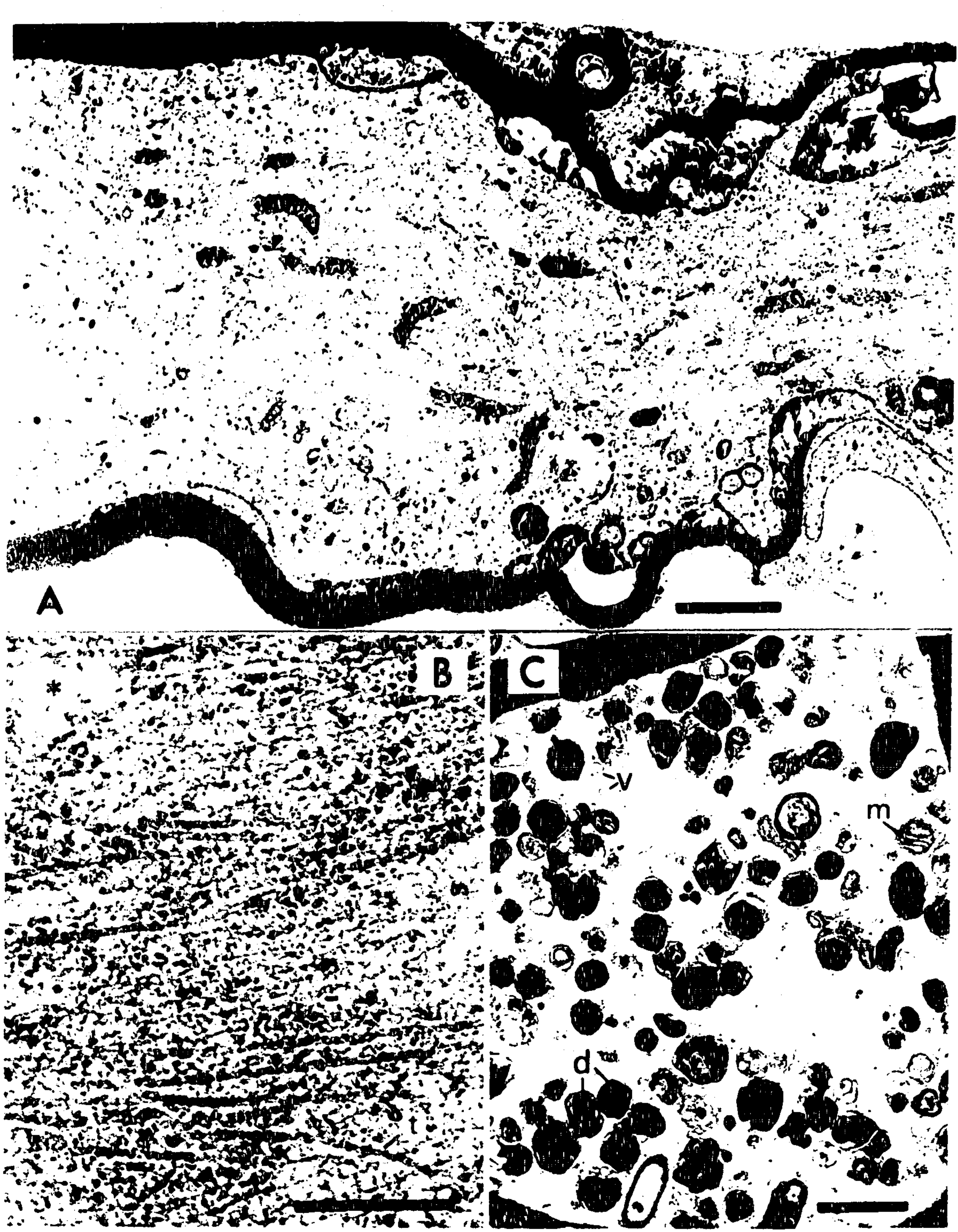

A
s
dm
ax
g
dm
d
my
B
d
g
C
my

Figure 4.
Details of organelles at the junction of parent and daughter axons; all electron micrographs are of longitudinal sections. **A**, section through the proximal side (to the left) of a junction at 7 days regeneration. Fine granular material not prominent in normal axoplasm is present throughout the section. Mitochondria and a few other large organelles are also present. **B**, a detail of the fine (approximately 10 nm diameter) granular material at the proximal side of the junctional region at 28 days regeneration. Microtubules (t) and neurofilaments course among the granules. The asterisk indicates an area of axoplasm with an electron denseness comparable to that of the axoplasm far from the junctional region. **C**, section through the accumulation of large membranous organelles at the periphery of the junctional region at 34 days regeneration. The accumulation of organelles consists of small vesicles (v), mitochondria (m), and dense bodies (d). Scale bars: **A**, 1 μm; **B**, 0.5 μm; **C**, 1 μm.


A
B
C
v
m
d
t
*

About 2 to 5 weeks after the crush, the distal stump of the parent axon at the junctional region had degenerated and was no longer seen in sections through the junctional region (Fig. 3B). The regenerating portion of nerve distal to the junction had become thinly myelinated. The node of Ranvier so formed was unusually long. For example, in nerves that had regenerated for 18 days, the length of the nodal gap measured 11-17 µm (n=5) (Mean nodal gap length in normal axons of the same nerve was 2.2 µm, n=10). The nodal region contained accumulations of large membrane bounded organelles resembling those which normally undergo retrograde axonal transport (Smith, 1980; Tsukita and Ishikawa, 1980; Fahim et al., 1985)(Fig. 4C). The organelles occupied a zone around the periphery of the axis cylinder. Vesicles with diameters of approximately 50 nm also occurred among the accumulation of larger organelles. Cytoskeletal elements rarely occurred with the accumulation of organelles in this region. The central core of the axon contained a normal distribution of cytoskeletal elements. An accumulation of 10 nm granules which sometimes contained a few glycogen granules was present proximal to the junction (Fig. 4B).

At 5 to 20 weeks of regeneration, the junctional region was still easily recognized by the unequal thickness of myelin on each side of the junction. The nodal gap had become narrower, although some junctional nodes were still as long as 5 µm. Accumulations of large organelles had diminished. However, proximal to the junction, the accumulation of 10 nm granules was still present and spanned as much as 30 µm in one axon examined (Fig. 3C).

Nodes of Ranvier immediately proximal to the junction were examined (Fig. 5A). Although profiles similar to the granules of the junction were seen within some nodes, these granules were much less abundant and mostly found in the nodal region. Large membrane-bounded organelles, although they were sometimes present, did not appear in large clusters. Newly formed nodes of Ranvier immediately distal to the junction were also examined (Fig. 5B). The cytoplasmic content of these nodes appeared normal and did not contain any aggregate of organelles.

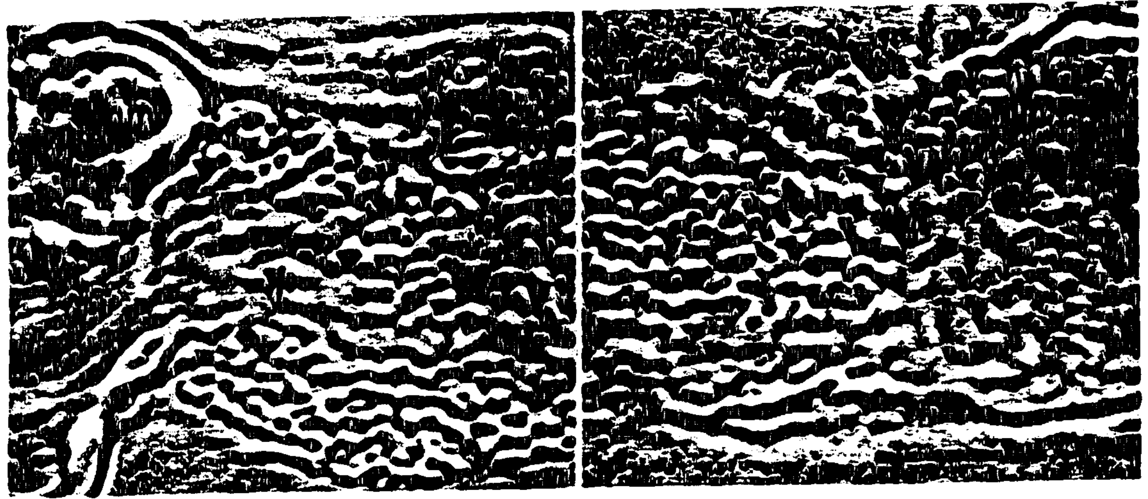
Computer-enhanced video microscopy of single myelinated axons in the region of the junction showed features of living fibers that corresponded well with the ultrastructural findings. The junctional regions were easily recognized in all the axons that had regenerated to a maximum period of 20 weeks. The parent axon was thickly myelinated while the daughter axon became thinly myelinated at regeneration times 2 weeks or more.

