THE UNIVERSITY OF CALGARY

ORTHOGRADE AND RETROGRADE AXONAL TRANSPORT OF LABELLED PROTEIN IN INTACT AND INJURED PERIPHERAL NERVE

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "ORTHOGRADE AND RETROGRADE AXONAL TRANSPORT OF LABELLED PROTEIN IN INTACT AND INJURED PERIPHERAL NERVE" submitted by Victor Thomas Bulger in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

The purpose of this research was to investigate the hypothesis that retrograde axonal transport functions as a feedback regulator of nerve cell body metabolism. This theory postulates that levels of cell body synthesis are responsive to changes in peripheral metabolic requirements, which are signalled via retrograde transport. This was tested by determining alterations in orthograde and retrograde axonal transport of ^{3}H-L-leucine-labelled protein in acutely- and chronically-injured rat sciatic nerve.

The ability of the retrograde transport system to signal a change in peripheral requirements was studied in acute experiments. A premature return of labelled protein was found in ligated nerves. Following concurrent nerve ligation and precursor injection, labelled protein reversed its direction of transport at a maximum of 1.8-2.4 hours after arrival at the ligature. The minimum retrograde transport velocity of the protein was 112-133mm/day. Electrophoretic characterization of labelled protein transported in the peak of retrograde-transported activity in intact and ligated motor axons revealed major differences in relative amounts of constituent polypeptides. A premature return of labelled protein was also found after blockage of orthograde transport by sub-epineurial injection of colchicine, the effects of which mimic axotomy. The premature return of protein in injured nerves was considered to be a possible signal of axotomy to the neurone cell body. A classically-defined chromatolytic response was absent from most axotomized sciatic motoneurones at up to 5 days after nerve ligation, but this evidence was not considered sufficient to cast doubt upon the postulated axotomy signal.

The responsiveness of cell body metabolism to peripheral feedback was studied in chronic experiments. Chronically-injured and regenerating sciatic motor axons demonstrated length-dependent alterations in orthograde protein transport. Orthograde transport at 9-11 hours after precursor injection decreased to 70% of control values within 1 day of nerve ligation. This reduced level of transport was maintained in non-regenerating nerves for up to 30 days. In regenerating nerves orthograde transport returned to control values by 30 days after ligation, coincident with functional reinnervation. The above-normal magnitude of retrograde transport found at 9-11 hours after precursor injection in acutely-ligated nerves was maintained in non-regenerating nerves. In regenerating nerves peak retrograde transport occurred at later times after precursor injection, reflecting increasing nerve length. By 30 days retrograde transport in regenerating nerves was similar to that found in intact nerves. These changes suggested that cell body protein synthesis is responsive to the changing metabolic requirements of regenerating nerves.

The results of this research therefore support the hypothesis that retrograde axonal transport mediates feedback regulation of nerve cell body metabolism.

Some additional observations were made on the transport reversal process that develops at a ligature. The same fraction of orthograde-transported protein was returned from ligatures applied to sensory axons and motor axons ligated at different distances from the spinal cord, although greater absolute amounts were returned from more proximal ligatures. Electrophoretic profiles of labelled protein from the peak of prematurely-returned protein in ligated motor axons resembled those

previously obtained for fast orthograde-transported protein. This evidence suggested that orthograde-transported protein accumulating at a ligature may be non-selectively reversed and returned to the neurone cell body. The amount of protein that is returned is length-dependent, and may serve to inform the cell body of the site of axotomy.

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TO

A.G.E. NORTH,

FOR

FRIENDSHIP AND WISDOM,

THIS WORK IS

DEDICATED

TABLE OF CONTENTS

	Page
ABSTRACT	iii
ACKNOWLEDGEMENTS	vi
DEDICATION	vii
LIST OF TABLES	xii
LIST OF FIGURES	xiii
LIST OF PLATES	xv
CHAPTER I. INTRODUCTION	1
1. Historical Background	1
2. Characteristics of Orthograde Axonal Transport	4
3. Characteristics of Retrograde Axonal Transport	8
4. Research Proposal	11
CHAPTER II. MATERIALS & METHODS	14
1. Experimental Animals	14
2. Reagents	14
3. Labelling of Transported Proteins	14
i) Preparation of Precursor	14
ii) Surgical Preparation for Precursor Injection	15
iii) Precursor Injection	16
iv) Post-Operative Care and Observations	17
4. Sciatic Nerve Ligation	18
i) Why	18
ii) How and When	18
iii) Where	18
iv) Timed Accumulation of Labelled Protein	19

Page

5. Analysis of Protein Transport. 19

i) Location of Accumulated Labelled Protein. 19

ii) Quantification of Labelled Protein Accumulation By Scintillation Counting 21

iii) Quantitative Analysis of Labelled Protein Transport. 23

iv) Calculation of a Correction Factor 25

v) Quantitative Analysis of Total Accumulated Activity . 27

CHAPTER III. REVERSAL OF AXONAL TRANSPORT AT A NERVE CRUSH . . . 29

1. Introduction 29

2. Materials and Methods. 30

i) Intact Axons. 30

ii) Injured Axons. 31

iii) Transport Determination 32

3. Results. 32

i) Orthograde Transport in Intact and Injured Axons. . . 32

ii) Retrograde Transport in Intact Axons 32

iii) Retrograde Transport in Injured Axons 33

iv) Retrograde Transport in Motor Axons Injured 6 Hours Post Injection. 37

v) Labelled Protein Transport Into the Sciatic Nerve in the First 30 Hours P.I. 39

vi) Return of Labelled Protein from Intact and Injured Nerves. 43

4. Discussion 43

CHAPTER IV. QUALITATIVE DIFFERENCES IN NORMALLY & PREMATURELY-RETURNED PROTEIN. 52

1. Introduction 52

Page

2. Materials and Methods. 53

i) Collection of Labelled Protein. 53

ii) Electrophoresis Protocol 53

iii) Protein Characterization. 55

3. Results. 56

4. Discussion 61

CHAPTER V. RETROGRADE TRANSPORT OF PROTEIN AFTER COLCHICINE TREATMENT. 67

1. Introduction 67

2. Materials and Methods. 68

i) Colchicine Treatment of Nerves. 68

ii) Evaluation of Colchicine Effects 70

3. Results. 70

4. Discussion 71

CHAPTER VI. NERVE CELL BODY RESPONSE TO AXOTOMY. 76

1. Introduction 76

2. Materials and Methods. 77

3. Results. 78

i) Control Cells 79

ii) Axotomized Cells 79

4. Discussion 83

CHAPTER VII. TRANSPORT IN REGENERATING & NON-REGENERATING NEURONES 86

1. Introduction 86

2. Materials and Methods. 87

3. Results. 88

i) Behavioral. 88

Page

ii) Anatomical 89

iii) Protein Transport 90

4. Discussion 97

CHAPTER VIII. CONCLUSIONS. 105

REFERENCES. 110

LIST OF TABLES

TABLE Page

2.1 Comparison of Relative Accumulation Values Obtained from 2 Hour and 3 Hour Collection Intervals. 26

5.1 Orthograde and Retrograde Protein Transport in Sciatic Nerves Injected with Saline or Colchicine. . . 73

LIST OF FIGURES

FIGURE		Page
2.1	Diagram of the Rat Sciatic Nerve.	20
2.2	Representative Activity Profile Along Sciatic Motor Axons 10 Hours After Precursor Injection	22
3.1	Retrograde Transport in Normal and Injured Sciatic Motor Axons.	34
3.2	Retrograde Transport in Normal and Injured Sciatic Sensory Axons.	34
3.3	Retrograde Transport in Motor Axons Ligated at the Time of Precursor Injection or 6 Hours Later	38
3.4	Comparison of Orthograde and Retrograde Transport in Ligated Sensory Axons	40
3.5	Total Labelled Protein Accumulation at the Injury Site.	41
3.6	Return of Labelled Protein by Retrograde Transport. . .	44
4.1	Molecular Weight Calibration Curve for 4% Polyacrylamide Gel Columns	58
4.2	Distribution of Labelled Polypeptides and Coomassie-Blue Stained Materials on 4% Polyacrylamide Gels.	59
5.1	Transported Protein Distribution After Sub-Epineurial Colchicine Injection.	72
7.1	Transported Protein Distribution in Regenerating and Non-Regenerating Motor Axons	91
7.2	Orthograde Transport in Chronically-Injured Motor Axons.	93
7.3	Retrograde Transport in Chronically-Injured Motor Axons.	93

FIGURES

Page

7.4 Retrograde Transport in Injured Motor Axons at Different Stages of Regeneration 95

7.5 Relative Incorporation of Precursor into Proteins of Regenerated and Intact Portions of Regenerating Motor Axons. 96

LIST OF PLATES

PLATE		Page
6.1	Control Sciatic Motoneurones	80
6.2	Control Motoneurone.	80
6.3	Control Motoneurone.	80
6.4	3 Day Axotomized Sciatic Motoneurones.	81
6.5	4 Day Axotomized Motoneurone	81
6.6	4 Day Axotomized Motoneurones.	81
6.7	5 Day Axotomized Motoneurones.	82

CHAPTER I

INTRODUCTION

1. HISTORICAL BACKGROUND

Neurones are cells unique both physiologically and morphologically. Functionally distinctive due to selective excitability characteristics, they have evolved into the complex information processing systems of higher organisms through structural modifications permitting rapid signal conduction and integration. As a rule, after integration of incoming signals at the receptive surface of the neurone, impulses are transmitted to subsequent neurones or effectors after conduction across distances of up to several meters along cytoplasmic extensions known as axons. While greatly facilitating coordinated nervous system function these elongated processes pose a maintenance problem for the neurone. Since in vertebrates, at least, axoplasm and axonal organelles are essentially incapable of macromolecular synthesis (Droz & Koenig, 1969), their functional and structural integrity depend upon metabolic communication with the synthetic machinery in the cell body.

The necessity for molecular transfer between the nerve cell body and its axon was first proposed by Waller (1850) to explain the degeneration of the distal nerve segment after peripheral nerve section. A similar transfer process was assumed by Scott (1906), who considered that the fatigue of an isolated nerve-muscle preparation by nerve stimulation

resulted from depletion of a "secreted" substance originating in the spinal cord cells. Since then the time course of neuromuscular transmission failure and the onset of denervation changes in muscle fibres after nerve section have been shown to depend upon the length of the remaining distal nerve stump (Usherwood, 1963; Miledi & Slater, 1970; Thesleff, 1974). More recently still, in an extension of Waller's observations, it has been demonstrated (Joseph, 1973; Lubinska, 1977) that after nerve section degeneration proceeds distally from the site of section at a constant rate. Clearly the evidence suggests the existence in nerves of a transport system functioning to supply axons and terminals with metabolic products synthesized in the cell body.

The first work to conclusively demonstrate the orthograde movement of material in nerves was that of Weiss & Hiscoe (1948). In regenerating peripheral nerve in rats and other mammals a ballooning of axons was found proximal to an applied constriction. Upon removal of the constriction the expanded region of axoplasm moved distally along the nerve at a velocity of 1-2mm/day. This was interpreted to show the movement of a column of axoplasm.

Radioactive labelling of neuronal phospholipids by ^{32}P injected either intraperitoneally into guinea pigs (Samuels *et al.*, 1951) or directly into the spinal cord of cats in the vicinity of ventral horn motoneurones (Ochs & Burger, 1958; Ochs *et al.*, 1962) permitted demonstration of a proximo-distal phospholipid gradient along the sciatic nerve. Appearance of labelled phospholipids in the nerve could be prevented by spinal cord compression before precursor injection, which resulted in asphyxiation of the sciatic motoneurones (Ochs &

Burger, 1958). The slope of the phospholipid gradient changed with time, suggesting movement away from neurone cell bodies at a velocity of 3-4mm/day. A similar gradient of ^{32}P-labelled phospholipids was found in feline vagus and hypoglossal nerves moving at an estimated velocity of 40-72mm/day (Miani, 1963). This gradient was not due to labelled nerve compounds synthesized locally by neuroglial cells. The labelled material was thus suggested to be transported intra-axonally. Autoradiographic evidence supported this hypothesis (Droz and Leblond 1962, 1963). After i.p. injection, in rats, ^{3}H-leucine entered spinal cord neurones and was synthesized into protein (Leblond *et al.*, 1957). At increasing times after injection peripheral nerves were removed and prepared for autoradiography, and demonstrated intra-axonal activity peaks moving distally along the nerves at velocities of several millimetres per day. At about the same time Lubinska *et al.* (1964), reporting on the accumulation rate of acetylcholinesterase (AchE) in sectioned peripheral nerve, estimated its velocity of transport in motor axons to be 30-60mm/day.

The theory of Weiss & Hiscoe (1948) that proposed a bulk flow of axoplasm was inadequate to account for the selective, rapid transport of enzymes and phospholipids. It was evident that more detailed study of the characteristics of axonal transport was required.

The reports of Lasek (1966, 1968), Kerkut *et al.* (1967), and Ochs *et al.* (1967) represent a watershed in the study of axonal transport. These works introduced the technique of localized precursor microinjection to selectively label neuronal proteins, which considerably reduced the errors of non-specific labelling. Pulse-labelling of protein synthesis

produced transported proteins with high specific activity, permitting demonstration of a phase of protein transport moving at a velocity of several hundred millimetres per day. The role of this mechanism in the developing and mature neurone has since been intensively studied.

2. CHARACTERISTICS OF ORTHOGRADE AXONAL TRANSPORT

Axonal transport has been studied by light and electron microscopy, and by detection of transported enzymes and newly-synthesized molecules identified by precursor labelling. Direct observation by Nomarski and darkfield optics has permitted characterization of the transport of a population of large (>0.5μM diameter) axonal organelles (Cooper and Smith, 1974; Leestma, 1976). Transport has been characterized by following the distribution of labelled molecules after precursor injection, and by quantifying accumulation of labelled molecules and identified enzymes at sites of nerve section or ligation. The techniques of electron microscopy have been utilized to investigate the structural correlates of transport in axons. Comprehensive reviews of axonal transport have been published by Lasek (1970), Jeffrey & Austin (1973) and Heslop (1975).

In central and peripheral neurones newly-synthesized materials that are transported away from the soma include proteins, glycoproteins, lipids, mucopolysaccharides, neurotransmitters and related enzymes (see Jeffrey & Austin, 1973). Organelles such as mitochondria, adrenergic dark-core vesicles, and elements of the continuous system of smooth endoplasmic reticulum (SER) as well undergo orthograde transport (Dahlstrom, 1965; Droz *et al.*, 1973). Although these components are

individually transported at different velocities, axonal transport is considered to manifest itself in two distinct, fast and slow, phases.

Radiolabelling experiments have demonstrated that the fast orthograde wavefront of labelled protein moves at a velocity of approximately 400mm/day, the maximum rate found for orthograde transport (Ochs, 1972). Acetylcholinesterase is transported at this velocity as well (Ranish & Ochs, 1972). A plateau of activity following behind this wavefront is composed of material transported more slowly (Karlsson and Sjostrand, 1971b, 1972) and/or later exported from the cell body (Forman *et al.*, 1972; Grafstein *et al.*, 1975). In this plateau the neurotransmitter noradrenaline is transported at a velocity of 120mm/day (Livett *et al.*, 1968). Ultracentrifugation studies have localized labelled fast transported materials in particulate cell fractions (McEwen & Grafstein, 1968; Sjostrand & Karlsson, 1969), demonstrating the incorporation of precursor into membranous structures. This has been confirmed by high resolution electron microscope autoradiography (Droz *et al.*, 1975), which found labelled fast transported protein associated with profiles of SER.

The slow phase of transport moves with a velocity of <10mm/day and contains most of the incorporated activity following labelled precursor injection (McEwen & Grafstein, 1968; Sjostrand & Karlsson, 1969). These slow transported proteins are acid-soluble, and contribute to the renewal of the axoplasmic matrix (McEwen & Grafstein, 1968; Sjostrand & Karlsson, 1969). Since the rate of nerve regeneration corresponds to the velocity of slow transport the latter may represent the "bulk flow" of axoplasm postulated by Weiss & Hiscoe (1948).

Autoradiographic evidence demonstrates that labelled material transported with the SER is incorporated into the preterminal axolemma, the presynaptic membrane and synaptic vesicles (Droz *et al.*, 1973; Bennett *et al.*, 1973). The half-life of some labelled molecules in nerve terminals can be as short as 12 hours (Cuenod & Schonbach, 1971; Droz *et al.*, 1973), emphasizing the necessity for rapid delivery of materials synthesized in the cell body. Neurotransmission thus depends upon axonal transport as much for structural maintenance of the nerve terminal as for delivery of transmitters and related enzymes.

Orthograde-transported materials are also implicated in intercellular communication, the so-called trophic phenomena. One such example is the effect of an axonally-transported factor on the metabolism and response characteristics of muscle cells. In the absence of disturbances to either electrical conductivity or transmitter release denervation changes are noted after transport block (Albuquerque *et al.*, 1972; Fernandez & Ramirez, 1974; Inestrosa & Fernandez, 1976; Kauffman *et al.*, 1976). There is some evidence demonstrating the transfer of trophic materials from nerve terminals to other cells. Musick & Hubbard (1972) have shown that protein is released from motor nerve terminals, and autoradiographs have been presented (Appeltaeur & Korr, 1975) indicating entry into muscle fibres of labelled material originating in motor nerves. Neurotrophic regulation has also been proposed to maintain peripheral nerve innervation patterns in maturity. In the salamander the skin of the hindlimb is innervated by sensory nerves from three segmental ganglia. Surgical interruption of the middle nerve root results in collateral sprouting of the two adjacent nerves

into the denervated area of skin. The same collateral sprouting is found if the middle nerve is exposed to a drug that disrupts axonal transport without affecting impulse transmission (Aguilar *et al.*, 1973). Drug application does not result in degeneration of sensory terminals (Diamond *et al.*, 1976), suggesting that an orthograde-transported factor suppresses sprouting of adjacent axons. Other trophic influences of nerve have been recently reported in a volume edited by Drachman (1974).

The mechanism of fast orthograde transport is not completely understood. It is an energy-dependent (Ochs & Hollingsworth, 1971; Ochs & Smith, 1971), temperature-sensitive (Gross, 1973) process that is independent of axonal diameter (Ochs, 1972), and continues after isolation from the nerve cell body (Lubinska *et al.*, 1964; Bray *et al.*, 1971). The major structural elements of the axon, microtubules and microfilaments, were originally suspected of involvement in transport due to their role in other cytoplasmic transport processes (eg. mitotic spindle) (Schmitt, 1968). Their involvement is suggested by light microscopic evidence showing that transported particles follow distinct intra-axonal paths (Kirkpatrick *et al.*, 1972; Cooper & Smith, 1974), and by electron microscopic demonstration of cross-bridges between axonal microtubules and transported organelles (Smith, 1971; Smith *et al.*, 1975). Inhibition of axonal transport results when nerves are exposed to microtubule-disrupting drugs (Banks *et al.*, 1971; Byers *et al.*, 1973; Paulson & McClure, 1974).

On the basis of its localized, energy-dependent characteristics, and because of the biochemical similarity between microtubule subunits

and actin (Olmstead & Borisy, 1973), the sliding filament theory of muscle contraction has been adapted as a model for fast axonal transport (Schmitt, 1968). The saltatory movements of axonally-transported particles observed under the light microscope (Kirkpatrick *et al.*, 1972; Cooper & Smith, 1974) are thought to reflect the unit events of transport. Ochs (1971) has based a unitary hypothesis of axonal transport on this model and proposed that transported materials are attached to transport filaments. A sequence of actomyosin ATPase-like interactions are thought to move these filaments along a microtubule "track". Alternatively, Gross (1975) has postulated that axonal transport functions via a less specific mechanism. He proposed that transport occurs within a streaming phase of axoplasm produced by microtubular ATP hydrolysis. The observed heterogeneity of transported material could result from such a method of transport. Both hypotheses have limitations, suggesting that a model combining particle-substrate and fluid-carrier interactions would better describe the mechanism of orthograde transport.