During the short period of regeneration (3 days to 2 weeks), axonal sprouts extended along the outside surface of the distal stump. Within the parent axon proximal to the junction, accumulations of small sub-resolution particulate bodies (<50 nm) could be detected. At the intermediate period (2 to 5 weeks, Fig. 6), the junctional region was similar in appearance to that of a node of Ranvier except for the unusually long length of the unmyelinated region. An accumulation of large organelles occupied the periphery of the nodal region. These organelles were stationary. A bidirectional organelle traffic was present through the central core of the axocylinder in the region of the junction. At longer regeneration

Figure 5.
Electron micrographs of longitudinal sections through the nodes of Ranvier. **A**. Node (parent) immediately proximal to the junctional node; 18 days regeneration. **B**. First node (daughter) distal to the junctional node; 34 days regeneration. The cytoplasm of the nodal regions shown in **A** and **B** does not contain any accumulation of organelles which is typical of the cytoplasm of the junctional node at these regeneration times. Scale bars, **A**, 1 µm; **B**, 2 µm.

A
B

Figure 6.
A montage of video-enhanced, differential interference contrast micrographs of a junctional node between the parent and daughter axons at 21 days after the crush. The proximal side of the junction is shown on the left and the distal side of the junction is shown on the right. The parent axon (to the left) has a thick myelin sheath (pm) while the daughter axon (to the right) has a thin myelin sheath (dm). Organelles such as those shown by the arrowheads accumulate at the periphery of the junctional node. The central core of the junctional node (*) contains transporting elements (microtubules) but is relatively free of organelles. The myelin sheath (dm) covering the daughter axon is thin compared to the myelin sheath (pm) of the parent axon. The video micrographs were taken at different focal planes. The length of the junctional node shown is similar the length observed in the specimen. The video image resembles closely the electron microscopic image shown in Figure 3B. Scale bar, 5 μm.



times (5 to 20 weeks), the appearance of the junctional region was similar to that of the intermediate times, however, organelle accumulations at the junction became less common. At 20 weeks of regeneration, only a few fibers contained clusters of organelles at the junction.

In summary, the particulate organelle content of the junction between parent and daughter axons (50 nm vesicles, large membrane-bounded organelles, and 10 nm granules) was characteristic of this region during regeneration.

4.2.2 Continuity of microtubules across the junction

The results described above show that in Xenopus peripheral nerve regeneration is very successful; hence a failure to regenerate cannot be used for explaining the observed accumulation of transported proteins at the crush site (see Results in Chapter 5). Therefore, the possibility that there was a defect in the transporting filaments, microtubules, at the junctional region was examined.

The location of the parent-daughter fiber junction of each individual axon was determined from a reconstruction of axonal morphology on the basis of light and electron microscopic examination of semi-serial sections. Cross-sections of axons (21 days regeneration) at location less than 200 µm each side of the junctions were used for the present analysis. A total of fifteen myelinated fibers with diameters ranging from 2 to 19 µm were examined. Two of these fibers were not suitable for analysis due to their small sizes. One could not be traced successfully. Eleven of the remaining twelve fibers had regenerated a single daughter axon. The number of

microtubules in the axis cylinder on both sides of the junction was counted. The remaining fiber had regenerated two daughter axons of similar size. The number of microtubules distal to the junction, in this case, was taken as the sum of the numbers of microtubules in the two daughter axons. The result, for each axon, was expressed as a ratio of the number of microtubules in the daughter axon(s) to the number of microtubules in the parent axon (Table 7). The mean (±SEM) of these ratios was 0.99 (±0.05) for n=12.

The results, together with the work on morphological examination of longitudinal sections through the junction described in Section 4.2.1, suggest that microtubules are continuous through the junction.

4.3 Conclusion

The structural work described here shows that the junction between the parent and the daughter axons in regenerating myelinated nerve is structurally unique. The internal structure of the junction is characterized by accumulations of organelles. The types of organelles present at the junction changes with regeneration time in a manner that suggests that the organelle accumulation is related to the participation of axonal transport in degenerative and regenerative processes. No evidence was obtained to suggest that the structure of the junctional region was dependent on the manner in which the nerve was desheathed prior to fixation.

Table 7

Numbers and ratios of microtubules in regenerating axons to number of microtubules in parent axons

Axon number	Number of microtubules in parent axon	Number of microtubules in daughter axon	Ratio
1	902	792	.88
2	360	384	1.07
3	431	396	.92
4	389	408	1.05
5	490	377	.77
6	382	299	.78
7	271	303	1.12
8	188	180	.96
9	261	336	1.29
10	433	390	.90
11	426	427	1.00
12	396	446	1.13
Mean			.99
SD			.15
SEM			.05

5 RAPID AXONAL TRANSPORT OF RADIOLABELED PROTEIN

Initially, in this study, the questions that were posed were: what are the characteristics of the rapid axonal transport of protein in regenerating nerve, and do these characteristics change in any way across the junction of the parent and daughter regions of the nerve? As the investigation progressed, it became clear that some of the results that related to the properties of the junctional region were influenced by the manner in which the nerve sheath was removed. Therefore, this investigation includes a study of the effect of nerve desheathing on the properties of rapid axonal transport in regenerating nerve.

5.1 Methods

5.1.1 Nerve preparation and pulse creation

The procedures by which a pulse of radioactively labeled protein was created in the sciatic nerve and by which the anterograde and retrograde transport of the pulse was detected have been described in detail (Snyder, 1986a). These methods may be summarized as follows.

The regenerating sciatic nerve was removed in continuity with its 9th dorsal root ganglion and a few millimeters of the spinal roots. The length of the sciatic nerve removed extended beyond the site of the crush, usually by several centimeters. A tie with a long thread was placed at the distal end of the nerve to provide a means of handling the nerve (distal ligature). The nerve preparation was transferred to a two-compartment tray so that the ganglion and its

spinal roots were contained in one compartment (ganglion compartment) and the sciatic nerve extended into the second compartment. The connection between the two compartments was a hole 1.5 mm in diameter and 3 mm in length, through which the nerve passed; this was sealed with silicone grease. The seal was found to be an acceptable diffusion barrier between the two compartments and did not caused any adverse effect on axonal transport. These operations were performed with the nerve preparation bathed in an oxygenated physiological saline (for composition, see Section 3.1.2).

The saline solution in the ganglion compartment was then replaced by 25 µl of saline containing 0.15 to 0.25 mCi of L-[^{35}S] methionine (specific activity 1,500 Ci/mmol, Amersham Corp.). After an initial incubation period of 1 hour to allow the label to enter into the cell bodies and to be incorporated into protein, the radioactive solution in the ganglion compartment was removed and replaced with plain saline. Following a further incubation period of about 3.25 hours for the incorporation of label to be completed and for the labeled protein to be transported into the sciatic nerve, the sciatic nerve was severed 4 to 5 mm distal to the ganglion. The sciatic nerve, now containing a pulse of labeled protein, was removed from the two-compartment tray and rinsed for 5 minutes with fresh saline solution to which non-radioactive methionine (1.0 mmol/l) was added. The addition of non-radioactive methionine to this and subsequent rinses reduced the incorporation of free ^{35}S-methionine in non-neuronal cells of the nerve. The nerve was then adjusted to its normal length using the method of Chan et al. (1980).

Desheathing (see Section 2.4), when required, was performed at this time. The purpose of removing the perineurial sheath from the nerve prior to recording is primarily to increase the efficiency of detection, especially when a low energy ß-emitter such as ^{35}S is involved. In this work, the perineurial sheaths were handled by one of the following procedures:

a) sheath was left intact on the nerve;

b) slit-desheath;

c) sheath was slit along the whole length of the nerve but not removed;

d) desheathed by eversion;

The nerve was rinsed again until 2.25 hours had elapsed from the time that the ganglion had been removed. At this time, a 10 mm proximal segment of sciatic nerve was cut off. The procedures of rinsing and removing an extra length of nerve produced a nerve which contained a discrete pulse of radiolabeled protein with little or no contamination caused by diffusion. The nerve preparation was finally transferred, at the standard length, to a one-compartment tray which contained a 3.6 µm thick mylar floor forming the exit window for ß-particles. Throughout the following recording period, the tray was perfused with about 170 ml of saline (containing non-radioactive methionine) at a temperature of 23±0.5°C. A coarse gauze stretched across a plastic frame gently pressed the nerve against the mylar exit window. The tray was placed on top of the mylar entrance window of a position-sensitive detector, the multiple proportional counter (MPC), so that the ß-particles emitting from the nerve could enter the detector.

All procedures, except otherwise stated, were performed at room temperature (20-22°C).

5.1.2 Liquid scintillation analysis

At the conclusion of each experiment, the nerve was processed for liquid scintillation counting (see Section 3.1.2).