3. CHARACTERISTICS OF RETROGRADE AXONAL TRANSPORT

A process of retrograde axonal transport is also present in nerves. It was first suggested to exist by Lubinska *et al.* (1964), who found transported AchE accumulation at both ends of an isolated segment of dog peroneal nerve. Since then the peripheral neurone has been demonstrated to reverse at an injury site orthograde-transported organelles (Zelena, 1968; Banks *et al.*, 1971), enzymes (Lubinska & Niemierko, 1971), and labelled proteins (Bray *et al.*, 1971; Edstrom and Hanson, 1973) originally synthesized in the cell body. The

phenomenon of retrograde transport is not, however, solely confined to injured nerves. Return of orthograde-transported material also occurs in intact nerves (Bray *et al.*, 1971; Frizell & Sjostrand, 1974b; Abe *et al.*, 1974), although neither the location nor the mechanism of reversal are known.

The following evidence suggests that part of the function of retrograde transport is the recycling of peripheral axon components. Labelled axonal proteins have a half-life ranging from less than 24 hours to 40-50 days (Droz, 1975). Correspondingly, 50-60% of labelled orthograde-transported protein is returned to the cell body in the first 24-99 hours after precursor injection (Frizell & Sjostrand, 1974b; Bisby, 1977b). Early-returning protein may therefore be that portion of orthograde-transported material undergoing rapid turnover in the axon.

Besides carrying endogenous molecules the retrograde transport system returns to the cell body exogenous material taken up at nerve terminals. These include molecules of importance to the neurone such as nerve growth factor (NGF) (Stoeckel *et al.*, 1975, 1976), molecules of no physiological importance such as horseradish peroxidase (HRP) (LaVail & LaVail, 1972), and some of pathological consequence, including the herpes simplex virus (Kristensson, 1970) and tetanus toxin (Stoeckel *et al.*, 1975). The mechanism of nerve terminal uptake is not entirely understood. It does possess some selectivity, since motor nerve terminals will take up HRP, but not NGF (Stoeckel *et al.*,1975). However, uptake of HRP is proportional to electrical activity of the nerve (Holtzman *et al.*, 1971; Litchy, 1973), suggesting that the nerve

terminal environment may be sampled by endocytosis during terminal membrane recycling (Heuser & Reese, 1973).

The importance of retrograde transport-delivered NGF to the developing sympathetic nervous system (Levi-Montalcini & Angeletti, 1968) prompts speculation that combination of nerve terminal uptake with the retrograde transport system permits retrograde intercellular communication. Labelled amino acids or derived proteins are also taken up by motor nerve terminals and retrograde-transported (Kerkut *et al.*, 1967; Watson, 1968b), demonstrating that various post-synaptic molecules may have access to nerve cell body synthetic machinery. In fact, there is ample evidence demonstrating bidirectional trophic effects in the nervous system. The loss of spinal motoneurones during development (Hughes & Egar, 1972; Prestige & Wilson, 1972) coincides with the elimination of polyneuronal input to single muscle fibres (Bennett & Pettigrew, 1974, 1975; Pilar and Landmesser, 1974). Hughes and Tschumi (1958) have similarly demonstrated the importance of the peripheral nerve field to normal development of sensory ganglia. As mentioned earlier, the mature innervation patterns are then maintained by orthograde transported factors that inhibit sprouting by other neurones (Bennett & Pettigrew, 1974; Diamond *et al.*, 1976).

Studies of retrograde transport of HRP have found it to be retrograde transported at a velocity of 72-120mm/day in the chick visual system (LaVail & LaVail, 1972) and in the rat or mouse hypoglossal nerve (Kristensson *et al.*, 1971; Kristensson & Olsson, 1974, 1975). In the rabbit hypoglossal nerve labelled protein is retrograde transported at 140mm/day (Frizell & Sjostrand, 1974b). Stoeckel *et al.* (1976) have

shown that NGF returns to sympathetic neurones of the rat superior cervical ganglion at a velocity of 50-75mm/day. In sympathetic axons of the rabbit sciatic nerve the retrograde transport velocity of the enzyme dopamine-β-hydroxylase is 288mm/day (Brimijoin & Helland, 1976). Although resulting in part from different measuring methods, retrograde transport velocity is thus seen to vary for different materials, similar to orthograde transport.

Many characteristics are shared by the two transport systems. Retrograde transport is also energy-dependent (Edstrom & Hanson, 1973), temperature sensitive (Brimijoin & Helland, 1976), and is inhibited by microtubule disruption (Kristensson *et al.*, 1970; Kristensson & Sjostrand, 1972; Abe *et al.*, 1974). The ultrastructural correlates of retrograde transport have been more difficult to establish. Labelling experiments initially label newly-synthesized orthograde transported materials, and their prolonged efflux from the cell body hinders autoradiographic localization of retrograde transported materials. However, mindful of this warning, Griffin *et al.*, (1976) have by autoradiography found labelled retrograde transported protein accumulated at a nerve ligature to be associated with SER membranes. Support for this interpretation comes from studies using HRP, which is found within the lumen of axonal SER (LaVail & LaVail, 1972, 1974).

These similarities in orthograde and retrograde transport characteristics suggest that a single mechanism may be responsible for the bi-directional axonal transport of materials and organelles.

4. RESEARCH PROPOSAL

As mentioned, current axonal transport theory holds that orthograde

and retrograde systems function reciprocally, the former delivering newly-synthesized materials to the neuronal periphery, and the latter returning turned-over axonal components for degradation. Retrograde transport of these materials has also been proposed to constitute a feedback loop for regulation of cell body metabolic pathways (Ochs, 1974). Ongoing synthesis levels would thus be determined by the amounts of returning metabolites. For example, changes in peripheral requirements due to changes in functional activity or length (growth, or, conversely, traumatic shortening) would be reflected in the amounts of returned material. These alterations would in turn increase or decrease cell body synthesis of specific molecules until a new metabolic equilibrium was established.

The feedback function of the retrograde transport system would be supported by evidence showing that (i) peripheral return is altered in response to changes in axonal metabolic requirements, and (ii) nerve cell body metabolism responds to alterations in peripheral return. A demonstration of the first condition has already been presented. Bray *et al.* (1971), when studying retrograde transport in chicken sciatic nerve, noted that returning labelled protein began to accumulate earlier at a more proximal ligature when its orthograde transport to the nerve terminals was blocked by a distal ligature. This demonstrated a change in the return of labelled protein in response to ligation, a procedure which should have drastically altered axonal metabolic requirements.

This project was initiated with the intent of more thoroughly studying the retrograde transport of labelled protein in intact and ligated peripheral nerve. The rat sciatic nerve was chosen as the

experimental model because basic protein transport characteristics in this system were already known, and techniques for labelling and evaluating protein transport in sciatic nerve axons had already been developed (Bisby, 1976b, 1977b). Investigation of retrograde protein transport in intact and ligated motor and sensory axons could reveal changes in retrograde transport after axotomy that serve to inform the nerve cell body of changes in peripheral metabolic requirements. Additional information might also be obtained about characteristics of retrograde protein transport, and about the transport reversal process that occurs in both intact and injured nerves. Other experiments could more closely study the transport reversal process by observing the effects of a microtubule-disrupting drug on protein transport.

In response to peripheral injury nerve cell bodies do respond metabolically (Brattgard *et al.*, 1957) and ultrastructurally (Lieberman, 1971) in such a manner as may result in nerve regeneration (Grafstein, 1975). Regenerating nerves provide a good model for the study of cell body metabolic response to alterations in retrograde-transported feedback since the feedback should be constantly changing. Experiments could thus be performed on chronically-injured nerves in which the cell body response to elongation could be monitored by determining changes in orthograde and retrograde labelled protein transport. The cell body response to nerve injury could also be evaluated by observation of morphological changes in axotomized neurones.

CHAPTER II

MATERIALS & METHODS

1. EXPERIMENTAL ANIMALS

Male or female Sprague-Dawley rats weighing 180-400 grams, obtained from the breeding stock of the Medical Vivarium, University of Calgary, were used for all experiments. Animals were housed in the Vivarium and maintained on an ad lib standard rat pellet and water diet. Pentobarbital anaesthesia (Diabutal, Diamond Laboratories; 50mg/kg, by intraperitoneal injection) was administered prior to all surgical procedures.

2. REAGENTS

All solutions were prepared with reagent-grade chemicals and distilled water.

3. LABELLING OF TRANSPORTED PROTEINS

i) Preparation of Precursor

Injection of ^{3}H-L-leucine into the vicinity of neuronal cell bodies contributing axons to the sciatic nerve has been shown to result in neuronal uptake, subsequent protein synthesis, and transport of labelled protein down the sciatic nerve (Ochs *et al.*, 1967; Lasek,

1968; Bisby, 1975). In the work reported here, protein transported in motor and sensory axons of the rat sciatic nerve was labelled by microlitre injections of ^{3}H-L-leucine into the vicinity of the lumbar spinal cord motoneurone pool and the L5 dorsal root ganglia (DRG), respectively.

4,5-^{3}H-L-leucine (New England Nuclear, 30-50 Ci/mmole, or Amersham-Searle, 30-60 Ci/mmole) was concentrated by evaporation under vacuum and redissolved in Ringer's solution to a concentration of 10μCi/μl. The labelled solution was introduced into volume-calibrated glass micropipettes by capillary action. Micropipette tip diameters of 30-50μM were used to minimize damage to the spinal cord during injection. A teflon coating was applied to the micropipette tips by aerosol spray (Crown) to reduce escape of label back up the outside of the micropipette during injection. Air pressure served as the driving force for injection by connection of a 5ml syringe to the micropipette through a length of polyethylene tubing attached to a 20 guage needle.

ii) Surgical Preparation for Precursor Injection

The spinal cord was exposed by dorsal laminectomy at the level of the L1-L2 vertebrae for precursor injection into the sciatic nerve motoneurone pool. In the rat most of the neurones lie in the ventrolateral and dorsolateral ventral horn of the spinal cord at this level (Romanes, 1964). Their position is revealed on the surface of the cord by a "V" pattern formed by dorsal roots entering the spinal cord, and is thus easily located. A similar situation has

been noted in the cat (Ochs & Burger, 1958).

The L5 DRG were exposed for labelling of protein transported in sensory axons of the sciatic nerve. Dissections usually revealed this ganglion to be the major contributor of fibres to the sciatic nerve, although there was considerable variation from animal to animal in the contribution from the L4 and L6 ganglia. Animals were always bilaterally symmetric in this regard.

iii) Precursor Injection

The lower spinal column was immobilized in a spinal frame for precursor injection, suspended between a vertebral clamp and bars placed rostral to the iliac crests. Injection micropipettes were held in a Kopf 1271 electrode holder and mounted on a Kopf 1761 micromanipulator for positioning in injection sites. A Bausch and Lomb AK71 illuminated dissection microscope assisted in positioning the micropipettes.

For ganglion injections the micropipette was lowered over the centre of the L5 ganglion until the tip had just penetrated the connective tissue capsule. A 1µl injection was made over a period of 30-45 seconds. Successful injections (i.e. those resulting in high labelling levels of transported protein) were usually indicated by coincident swelling of the ganglion. The micropipette was left in the ganglion for an additional 1-2 minutes to reduce escape of the precursor along the injection track.

To label protein transported in motoneurone axons, four 1µl injections were made 1mm apart at sites 0.8-1.0mm lateral to the dorsal median sulcus at a depth of 1.5-1.8mm. Each injection was

made over 20-30 seconds, after which the micropipette was left in place for a further 1.0-1.5 minutes. During injection into the right side of the spinal cord electromyograms were recorded from the right tibialis anterior muscle using needle electrodes. EMG activity was displayed on a Tektronix 5031 dual beam storage oscilloscope and relayed to an audio monitor. Injections that resulted in high levels of transported protein labelling were accompanied by audible motor unit activity and usually by overt movements of the hindlimb. This permitted the easy identification of successful injection sites. Injections into the left side of the spinal cord were made using the same co-ordinates as were used on the right. Movements of the left hindlimb were often seen during precursor injection, indicating a successful injection.

iv) Post-operative Care and Observations

After precursor injection the spinal cord was covered with a length of Ringer's-soaked Gelfoam (Upjohn), the skin was closed with surgical clips and the animal was placed under a heat lamp to maintain body temperature. After recovery from anaesthesia it was returned to a cage and provided with food and water ad lib. Average mortality was 5-10% and could be attributed to either anaesthetic overdose or excessive accumulation of secretions in the respiratory tract.

Mobility was decreased post-operatively due to the radical resection of vertebral musculature. Ambulation was usually not affected by surgery and precursor injection. When spinal cord injury occurred during surgical preparation it was usually indicated by bruising of the cord. The result of injury was gait impairment and,

in agreement with Ochs & Burger (1958) and Ochs *et al.* (1962), insignificant levels of labelled protein in the sciatic nerve.

4. SCIATIC NERVE LIGATION

i) Why

Nerve ligation served multiple functions. First, a single ligature produced a site of injury at which the reversal of protein transport would occur (Bray *et al.*, 1971; Lubinska & Niemierko, 1971; Abe *et al.*, 1974). Second, ligation permitted quantification of axonal transport by causing accumulation of transported protein. Third, for long term studies, chronic nerve ligation produced a shortened, non-regenerating nerve, while a regenerating nerve resulted when the ligature was loosened after the nerve was crushed.

ii) How and When

At various times after precursor injection animals were reanaesthetized, and the sciatic nerves were exposed either in the upper or lower thigh by retraction of the biceps femoris muscle, or in the sciatic notch by division of the gluteal muscles and the iliac arch. The nerve was then crushed in one or more places by knotting around it a length of 4/0 surgical thread.

iii) Where

Single ligatures, to serve as reversal sites, were applied 90±3mm or 70±3mm from the midpoint of injection sites in the spinal cord (parallel with the origin of sciatic nerve ventral roots), and

60±3mm from the L5 DRG (Fig. 2.1). In long term experiments single ligatures were applied 90±3mm from the origin of the ventral roots.

The quantification function was served by a pair of ligatures (hereinafter called collection crushes) tied 6-10mm apart on the nerve, adjacent to which transported protein would accumulate. In previously ligated nerves the distal collection crush was made 15mm proximal to the reversal ligature. Collection crushes were made at similar locations on intact nerves. In regenerating and non-regenerating nerves the distal collection crush was made 15mm proximal to the original injury site.

iv) Timed Accumulation of Labelled Protein

After application of single ligatures, the skin was closed by surgical clips or sutures, and animals were placed under a heat lamp until they had recovered from anaesthesia. After collection crushes were applied, the skin was closed by surgical clips and the animals were allowed to survive for 2 or 3 hours, while transported protein accumulated adjacent to collection crushes. Animals were then killed by neck fracture.

After the animals were killed the sciatic nerves were immediately removed and loosely-attached connective tissue was removed from them with fine forceps. Nerves were then stretched to full length on cardboard strips and frozen at $-25^{o}C$ to arrest protein transport.

5. ANALYSIS OF PROTEIN TRANSPORT

i) Location of Accumulated Labelled Protein

Figure 2.2 is a representative profile of labelled protein activity

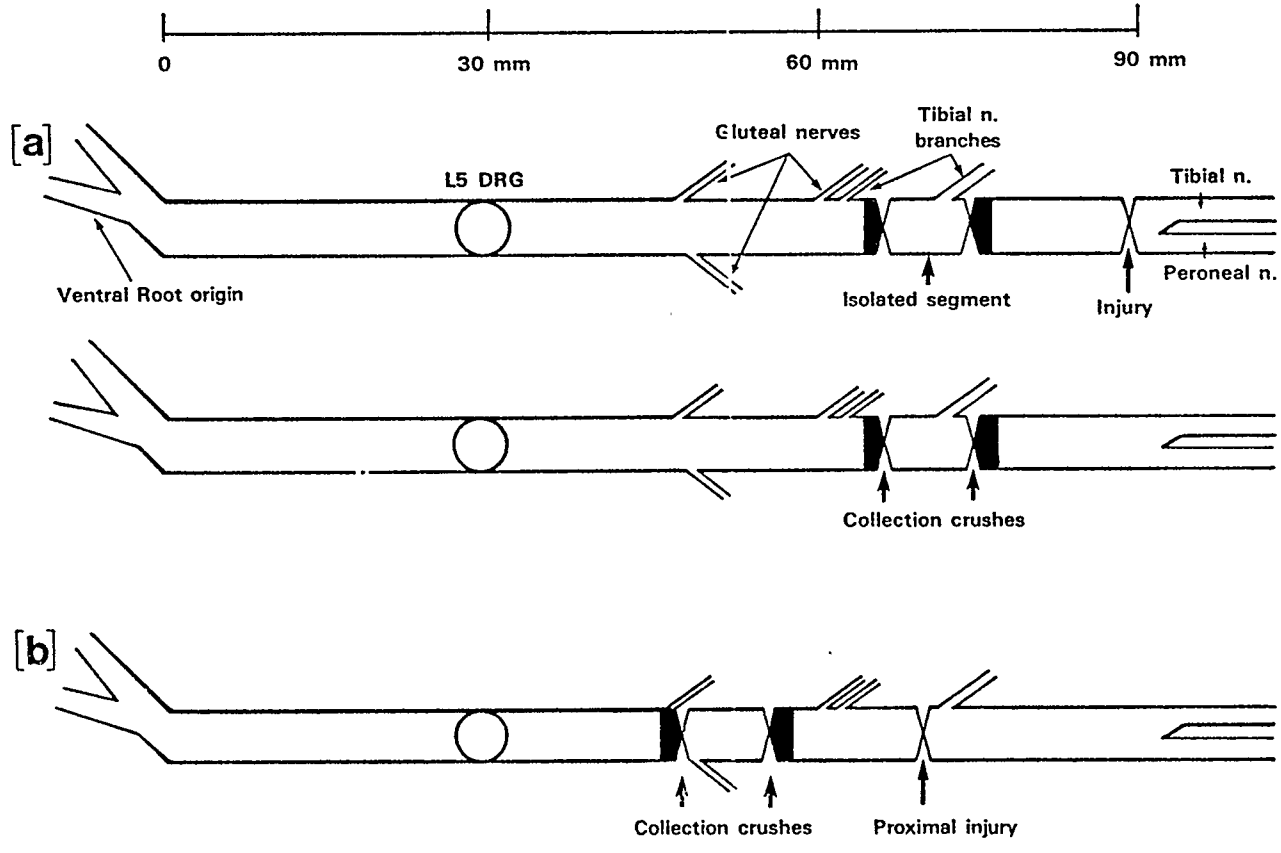

Figure 2.1 Diagram of the rat sciatic nerve, indicating locations and terms used in the text. (A) location of accumulation segments (shaded) in injured and intact nerves. (B) location of accumulation segments in nerves after proximal injury.

in motor axons of a sciatic nerve 10 hours after precursor injection. The injury ligature was applied at the time of precursor injection, and the collection crushes were made 8 hours later. Note the log activity scale used to telescope the magnitude of protein accumulations. Accumulated activity is contained almost exclusively in the first 2mm segment of nerve adjacent to a ligature. This has been confirmed by autoradiography (Bisby, unpublished observations).

ii) Quantification of Labelled Protein Accumulation by Scintillation Counting

Working outwards from the ligatures, nerves were therefore cut into 2mm lengths using a razor blade, and each segment was placed in a glass scintillation vial, to which was added 2ml of 10% trichloroacetic acid (TCA). After incubation for 8-12 hours at room temperature to remove unbound (non-protein) label the TCA was removed by aspiration, and TCA-insoluble material was dissolved in 0.5ml of Protosol (New England Nuclear) or Liquid Tissue Solubilizer (Baker) at 55^{o}C for 3-5 hours. After addition of 10mls of a toluene-Liquifluor (New England Nuclear) scintillation fluid, containing 4.0 grams PPO and 50mg POPOP per litre of toluene, vials were left in a cupboard overnight to reduce chemo- and photoluminescence. Background activity was estimated by use of two blanks in each counting run; these entered the experimental protocol after the TCA aspiration step.

Most vials were counted in either a Beckman LS 3155T or a Beckman LS 233 scintillation counter. Two Packard 3375 Tri-Carb Scintillation Spectrometers were also used to count nerve samples in 15-20% of the experiments performed. Each vial was counted for 10 minutes or to

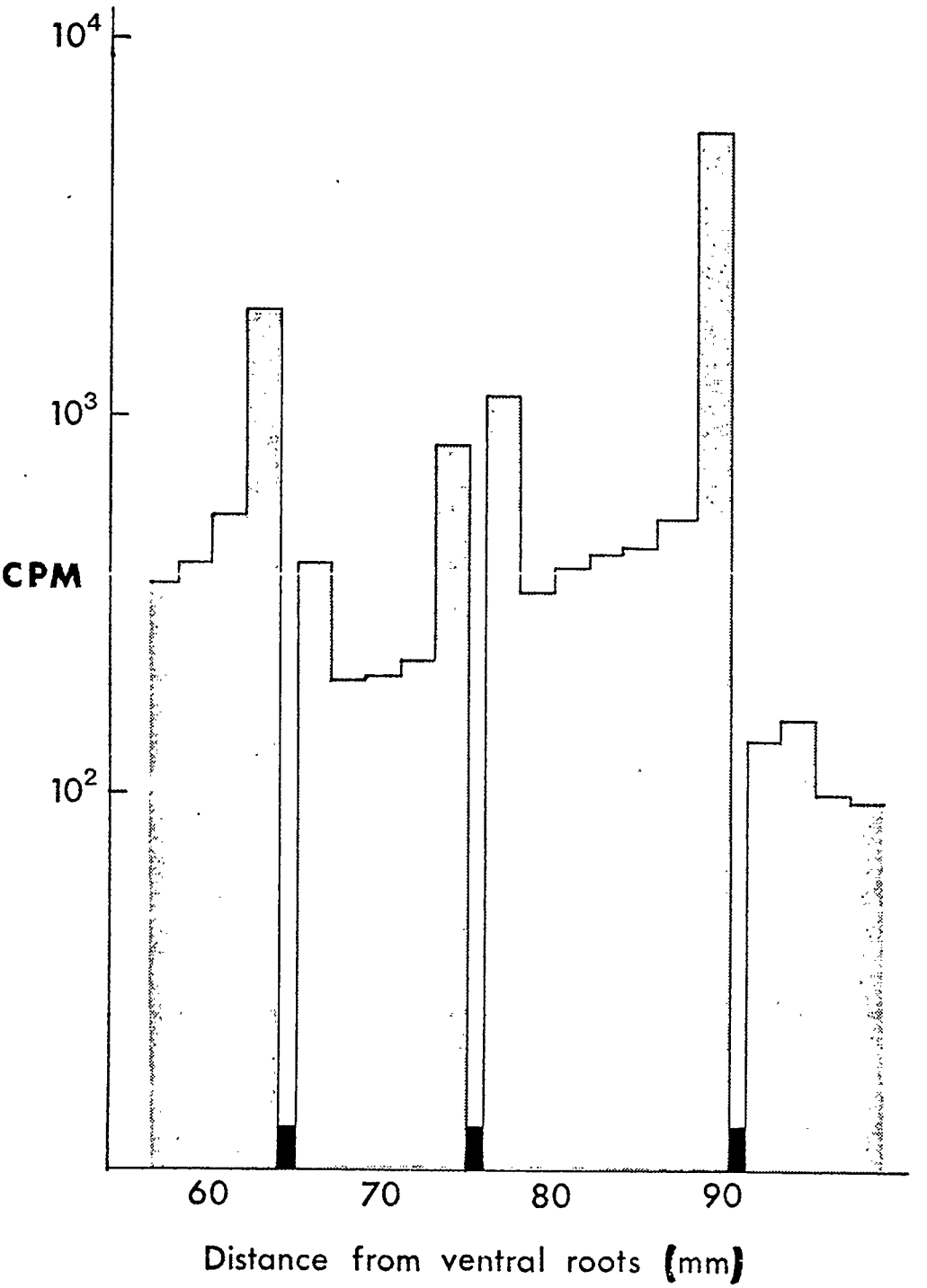

Figure 2.2 Representative activity profile along sciatic motor axons 10 hours after concurrent precursor injection and distal ligation. Collection crushes were applied 8 hours later. Vertical axis: TCA-insoluble activity per 2mm nerve segment. Horizontal axis: distance from sciatic ventral roots. Note most transported activity accumulated in 2mm segment adjacent to ligatures.

5% error.

Quenching was determined by the method of external standardization, and was found to be uniform for all nerve samples. Fifty vials, ten from each of five different experiments, were counted in a Beckman LS 233 scintillation counter. By external standardization they were counted with efficiencies of (mean ± S.E.M.): 38.1% ± .42%, 37.9% ± .59%, 37.4% ± .23%, 37.0% ± .40%, and 37.1% ± .58%. Results were therefore calculated using c.p.m. values from which the background activity, averaging 30 c.p.m. per vial, had been subtracted.

iii) Quantitative Analysis of Labelled Protein Transport

Transport of labelled protein was determined by measuring the accumulation of TCA-insoluble activity in nerve segments proximal and distal to collection crushes. Accumulation of labelled protein at crush sites is not due to the uptake of blood-borne precursor, nor from electrophoresis of charged molecules responding to an injury potential (Bisby, 1976b). Accumulations proximal to collection crushes therefore reflected orthograde protein transport, while retrograde transport was reflected in protein accumulation distal to collection crushes.

Protein transport was calculated from accumulation data by means of the following formula:

$$\text{RELATIVE ACCUMULATION} = \frac{(a/b) - 1}{T} + 1$$

where a = activity (cpm) in 2mm nerve segment adjacent to collection crush (accumulation segment)

b = mean activity per 2mm in isolated segment lying between collection crushes and an equal length of nerve lying proximal or distal to the accumulation segment

T = interval (hours) of accumulation

This formula was devised to standardize the measurement of protein accumulation, and thus of protein transport, and was required for several reasons.

First, comparable amounts of activity were not transported in all nerves. This was first reported by Ochs *et al.* (1967) and is probably due both to errors inherent in the injection technique, and to anatomical variability. The inconstancy of the branching pattern of the sacral plexus, noted earlier, could result in the transport of labelled protein in differing numbers of sensory nerves, which would be seen as variability in the amount of transported protein. Differential precursor uptake by neurones could also be a source of variability, due to changes in the location of injection sites relative to sciatic sensory and motoneurone cell bodies, and given the possibility of leakage of the precursor away from the injection site. Accumulation of transported protein at collection crushes was therefore expressed relative to a measure of the level of activity existing in the nerve before the crushes were made. Thus in a nerve in which only small amounts of labelled protein had been transported, accumulations of transported activity (a) would be small, but so also would be the pre-existing level of activity (b).

Second, a proximo-distal gradient of non-mobile activity remains in the rat sciatic nerve after passage of the wavefront of orthograde transported activity (Bisby, 1977b). Thus the "background" activity (b) in the nerve was calculated as the mean of activity in nerve segments above and below the accumulation segment, which would more accurately estimate the non-mobile activity in the accumulation segment.

Third, transported protein accumulated at collection crushes

for either 2 or 3 hours. Standardized accumulation data was therefore expressed as relative accumulation per hour. The longer collection interval was used in an attempt to yield a more accurate value for transport at later times after precursor injection, when less protein is transported down the axons (Ochs & Ranish, 1969; Bisby, 1976b).

Axonal transport is therefore represented as it is reflected in the accumulation rate of labelled protein at ligatures applied to the sciatic nerve at various times after precursor injection. The rate of protein accumulation is dependent upon both the velocity and the amount of mobile protein. In this work the term "rate" will not denote simply velocity of transport, which other authors have used it to mean (Grafstein & Murray, 1969; Lasek, 1970; Lubinska & Niemierko, 1971).

iv) Calculation of A Correction Factor

After experiments using 2 hour and 3 hour collection intervals had begun, it was discovered that accumulation of orthograde transported protein was not a linear function of time. Further experiments were therefore performed to compare 2 hour and 3 hour relative accumulation (R.A.) values for orthograde and retrograde transport. R.A. values were calculated for labelled protein transport in intact and ligated sensory axons at an early (12-17 hours) and at a later (30-39 hours) time period after precursor injection and in ligated motor axons at an early (13-16 hours) time period after precursor injection (Table 2.1). Orthograde transport was shown in all cases to be underestimated by R.A. values calculated from accumulation data obtained from 3 hour collection intervals. R.A. values for retrograde protein transport

TABLE 2.1 Comparison of relative accumulation values obtained from 2 hour and 3 hour collection intervals in intact and ligated sensory axons at two time periods after precursor injection and in ligated motor axons at an early time period after precursor injection. Orthograde transported protein accumulated for 2 hours or 3 hours at a proximal collection crush approximately 35mm from the L5 DRG. Retrograde transported protein accumulated for 2 hours or 3 hours at a distal collection crush approximately 45mm from the L5 DRG. Expressed as relative accumulation (R.A.±S.E.M., n=number of nerves).

A) SENSORY AXONS

i) Intact	14-16 Hours	14-17 Hours	30-32 Hours	36-39 Hours
Retrograde Transport (n)	1.31 ± .06(6)	1.32 ± .02(4)	1.19 ± .05(6)	1.08 ± .08(4)
Orthograde Transport (n)	1.82 ± .10(6)	1.60 ± .10(4)	1.54 ± .03(6)	1.34 ± .02(4)

ii) Ligated	12-14 Hours	11-14 Hours	30-32 Hours	30-33 Hours
Retrograde Transport (n)	1.63 ± .15(6)	1.60 ± .15(5)	1.21 ± .03(6)	1.26 ± .01(6)
Orthograde Transport (n)	1.89 ± .09(6)	1.68 ± .12(5)	1.54 ± .04(6)	1.35 ± .05(6)

B) MOTOR AXONS

i) Ligated	13-15 Hours	13-16 Hours
Retrograde Transport (n)	1.53 ± .20(6)	1.46 ± .11(4)
Orthograde Transport (n)	2.12 ± .08(6)	1.86 ± .04(6)

were the same for 2 hour and 3 hour collection intervals.

The error in orthograde R.A. values for the periods tested was at most 15% and was not statistically significant at the early collection period in sensory axons. However, correction of this error was required to permit the derivation of obtained data in Chapter III (see Figs. 3.4-3.6). A correction factor was therefore calculated as the ratio of 2 hour R.A. values to 3 hour R.A. values in the two collection periods shown in Table 2.1. This ratio was 1.14 and 1.15 for the early and later collection periods in intact sensory axons, 1.14 for both periods in ligated sensory axons, and 1.14 in ligated motor axons at the early collection period.

The uniformity of this ratio at the two collection periods in sensory axons was interpreted to suggest that the 1 hour accumulation difference in R.A. values was constant. Thus R.A. values for all 3 hour collection intervals from 12-39 hours after precursor injection were recalculated by use of the above ratio. Furthermore, that the same ratio was found in ligated motor axons at the early time period inferred that the 1 hour accumulation difference was a characteristic of orthograde protein transport not restricted to sensory axons. Therefore 3 hour R.A. values for orthograde transport in motor axons were also recalculated using this ratio as a correction factor.

v) Quantitative Analysis of Total Accumulated Activity

Total labelled protein activity accumulated at a reversal site over a prolonged period was calculated by use of the following formula:

$$\text{TOTAL ACCUMULATION} = a/b$$

where a = activity (cpm) in 2mm accumulation segment

b = mean activity per 2mm in isolated segment and an equal length of nerve proximal to the accumulation segment

CHAPTER III

REVERSAL OF AXONAL TRANSPORT AT A NERVE CRUSH

1. INTRODUCTION

Retrograde axonal transport has been demonstrated for exogenous molecules taken up by axon terminals (Kristensson *et al.*, 1971; Stoeckel *et al.*, 1975), and for endogenous enzyme molecules (Lubinska & Niemierko, 1971; Brimijoin & Helland, 1976) and organelles (Banks *et al.*, 1969; Kirkpatrick *et al.*, 1972; Cooper & Smith, 1974). Labelled protein originally synthesized in the cell body and transported in an orthograde direction later accumulates distal to nerve crushes (Bray *et al.*, 1971; Edstrom & Hanson, 1973; Sjostrand & Frizell, 1975; Bisby, 1976a, b, 1977b), showing that it is undergoing retrograde transport. The retrograde transport of endogenous molecules probably performs a recycling function, returning to the synthetic apparatus in the cell body the constantly turning-over constituents of the nerve periphery. It might, however, also provide the cell body with information regarding the functional status of the axon and terminals. Ochs (1974) has suggested that neuronal synthesis may be regulated by such feedback control.

Although the site of transport reversal in intact axons is not known, reversal does occur at a site of axon injury (Bray *et al.*, 1971; Lubinska & Niemierko, 1971; Abe *et al.*, 1974). After an injury such as a crush, the soma reacts by undergoing a series of structural

and biochemical changes which may lead in some cases to cell death, and in other cases to attempted or successful axon regeneration (Lieberman, 1971). Clearly, a signal must reach the cell body to initiate this axotomy response, which will be referred to as chromatolysis for brevity, although, strictly speaking, this term describes only the change in cell body staining characteristics after axonal injury. Cragg (1970) has reviewed some of the most likely chromatolytic signals.

The intent of the work described in this chapter was to compare the retrograde transport of labelled endogenous protein in intact and crushed axons to determine whether the reversal of transport that occurs at an axon injury could serve as a signal for chromatolysis. This study was confined to the immediate post-axotomy period because of evidence showing that the first changes in the soma occur within a day of axotomy (Cerf & Chacko, 1958; Torvik & Heding, 1969).

2. MATERIALS & METHODS

In all experiments precursor was injected at time zero into the vicinity of sciatic nerve motoneurones or the L5 dorsal root ganglia (DRG). Methods used for precursor injection, nerve ligation, and analysis of protein transport have been described (Chapter II).

i) Intact Axons

At intervals after precursor injection collection crushes were made on the sciatic nerve at points 65 and 75 ± 3mm from the origin of the ventral roots (Fig. 2.1(a)). Accumulation of label adjacent to ligatures for 2 or 3 hours allowed determination of orthograde and

retrograde transport of labelled protein in intact nerves at increasing intervals post injection (p.i.).

ii) Injured Axons

Injured axons were produced by applying a single sciatic nerve ligature, either at the time of precursor injection, or 6 hours later, approximately 90mm from the origin of the ventral roots (Fig. 2.1(a)). Collection crushes were made at intervals after precursor injection, with the distal ligature made 15mm proximal to the injury crush. Accumulation periods of 2 or 3 hours were used, as before.

In some animals, at the same time that precursor was injected into the ventral horn, an injury ligature was tied more proximally, about 65mm from the origin of the ventral roots (Fig 2.1(b)). At 5 or 6 hours p.i., collection crushes were made 15 and 25mm proximal to the injury crush and a further 2 or 3 hours was allowed for protein accumulation. These experiments were performed to determine the magnitude of protein reversal from a more proximal point on the nerve. Before comparing return from proximal and distal nerve injuries, it was necessary to control for the previously mentioned proximo-distal gradient of non-mobile activity (see Chapter II 5.(iii)). Therefore in some nerves both proximal and distal sets of collection crushes were made at 15 hours p.i. This corresponded to a time 11.4 hours and 10.0 hours, respectively, after the orthograde wavefront had passed the proximal and distal collection crushes in motor axons. The ratio of activity in the two isolated segments was used to calculate the magnitude of return from the two injury sites in common units.

iii) Transport Determination

Transport of labelled protein was expressed in terms of relative accumulation adjacent to collection crushes made at intervals up to 38 hours p.i. The determination of relative accumulation has been explained (Chapter II). It should be noted that 3 hour relative accumulation values for orthograde protein transport have been recalculated to correct for non-linearity of accumulation with time (see Chapter II 5.(iv)). Recalculated data appears in Figures 3.4-3.6.

To determine the total activity conveyed by orthograde or retrograde transport, the curves obtained for accumulation rates were integrated. Curves were reproduced on constant weight paper and areas beneath the curves were weighed. For calculation purposes these areas were expressed in terms of accumulation units, one unit representing a relative accumulation of 1 maintained for 1 hour.

3. RESULTS

i) Orthograde Transport in Intact and Injured Axons

The relative accumulation measured at the proximal collection crush revealed the rate of orthograde protein transport in the sciatic nerve. Orthograde transport was not significantly different in intact and injured axons and thus could not be responsible for any changes in retrograde transport in injured axons.

ii) Retrograde Transport in Intact Axons

The relative accumulation rate of activity distal to the distal collection crush at different time intervals after precursor injection

is shown in Figures 3.1 and 3.2, for motor and sensory axons, respectively. This accumulation is considered to represent retrograde axonal transport. Preliminary results showed that orthograde transported protein first reached the site of the distal collection crush 3.5 hours after DRG injection of precursor and 5.0 hours after spinal cord injection. An increase in the accumulation of label between the first and second collection periods indicates that retrograde transport had begun by 7-9 hours p.i. in sensory axons and by 9-11 hours p.i. in motor axons. That is, in normal axons, return toward the soma of newly-synthesized protein began within 6 hours of its arrival in the periphery. The reason for initial values for distal accumulation of less than unity, representing a loss of activity from the accumulation segment, was probably due to orthograde transported protein leaving the accumulation segment and not being replaced because of the ligature proximal to the segment.

Direct comparison of retrograde transport in motor and sensory axons can be made by shifting the sensory curve 1.5 hours to the right, since labelled protein reached the site of the collection crushes this much earlier after DRG injection than after spinal cord injection. Retrograde transport in intact motor axons was significantly higher than in intact sensory axons between 12 and 24 hours p.i. The greater retrograde transport of endogenous protein in motor axons has been previously described at longer intervals after precursor injection as well (Bisby, 1977b).

iii) Retrograde Transport in Injured Axons

Figures 3.1 and 3.2 also compare retrograde transport in intact

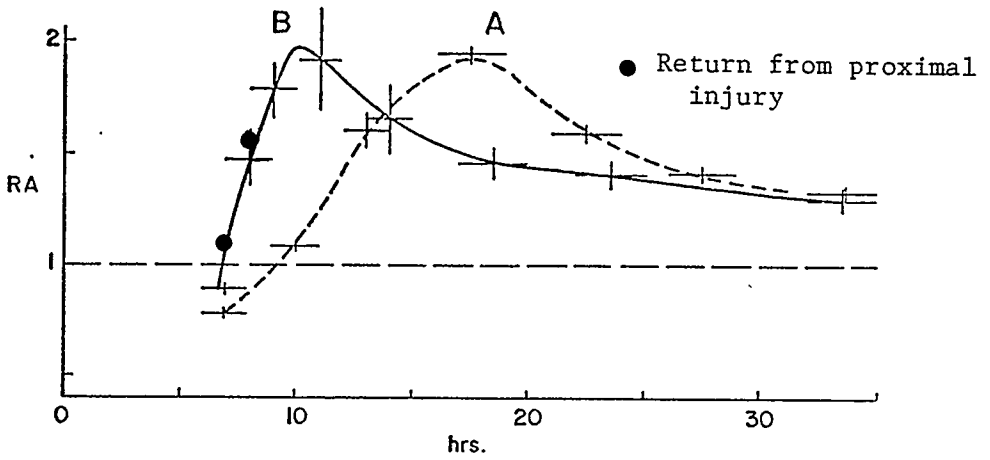

Figure 3.1 Retrograde transport in normal (A) and injured(B) sciatic motor axons. Vertical axis: relative accumulation as defined in text. Horizontal axis: hours after concurrent precursor injection and nerve ligation. Filled circles represent relative accumulation after reversal at a proximal injury ligature. Each data point covers the 2 or 3 hour period of accumulation, and shows the mean ± S.E.M. for at least 5 nerves.

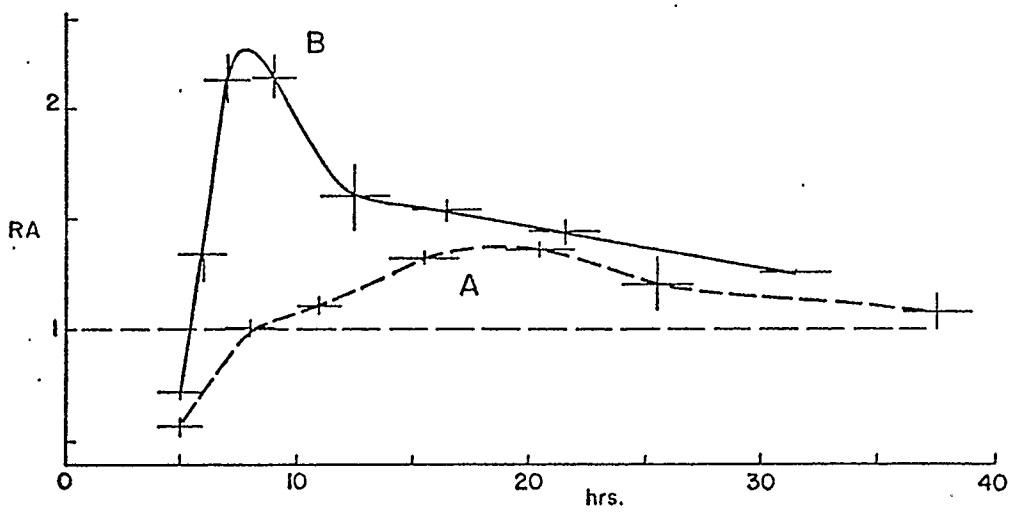

Figure 3.2 Retrograde transport in normal (A) and injured (B) sciatic sensory axons. Legend is the same as for Fig. 3.1.

and injured axons. In both motor and sensory axons injury to the axon resulted in an increased distal rate of accumulation of labelled protein at earlier times after precursor injection than in intact axons. Preliminary experiments showed that the wavefront of transported protein reached the site of injury 4.3 hours after DRG injection and 5.8 hours after spinal cord injection. There was significant retrograde transport, as shown by increased accumulation, at 5-7 hours and 7-9 hours p.i., respectively, in sensory and motor axons. The distance between the injury and the distal collection crush was 15mm, so that a minimum estimate of the velocity of retrograde transport can be made, assuming (i) that reversal of transported protein occurred immediately the protein arrived at the crush, and (ii) that retrograde transported protein arrived at the distal collection crush at the end of the 2 hour collection period.

$$\text{Velocity for sensory axons} = \frac{15 \times 24}{(7-4.3)} = 133\text{mm/day}$$

$$\text{Velocity for motor axons} = \frac{15 \times 24}{(9-5.8)} = 112\text{mm/day}$$

If there was a delay in reversal, or an earlier arrival of protein, the minimum velocity estimate must be increased.