5.1.3 Multiple proportional counter, data collection, and analysis

A full description of the multiple proportional counter (MPC) is beyond the scope of this thesis. Detailed descriptions of the use of position sensitive detectors of radiation in the assessment of axonal transport have been published (Snyder and Smith, 1982, 1984; Snyder, 1986a). The MPC (Snyder, 1984) consists of a series of single proportional detectors spaced at 3.18 mm intervals. Radiation that escapes the preparation and that penetrates the exit window of the nerve tray, enters the MPC via a 10 µm thick mylar entrance window. The counts detected by each proportional counter are recorded by a multichannel analyzer and stored for subsequent analysis on punched paper tape.

In this work, counts versus position spectra were recorded from the labeled nerve for 21-25 hours in 1000-second blocks. Three to five records obtained just prior to the placement of the nerve in the tray provided an average background spectrum which was subsequently subtracted from all records. Data obtained was rearranged into a form so that the counting rate for each 3.18 mm segment of the nerve could be plotted as a function of time (counts-

versus-time plot). All spectra were expressed as counts per hour. Subsequent analyses were made using these plots.

Transport rates and the time required for the reversal of transport to occur close to a lesion (the turnaround time) were determined for each preparation using techniques that have been described (Snyder, 1986a). In brief, five times, together with their standard deviations, were calculated from each counts-versus-time plot for each segment. The times were those when the anterograde pulse first reached 10% of its maximum peak counting rate (0.1 AF), its maximum counting rate (AP), when it fell to 10% of the stationary plateau (0.1 AS), and the times at which the retrograde pulse first reached 10% of its maximum counting rate (0.1 RF) and its maximum counting rate (RP). The arrival time of any part of the pulse of radioactivity at any nerve segment had a statistical error that originated in the counting statistics; this error was calculated in the assessment of arrival times. The calculated times were then plotted as a function of position along the nerve (time-versus-position plot). A straight line was fitted to each of the five sets of times, using a least-squares technique with data points weighted inversely as the square of the error in arrival time. The reciprocal slopes of the straight line fits gave transport rates for the respective parts of the pulse. Extrapolation of the lines to the position of the crush or the distal ligature yielded the turnaround time, defined as the difference in time between the arrival of the anterograde pulse and the departure of the retrograde pulse from the crush site or the distal ligature.

Deposition in the crush region was estimated from the amount of TCA-insoluble radioactivity in a 6 mm segment (two 3-mm segments) of nerve containing the crush site. This was expressed as a percentage (% deposition) of the activity contained in the pulse that traversed the segment. The activity contained in the pulse was taken as the TCA-insoluble activity in the crush segment plus the activity in all segments distal to the crush.

The % deposition in the crush region was defined as:

$$\% \text{ deposition} = \frac{C_{(C-1)} + C_{(C)}}{C_{(C-1)} + C_{(C)} + C_{(C+1)} + C_{(C+2)} + \ldots}$$

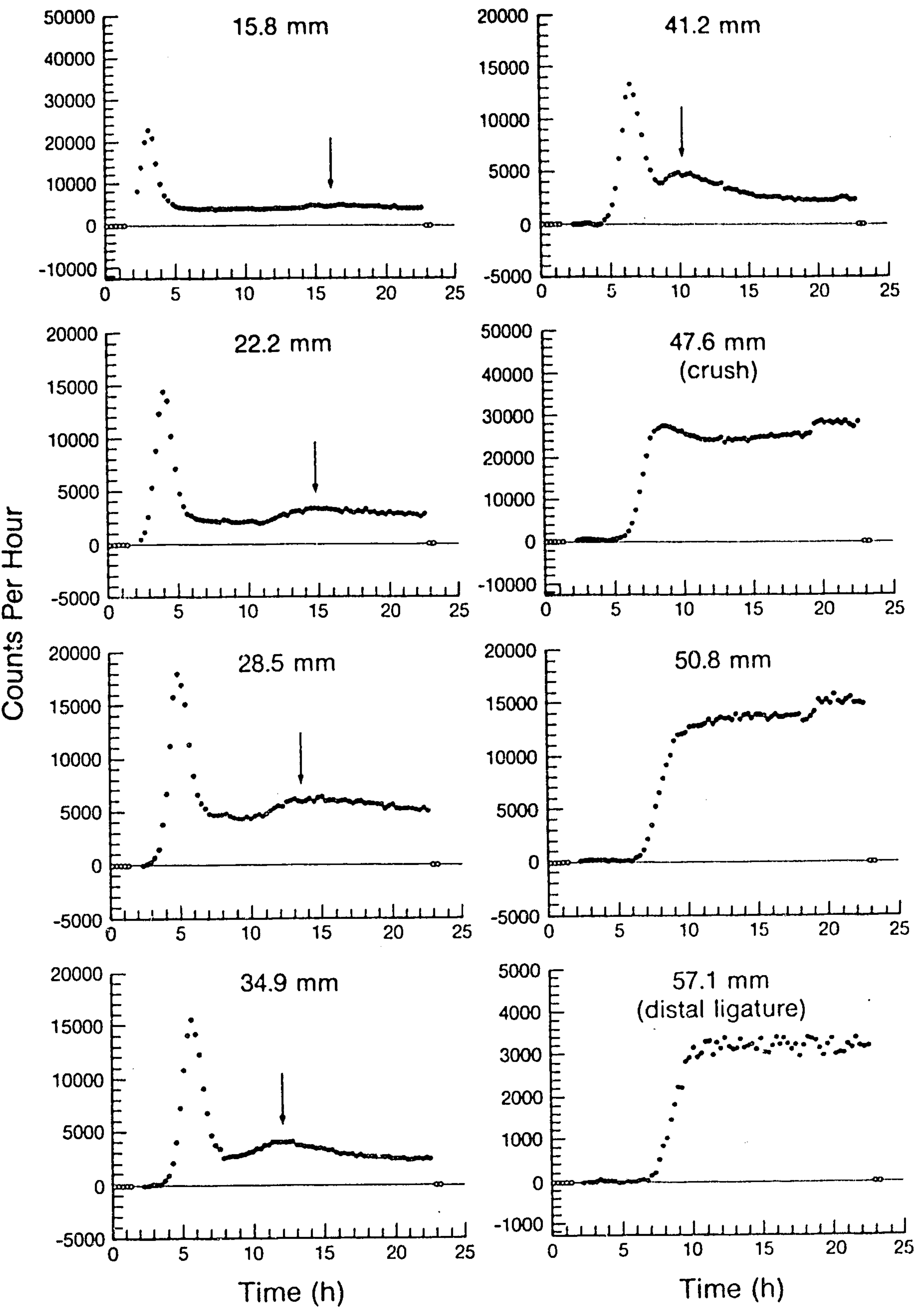
where (c) represents the segment containing the crush, (c-n) is the nth segment proximal to the segment (c), (c+n) is the nth segment distal to the segment (c), and $C_{(i)}$ represents the amount of radioactivity (counts per hour obtained from liquid scintillation analysis) of segment i.

5.2 Results

5.2.1 General observations

All nerves examined exhibited fast anterograde transport of a pulse of radioactivity along the nerve. A typical counts-versus-time plot (Fig. 7) for a segment of the parent nerve was represented by an initial quiescent period during which the labeled protein had not reached that particular segment. This was followed by an increase in counting rate when the anterogradely-moving radiolabeled pulse arrived. The counting rate peaked and subsequently decreased when

Figure 7.
Plots of counts-versus-time showing the propagation of a pulse of radiolabeled protein in a sciatic nerve 26 days after a crush lesion. Each plot (filled circles) shows the time course of the activity through a 3.18 mm segment of nerve. Open circles show the background activity of the nerve chamber recorded before and after the nerve run. The distance of the segment from the proximal end of the nerve is given in mm on each plot. For this particular nerve, nineteen plots were obtained; only eight are shown. A pulse of radiolabel moved in the anterograde direction and gave rise to an accumulation of label at the crush site between 6-8 h; later, a smaller pulse of label (arrows) moved in the retrograde direction.


15.8 mm
41.2 mm
22.2 mm
47.6 mm
(crush)
28.5 mm
50.8 mm
34.9 mm
57.1 mm
(distal ligature)
Counts Per Hour
Time (h)
Time (h)

the pulse exited the segment. Residual activity remained behind the pulse in each segment, this is thought to represent protein that is deposited from the rapidly moving phase to a stationary phase. At later times in some preparations a second pulse-like increase in counting rate occurred; this was shown to be due to retrogradely transport label.

5.2.2 Nerve preparations in which the sheath was handled by different methods

Fifteen nerves which had regenerated for 20-22 days were used. Radiolabeled proteins were detected in nerve segments distal to the crush site indicating that regeneration had occurred.

Based upon the results obtained by liquid scintillation and MPC analyses, two different types of behavior in rapid transport were identified. One type of behavior was typical of nerve preparations (Group 1) in which the sheath was either not removed (n=4), slit but not removed (n=1), or slit-desheathed (n=3). The second type of behavior was obtained from nerves (Group 2) in which the sheaths were removed by eversion (n=7).

With both Group 1 and Group 2 nerves, the patterns of anterograde transport along the parent nerve were similar. These patterns of pulse movement were essentially identical to that of normal nerves (Snyder, 1986a). Deposition in the parent nerve, which may be inferred from above background counting rate in a segment following the passage of the pulse (Fig. 7), is also identical to that of normal nerves (Snyder, 1986a).

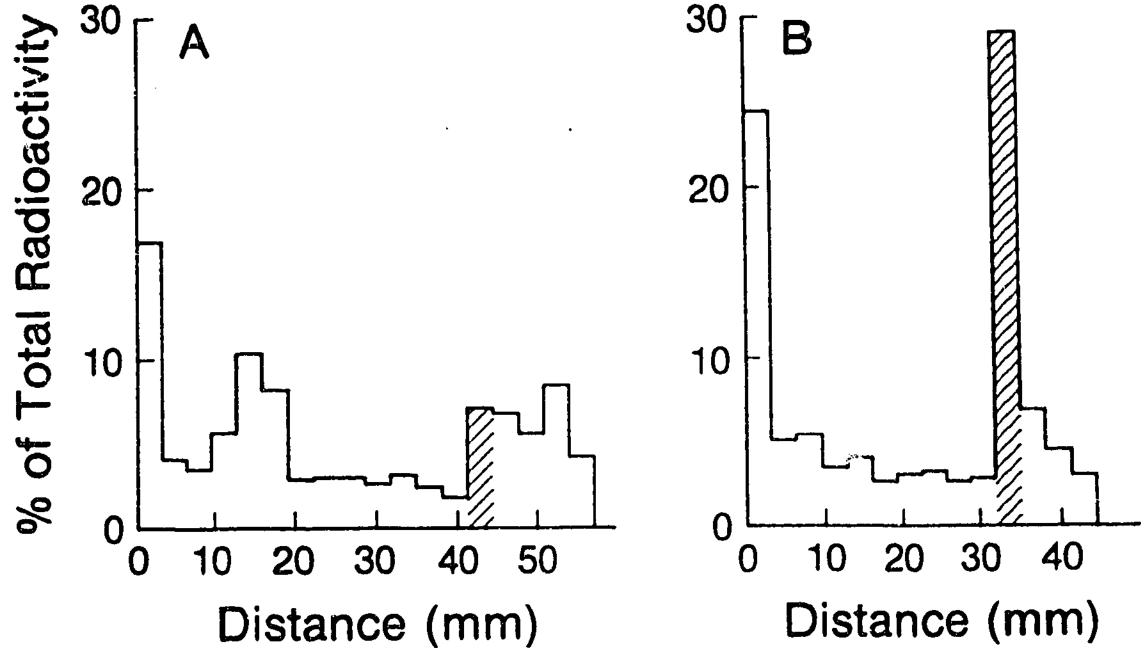
Unlike the parent nerves, there were differences between Group 1 and Group 2 preparations in transport characteristics in the region of the original lesion and along the daughter nerve. Group 1 nerves typically showed an approximately uniform, and statistically significant ($P<0.005$), 2-5 times increase in the amount of label deposited in a unit length at the crush site and in the daughter nerve compared to that deposited in the parent nerve (Fig. 8A). In addition, two nerves (out of 8) showed a larger deposition in the segment of the nerve containing the original crush site than the immediate adjacent segments (not shown). Group 2 nerves, on the other hand, all showed significantly greater deposition in the crush region than on either side of the crush and also showed monotonically decreasing deposition in the daughter nerve (Fig. 8B). Deposition in the crush region of Group 2 nerves was estimated to be 70±7% (mean±SEM), compared to 28±3% for Group 1 nerves.

The results are interpreted to indicate that the mechanical stress imposed on the nerve during the removal of the perineurial sheath by eversion might be the cause of the increase in deposition of radiolabels in the region of the original crush.

5.2.3 Nerves regenerated for 1 to 80 days, desheathed by eversion (Group 2).

The effect of desheathing by eversion on deposition and retrograde transport was investigated using nerves that had regenerated for various periods.

Figure 8.
Distribution of TCA-insoluble radiolabel determined by liquid scintillation analysis. **A**, perineurial sheath removed after being slit (Group 1). **B**, sheath removed by eversion (Group 2). Segment containing the crush denoted by cross hatching.


% of Total Radioactivity
A
B
30
20
10
0
0 10 20 30 40 50
0 10 20 30 40
Distance (mm)
Distance (mm)

a) deposition

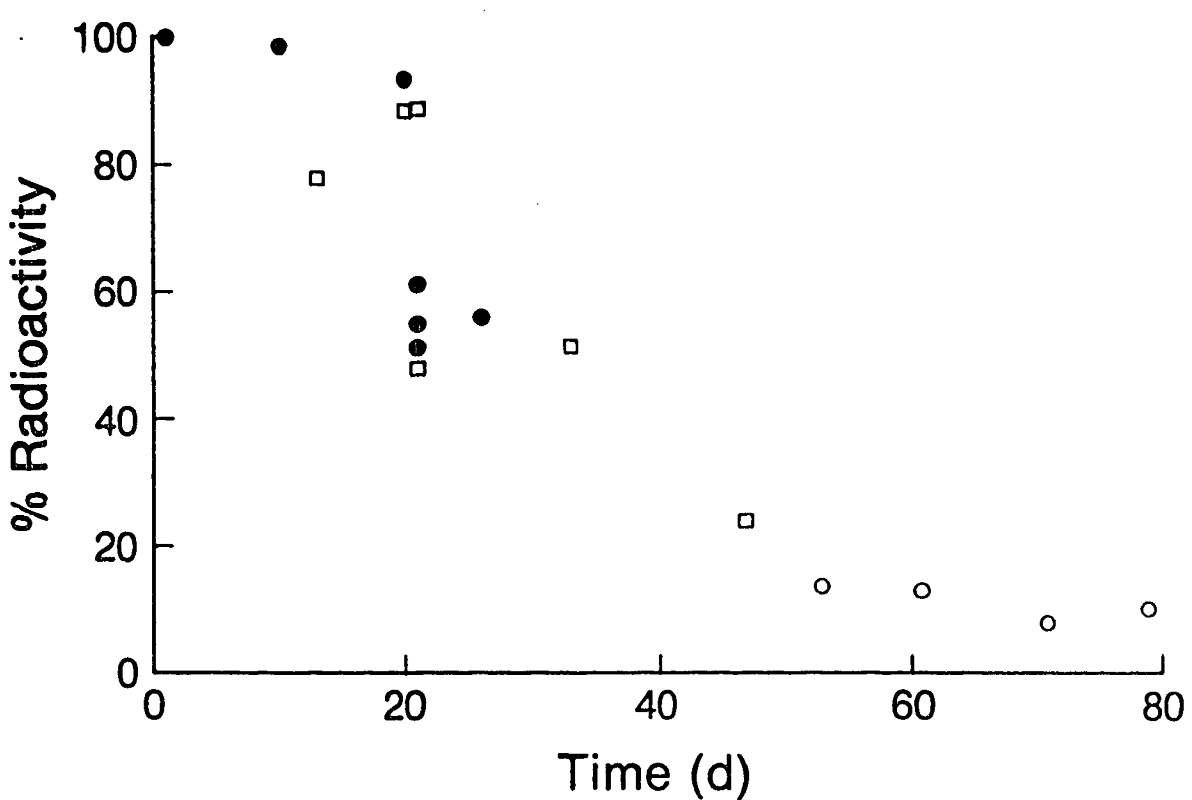
Deposition in the region of the crush decreased from essentially 100% at times less than 10 days to levels typical of the parent nerve by about 50 days (Fig. 9). Thus, the time-related pattern in deposition suggests that the sensitivity of this region to mechanical stress decreased with increasing maturity of the regenerating nerve.

b) retrograde transport

The detection of a retrograde pulse was made possible by examining the counts-versus-time plots (Fig. 7). In 11 of the 17 preparations, in addition to an anterograde pulse, a retrograde pulse was detected in the parent segment. A retrograde pulse was indicated by an increase followed by a decrease in counting rate similar to that of an anterograde pulse, but this occurred at a time later than that of an anterograde pulse and occurred in a time sequence in the series of segments indicating retrograde propagation. In the remaining 6 preparations, no retrograde pulse was detected. There are at least three possible explanations to account for the absence of a retrograde pulse in 6 of of 7 preparations: a retrograde pulse did not exist, or the pulse did not contain sufficient radiolabeled protein to be detected by this analysis, or the retrograde pulse was too dispersed to form the discrete pulse that was necessary for it to be recognized by this technique.

No retrograde pulse was detected in the daughter segment of regenerating nerves by this analysis.