An estimate may also be made of the maximum time taken for reversal of transported protein at the injury ligature if we assume (i) that protein arrives at the collection crush at the end of the collection period, and (ii) that the maximum velocity of retrograde transport is 428mm/day in motor axons, and 393mm/day in sensory axons, the same as the velocity of orthograde transport (Bisby, 1977b). The latter is reasonable given the evidence that the velocity of retrograde

transport has nowhere been found to exceed that of orthograde transport in the same model (Kerkut *et al.*, 1967; Lubinska & Niemierko, 1971; Ranish & Ochs, 1972; Cooper & Smith, 1974). Since the distance between origin and collection crush is 15mm the maximum time for reversal to begin in motor axons is:

$$(9-5.8) - \frac{(15 \times 24)}{428} = 2.4 \text{ hours}$$

and in sensory axons is:

$$(7.4.3) - \frac{(15 \times 24)}{393} = 1.8 \text{ hours}$$

If the velocity of transport was less, or if protein arrived earlier at the collection crush, the estimate must be reduced.

Peak retrograde transport occurs in both motor and sensory axons approximately 5 hours after arrival of the wavefront at the injury. In intact motor axons peak retrograde transport occurs at 16-19 hours p.i., while in injured motor axons it occurs at 9-11 hours p.i. The increase in retrograde transport found in intact motor axons at 14-24 hours p.i. does not occur in injured nerves, showing that axotomy, as well as causing a premature return of protein, also blocks the normal pattern of protein return from the nerve periphery. In intact sensory axons retrograde transport never exceeds that in injured axons.

Retrograde transport was also measured at two early periods after precursor injection in motor axons that were ligated more proximally than those already mentioned. The accumulation rates found at these two times are shown in Figure 3.1. They have been shifted 1.5 hours to the right to adjust for the difference in arrival time at the collection crushes in the two sets of experiments. Both lie on the curve that

describes return from a distal injury. Since the magnitude of return was calculated as the ratio of accumulated activity to local pre-crush activity at the two collection crush locations, it may not be equal in both cases. The actual amount of label returned from a high crush will only equal that returned from a low crush if the "background" at the two levels of collection crushes is comparable. When two sets of collection crushes were made on the same nerve after passage of the orthograde wavefront, the isolated segment between the high set of collection crushes contained 1.68 times the amount of labelled protein per mm found between the collection crushes made distally (±.08 S.E.M.; n=15). Thus, although the same relative amount of label is reversed from two locations on the nerve, a greater absolute amount returns to the cell bodies after a more proximal injury.

iv) Retrograde Transport in Motor Axons Injured 6 Hours Post Injection

Figure 3.3 compares the time course of accumulation in axons injured either at the time of precursor injection or 6 hours later. There is a 1 hour lag in the initial part of the two curves, except during the 6-8 hour collection period, when presumably protein reversed at the injury had not yet reached the collection crushes. This 1 hour period could indicate that some time is required by a nerve to develop the ability to reverse the direction of transported material at an injury. This is not the same as the time taken for transported material to reverse its direction of transport, estimated in the previous section. Orthograde transported protein first reached the site of injury 5.8 hours p.i. In the case of axons injured at 0 hours

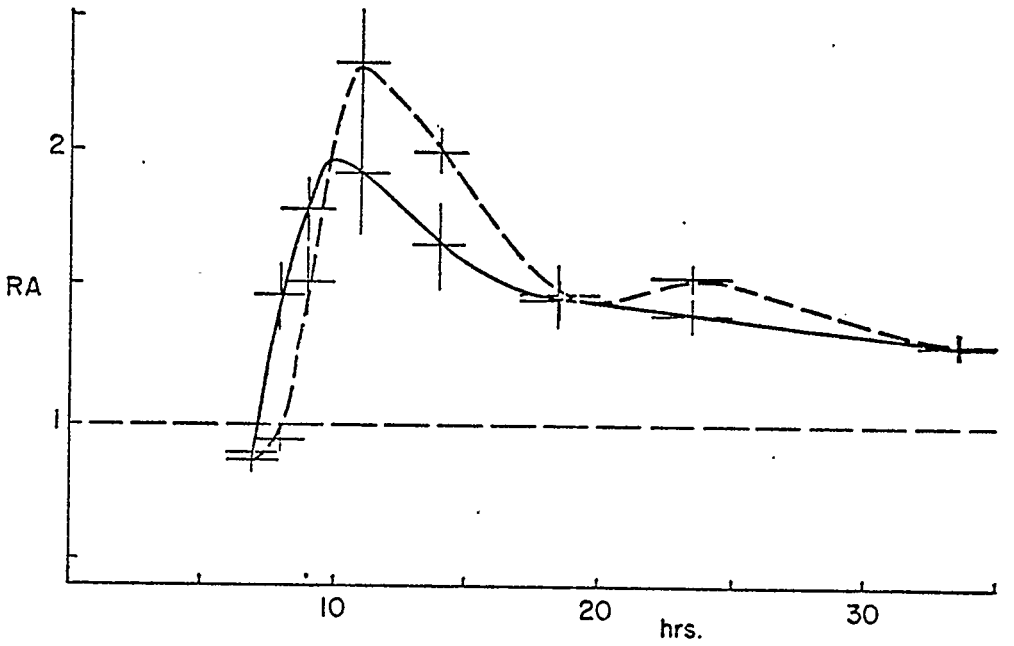

Figure 3.3 Retrograde transport in motor axons ligated at the time of precursor injection (0 hr) or 6 hrs later. Vertical and horizontal axes as in Figs. 3.1 and 3.2. —— ligated at 0 hr (same as Fig. 3.1(B)). --- ligated at 6 hrs. Note the 1 hour lag between the early portions of the two curves.

p.i. the mechanism for reversal was assumed to be fully developed by 5.8 hrs p.i., when labelled protein first reached the injury. It took an additional time for the orthograde-transported protein to reverse direction and return to the distal collection crush. In the case of axons injured at 6 hrs p.i. the lag period of 1 hr in the retrograde transport curve means that reversal must have occurred at (6.8 + X) hrs p.i. But, when the injury was made at 6 hrs p.i., labelled protein was already present at the injury site. Therefore, if the mechanism for reversal developed instantaneously on injuring the nerve, reversal should have begun at (6 + X) hrs p.i. The additional delay of 0.8 hrs in initiating reversal may thus represent time required by the nerve to develop the ability to reverse transport.

v) Labelled Protein Transport Into the Sciatic Nerve in the First 30 Hours P.I.

Figure 3.4 shows the relative accumulations at the proximal and distal collection crushes for injured sensory axons. The broken line represents the difference between proximal and distal accumulations. Since proximal accumulation is a measure of orthograde transport and distal accumulation a measure of retrograde transport, the difference curve gives a measure of the net delivery of activity to the axon distal to the collection crushes. Assuming that the majority of this activity is accumulating immediately proximal to the site of injury, integration of the difference curve gives the calculated total accumulation of activity at the injury.

In Figure 3.5 the calculated total accumulation at the injury site (broken line) is seen to correspond closely to the measured total accumulation (data points) for sensory axons. This may demonstrate

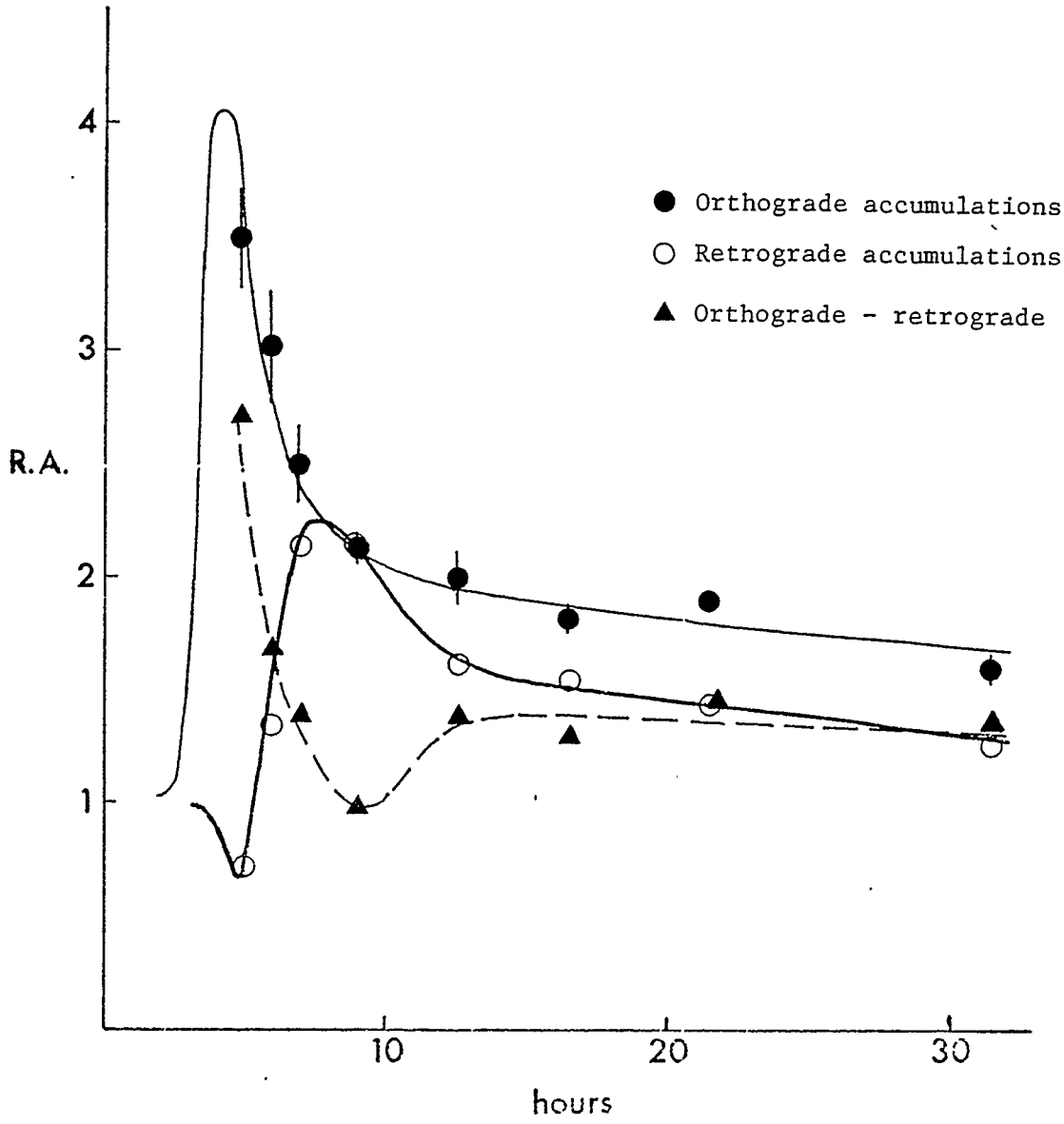

Figure 3.4 Comparison of orthograde and retrograde transport in ligated sensory axons. Vertical axis: relative accumulation. Horizontal axis: time in hours since precursor injection. ●—● orthograde transport accumulation. Mean ± S.E.M. for at least 6 nerves. Note that orthograde accumulation is assumed to begin 3 hours after precursor injection, when the wavefront of transported protein reached it. The shape of this curve between the onset of accumulation and the first data point was determined from the shape of the advancing protein wavefront. ○—○ retrograde transport accumulation (same as Fig. 3.2(B)). ▲—▲ calculated difference curve, representing balance between orthograde and retrograde transport, that is, the rate of accumulation of activity at the injury site.

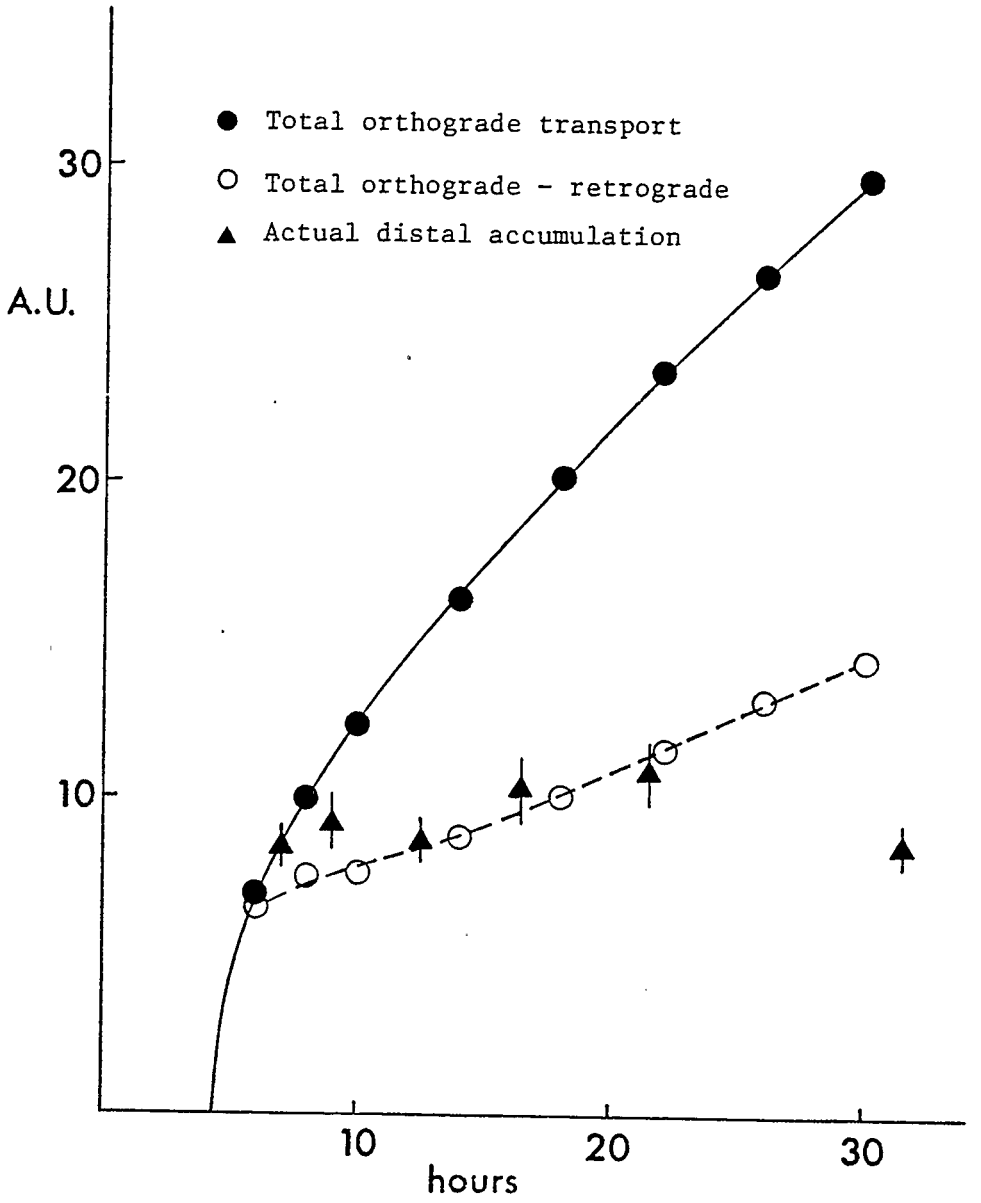

Figure 3.5 Total labelled protein accumulation at the injury site. Vertical axis: accumulation units; a relative accumulation of 1 maintained for 1 hour = 1 A.U. Horizontal axis: time in hours since precursor injection. ▲—▲ total accumulation in 2mm nerve segment proximal to injury ligature. Mean ± S.E.M. for at least 6 nerves. ○—○ total accumulation calculated from integration of the difference curve in Fig. 3.4. ●—● total orthograde transport calculated from integration of the orthograde transport curve in Fig. 3.4, representing delivery of activity to the injury site.

that most of the labelled protein transported past the site of the collection crushes (before they were applied) reached the injury ligature 15-25mm distally. It has been shown that protein in the orthograde wavefront of activity is incorporated into the axon (Cancalon & Beidler, 1975; Gross & Beidler, 1975), but these results suggest that only a small proportion of wavefront activity is incorporated per unit length of axon. Conversely, integration of the difference curve may not accurately estimate total activity remaining at the injury ligature.

Both measured and calculated curves begin with a rapid increase in accumulated activity up to 4 hours after arrival of the labelled wavefront at the crush site, followed by a slower increase of activity up to 21 hours p.i. At 31 hours p.i. there was a measured decrease in accumulation, whereas the calculated curve predicts a sustained increase. This suggests that between 21 and 31 hours p.i. an additional process developed at the injury site that reduced orthograde-transported accumulation more than could be accounted for by retrograde transport alone. A possible explanation could be the degeneration of injured axons back to the first or second more proximal node of Ranvier from the injury site (Kreutzberg, 1972). If this were accompanied by resorption of axoplasm into more proximal portions of the proximal stump the accumulation segment would contain less labelled protein than calculated by summation of orthograde and retrograde transport effects. Examination of nerve activity profiles at 21 and 31 hours p.i. could neither confirm nor refute this possibility.

Also shown in Figure 3.5 is the integral of the orthograde transport curve shown in Figure 3.4. Accumulation of activity at the injury

site falls short of what would be predicted as the basis of orthograde delivery alone, most probably because of subsequent retrograde transport of activity from the injury. It is possible, however, that measurements of accumulation rate may give an inaccurate estimate of the true magnitude of orthograde transport.

vi) Return of Labelled Protein from Intact and Injured Nerves

The relative magnitude of retrograde transport in intact and injured motor and sensory axons is shown in Figure 3.6. Here the integrals of the retrograde transport curves have been plotted as a percentage of the integrals of the orthograde transport curves for intact and injured motor and sensory axons. As previously mentioned, there is no significant difference in orthograde transport in normal and injured axons. These curves represent the proportion of labelled protein originally transported into the axon that is returned towards the cell body by retrograde transport in the first 30 hours p.i. In injured axons more of the transported activity is returned than in intact axons, although in motor axons the difference between injured and intact axons is much less pronounced than in sensory axons. By 30 hours p.i. the same proportion of orthograde transported protein has returned from injured and intact motor axons.

4. DISCUSSION

The effect of nerve injury on protein transport in motor and sensory axons has been evaluated by determining changes in the accumulation rate of labelled protein up to 30 hours after precursor injection. In the

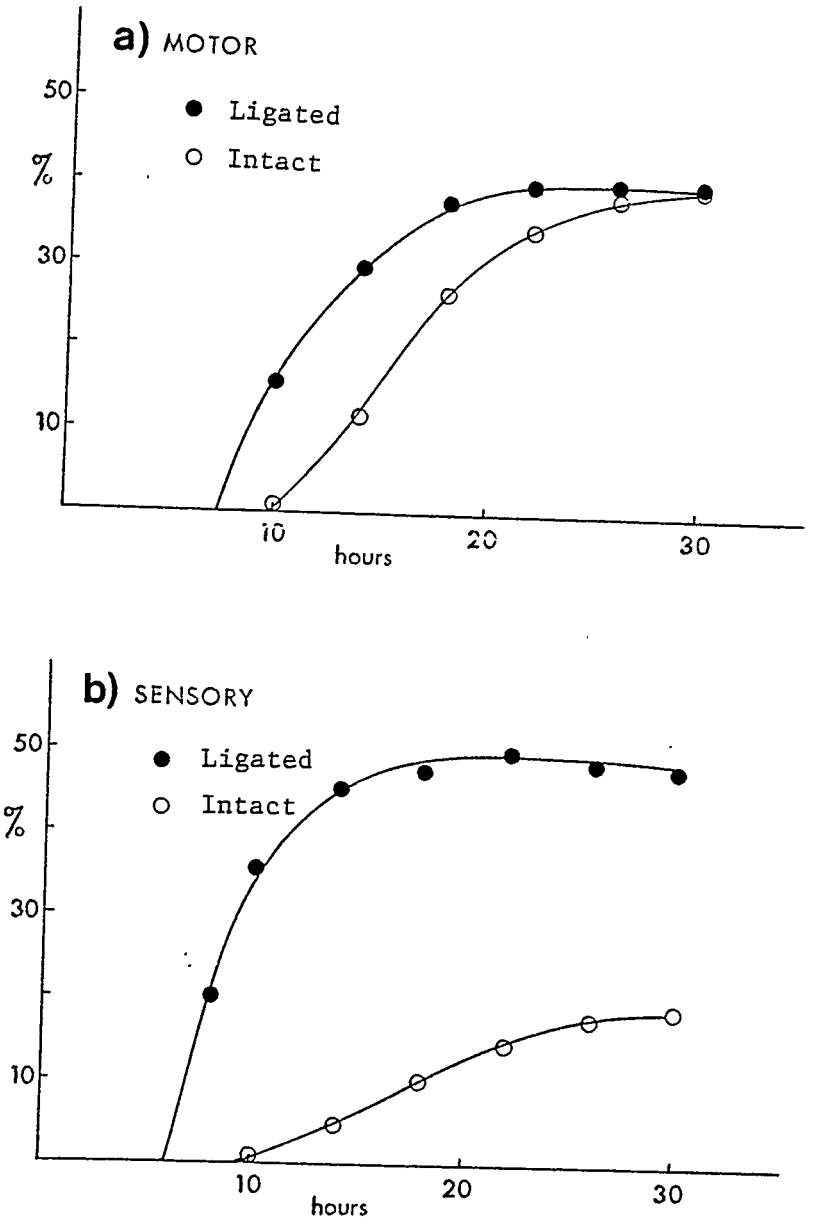

Figure 3.6 Return of labelled protein by retrograde transport. Vertical axis: integral of retrograde transport curves as a percentage of the integral of the orthograde transport curve. Horizontal axis: time in hours since precursor injection. (A) Motor axons. (B) Sensory axons. Points represent times at which percentage return was compared in intact and injured axons.

past this method of estimating transport has been used to determine transport velocity of endogenous enzymes (Lubinska & Niemierko, 1971; Ranish & Ochs, 1972; Jablecki & Brimijoin, 1975) and the transport characteristics of labelled protein (Edstrom & Hanson, 1973; McLean *et al.*, 1975; Bisby, 1976b, 1977b). The series of accumulation periods used here is considered to accurately reflect protein transport in motor and sensory axons of the rat sciatic nerve in the first 35-38 hours p.i. Incubation of accumulation segments in TCA removed unbound precursor, assuring that accumulated activity represented labelled polypeptides. Also, electrophoretic evidence has shown that transported proteins may accumulate at a ligature for at least 6 hours without significant breakdown (Bisby, 1977a). Accumulation of retrograde transported protein was found to be a linear function of time for at least 3 hours, and 3 hour accumulation values for orthograde transport were corrected for non-linearity (see Methods (iii), this chapter). In support of the latter, a close correspondence was found between total accumulation at an injury ligature and the integral of the difference curve calculated from orthograde and retrograde accumulation rate curves (Fig. 3.5).

In intact motor and sensory axons return of labelled protein had begun within 6 hours of its reaching the nerve periphery. This suggests that a portion of newly-synthesized protein has a very rapid turnover rate in the peripheral neurone. Previous investigations into the fate of fast transported protein have reported similar findings. In peripheral nervous system axons, labelled retrograde transported protein has been found to accumulate distal to applied ligatures within 12 to 24 hours of precursor injection (Bray *et al.*, 1971; Frizell & Sjostrand, 1974b;

Bisby, 1976b, 1977b; Griffin *et al.*, 1976). Similarly in central nervous system axons the half-life of a portion of fast transported protein has been estimated, on the basis of the rate of loss of radioactivity from nerve terminals, to be from 12 hours (Cuenod & Schonbach, 1971) to 17 hours (Droz *et al.*, 1973; Koenig *et al.*, 1973).

Frizell & Sjostrand (1974b) have proposed that the early return of transported protein represents a method for retrieval of "surplus material" synthesized by the nerve cell body, but this is not very likely. The metabolic extravagance implied by such casual regulation of synthesis would seem to be energetically disadvantageous. It has been shown by Watson (1968a), and will be further demonstrated in Chapter VII, that cell body synthetic processes may be closely linked to axonal length. At this time all that can be said is that a proportion of fast transported protein undergoes a turnover within hours of arriving in the periphery of motor and sensory axons. Perhaps it reflects a return of axonal organelles into which newly-arrived protein had been incorporated. Autoradiographic evidence supports this possibility: early-returning labelled protein is associated with elements of smooth endoplasmic reticulum and with mitochondria (Griffin *et al.*, 1976).

The greater amount of protein returned from intact motor axons compared to intact sensory axons, shown here at 15-25 hours p.i., and previously at 40-52 hours p.i. (Bisby, 1976b), provokes speculation that differences in protein transport reflect the functional differences of these axons. There is evidence to show that different amounts (Lasek, 1968; Ochs, 1972) and types (Anderson & McClure, 1973; Bisby, personal communication) of labelled protein are transported into central and peripheral processes of DRG neurones. Different axonal

turnover times would not, therefore, be unlikely. However, differences in the innervation pattern of sciatic sensory and motor axons could as well account for the dissimilar return of labelled protein from the two axon types.

The major finding of this study is the premature return of orthograde-transported protein in ligated nerves. The nerve develops the ability to reverse transported material about 0.8 hours after ligation, and orthograde-transported protein begins to return toward the cell body with a minimum velocity of 112-133mm/day at a maximum of 1.9-2.3 hours after arrival at a ligature.

The time lags preceeding transport reversal are probably required for vacating of the transport mechanism by retrograde-transported material returning from the periphery, and for uptake by the retrograde transport system of labelled material accumulating at the injury. Ultrastructurally, the axoplasm within 0.2-0.5mm of nerve section or crush becomes crowded with transported organelles: smooth endoplasmic reticulum, transport vesicles, and mitochondria (Blumke *et al.*, 1966; Zelena, 1968; Morris *et al.*, 1972). An accumulation of transported organelles is found within 3 hours of nerve injury (Zelena, 1968), so that by the time labelled protein had reached the injury ligature, 4.3 or 5.8 hours after precursor injection, the reversal site would have been densely packed with organelles. It is possible that the uptake of material by the retrograde transport system at a crush site in the first hours after injury is entirely arbitrary. Ochs (1975) has said as much, proposing that transport reversal at a ligature at least partly results from "local (structural) changes" produced by ligation. Perhaps at an injury material becomes detached from the

orthograde transport system and is picked up at random by the oppositely-directed system, which no longer carries material returning from beyond the ligature. The higher concentration of axonal components due to the compression effects of injury could serve to facilitate reversal. The similarity in proportional return from both ligated sensory axons and motor axons ligated at two levels (Figs. 3.1 & 3.2) infers just such a non-specific mechanism. In motor axons the proportion of transported activity returned from ligatures made at two levels of the nerve was the same, even though different absolute amounts of labelled protein were returned. This suggests that the amount of material reversed at a ligature is a function of the amount arriving by orthograde transport, and further supports the reversal mechanism being a non-specific characteristic of axons.

The minimum velocity estimate for retrograde protein transport of 112-133mm/day is within the range of previously reported values. Labelled proteins are retrogradely transported at 140mm/day in rabbit hypoglossal nerve (Frizell & Sjostrand,1974b) and at 60mm/day in frog sciatic nerve at 18°C (Edstrom & Hanson, 1973), which translates into a velocity of 230-260mm/day at 37°C (Ochs & Smith, 1971b). Other substances are retrograde-transported at similar rates: acetylcholinesterase has been found to return towards the cell body at rates of 130mm/day (Lubinska & Niemierko, 1971) and 220mm/day (Ranish & Ochs, 1972); dopamine-β-hydroxylase is transported at 288mm/day (Brimijoin & Helland, 1976); and horseradish peroxidase returns at 120mm/day (Kristensson *et al.*, 1971).

The biochemical and structural changes that occur in the nerve cell body in response to peripheral nerve injury (Lieberman, 1971) are

probably initiated by a signal transmitted by retrograde axonal transport. First, the onset of the axotomy response depends on the length of the proximal nerve stump (Humbertson, 1963; Watson, 1968a; Kristensson & Olsson, 1975). Second, blockade of axonal transport with colchicine produces typical chromatolytic changes in the cell body (Pilar & Landmesser, 1972; Cull, 1975; Purves, 1976). There are several candidates for the axonally-transported signal. Since extra-axonal molecules such as horseradish peroxidase and nerve growth factor can be taken up by nerve terminals and retrograde-transported (Kristensson *et al.*, 1971; Stoeckel *et al*,1975), axotomy would prevent such molecules as might be derived from innervated tissue from reaching the cell body. Watson (1968a), however, has shown that there is an additional, axonal, signal that is involved in the cell body response to injury. Mechanical trauma alters the permeability barriers of the nerve so that injured axons can take up and transport exogenous molecules at the point of injury (Kristensson & Olsson, 1974). The arrival of abnormal molecules at the cell body has been proposed to be the signal for chromatolysis (Berry & Riches, 1974). The work reported here presents an alternative hypothesis: that the signal is the return to the cell body of endogenous molecules that are normally destined for the axon, reversal of axonal transport having occurred at the site of injury. The time of arrival of the signal would be length-dependent. Since chromatolysis is an indirect response to axotomy (see Chapter VI) the discrepancy between the estimated velocity of the retrograde-transported signal and the onset of chromatolytic changes (Watson, 1968a; Kristensson & Olsson, 1975) should not be considered of critical import.

This hypothesis provides an explanation for two features of

chromatolysis pointed out by Cragg (1970): first, there is an inverse relationship between the severity of the chromatolytic reaction and the length of the proximal axon stump; second, chromatolysis of DRG cells after section of the dorsal roots in non-existent, or less severe than after section of peripheral nerves. Fast-transported protein is distributed both to terminals and non-terminal parts of the axon, so that in a neurone with a long axon the majority of the fast-transported protein leaving the soma is incorporated into the non-terminal axon (Gross & Beidler, 1975; Cancalon & Beidler, 1975; Ochs, 1975). Consequently, a decreasing amount of fast-transported protein remains mobile at increasing distances from the cell body. Thus the amount of protein originally exported from the soma which is prematurely returned after axotomy will depend on the position of the injury site. This was demonstrated by comparison of retrograde transport from injury ligatures made 65 and 90mm from the origin of the sciatic nerve ventral roots, where more protein is returned to the cell body from the more proximal injury. A complimentary situation is found in DRG cells: Ochs (1972) has shown that only one-third to one-fifth as much labelled protein is transported in the ventral roots as in the peripheral axon after precursor injection into the DRG. An injury to the dorsal roots will therefore result in the return to the soma of a smaller fraction of the total exported protein than after a peripheral injury, and the response of the DRG cells will be less severe.

What does the premature return of protein after axotomy actually mean to the cell body? It was shown that an increased amount of newly-synthesized protein was reversed at a ligature, but it is not likely

that magnitude is itself important as a signal. Most probably information about axotomy is carried by changes in the quantities of different types of protein returned to the cell body. For instance, it is probable that a greater proportion than usual of proteins carried in the orthograde wavefront are returned to the cell body after reversal at a ligature. Chapter IV will deal with the composition of the prematurely-returned protein.

Axotomy produced by a ligature results in an accumulation of orthograde-transported protein proximal to the ligature. In addition, a considerable amount of newly-synthesized rapidly-transported protein reverses direction, presumably at the site of accumulation, and is returned to the cell body. The increased return of protein to the cell body may convey information about the occurrence and the site of axotomy and precipitate an appropriate response.

CHAPTER IV

QUALITATIVE DIFFERENCES IN NORMALLY & PREMATURELY-RETURNED PROTEIN

1. INTRODUCTION

Sciatic nerve ligation causes a premature return to motor and sensory cell bodies of labelled orthograde-transported protein (Chapter III). This phenomenon may represent a mechanism by which the cell body is informed of axonal injury and to which it responds with a series of metabolic and structural changes (Grafstein, 1975).

The actual signalling function of the abnormally-returning protein could be performed by the bulk return of a greater-than-normal amount of newly-synthesized material. However, current concepts of metabolic regulation assert that the control of synthetic reactions is mediated via the negative feedback effects of specific metabolities (Monod *et al.*, 1963; Koshland & Neet, 1968). It is more likely, then, that one or more components of the prematurely-returned material constitutes the axotomy signal.

Fast-transported material from a number of neuronal systems has in the past been characterized by SDS-polyacrylamide gel electrophoresis (Anderson & McClure, 1973; Edstrom & Mattsson, 1973; Willard *et al.*, 1974). This technique was adapted (Bisby, 1977a) to determine if differences exist in the composition of protein returning from injured and intact sciatic motor nerves that may serve to inform the cell bodies of axotomy.

2. MATERIALS & METHODS

i) Collection of Labelled Protein

Two series of experiments were performed. In both, proteins transported in sciatic nerve motor axons were labelled as previously described (Chapter II). In one series sciatic nerves were injured by bilateral ligation approximately 90mm from the origin of the ventral roots at the time of precursor injection. Nine hours later a second, collection ligature was applied 15mm proximal to the first, and left in place for 3 hours, when the animal was killed. In this interval protein comprising the early peak returning from the injury ligature (Fig. 3.1) would accumulate distal to the collection ligature.

In the second series, nerves remained intact until 22 hours p.i., when a collection ligature was applied 75mm from the origin of sciatic ventral roots. This was left in place for 3 hours, then the animal was killed. Retrograde-transported protein accumulating distal to this ligature comprised the early peak return from intact nerves (Fig. 3.1).

Labelled protein transported in an orthograde direction at both sampling times accumulated proximal to applied collection ligatures.

ii) Electrophoresis Protocol

Nerves were rapidly removed, placed on cardboard strips, and frozen in solid CO_2 until extraction of protein. Accumulation segments (2mm lengths) were cut from the nerves, and in some cases the epineurial sheath was removed with fine forceps under a dissecting microscope to facilitate homogenization. Analysis of protein returning from a

ligature was performed on pooled accumulation segments from 8 nerves, while accumulation segments from 12 intact nerves were pooled because of the lower levels of activity transported at the later time.

Segments were pulverized in a steel punch cooled with solid CO_2. The resultant powder was homogenized at room temperature in a Teflon-glass homogenizer (Kontes; clearance .004-.006 inches) by 20 strokes of a motor-driven pestle in 100µl of solubilizing solution (sodium phosphate buffer, 10^{-2}M, pH 7.2; sodium dodecyl sulphate (SDS), 3% W/V; mercaptoethanol, 3% W/V; and ethylenedinitrilotetraacetic acid (EDTA), 10^{-3}M). The homogenate was transferred to a micro test tube, and a further 100µl of solubilizing solution used to wash the homogenizer was added. The homogenate was then incubated at 95°C for 10 minutes and centrifuged for 10 minutes at 8000 x g. The supernatant was decanted and further centrifuged for 30 minutes at 8000 x g. This extraction procedure was found to solubilize 90-95% of the total ^{3}H activity in nerve segments (Bisby, 1977a).

The final supernatant was mixed with 1/5 of its volume of tracing dye (Bromphenol blue 0.5%; sucrose 30%; Biorad) and layered on top of commercially prepared 4% polyacrylamide gel columns (Biorad). Gels had been previously conditioned by electrophoretic introduction of buffer solution (Tris/acetic acid, 0.025M, pH 6.1; SDS, 0.1% W/V).

In order to obtain enough activity for scintillation counting, samples were equally divided between several gels, and equivalent gel fractions were pooled for counting subsequent to electrophoresis. To prevent overloading the gels with protein, homogenate from a maximum of 6.7mm of nerve was applied to each gel. The total sample volume

applied to each gel varied from 50-70µl.

Electrophoresis was performed in a water-cooled Biorad 150A electophoresis cell, driven by a Biorad 400 power unit. Samples were run into the gel for 15 minutes at 4mA per gel, then the run was continued for a further 2-3 hours at 7-8mA per gel. At the end of the run gels were removed from the glass columns and the position of the dye wavefront was marked by insertion of a short length of stainless steel wire.

iii) Protein Characterization

Gels were fixed in 40% isopropanol and 10% acetic acid for 1 hour, with a change of fixative after 30 minutes. After staining for 30 minutes at 60°C in a solution of 0.05% Coomassie Blue, 10% isopropanol, and 10% acetic acid, gels were destained overnight in 10% isopropanol and 10% acetic acid. Stained gels were scanned at 590mm with a scanner attachment to a Beckman Acta CV spectrophotometer to localize protein constituents.

After destaining, gels were sliced into 2mm lengths; the number of slices per gel was constant within a single sample run, but varied from 46-48 in different sample runs. Equivalent gel slices from the same sample run were pooled in scintillation vials. Protein was extracted with a 1 ml/slice solution of 50% toluene, 45% Protosol (N.E.N), and 5% distilled water. Vials were incubated at 60°C for 90 minutes, cooled to -25°C for 60 minutes, and left at room temperature for 24 hours. At that time 10mls of a toluene-Liquifluor (N.E.N.) scintillation fluid was added (4gms PPO, 50mg POPOP per litre toluene). After 24 hour storage in the dark to reduce chemoluminescence, vials

were counted in a Beckman LS 3155T Liquid Scintillation System. Quenching was corrected by the method of external standardization, and counting efficiency was found to be 35-40%, and uniform for a given batch of samples.

The transformed activity data was used to produce electrophoretic profiles of transported protein in intact and ligated sciatic nerves.

A molecular weight calibration curve was produced using the following protein standards: chymotrypsinogen (25.7×10^3 daltons), ovalbumin (4.5×10^4), bovine serum albumin (7.0×10^4), and phosphorylase A (9.6×10^4). 10µl of a 10mg/ml solution of each standard was added to 10µl of solubilizing solution and incubated at 95°C for 10 minutes. After samples cooled to room temperature, 5µl of tracking dye was added, and each standard was applied to the top of a preconditioned 4% polyacrylamide gel column. Electrophoresis, fixing, staining, and destaining conditions were the same as for nerve proteins. An R_f value was determined for each standard from the ratio of distance travelled by the protein peak divided by the distance travelled by the tracking dye front. The bovine serum albumin standard yielded R_f values for its mono-, di-, tri-, and tetrameric forms.

3. RESULTS

Three electrophoretic analyses were performed on transported proteins from ligated sciatic nerves, and two were performed on transported proteins from intact nerves. Considerable variability was encountered in the degree to which specific protein activity peaks were resolved. This variability was not due to differences in the amount of labelled

protein analysed, as the total accumulated activity was fairly constant from one sample to the next, nor was it due to differences in the number of gels used per sample run, since a constant volume of nerve homogenate was run on each gel.

Figure 4.1 shows the molecular weight calibration curve for the 4% polyacrylamide gels used for electrophoresis. A straight line was fitted to the data points by linear regression analysis (correlation coefficient = .99). The extrapolated limits of resolution of the gels were 30,000-350,000 daltons.

Figure 4.2 compares the best resolved electophoretic profiles of protein returning to motoneurone cell bodies at 9-11 hours p.i. from ligated nerves (a), and at 22-25 hours p.i, from intact nerves (b). Protein returning at 9-12 hours is characterized by three dominant peaks: I (R_f 0.42), II (0.68), and III (0.96), corresponding to molecular weights of 127K (10^3 daltons), 68K, and 35K, respectively. These peaks, along with a "shoulder" of transported activity extending from R_f 0.2-0.31 (165K-210K), demonstrate the retrograde transport of large quantities of select types of proteins. This differs greatly from the situation found in intact nerves. Although the dominant retrograde transported proteins found in ligated nerves are also found in intact nerves they are, except for those comprising the 35K peak, transported in much reduced quantities relative to other proteins. Retrograde transport in ligated nerves appears to return to nerve cell bodies increased amounts of fewer types of proteins than in intact nerves.