Figure 9.
Deposition of TCA-insoluble radiolabel in the crush region of Group 2 nerves expressed as a percentage of the label which entered the region versus time after crushing the nerve. Filled circles indicate preparations in which a retrograde pulse of label originated from the crush region. Open circels, preparations in which a retrograde pulse originated at a ligature placed on the daughter nerve. Open squares, preparations with no evidence of a retrograde pulse.

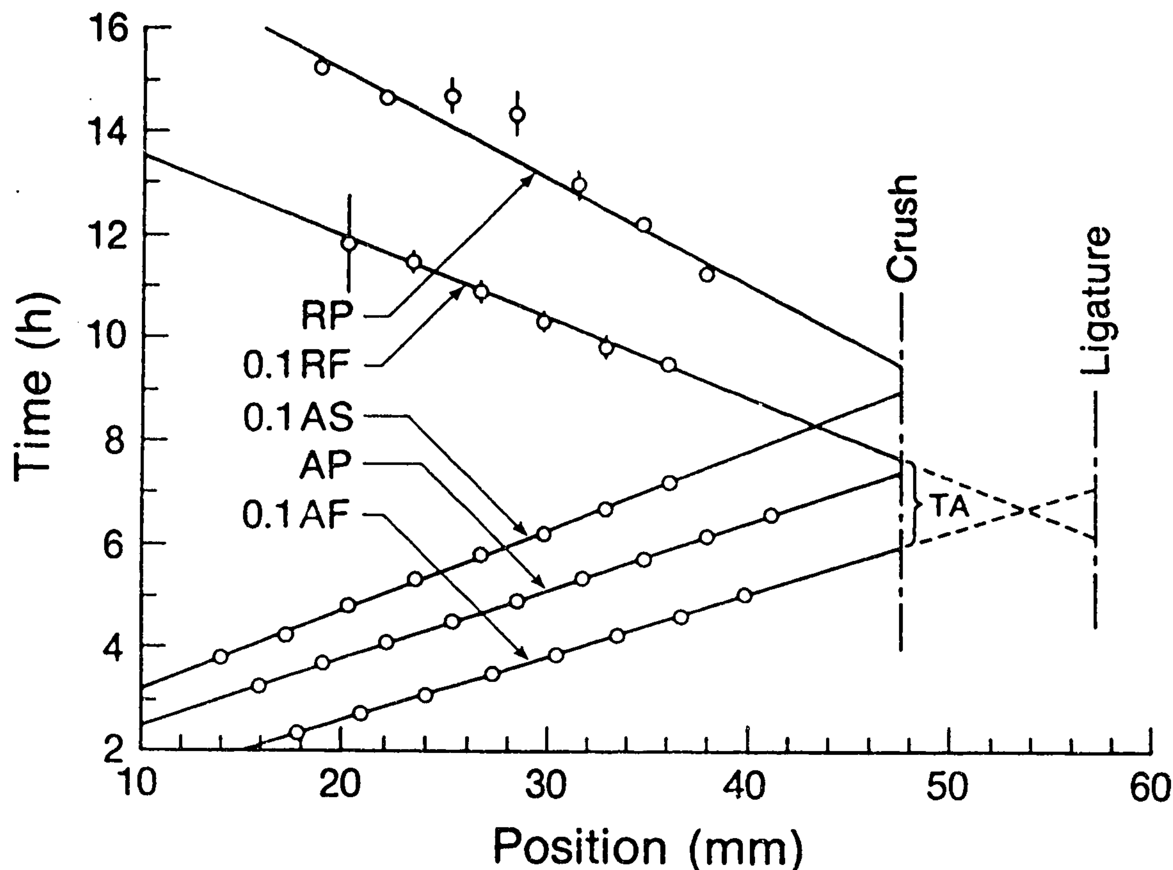

% Radioactivity
Time (d)
0
20
40
60
80
100

In those nerve preparations which showed a retrograde pulse, it is possible to estimate the site at which the retrograde pulse originated by extrapolation of the data points in the position-versus-time plot. For the example given in Fig. 10, it is obvious that the retrograde pulse could not have originated from sites along the proximal segment of the parent nerve. It is not likely that the pulse could have come from the distal portion of the parent nerve since MPC data showed that radiolabels were transported up to the region of the lesion. Neither is it possible that the pulse could have arisen just proximal to the distal ligature since the time required for the reversal of the anterograde pulse would have been negative. However, if a turnaround time of anterograde transport similar to that of the measured turnaround time at a ligature in normal nerves is assumed (Snyder, 1986a), then the retrograde pulse would have had its origin at or close to the crush site. On this basis, it seems that a reversal of anterograde transport was most likely to occur at the crush site where anterogradely transported protein accumulated.

The analyses of results from all the nerves, using similar reasoning, showed that at regeneration times of up to about 30 days, a retrograde pulse was produced probably from a reversal of an accumulation of anterogradely transported protein at the region of the crush. At regeneration times greater than 50 days, the retrograde pulse did not originate from the region of the crush, rather, it was formed by the reversal of anterogradely

Figure 10.

Transport-rate plot for the preparation whose counts-versus-time plots are shown in Figure 9. The nerve was ligatured distally at 57.1 mm. Data points show the times at which defined features of the anterograde pulse of radiolabel reached each segment of nerve. The times were those when the anterograde pulse first reached 10% of its maximum peak counting rate (0.1 AF), its maximum counting rate (AP), when it fell to 10% of the stationary plateau (0.1 AS), and the times at which the retrograde pulse first reached 10% of its maximum counting rate (0.1 RF) and its maximum counting rate (RP). No error bars indicates that the standard deviation is smaller than the size of the symbol. Extrapolation of the 0.1 AF and 0.1 RF lines to the position of the distal ligature shows that the retrograde pulse could not have been negative.



Time (h)
Position (mm)
RP
0.1 RF
0.1 AS
AP
0.1 AF
Crush
Ligature
TA
16
14
12
10
8
6
4
2
10
20
30
40
50
60

transported proteins that accumulated just proximal to a distal ligature on the daughter nerve (Fig. 9).

5.2.4. Transport rates

Transport rates in both the parent and daughter axons of Group 1 nerves (20-22 days) and the parent axons of Group 2 nerves (1-80 days) were determined from the slopes of the fitted lines of the time-versus-position plots. No differences in transport rates were obtained between the nerves at various regeneration periods or between nerves desheathed by different methods. Therefore, the results from all the nerves were combined to calculate the mean transport rates (Table 8). These rates were compared to rates estimated for normal, unoperated sciatic nerves of the same animal (Snyder, 1986a). The results show that the parent nerve exhibited anterograde and retrograde rates similar to those of normal, unoperated controls. In addition, the anterograde transport rate in daughter nerve was similar to the rate in the parent and normal, unoperated nerves.

5.3 Conclusion

The studies of axonal transport by radiolabeling axonally transported proteins show that transport rates in regenerating nerves are similar to those determined in normal non-elongating nerves. Anterograde transport rate is not affected across the crushed region. However, increased deposition is observed in the daughter segment of the nerve. Desheathing the nerve by eversion seems to cause an increased deposition of radiolabels in the crush region. This

Table 8

Mean ± SEM of transport rates in regenerating nerves at 23°C (mm/day)

Nerve type	Anterograde rate (0.1 AF)	Retrograde rate (0.1 RF)
Group 1 parent nerves (21d regeneration)	180.62±4.1 (n=5)	-
Group 2 parent nerves (1-80d regen)	181.5±2.7 (n=17)	160.4±5.7 (n=11)
Group 1 daughter nerves (21d regen)	182±18.3 (n=3)	-
Group 1 and Group 2 parent nerves (1-80d regen)	181.21±2.2 (n=22)	160.4±5.7 (n=11)
Normal nerves (Snyder, 1986b)	180.8±2.2	158.0±7.3

effect diminishes as regeneration time increases. The increased deposition of protein in the crush region and the daughter segment may reflect an active mechanism in the axoplasm that controls the amount of axonally transported protein to be retained for local uses.

6 ORGANELLE TRANSPORT ACROSS THE JUNCTION OF PARENT AND DAUGHTER AXONS

The results obtained from radiolabel transport experiments indicate that an arrest of axonal transport could occur in the region of the original crush of the regenerating nerves. This phenomenon was observed in some Group 1 nerves at the regeneration time examined (20-22 days) and in an exaggerated form in Group 2 nerves at regeneration times up to 30 days. The results did not, however, provide any information as to the cellular basis of the phenomenon of accumulation of protein in the region of the crush. It is possible that the mechanical process of desheathing by eversion resulted in a complete interruption of protein transport of some axons, which implied that the accumulation of protein at the region of the crush represented an experimental artifact; or the accumulation of protein may be related to the properties of the junction between the parent and daughter axons. These questions were investigated by videomicroscopy of individual regenerating axons.