Profiles of orthograde transported protein at 9-12 hours p.i.

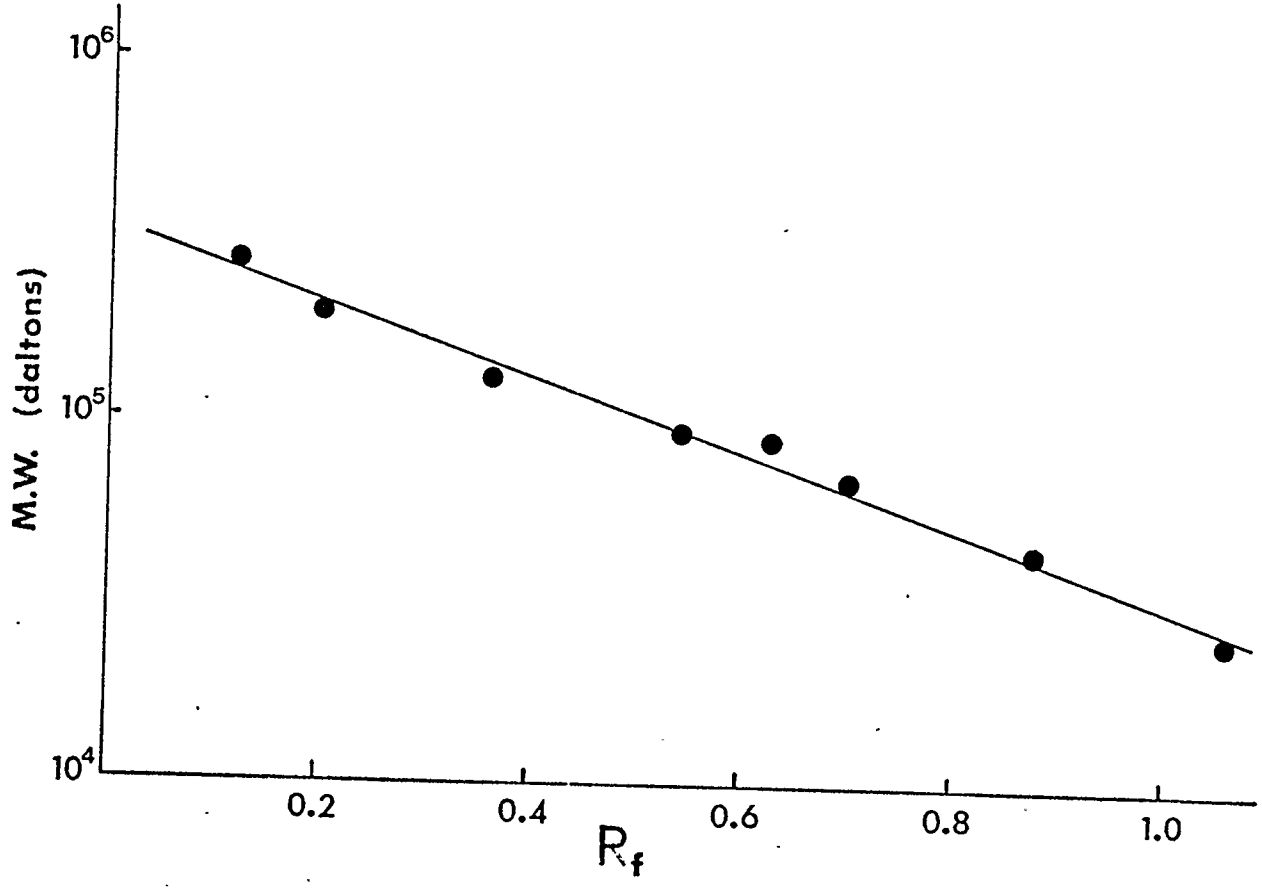

Figure 4.1 Molecular weight calibration curve for 4% polyacrylamide gel columns. Vertical axis: molecular weight of protein standards. Horizontal axis: R_f values.

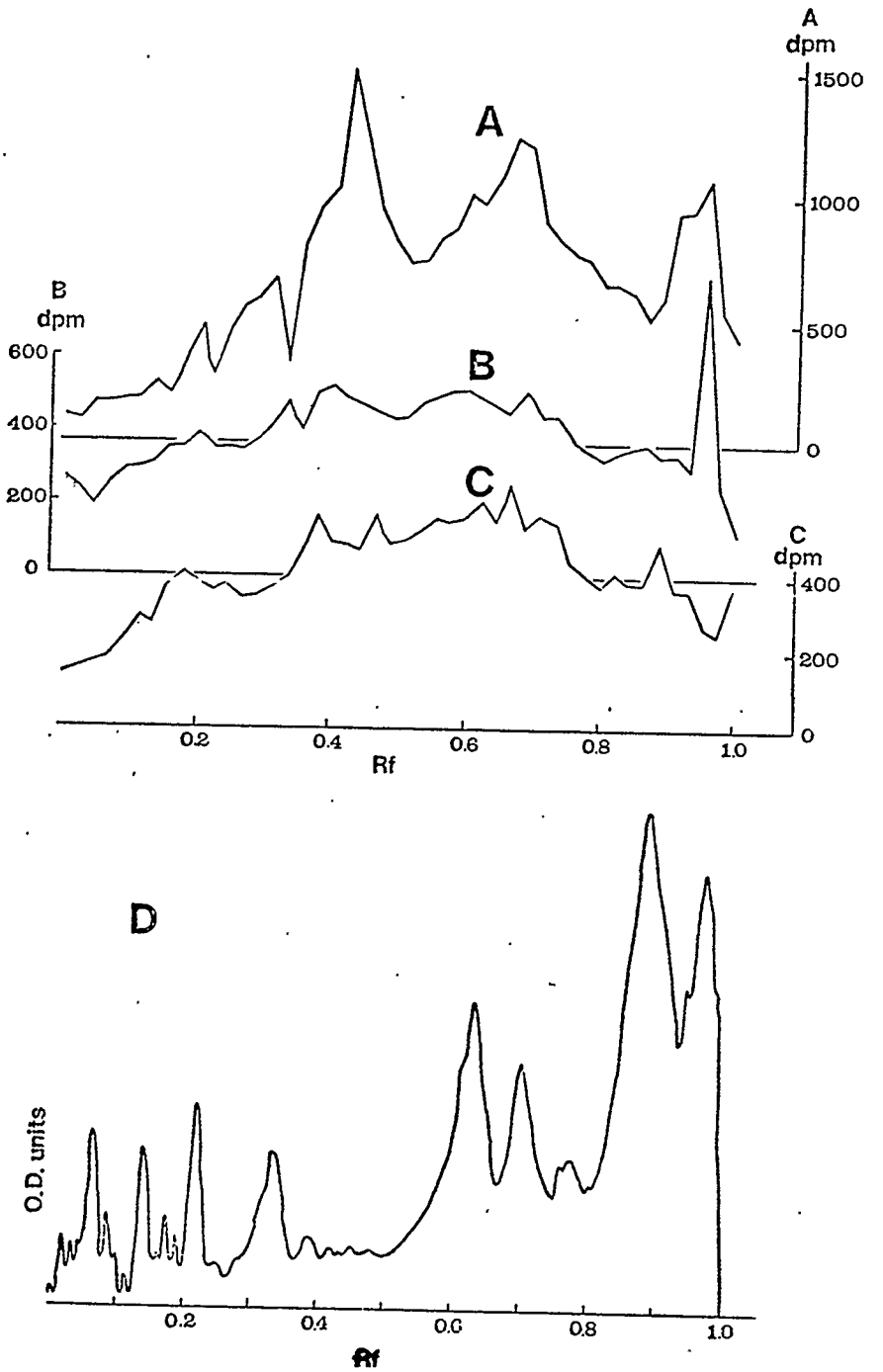

Figure 4.2 Distribution of labelled polypeptides and Coomassie-blue stained materials on 4% polyacrylamide gels. Vertical axis: A,B, and C = dpm of labelled protein in sciatic motor axon accumulation segments; D = arbitrary optical density units. Horizontal axis: Rf values. A. labelled protein in accumulation segments distal to 9-12 hour collection crush in injured nerves. B. labelled protein in accumulation segments distal to 22-25 hour collection crush in intact nerves. C. labelled protein in accumulation segments proximal to 22-25 hour collection crush in intact nerves. D. optical density scan at 590 nM of a Coomassie-blue stained 4% gel.

were poorly resolved and are therefore not reproduced here. However, the same methods have been used to characterize ^{3}H-leucine labelled protein accumulating from 4-10 hours p.i. at a ligature on the rat sciatic nerve (Bisby, 1977a). The electrophoretic profile obtained is essentially the same as that found for protein returning from a nerve ligature at 9-12 hours p.i. (Fig. 4.2(a)),where three protein peaks predominate. By 22-25 hours p.i. (Figure 4.2(c)) the dominant fast orthograde transported protein peaks are present in reduced amounts relative to other proteins. The changing composition of orthograde transported protein with increasing time after precursor injection is well established (Karlsson & Sjostrand, 1971a; Willard *et al.*, 1974; Hoffman & Lasek, 1975).

Generally, then, the relative amounts of different proteins constituting the returning peak of activity are dissimilar in intact and ligated nerves. In fact they more closely resemble the distributions found in orthograde-transported material in the same time interval. The cell bodies of ligated nerves thus receive a premature peak of returning protein, the composition of which is essentially unchanged from that transported into the axons 9-12 hours previously.

Figure 4.2(d) is a typical optical density scan of a stained gel after electophoresis of proteins extracted from nerve segments in which orthograde-transported protein had accumulated from 22-25 hours p.i. Scans of nerve protein proximal and distal to the collection ligature from both accumulation intervals were virtually identical. Little correspondence exists between transported ^{3}H-labelled proteins and stained nerve proteins. There are many more distinct stained

bands than there are activity peaks, and the major activity peaks are not represented significantly in any of the stained protein bands. Also the areas of most intense continuous staining are in the region of low molecular weight proteins (R_f 0.5-0.8, and R_f 0.5-1.0), while labelled proteins are more uniformly distributed along the molecular weight axis.

4. DISCUSSION

SDS-polyacrylamide gel electrophoresis was used to characterize fast-transported proteins from intact and ligated sciatic nerves. SDS is an anionic detergent that solubilizes the predominantly membrane-bound, and hence insoluble, fast-transported protein (McEwen and Grafstein, 1968; Karlsson & Sjostrand, 1971b, c; Edstrom & Mattsson, 1973). Constituent polypeptides migrate down the gels at a rate inversely proportional to the logarithm of their molecular weight (Shapiro *et al.*, 1967; Weber & Osborn, 1969). The separation thus obtained has been used in the characterization of transported proteins from a number of different neuronal systems (Anderson & McClure, 1973; Barker *et al.*, 1975; Cancalon & Beidler, 1975; Siegel & McClure, 1975).

Here the technique was used to compare labelled retrograde-transported proteins returning from intact and ligated sciatic nerves. In these experiments difficulty was experienced in reproducably resolving labelled protein profiles. This is most likely to have resulted from the pooling of up to three 2mm gel slices for each point of an electophoretic profile, since electrophoretic profiles of slow-transported proteins, which are obtained without pooling, demonstrate much improved

peak resolution (Bisby, unpublished observations). Bisby (1977a) has shown that resolution is not affected by proteolysis or ^{3}H exchange: proteolysis does not occur during the accumulation period, and neither proteolysis nor ^{3}H exchange occurs during extraction.

The major retrograde-transported protein peaks from ligated nerves, with molecular weights of 35K, 68K, and 127K, are considered to represent polypeptides normally transported in the sciatic nerve, since they have been shown in the wavefront of fast orthograde transport in the rat sciatic nerve (Bisby, 1977a). Although their actual function may only be guessed, they are probably fairly important neuronal constituents. One or more of the three protein peaks have as well been demonstrated in fast-transported protein from the cat sciatic nerve (Anderson & McClure, 1973), rabbit optic nerve (Willard *et al.*, 1974), the toadfish sonic motor nerve (Barker *et al.*,1975), and the garfish olfactory nerve (Cancalon *et al.*, 1976). Because proteins and glycoproteins with similar molecular weights have been found as components of synaptic vesicles and synaptosomal plasma membranes (Morgan *et al.*, 1973; Langley & Kennedy, 1977), nerve terminal function could depend upon their provision by axonal transport.

As mentioned before, the composition of orthograde transport profiles change with time. The quantity of the dominant proteins transported earlier is much reduced, and although their locations are still evident, unresolved proteins account for most of the activity in the later profile. The time-dependent change in orthograde-transported proteins could be due to: (i) differences in the transport velocities of various proteins, or (ii) differences in the time of protein efflux from the cell body. Although the velocity of protein transport during

the two accumulation intervals cannot be estimated, it is probably not the only determinant of the observed compositional changes. Whereas the transport filament model of transport (Ochs, 1974) predicts that transport velocities are inversely related to molecular weight, at neither interval are the majority of accumulated proteins of only high or low M.W. The carrier stream model of Gross (1975), in which the transport mechanism behaves like a chromatography column (i.e. velocity is proportional to molecular weight), is likewise inadequate to explain composition changes in terms of different transport velocities. On the other hand, cell body release and axonal transport of labelled glycoproteins (Forman *et al.*, 1972; Marko & Cuenod, 1973) and phospholipids (Grafstein *et al.*, 1975) does continue for some days after precursor injection. Orthograde-transported proteins accumulating at 22-25 hours p.i. (Fig. 4.2(c)) may therefore represent material which is transported later from the nerve somata.

The discrepancy between profiles of labelled proteins and stained nerve proteins is twofold. First, many more protein peaks than activity peaks are found, probably as a result of poor resolution stemming from gel pooling. Techniques such as autoradiography (Willard *et al.*, 1974) or fluorography (Barker *et al.*, 1977) that permit analysis of electrophoresed protein by optical scanning, and hence are not hindered by the artificial discontinuity introduced by gel pooling, characteristically resolve 14-24 fast-transported proteins. Second, the majority of nerve proteins are of low molecular weight (<100K), compared with the range of molecular weights found for transported proteins, and do not correspond to transported activity peaks.

A recent survey of nerve protein characterizations (Bisby, 1977a) has made the same observation. Since stained nerve proteins from both

sides of a collection crush are identical for both accumulation intervals, it must be concluded that fast transported proteins are not major components of whole nerve. The major proteins may not even be of neuronal origin. Theiler & McClure (1977) have shown a close correspondence between stained nerve proteins and those synthesized by Schwann cells.

Most significantly, it has been demonstrated that the proportions of protein constituents in the early-returning retrograde transport peak in a ligated nerve differ from those found in the transported peak returning from an intact nerve. An intact nerve periphery returns to the cell body a heterogeneous mixture of proteins with molecular weights of 30K-350K, its composition probably dependent upon protein turnover rates in the periphery. A greater magnitude of labelled protein returns from a ligated nerve, dominated by the dominant proteins of fast orthograde transport. Proteins from intact nerves that were retrograde-transported at 9-12 hours p.i. were not characterized because of the low levels of activity transported at that time (Fig. 3.1), so actual comparison of proteins returning at the same time from intact and ligated nerves was not made. Nonetheless, even if these proteins were returned in the same proportions from the two types of nerve a much larger quantity would reach the axotomized cell bodies.

These results demonstrate that the retrograde transport system may function to mediate the regulation of nerve cell body synthetic activity via negative feedback effects of returning metabolic products. Although the initiator of the complex sequence of biochemical and structural changes that follow axonal injury (Grafstein, 1975) is not identifiable, the return of abnormal proteins, or abnormal quantities

of proteins, in ligated nerves could conceivably produce drastic metabolic responses. The proposal (Chapter III) that abnormal return of protein from a nerve injury site may constitute a signal for chromatolysis is thus given added support.

Also of interest is the similarity in electrophoretic profiles of fast othograde- and premature retrograde-transported proteins in ligated nerves. That molecular weight appears to play no part in determining "reversibility" suggests that the turnaround process operating to shunt transported material from orthograde to retrograde transport systems is non-selective. This may not be entirely correct, however, since not all transported materials may undergo reversal at a crush site. Banks *et al.* (1969) have shown that noradrenaline (NA) transported in granular vesicles does not accumulate at a proximal collection ligature following distal ligation of the cat hypogastric nerve. Although Haggendal *et al.* (1975) have found return of NA from a peripheral injury, its concentration is very low compared to the quantity that is orthograde-transported. This reduced reversibility is perhaps due to the form in which NA is transported (i.e. within vesicles).. Both labelled orthograde-transported proteins and retrograde-transported horseradish peroxidase have been shown to be primarily associated with axonal profiles of smooth endoplasmic reticulum (SER) (Droz *et al.*, 1975; LaVail & LaVail, 1974). Perhaps SER components that accumulate at a nerve crush (Blumke *et al.*, 1966; Morris *et al.*, 1972) are more readily reversed than are discrete vesicles.

Additionally, though the packaging in the SER of fast-transported protein is not understood, the bulk turnaround of all orthograde-transported proteins seems to suggest that different proteins are

packaged and transported in bulk. That is to say, at a time determined by a protein's axonal turnover rate, it, along with similar axonal components, is transported from the cell body either as part of the SER membrane, or within its lumen. If this is so, then the disbursement of transported materials in the nerve must depend upon local mechanisms, and not upon differences in the type of transported organelle.

CHAPTER V

RETROGRADE TRANSPORT OF PROTEIN AFTER COLCHICINE TREATMENT

1. INTRODUCTION

Axonal transport is an energy-dependent process that probably occurs via a sequence of localized, step-like reactions (Schmitt, 1968; Banks *et al.*, 1969; Ochs, 1974; Cooper & Smith, 1974). On the basis of morphological evidence axonal microtubules are considered to be the prime structural elements mediating transport. Microtubules are found in close association with transported organelles, including mitochondria, a possible energy source (Raine *et al.*, 1971; Cooper & Smith, 1974; Smith *et al.*, 1975), and disruption of microtubule integrity results in the inhibition of transport and accummulation of transported material (Dahlstrom, 1968; Kreutzberg, 1969; Fernandez *et al.*, Byers *et al.*, 1973; Hammond & Smith, 1977). Inhibition of transport by an analogous series of drugs has been found to correlate with microtubule-disrupting ability (Paulson & McClure, 1974).

The compounds colchicine and vinblastine have been most frequently used to disrupt axonal microtubules. Colchicine treatment of peripheral nerves results in effects similar to those found after axotomy both at the innervated end organ (Aguilar *et al.*, 1974; Fernandez & Ramirez, 1974; Inestrosa and Fernandez, 1976) and at the nerve cell body (Pilar & Landmesser, 1972; Cull, 1975; Purves, 1976). Concurrently,

colchicine treatment causes inhibition of protein transport (Karlsson & Sjostrand, 1969; James *et al.*, 1970; McLean *et al.*, 1975) and accumulation of axonal organelles similar to that found proximal to a nerve crush (Hokfelt & Dahlstrom, 1971; Rodriguez-Echandia *et al.*, 1973).

Because the axotomy signal may be the abnormal return of transported material found in injured nerves (Chapter III & IV), I investigated whether a similar return of labelled protein occurs in colchicine-treated nerves, which could account for its axotomy-like effects.

2. MATERIALS & METHODS

i) Colchicine Treatment of Nerves

Sciatic nerves were exposed to colchicine by two methods. The first involved placing around the nerve a silastic cuff prepared according to the method of Albuquerque *et al.* (1972). Solid colchicine (0.1% w/w) was mixed thoroughly with Silastic type A moldmaking rubber (Dow Corning), and vulcanized into cylindrical cuffs of 1.0mm inner diameter, 4.0mm outer diameter, and 5-6mm in length. Control cuffs were made similarly, but did not contain colchicine. A 1mm wide slit was cut in the side of the cuffs to allow placement on nerves. Labelled protein transport was compared in nerves to which control cuffs and colchicine-embedded cuffs had been applied. This method proved unsatisfactory in demonstrating colchicine effects on axonal transport for two reasons. First, the cuffs were placed on the nerves 24 hours before precursor injection to permit the slow development of colchicine effects on axonal transport (Karlsson *et al.*, 1971;

Paulson and McClure, 1974). Comparable transport blocking effects were found in both control and experimental nerves, probably due to mechanical damage of the nerve caused by the cuffs alone. Second, the amount of drug that would have diffused out of experimental cuffs during the 24 hour incubation period (Kauffman *et al.*, 1974) was probably insufficient to affect transport (Dahlstrom, 1968; Kreutzberg, 1969; Rodriguez-Echandia *et al.*, 1973).

The second method, which did prove satisfactory, utilized the technique of sub-epineurial injection of a colchicine solution into the sciatic nerve. This method was first used by Dahlstrom (1968) and was found to cause accumulation of catecholamine storage granules in sympathetic nerves. Similar transport-blocking effects have been produced in cholinergic nerves after sub-epineurial colchicine injection (Kreutzberg, 1969; Heiwall *et al.*, 1976).

Injections of 4µl of either normal saline or a 30mM colchicine solution were made into sciatic nerves 80-85mm from the origin of the ventral roots. Half of the injection was made into each of the two nerve bundles (tibial and common peroneal) that constitute the sciatic nerve at this level. For injection, graduated glass micropipettes with tip diameters of 100-150µM were connected to a 10µl microsyringe (Hamilton) by an absolute alcohol interface through a length of polyethylene tubing. A teflon coating (Crown) was sprayed on micropipette tips to prevent leakage back along the micropipette during injection. Micropipette tips were inserted tangentially along the nerve for injection, which was made over the course of 25-30 seconds, and were left in place for 1-2 minutes to reduce leakage from the nerve.

Incisions were then closed with surgical clips and animals were maintained at normal body temperature until recovery from anaesthesia.

ii) Evaluation of Colchicine Effects

Precursor injection into the spinal cord motoneurone pool and colchicine injections into the sciatic nerve were made concurrently. The colchicine effect on transport should have manifested itself by the time labelled protein had reached the nerve injection site (Rodriguez-Echandia *et al.*, 1973; Heiwall *et al.*, 1976). To evaluate reversal of labelled protein at the colchicine injection site collection crushes were made 15 and 25mm proximal to the injection site at 9 hours p.i. and left in place for 2 hours, when the animal was killed. The 9-11 hour interval was found to represent the time of peak retrograde transport in ligated motoneurone axons (Fig. 3.1). Relative accumulation of transported protein proximal and distal to collection crushes was calculated as before (Chapter II).

3. RESULTS

Preliminary experiments were performed to determine the distance that injected solutions would diffuse along the nerve trunk by 11 hours after sub-epineurial injection. Micropipettes were inserted tangentially into the two nerve bundles composed of sciatic nerve axons, and 2µl of a toluidine blue solution were injected into each bundle. Before the leg incision was closed, inspection of the nerve revealed diffusion of the dye limited to 1-2mm in either direction from the injection site. By 11 hours most of the dye had disappeared

from the nerve, probably due to leakage from the injection site. Under a dissecting microscope traces of the dye were observed to extend 3-4mm in either direction along the nerve. There was thus at least a 6-8mm length of nerve upon which injected solutions could have an effect.

Representative radioactivity profiles of nerves injected with saline or colchicine to which 9-11 hour p.i. collection crushes were applied are shown in Figure 5.1. Orthograde transport past the saline injection site was unimpeded, while a partial blockage of transport in colchicine-injected nerves resulted in labelled protein accumulation adjacent to the nerve injection site. As expected, the length of nerve along with orthograde transport is inhibited by injected colchicine extends about 6mm proximal to the injection site. Also clearly shown is the greater magnitude of accumulation of retrograde transported protein distal to the collection crushes in the colchicine-injected nerve.

The magnitude of orthograde and retrograde transport at 9-11 hours p.i. in saline- and colchicine-injected nerves is compared in Table 5.1. Accumulation of orthograde transported protein was the same under both conditions, but significantly more retrograde transported protein accumulated in nerves injected with colchicine. The amount of protein returned from colchicine-injected nerves was not, however, as great as in ligated nerves during the same time interval.

4. DISCUSSION

Although colchicine treatment of nerves has been used extensively

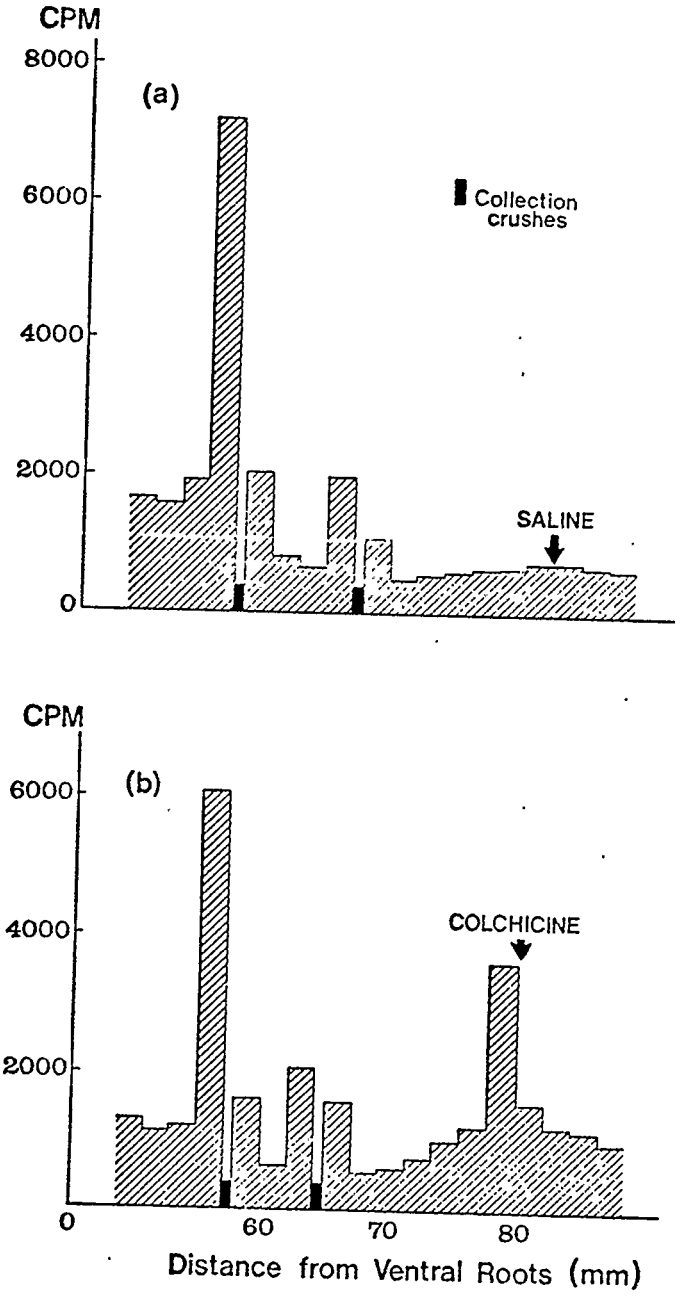

Figure 5.1 Transported protein distribution after sub-epineurial colchicine injection. 2ul of Normal saline or colchicine (30 mM) was injected into peroneal and tibial branches of the sciatic nerve at the time of precursor injection. Collection crushes were in place from 9-11 hours to determine protein transport. Vertical axis: TCA-insoluble activity per 2mm nerve segment. Horizontal axis: distance from sciatic ventral roots.

TABLE 5.1 Orthograde and retrograde transport in sciatic nerves injected with saline or colchicine, or in ligated nerves, at 9-11 hours p.i. Accumulation of labelled protein at collection crushes expressed in relative accumulation units (R.A.±S.E.M., n=number of nerves).

	ORTHOGRADE (n)	RETROGRADE (n)
Saline Injected	3.07 ± .05(6)	1.03 ± .02(6)
Colchicine Injected	2.96 ± .13(7)	**1.23 ± .06(7)
Ligated	+ 3.00	+ 1.95

** $p<.01$ (One-tailed Student's t-test)

+ Estimated from orthograde and retrograde transport curves obtained from nerves ligated at 0 hour P.I. (Chapter III)

to block the orthograde axonal transport of neurotransmitter-related enzymes (Dahlstrom, 1968; Kreutzberg, 1969; Banks *et al.*, 1971; Heiwall *et al.*, 1976), labelled protein (Fernandez *et al.*, 1970; Karlsson *et al.*, 1971; Paulson & McClure, 1974; McLean *et al.*, 1975), and transported organelles (Hammond & Smith, 1977), reversal of transported material at a colchicine block has never been examined. Such a reversal could, by simulating a nerve crush, serve to produce nerve cell body changes in the same manner as may the prematurely-returned protein in axotomized nerves (Chapter III & IV).

Sciatic nerve axons were most effectively exposed to colchicine by sub-epineurial injection. This technique does not produce the mechanical damage produced by a cuff and directly exposes axons to the drug. Dose levels were calculated from previous studies (Dahlstrom, 1968; Fernandez & Ramirez, 1974; Heiwall *et al.*, 1976) so as to produce axonal transport effects by the time labelled protein had reached the nerve injection site. Previous work in the visual system (Karlsson *et al.*, 1971; Paulson & McClure, 1974), however, found that long (24 hour) incubation times were required for maximal transport inhibition by colchicine after intraocular injection. The incomplete blockage of orthograde transport (Fig. 5.1) may thus result from the single or combined effects of short incubation time and exposure to a low drug dose due to its leakage out of the nerve.

The important point made by these results is that the inhibition of protein transport by colchicine is followed by reversal and return to the motoneurone cell bodies of an abnormally large amount of newly-synthesized protein. Although the amount returned was less than that

found at the same interval after nerve ligation, this could be in part due to the incomplete inhibition of orthograde transport which would result in a reduced amount of "reversible" protein. Assuming that the velocity of returning protein was similar to that of protein reversed at a ligature (Chapter III) it would arrive at the motoneurone cell bodies prior to the onset of the chromatolytic changes demonstrated after colchicine "axotomy" (Pilar & Landmesser, 1972; Cull, 1975; Purves, 1976). These results further support the proposition that the functional status of the axon is monitored by the cell body via the return of transported material (Chapter III), with changes in the amounts or constitution of returning material perhaps serving to initiate a response in the soma (Chapter IV).

CHAPTER VI

NERVE CELL BODY RESPONSE TO AXOTOMY

1. INTRODUCTION

The response of neuronal cell bodies to axotomy consists of both metabolic (Brattgard *et al.*, 1957; Watson, 1965a, 1968a) and morphological changes (Lieberman, 1971; Grafstein, 1975). On the basis of response latency the metabolic alterations are considered to be primary effects of nerve injury (Cerf & Chacko, 1958; Torvik and Heding, 1969). Structural changes are extremely variable, depending upon the experimental model (Lieberman, 1974; Torvik, 1976), and are considered secondary phenomena.

At the light microscope level, where demonstrated, the most common changes in axotomized neurones include displacement of the nucleus to the periphery of the cell body, dispersal of the stacks of basophillic Nissl substance (rough endoplasmic reticulum) such that staining is dustlike or particulate in appearance, and swelling of the cell body (Lieberman, 1971). This structural arrangement is thought to demonstrate the reordering of synthetic priorities in the neurone to permit regeneration (Grafstein, 1975). The time of onset and magnitude of these effects has been found to be dependent upon the distance of the injury from the cell body (Humbertson, 1963; Watson, 1968a).

It was proposed (Chapter III) that the signal initiating the neuronal regenerative response may be a premature return of transported protein from the site of injury. The minimum velocity of this signal is 112-133mm per day, and would thus reach the L5 dorsal root ganglia in less than 13 hours, and the sciatic nerve motoneurone pool within 20 hours. An attempt was made to demonstrate that structural alterations in axotomized sciatic nerve motoneurones were not initiated prior to the calculated arrival time of the proposed injury signal.

2. MATERIALS & METHODS

A modification of Einarson's (1932) gallocyanin method for staining Nissl substance (Drury & Wallington, 1967, p. 280) was used in an attempt to demonstrate the earliest structural changes in motoneurone somata after sciatic nerve ligation.

The left sciatic nerve was permanently ligated in a group of rats at the position of the distal crush previously described, 90mm from the exit of the motoneurone ventral roots. Animals were re-anaesthetized 2-5 days later and perfused intracardially with physiological saline for 3-5 minutes followed by buffered 10% formalin for 5 minutes. The spinal cord was then exposed by dorsal laminectomy, and the spinal column from T9-L4 was removed and left in fixative for 24 hours. The length of spinal cord constituting the lumbar enlargement was then isolated and left in fixative a further 24 hours. The segment of spinal cord in which were located sciatic nerve motoneurones (Chapter II) was isolated, dehydrated in ½ hour steps in graded ethanol solutions and benzene, and embedded in liquid paraffin at 56°C.

Cross-sections of spinal cord were cut 10μM thick on an American Optical 820 microtome and transferred to a 30°C water bath. From there sections were placed on glycerin-albumin coated glass slides and dried on a slide warmer. Sections were then rehydrated and placed in gallocyanin stain (pH 2.0) at 60°C for 30-45 minutes. Stained sections were dehydrated and sealed under glass cover slips with Permount (Fisher). All photographed sections were subject to the same preparatory protocol.

Previous work has found that chromatolytic alterations begin 3-5 days after sciatic nerve axotomy (Bodian & Mellors, 1945; Engh *et al.*, 1971; Price & Porter, 1972), and my preliminary results as well demonstrated no morphological alterations up to 3 days after axotomy. Representative control and axotomized cells were photographed at 3, 4, and 5 days after axotomy. Between 20-30 cells were photographed at each interval. Equal numbers of photographs were taken of large ventral horn cells from control and axotomized sides of the spinal cord. Photomicrographs were taken on a Zeiss Photomicroscope using black and white Ilford Pan F 35mm film (ASA 50). The film was developed in Rodinal (Agfa-Gevaert), fixed in Kodak Rapid Fixer, and air dried. All solutions used for developing and printing were kept at 20°C. Using a Durst M600 enlarger, negatives were printed on Kodak F5 paper, with Kodak Dektol developer, Kodak Indicator Stop Bath, Kodak Rapid Fixer, and Kodak Hypo Clearing Agent, then dried on a Premier model A Roto Dryer.

3. RESULTS

Nerve ligation abolished the hindlimb placing reflex, which is

the normal response to touching the dorsal surface of the hind paw when the animal is suspended. A branch of the sciatic, the superficial peroneal nerve, mediates this response. Additionally, in motoneurone axons protein is not transported beyond a ligature (Fig. 7.1(d)). Ligation was therefore thought to produce axotomy of all sciatic nerve motoneurones.

i) Control Cells

The large ventral horn cells contralateral to the ligated sciatic nerve served as controls. These cell bodies had a diameter of 30-40 μm, and demonstrated a range of shapes, from circular to multipolar (Pl. 6.1-6.3). Nuclei were located centrally and contained centrally-located nucleoli. The darkly-staining Nissl substance occurred in clumps and could be found either perinuclearly (Pl. 6.3), evenly distributed throughout the cytoplasm (Pl. 6.1), or even peripherally in some cases (Pl. 6.2). The intracellular organization in uninjured cells is therefore quite variable.

ii) Axotomized Cells

3 Days Plate 6.4 shows several sciatic nerve motoneurones 3 days after nerve ligation. Nuclei are still centrally-located and the Nissl substance persists in globular form. Although the nucleolus appears displaced in one of the cells (arrow), this degree of eccentricity was sometimes also found in control cells. Overall the 3 day axotomized neurones resembled control cells.

4 Days At 4 days after axotomy most cell bodies were still comparable in appearance to control cells. Two cell bodies whose

Plate 6.1 Control sciatic motoneurones. Note centrally-located nuclei and nucleoli, and granular Nissl substance. Gallocyanin stain. 400x.

Plate 6.2 Control motoneurone. 400x.

Plate 6.3 Control motoneurone. 400x.

Plate 6.4 3 day axotomized sciatic motoneurones. Possible nuclear displacement (arrow). 400x.

Plate 6.5 4 day axotomized motoneurone. Possible nuclear displacement opposite axon hillock (arrow). 400x.

Plate 6.6 4 day axotomized motoneurones. Nuclear eccentricity and Nissl substance displaced peripherally. 400x.

Plate 6.7 5 day axotomized motoneurone.
400x.

intracellular appearance demonstrated the greatest departure from normal are shown in Plate 6.5 and 6.6. In Plate 6.5 the cell nucleus is located at the periphery of the soma, opposite what could be the axon hillock (arrow); the Nissl staining is unaltered. The nucleus is slightly eccentric in the cell shown in Plate 6.6 (arrow), but here the clumps of Nissl substance are all located peripherally. Staining of the intervening cytoplasm is more diffuse, appearing granular or particulate. The structural alterations in these cells come closest to matching the classically-defined changes found in cells undergoing chromatolysis (Lieberman, 1971). However, it should once again be mentioned that these changes were found in less than 20% of the photographed cells.

5 Days Again at 5 days most photographed cells resembled control cells. Plate 6.7 shows a cell that could be undergoing chromatolysis. The cell cytoplasm is stained only diffusely by the Nissl stain, suggesting the disaggregation of rough endoplasmic reticulum found during chromatolysis.

4. DISCUSSION

The gallocyanin staining technique used here is considered to allow a highly selective demonstration of neuronal Nissl substance (Einarson, 1932), and is thought to owe its specificity for nucleic acids to use at a pH below the isoelectric point of most tissue proteins (Gabe, 1976, p. 541). The scant staining of the neuropil in the accompanying photographs attests to the stain's selectivity.

The neurones photographed were large diameter cells (30-40μM)

located in the ventrolateral and dorsolateral ventral horn of the L4 and L5 segments of the rat spinal cord. These cells have been found to constitute the motoneurone pool of the sciatic nerve (Bisby, 1975). A similar location in the spinal cord has been found for sciatic nerve motoneurones both in the cat (Romanes, 1964) and in man (Kappers *et al.*, 1960, p. 228-30).

Those structural changes found in motoneurones after sciatic nerve ligation were considered to demonstrate an axotomy response, since neurones have long been known to respond to axonal injury by undergoing alterations in the arrangement of cell body organelles (Nissl, 1892, cited in Lieberman, 1971). The alterations first found 4 days after axotomy consisted of movement of the cell nucleus away from the cytocentrum and dissolution of the clumps of Nissl substance. Structural changes were found in less than 20% of all cells photographed. Using the same model Engh *et al.*, (1971) reported that a maximum of 25% of ventral horn cells showed chromatolysis after mid-sciatic nerve section. Both sets of results are consistent with the popularly held view that rodent neurones generally are refractory to chromatolysis (Romanes, 1964; Lieberman, 1974).

In fact, the occurance of chromatolysis in any neurone depends upon many factors. Lieberman (1974) notes that its manifestation varies in different cell groups of the same animal, in the same cell group in different animals, and the same cell group in the same animal at different ages. Chromatolysis cannot therefore be considered a sine qua non of axotomized neurones.

The purpose of this study was to show that the onset of chromatolysis

in rat sciatic motoneurones did not preceed the arrival of the axotomy signal proposed in Chapter III. Since this signal would have reached the ventral horn cells within 20 hours of nerve ligation, the observation of chromatolytic changes at 4 days after axotomy does not contradict the proposed function of prematurely-returned protein. Although not an exhaustive study, the results presented probably reflect the secondary nature of the relationship between axotomy and chromatolysis.

The latter is known to be preceeded by, and perhaps dependent upon, prior biochemical changes. Cerf & Chacko (1958) found increased acid phosphatase activity in the dendrites of frog motoneurones 20 hours after ventral root section; these cells do not undergo chromatolysis (Edstrom, 1959). Torvik & Heding (1969) prevented chromatolysis in neurones of the mouse facial nucleus by inhibition of RNA synthesis 9 hours after axotomy. More recently, Dziegliewski *et al.*, (1976) have found increased amounts of phospholipids transported in rat sciatic motor axons 10 hours after axotomy. These examples assert that the nerve cell body is capable of rapid metabolic response to a rapidly-transported axotomy signal. Chromatolysis is therefore an indirect axotomy response, and is inadequate to identify the axotomy signal.