6.1 Method

Anterograde and retrograde organelle transport were evaluated on each side of the junction of randomly selected axons at two regeneration periods. The periods selected were 21-27 days and 55-57 days. The bases for selection of these two periods were to examine the behavior of bidirectional organelle transport at times when a large portion of anterogradely transported radiolabeled

protein accumulated and reversed transport at the crush site (21-27 days), and when radiolabeled protein traversed the same region without impediment (55-57 days).

6.1.1 Single axon preparations and video microscopy

The method for preparing single axons for video microscopy has been described in Section 3.1.3.

6.1.2 Image recording

Regenerating myelinated axons which exhibited reasonable bidirectional transport were used. This criterion of selecting axons was necessary in order to avoid selecting axons which might have been damaged during the isolation procedure or axons with mechanical blockage due to axons overlapping one another.

The enhanced images of the axon at locations 20 to 100 µm proximal and distal to the crush were recorded on 3/4 inch video tape for subsequent analysis. Organelle traffic was estimated from a television display at a magnification of X10,000 by counting moving images that crossed a 2.5 µm segment of axis cylinder in a period of several minutes.

6.2 Results

6.2.1 General description

Individual axons in the region of the junction were examined. All axons showed structural continuity at the region of the junction. The great majority of axons at all stages of regeneration exhibited an active bidirectional transport of organelles across the

junctional region. Organelles that traversed the junctional region apparently do so without any hinderance. In some axons which had regenerated for less than 30 days, there was obviously more organelle traffic proximal to the junction than distal to it. This apparent imbalance was not related, in these preparations, to the manner in which the nerve sheath had been removed. The procedure of isolating individual axons for examination by teasing them apart likely caused as much mechanical stress on the junction as did the removal of the nerve sheath by eversion. Therefore, it was not possible to study organelle transport at the junction in "stress-free" regenerating axons regardless of the manner by which the sheath was removed.

6.2.2 Organelle transport

The numbers of organelles traversing a small volume of axis cylinder (2.5 µm by 1 µm by a depth equal to the focal depth of the objective lens, approximately 0.5 µm) were counted for several minutes at locations from 20 to 100 µm each side of the junction.

If there were no loss or gain of organelles across the junction, then the number of anterograde organelles traversing a small unit volume of axoplasm distal to the junction, A_2, is related to the number of anterograde organelles traversing a similar volume, A_1, proximal to the junction by the following expression:

$$A_1(d_1)^2 = A_2(d_2)^2$$

where d_1 and d_2 are the diameters of the axis cylinders as measured from the television monitor at the locations at which A_1 and A_2 were obtained. Similarly, retrograde organelle traffic is represented by

$R_1(d_1)^2 = R_2(d_2)^2$.

Fig. 11 is a diagrammatic representation of organelle traffic at the junction.

The results are expressed as a percentage loss in anterograde traffic,

$[A_2(d_2)^2 - A_1(d_1)^2]/A_1(d_1)^2$,

and a percentage gain in retrograde traffic (Table 9),

$[R_1(d_1)^2 - R_2(d_2)^2]/R_2(d_2)^2$.

Examination of fibers which had regenerated for periods 21-27 days and 55-57 days did not show any time-related different between individual fibers of the same period. Therefore, results obtained from axons of the same regeneration period were combined for analysis. For the 21-27 days regenerating fibers (n=12), there was a mean loss in anterograde organelle traffic (±SEM) of 73.7±8.1%, while retrograde traffic showed a gain of 299±66.6%. Both mean values were significantly different ($P<0.001$) from the expectation of no loss or gain across the junction between the parent and daughter axons. In contrast, the group of fibers that had regenerated for 55-57 days showed a loss in anterograde transport of only 1.0±4.5% and a gain in retrograde traffic of 6.3±8.1% (n=10). Neither of these values was different from the expectation of zero loss or gain.

The results show good correlation with those obtained from radiolabeled protein study. During the regeneration period 21-27 days, approximately 70% of anterograde traffic was lost across the junction, while the retrograde traffic across the junction increased by

Figure 11.

A, Schematic diagram of the junction between parent and daughter axons in a regenerating myelinated nerve fiber. Myelin is indicated by shading. A_1, density of anterograde organelle traffic proximal to the junction. R_1, density of retrograde organelle traffic proximal to the junction. A_2 and R_2 indicate the densitites of organelle traffic distal to the junction. Using these traffic densities (see Section 6.2.2), the mean apparent gain in retrograde traffic and loss in anterograde traffic were calculated for groups of 12 fibers that had regenerated for 21–27 days and 55–57 days (**B**). Error bars indicate SEM. **C**, a schematic interpretation of the findings, Ant = anterograde; Ret = retrograde. The loss in anterograde organelle traffic and gain in retrograde traffic is attributed to a reversal of anterograde transport at the junction.

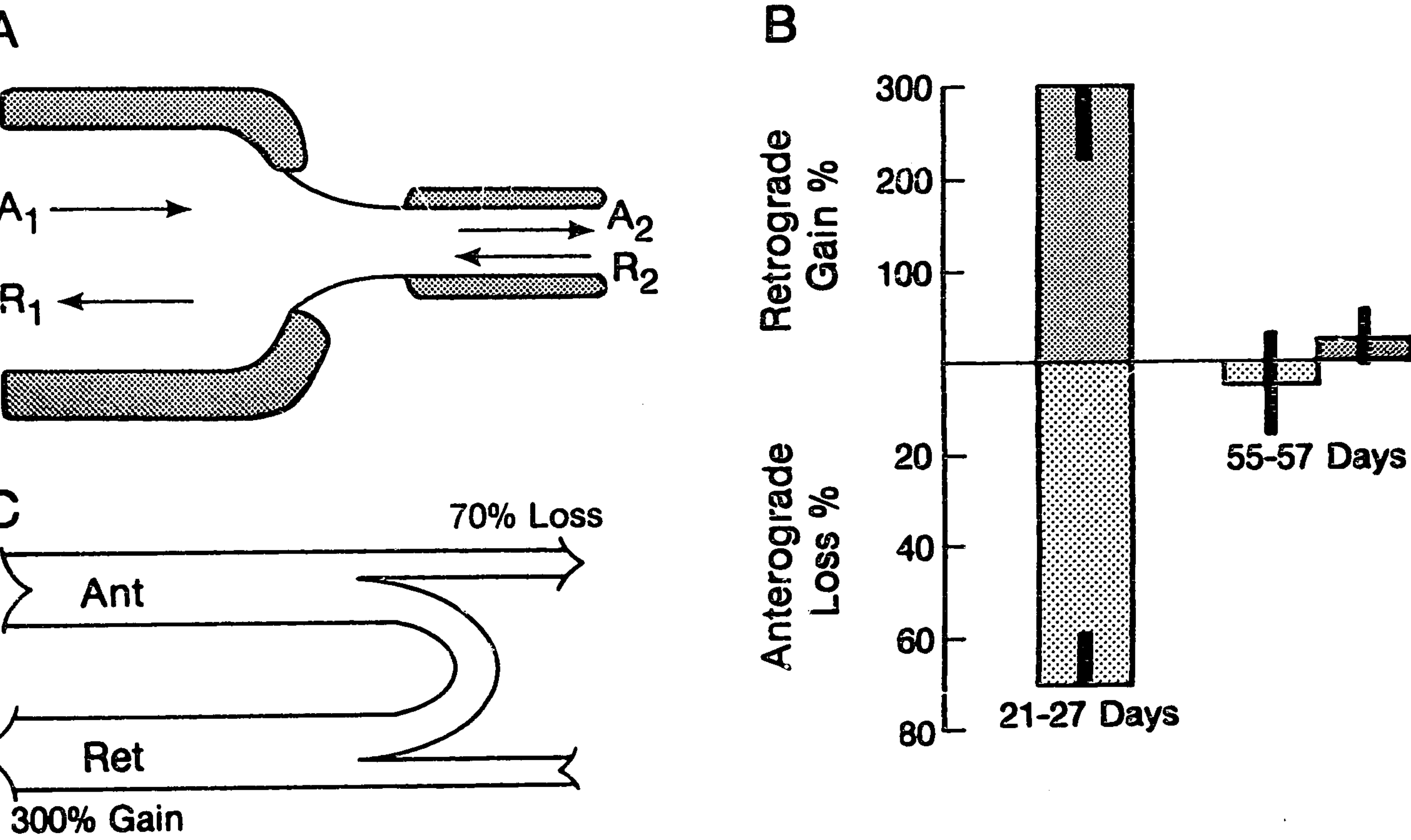

A
A1
R1
A2
R2
B
Retrograde
Gain %
Anterograde
Loss %
300
200
100
20
40
60
80
21-27 Days
55-57 Days
C
70% Loss
Ant
Ret
300% Gain

Table 9

Gain and loss of organelle traffic across the junction

	Regeneration times			
	21-27 days		55-57 days	
axon number	% anterograde loss	% retrograde gain	% anterograde loss	% retrograde gain
1	84.4	8.9	1.7	1.4
2	82.0	352	10.0	41.0
3	56.3	177	8.2	15.0
4	30.2	317	-8.1	12.5
5	85.2	254	-5.8	18.6
6	80.9	807	-21.7	10.0
7	70.7	146	18.9	39.0
8	92.7	489	-14.3	12.5
9	61.1	110	21.6	-50.0
10	83.7	591	-21.9	-46.0
11	83.7	103	21.4	12.5
12	-	233	2.4	8.9
MEAN	73.7	299	1.03	6.28
SD	18.1	230	15.66	27.9
SEM	5.47	66.6	4.52	8.06

about 300%. The results can be interpreted as follows: during the period 21-27 days, reversal of anterograde transport proximal to the junction resulted in an increase in retrograde transport and a loss in anterograde transport across the junction; during regeneration period 55-57 days, the reversal of anterograde traffic at the junction did not occur and normal behavior of organelle transport was restored.

7 DISCUSSION

7.1 Status of axonal regeneration

In this work, the axonal regeneration rate following crush injury in the sciatic nerve of Xenopus laevis was studied mainly by a morphological approach. The results show that the axons within the same nerve do not necessarily regenerate at the same rate. It was estimated that about 80% of myelinated fibers regenerated at a rate of 1 mm/day or faster at 20°C. A comparable rate of regeneration was estimated using a radiolabel method. These findings are in agreement with the rates obtained from peripheral nerves of other amphibians (Lubinska and Olekiewitz, 1950; Carlson et al., 1982; Edström et al., 1987; Edström and Kanje, 1988); regeneration in these studies occurred at approximately 1 mm/days at 20°C.

The results of this study suggest that most, and possibly all, of the axons produced sprouts that traversed the region of the crush successfully. No axonal "blind-ending" was observed. The completeness in initiation of regeneration was verified by an examination of a large number of fibers with videomicroscopy. Although the remarkable capability of regeneration possessed by the peripheral nerves of vertebrates and amphibians is well known, quantitative measures of the success of regeneration are scarce. The present work shows that regeneration is extremely successful in the adult Xenopus sciatic nerve after a crush injury.

In this work, a knowledge of both the regeneration rate and the completeness of regeneration is important if the properties of the junction between parent and daughter axons are to be inferred from

the distribution of axonally transported radiolabels in whole sciatic nerves. The morphological results provided evidence that the sciatic nerve successfully regenerates with no evidence of axonal degeneration or aberrant growth, therefore the properties of regenerating axons described in this work are not likely to be confused with the properties of axons that fail to regenerate successfully.

7.2 Structural properties of the junction between parent and daughter axons

In *Xenopus*, as in the rat sciatic nerve (Friede and Bischhausen, 1980; McQuarrie, 1985), sprouts initiate mostly at the first node of Ranvier immediately proximal to the lesion and at a time before the internode just proximal to the lesion completely degenerates. The implication from these observations is that this nodal region of the axon becomes structurally and functionally different from other regions of the axon.

The structure of the junction between parent and daughter axons described in this work is generally similar to that described in regenerating rat sciatic nerves (Zelená et al., 1968; Morris et al., 1972; McQuarrie, 1985). However, earlier morphological descriptions focus on times only shortly after injury (4 hours to 3 days), a period during which the axon undergoes both degenerative changes and regenerative changes. As a result, it is difficult to distinguish between regenerative processes, degenerative processes, and local axonal response to injury. The present work examined the region of the junction at regeneration times up to 20 weeks.

The morphological features of the junctional region can be briefly summarized as follows: at short regeneration times (3 days to 2 weeks), small clusters of 50 nm diameter vesicles typical of those that undergo anterograde transport (Smith, 1980; Tsukita and Ishikawa, 1980; Fahim et al., 1985) and uniformly distributed fine granular materials were present within the axoplasm on the proximal side of the junction. At the intermediate times (2 to 5 weeks), myelination of the new sprout had occurred. The junction so formed resembled a node of Ranvier with an exceptionally long nodal gap. Characteristic features within this nodal region included a rim of large organelles of types which are known to undergo retrograde transport (Smith, 1980; Tsukita and Ishikawa, 1980; Fahim et al., 1985). Small vesicles also occurred among the large organelles. Neurofilaments and bundles of microtubules coursed through the center of the axis cylinder. Granular materials continued to be present proximal to the junction. Quantitative investigation showed that the same numbers of microtubules were present on each side of the junction; this suggested that microtubules were continuous through the junction. At longer times of regeneration (5 to 20 weeks), densely packed granules were prominent proximal to the junction, however, the organelles that occupied the nodal region at earlier times had become scarce.

The organelle content of the nodes of Ranvier proximal and distal to the junctional region were examined and compared to those of the junctional region; these nodes did not contain the kind of organelle accumulations that were present in the junctional region.

These morphological findings show that the junction contains a unique composition of organelles (50 nm diameter vesicles, 10 nm granules, and large organelles) which is characteristic of this region of the regenerating axon.

The accumulation of large organelles that occurred at the region of the junction at intermediate regeneration times is similar to the peripheral accumulation of organelles that occurs in the junctional region of rat axons (Morris, 1972, Fig. 16). Similar organelles are normally present and retrogradely transported in the axoplasm of Xenopus axons (Smith, 1980). Therefore, the organelles that accumulate at the junctional node may represent retrogradely transported materials which are retained at the junction, and are not products derived from events associated with a local response to the crush lesion.

As to the origin of these organelles, it is suggested that retrogradely transported organelles are degenerative axonal structures from nerve terminals (Smith, 1980; Fahim, 1985). The organelles that accumulate at the junction may have had their origin in the distal region of the daughter axon, but it is also possible that they are retrogradely transported from other axonal sprouts which did not continue to grow. At the onset of regeneration, many sprouts are formed at the ends of individual axons (Morris, 1972; Friede and Bischhausen, 1980), but most of them disappear with increased time of regeneration (Shawe, 1955). Therefore, it is probable that the organelles represent retrograde transport of degradative material associated with the disappearance of these sprouts. The occurrence of myelination of the daughter axon at the same time may not be

entirely coincidental. In cultured neurites, the survival of the neurite depends on the local supply of nerve growth factor (Campenot, 1977). It is suggested that the Schwann cell may provide the neuron with nerve growth factor (Abrahamson, 1987). Sprouts which are myelinated may be locally supplied with nerve growth factor and continue to survive, while sprouts which are not myelinated are deprived of nerve growth factor and degenerate.

Regardless of the origin of these organelles, the reason that they remain in the junctional region for some time before they are eventually removed, presumably via retrograde transport, is not known. Evidence from the study shows that the delay in transport of these organelles is not caused by a structural deficiency in the transport system. Also, the delay cannot be satisfactorily explained by a limited capacity of the transport system since retrograde organelle traffic may be increased as much as three times in "stressed" axons.

It has been suggested that materials returning to the cell body may constitute a signal by which the cell body monitors axonal events at distant location (Bisby, 1987). The retention of retrogradely transported materials at the junction, either selectively or randomly, and the subsequent return of these materials to the cell body via retrograde transport, may have significant consequences on the nature of the signal that the cell body receives.

While many of the organelles that accumulate in the junctional region are identifiable as particulate components normally present in the cytoplasm, the identity of the 10 nm diameter granules that consistently occur proximal to the junction is not

known. A similar kind of accumulation of granules occurs at the junctional region of regenerating rat sciatic axons (McQuarrie, 1985, Fig. 7 top). In addition, granules of similar dimension occur in axons of Xenopus in the region of groups of microtubules (Smith, unpublished observation), proximal to some nodes of Ranvier of mammalian nerve (Berthold, 1968), and within clusters of microtubules in turtle axons (Schnapp and Reese, 1982). The size of these granules and their association with microtubules has led to the suggestion that they may represent the slowly transported, component b proteins. The present observations are consistent with the possibility that the accumulation of granules at the region of the junction is related to the dynamics of slow axonal transport.

The structural features suggest that the junction is a region of functional discontinuity at which the axonal transport of rapidly transported materials (vesicles and large organelles) and slowly transported proteins may be delayed. The cause of the interruption is unknown, but it is not related to the structural integrity of the transporting filaments.

7.3 Axonal transport in regenerating nerve

Both the proximal segment and daughter segment of a regenerating nerve exhibited similar fast rates of anterograde transport and these were essentially the same as transport rates in normal sciatic nerves (Snyder, 1986a). These findings are in agreement with studies on regenerating nerves of other vertebrates. The rate of fast transport did not change during regeneration (Frizell and Sjöstrand, 1974b; Ochs, 1976; Bisby, 1978; Griffin et al., 1981).

These results suggest that the underlying mechanisms of rapid axonal transport are essentially the same in regenerating axons and normal axons. Since the mechanisms and properties of axonal transport in the parent nerve did not seem to be affected by the manner in which the sheath was removed, it might be assumed that the transport rate of endogenous proteins which reversed direction at the region of the crush would be representative of retrograde transport in the parent portion of the regenerating nerve. The rate so obtained was also comparable to that reported for normal nerves (O'Brien and Snyder, 1982).

A portion of rapidly transported protein is normally deposited to a relatively stationary phase in vertebrate axons (Gross and Beidler, 1975; Ochs, 1975; Muñoz-Martinez et al., 1981, Snyder and Smith, 1985). The same phenomenon was observed in regenerating Xenopus nerves. The proportion of radiolabeled proteins deposited in the parent nerve was not different from that of normal nerve (O'Brien and Snyder, 1982). The daughter nerve, however, retained considerably more radiolabel, as in the case for glycoprotein in regenerating nerve (Tessler, 1980; Griffin et al., 1981), deposited protein is likely to be incorporated locally.

Results obtained from experiments with normal nerves seem to imply that deposition represents a passive process in which a constant proportion of the protein that passes through each segment of the nerve is retained (Muñoz-Martinez et al., 1981; Snyder and Smith, 1985). On the other hand, the pattern of deposition observed in the regenerating nerve suggests an active mechanism which controls the amount of material to be removed from the rapidly-

moving phase and retained in the stationary phase. A possible implication is that the control mechanism is local and the occurrence of regenerative processes in the daughter segment activate the control mechanism.

In some of the regenerating nerves studied, there was significantly more material deposited per unit length in the region of the original lesion than in either the parent or daughter nerves. This effect is similar to the long lasting deposition observed in the region of the lesion in regenerating nerves of other vertebrates (Forman and Berenberg, 1978, Bisby, 1978, Alberghina et al., 1983a,b; Danielsen et al., 1986). It is not likely that the observed increase of protein deposition in this region is produced as an artifact of experimental procedures. Furthermore, the present work shows that the effect is not caused by delay in regeneration, nor by events associated with failure of regeneration, nor by structural defects of microtubules in the region of the crush. The local accumulation of protein may also indicate that some non-passive mechanism causes deposition, and that this mechanism is uniquely active within the junctional region.

7.4 Induced changes in axonal transport in regenerating axons

The procedure of desheathing the nerve by eversion is used routinely in normal Xenopus nerve preparations to improve counting efficiency in studies of radiolabel transport. In studies of organelle transport, desheathing is a prerequisite to the isolation of single axons. In ultrastructural studies, removing the sheath facilitates the

infiltration of fixatives and embedding medium. There is no indication that the procedure alters the properties of axonal transport or causes structural damage in normal axons (O'Brien and Snyder, 1982; Snyder and Smith, 1985, Snyder 1986a,b). However, in preparations in which sciatic nerves had regenerated for up to approximately 30 days, this form of desheathing was associated in the present work with a large increase of protein deposition at the site of the original crush. The total amount of deposition in the region of the crush constituted about 70% of the rapidly transported label that entered the region.

The cellular basis of the above effect was investigated by videomicroscopy. The evidence obtained from the examination of single living fibers in the region of the junction indicates that the effect was not caused by complete damage to a large number of the axons, as all axons appeared structurally and functionally intact. This was confirmed by electron microscopic examination of axons in the region of the junction. No cytoplasmic defect within this region of the axon could be detected. A quantitative analysis of organelle transport across the junction of single living axons provided evidence that the deficit occurred in individual axons, and that the deficit was partial and was located at the junction of the parent and daughter axons.

It seems reasonable to assume that the changes in axonal transport in the region of the crush are induced by the procedure of desheathing. However, the occasional normal accumulation of label at the crush site (see Section 5.2.2) suggests that this effect may represent a normal phenomenon which is amplified by the stress imposed on the junction between the parent and regenerating axons.

The nature of the stress is not known, but stretching or compressing the nerve while removing the sheath are possible causes. In Aplysia neurons, it has been demonstrated that stretching can lead to an arrest of axonal transport (Koike, 1987). The investigator suggested that the deficit might have been caused by a disruption of the normal structure or arrangement of microtubules. The evidence presented in this study indicates that the accumulation of transported protein in the region of the original crush in these nerve preparations is not related to the structural integrity of microtubules in the region of the junction.

While it is generally acknowledged that reversal of transport occurs at the nerve terminal or at a lesion (Bray et al., 1971; Bisby and Bulger, 1977; Smith, 1987, 1988), this work describes a reversal of organelle transport within a continuous axonal channel in which no structural abnormality can be detected. Moreover, the behavior of both anterograde and retrograde organelles at the junctional region suggests that whatever the cellular alterations the process of removing the sheath by eversion might provoke, the effect of this alteration is apparently restricted to anterogradely moving organelles. This suggests that there may be two distinct mechanisms for regulating direction of transport in vertebrate axons (Smith, 1987, 1988).

Although it is reasonable to assume that the reversal of organelle transport at the junctional region of single axons correlates with the retrograde pulse of protein detected in these nerve preparations, the large deposition of protein at the region of the lesion cannot be satisfactory explained solely on the bases of

organelle transport. The present findings indicate that the organelles which failed to traverse the junctional region eventually reversed their direction of transport. Therefore, a possible explanation is that a large portion of protein, which was transported in structural association with organelles, was "unloaded" from the organelles and retained within the cytoplasm at the region of the junction, while the carrier-organelles reversed their direction of transport and did not accumulate at the junction.

It has been reported that rapidly transported protein is released from intact normal sciatic nerve (Hines and Garwood, 1977). The phenomenon was not confirmed by Tedeschi et al. (1981), but Snyder (1987) did show that rapidly transported proteins and polypeptides are released from normal, intact Xenopus sciatic nerves, and that the release of these materials is coincident with the passage of rapidly transported protein. In the desheathed, regenerating sciatic nerve of the bullfrog, a release of fast transported proteins and polypeptides has been detected in the daughter portion of the regenerating nerve with most of the materials seemingly originating from the junctional region (Tedeschi and Wilson, 1987). Tedeschi and Wilson proposed that these materials might serve as intercellular signals. These findings, together with results obtained from the present work, may be interpreted as follows: a proportion of axonally transported proteins is unloaded into the cytoplasm from the transporting mechanism. Some of these proteins may be retained within the cytoplasm, but some may be promptly released into the inter-axonal space. The mechanism controlling the quantity of protein to be released is unknown, but if the amount to be released

is dependent on the quantity of proteins that is contained within the immediate cytoplasm and that is not bound to the transporting mechanism, then it is to be expected that more material will be released from the daughter portion than from the parent portion. The region of the original lesion where a greater deposition of transported materials is induced by desheathing is therefore likely to release more material than either the parent or daughter axons.

7.5 General conclusion

This work was performed using three different methods. Each of these methods gave a different perspective on axonal transport in regenerating axons. The methods were: 1) the ultrastructural examination of fixed tissues using thin-section, transmission electron microscopy; 2) the study of the axonal transport of radiolabeled protein and 3) the detection of the movement of organelles in single living axons using enhanced video-microscopy. The ultrastructural method permitted the identification of various cellular components. Dynamic information on the average properties of axonal transport was obtained with the radiolabeling method. The optical method provided a high temporal and spatial resolution of the motion of organelles in individual nerve cells. Results obtained with these three methods allowed inferences that could not have been drawn from the results of one technical approach above.

This work provides evidence that the junctional region between the parent and daughter axons in regenerating nerve is not merely a segment of axonal channel with properties that are the

same as those of the axon to either side. The junctional region is a structurally and functionally distinct segment of the regenerating axon.

The role of the junctional region in regeneration is far from being understood. However, this work does allow some suggestions to be made. The junction between parent and daughter axons is a region at which almost step-like changes in the dynamics of both fast and slow axonal transport may take place. Some of these changes, such as the increased deposition of rapidly transported proteins, may be of importance for the incorporation of molecules into the structure of the growing axon. The junction may also play an important role in signalling the status of regeneration both within the individual neuron and between the axon and other surrounding cells. This work shows that under some experimental conditions, and at regeneration times up to several weeks, newly synthesized proteins and vesicles are reflected from the junction back towards the cell body. A similar, although perhaps not as pronounced, reversal of rapid axonal transport could act as a signal to the cell body that a continued output of growth-associated molecules is required. It is also possible that the transport back to the cell body of degenerative materials that initially accumulate at the junction may act as a similar signal. The complex process of nerve regeneration is known to involve intercellular as well as intracellular signalling. Newly synthesized polypeptides that are deposited in abnormally large amounts at the junction and within the daughter axon may be released into the extracellular space to act as intercellular signals.

These suggestions, some more speculative than others, may point the way to future work on the role of the junction between parent and daughter axons in axonal regeneration.

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