CHAPTER VII

TRANSPORT IN REGENERATING & NON-REGENERATING NEURONES

1. INTRODUCTION

The drastic metabolic changes found in the injured neuronal cell body (Brattgard *et al.*, 1957; Watson, 1965a, 1968a; Harkonen & Kauffman, 1973) are thought to represent the reorganization of synthetic priorities to permit regeneration (Grafstein, 1975). Injured neurones reduce production of neurotransmitter-specific enzymes (Koenig & Droz, 1971; Cheah & Geffen, 1973; Ross *et al.*, 1975) while simultaneously increasing RNA and protein synthesis (Watson, 1965a, 1968a; Harkonen & Kauffman, 1973; Kaye *et al.*, 1977). Ultrastructurally these priority changes may be reflected in transformations undergone by organelles located in the cell body that are involved in the synthesis of exported material (Murray & Forman, 1971; Price & Porter, 1972). Evidence demonstrating the manner in which the cell body response to axotomy is manifested in the delivery of axonally transported material, however, is contradictory.

The signal initiating the regenerative response may be the premature return of newly-synthesized protein from the site of nerve injury (Chapter III). This abnormal return could be either an acute axotomy response or a maintained phenomenon. A chronic abnormal return of protein could serve to continuously inform the injured cell body of the extent of missing axoplasm and thus perhaps maintain

the appropriate metabolic priorities throughout the course of regeneration. It has been suggested, however, that the neuronal regenerative response is a stereotyped reaction that proceeds oblivious to peripheral feedback (Grafstein, 1975). The duration of the postulated axotomy signal was therefore investigated.

2. MATERIALS & METHODS

At that location on the sciatic nerve used as the distal crush site, 90mm from the origin of the ventral roots (Chapter III), the nerve was crushed by ligation. Chronic ligation prevented nerve regeneration, while regeneration occurred when the ligature was loosened immediately after nerve crush. At 1, 2, 5, 10, 30, or 50 days after axotomy protein transported in sciatic nerve motoneurones was labelled as previously described.

In one series of experiments transport of labelled protein in regenerating and non-regenerating nerves was determined by calculating the relative accumulation rate of activity adjacent to collection crushes applied 65 and 75mm from the exit of sciatic ventral roots from the spinal cord at 9-11 hours after precursor injection (p.i.). This time interval corresponds to the period of peak retrograde transport of activity returned from a 0 hour ligature in motor axons (Chapter III).

In another series, involving regenerating nerves, changes in the time at which peak retrograde transport occurred was determined in nerves of increasing length. Collection crushes were applied as above for 2 or 3 hours at times from 7-23 hours p.i. in nerves

which had regenerated for 5, 10 or 30 days. The dynamics of transport over a range of times after precursor injection were thus determined in regenerating nerves, as had been determined for intact nerves in Chapter III.

Thirdly, incorporation of precursor into labelled protein by regenerating nerve was compared to incorporation by intact nerve. In a group of rats the sciatic nerve was crushed unilaterally and allowed to regenerate for 5, 10, 20, 30 or 50 days. Precursor was then injected bilaterally into the vicinity of sciatic motoneurone cell bodies. Collection crushes were made 65 and 75mm from the exit of sciatic ventral roots from the cord on both intact and regenerated nerves from 9-11 hours p.i., when animals were killed. For each regenerating nerve, activity per 2mm in a 6-8mm length of regenerated nerve was calculated as a ratio of activity per 2mm of isolated segment. The same ratio was calculated for the contralateral intact nerve to control for the proximo-distal gradient of axonal labelling (Bisby, 1977b). The ratio of labelled protein incorporation by regenerated and intact segments of regenerating nerves was then expressed as a percentage of the ratio found in intact nerves.

3. RESULTS

i) Behavioral

Loss of the placing reflex in the rat hindlimb was used as an index of axon interruption. This reflex is a normal response to touching the dorsal surface of the hindpaw when the animal is suspended, and is mediated by the sensory superficial peroneal nerve, a branch of the sciatic. Loss of the reflex was correlated with complete nerve

ligation by evidence demonstrating the absence of transported protein distal to the ligature in motoneurone axons. Reappearance of the reflex was observed by 30 days after the original injury in nerves allowed to regenerate. When regeneration was prevented by permanent nerve ligation, the reflex had not reappeared by 50 days after injury.

ii) Anatomical

Regeneration was further tested by the pinch-reflex test (Gutmann *et al.*, 1942; Konorski & Lubinska, 1945), which involves pinching the regenerating nerve in the lightly anaesthetized animal with fine forceps from its distal extremity proximally until a jump response is elicited. This technique relies on the high sensitivity of regenerating axons to mechanical disturbances (Lubinska, 1964). Regenerated axons are considered to extend to the point at which a response is first elicited.

In nerves permitted to regenerate this method found some axons to have proceeded to a point 2 or 3mm distal to the injury site by the second day after injury. By 5 days regeneration of the most rapidly-elongating axons extended past the point where branches of the sciatic entered the lower leg. This is consistent with previous work on rat sciatic nerve (Konorski & Lubinska, 1945; Bisby, unpublished observations) showing a 1-2 day latent period prior to the start of regeneration, which then proceeded at 3-4mm/day. At this rate (if all axons regenerated at the same rate) complete regeneration to their original terminations was estimated to require about 10-14 days.

In non-regenerating nerves a bulge developed proximal to the ligature within one day of injury. Distal to the ligature the degenerating

nerve gradually diminished in diameter and by 30 days consisted only of connective tissue. This distal stump usually separated from the nerve proximal to the ligature during removal. After 30 days some regeneration was observed to occur in all "non-regenerating" nerves, probably due to loosening of the ligature. For this reason transport in non-regenerating nerves could not be evaluated 50 days after injury.

iii) Protein Transport

As regeneration proceeded transported protein was found at increasing distances distal to the crush site. Figures 7.1(a)-(c) show the distribution of transported activity adjacent to a nerve crush 9-11 hours p.i. at 1, 2, and 5 days after injury. At 1 day most activity accumulated proximal to the crush site (Fig. 7.1(a)). Activity present distally is derived from limited regeneration and uptake by nerve sheath cells of blood-borne activity. The latter are very active metabolically after nerve injury (Altman & Das, 1964). Regeneration extends further at 2 days (Fig. 7.1(b)), although most transported activity still accumulated proximal to the crush site. By 5 days transport was only minimally blocked at the crush site (Fig. 7.1(c)), and most of the transported activity was found in the regenerating segment of nerve.

Transported activity was not found beyond the ligature in non-regenerating nerves (Fig. 7.1(d)).

Figure 7.2 shows 9-11 hours p.i. orthograde transport in regenerating and non-regenerating nerves at various times after nerve crush as reflected in the accumulation rate of labelled protein (see Chapter II 5.(iii)). Orthograde transport in intact nerves is represented by

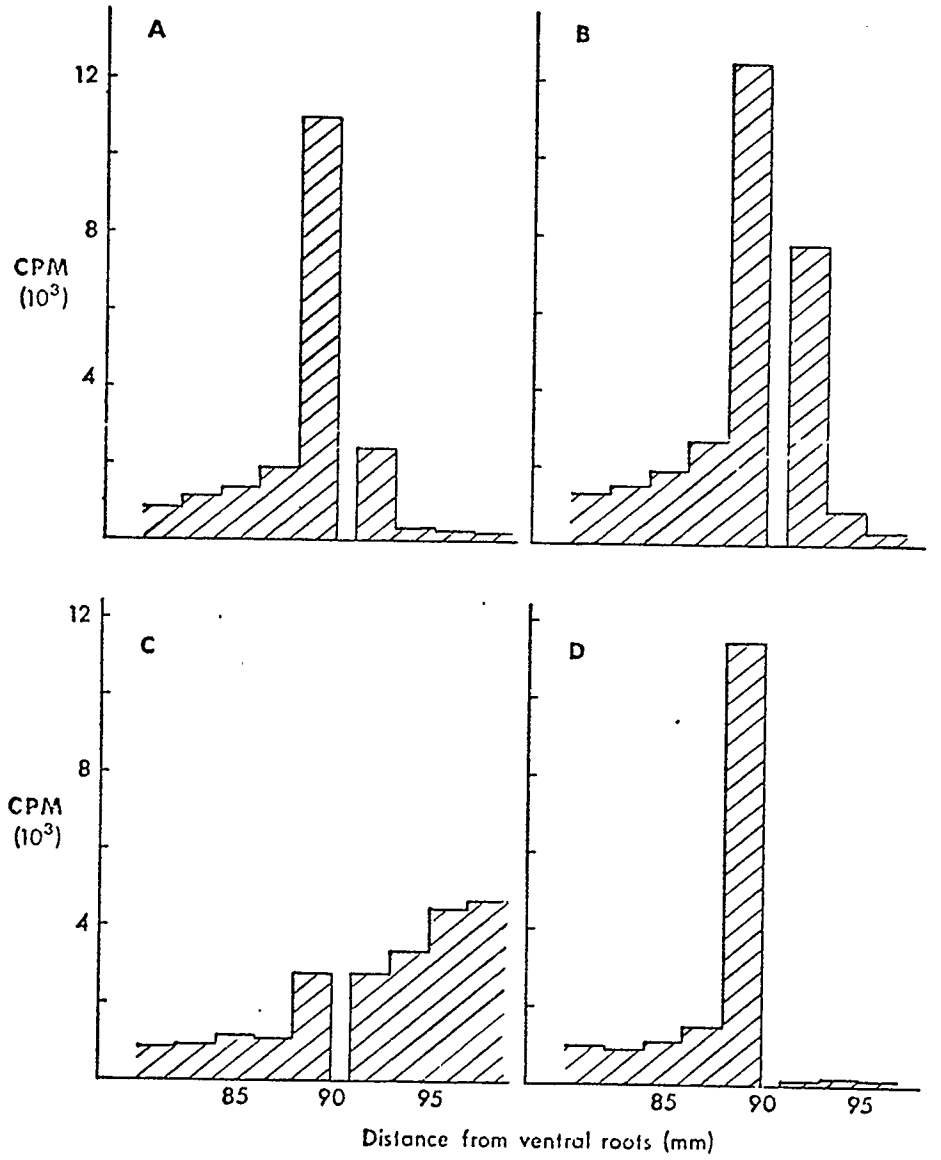

Figure 7.1 Transported protein distribution in regenerating and non-regenerating motor axons. Vertical axis: TCA-insoluble activity per 2mm nerve segment. Horizontal axis: distance from sciatic ventral roots. Space at 90mm represents original axotomy site. All nerves show protein distribution 11 hours after precursor injection. A. 1 day regenerating nerve. B. 2 day regenerating nerve. C. 5 day regenerating nerve. D. non-regenerating nerve 2 days after axotomy.

line C. Within 1 day of injury nerves show a sharp decrease in protein transport. This reduced level of transport is maintained in non-regenerating nerves (B) up to 30 days afterwards. In regenerating nerves (A), however, there is a recovery in orthograde transport, with return to values found in intact nerves by 30 days after injury. It should be noted that the most rapid increases in transport occur in the first 10 days after axotomy, during which some axons could have regenerated to their original length.

Retrograde transport in regenerating and non-regenerating nerves at 9-11 hours p.i. is similarly shown in Figure 7.3. Here line C represents retrograde transport in intact nerves at 9-11 hours. In both regenerating (A) and non-regenerating (B) nerves retrograde transport increases for the first 5 days after axotomy. Transport in non-regenerating nerves then decreases to and remains at the level found in newly-injured nerves for at least another 20 days. Transport decreases in regenerating nerves for 5-10 days and returns to levels found in intact nerves by 30 days.

The decline in retrograde transport to intact levels in regenerating nerves could be either real or apparent. On the one hand a regenerating nerve might incorporate more of the transported protein delivered to it and thus return less to the soma. On the other, as the decrease occurs in the period when most of the length of nerve would have regenerated, it could reflect purely a distance effect (i.e. as the nerve lengthened protein would have to travel further down the nerve before reversing direction and returning, so that the peak of retrograde transport would occur later than the 9-11 hour time interval). The time course of retrograde transport was therefore investigated in

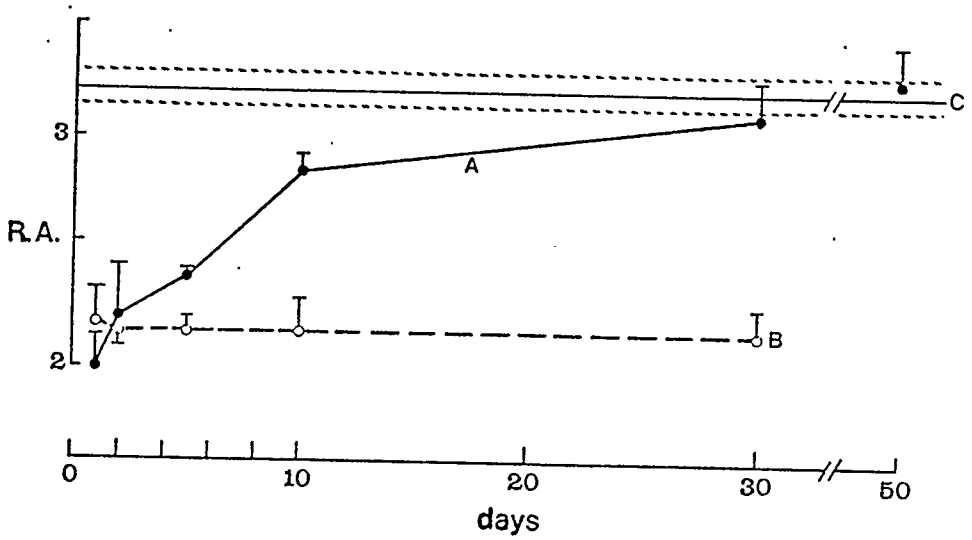

Figure 7.2 Orthograde transport in chronically-injured motor axons. Vertical axis: relative accumulation. Horizontal axis: time in days since axotomy. Data points show orthograde transport in regenerating (A), non-regenerating (B), and intact (C) motor axons 9-11 hours after precursor injection. Each point represents mean ± S.E.M. for at least 5 nerves.

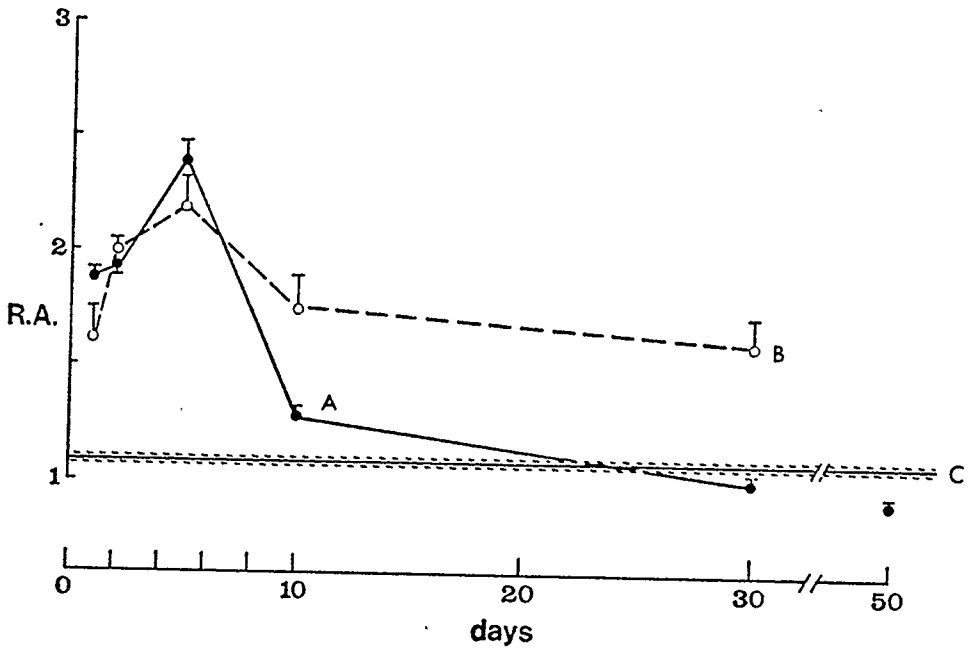

Figure 7.3 Retrograde transport in chronically-injured motor axons. Legend same as Fig. 7.2 for regenerating (A), non-regenerating (B), and intact (C) motor axons.

nerves allowed to regenerate for 5, 10 and 30 days (Fig. 7.4). The peak of retrograde transport at 5 days is at the same position in time as that in a nerve injured at the time of precursor injection but is increased in magnitude, as previously seen in Figure 7.3. By 10 days the peak magnitude has returned to the level found in newly-injured nerves but occurs 3 hours later. Some regenerated axons should have by this time attained their original length. At 30 days the peak is shifted still further to 18 hours p.i. It is diminished in magnitude, but otherwise resembles retrograde transport in intact nerves. The decrease in retrograde transport in regenerating nerves at 9-11 hours (Fig. 7.3) is thus only apparent, and indicates the increasing distance travelled by transported protein in regenerating nerves. The resemblance between retrograde transport curves obtained from 30 day regenerating and intact nerves may demonstrate the complete regeneration of the majority of injured motor axons. This time was coincident with the return of the placing reflex.

Figure 7.5 demonstrates the relative precursor incorporation levels in the regenerated and intact portions of regenerating sciatic nerve. Calculation of these values has corrected for the proximo-distal gradient of transported activity (Bisby, 1977b). After 5 days of regeneration there is 8-fold greater labelling of the regenerated segment of nerve. At 10 days activity in regenerated nerve segments is still 5 times greater than in intact segments of the same nerve. This magnitude of difference in incorporation is maintained until at least 50 days after nerve crush. However, comparison of levels of labelled protein in different portions of nerve may not accurately reflect differences in incorporation of transported activity. Since

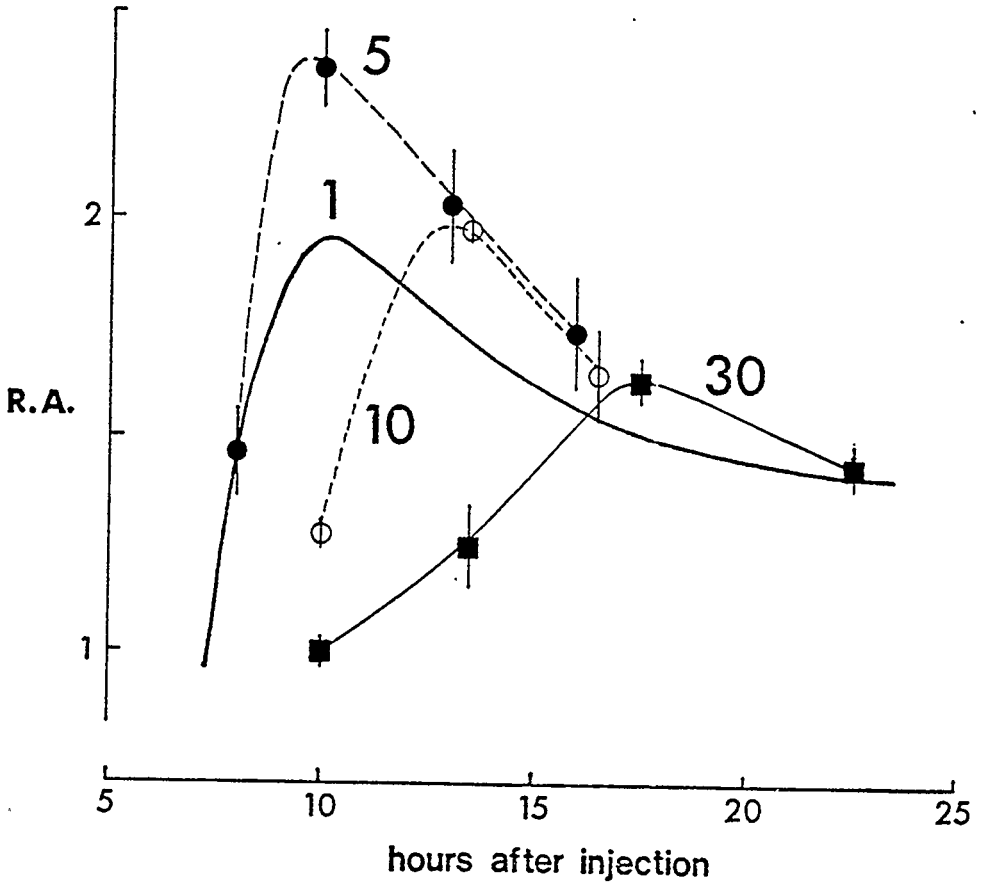

Figure 7.4 Retrograde transport in injured motor axons at different stages of regeneration. Vertical axis: relative accumulation. Horizontal axis: time in hours since precursor injection. 1 day curve same as Fig. 3.1(B). Each point represents mean ± S.E.M. for at least 6 nerves.

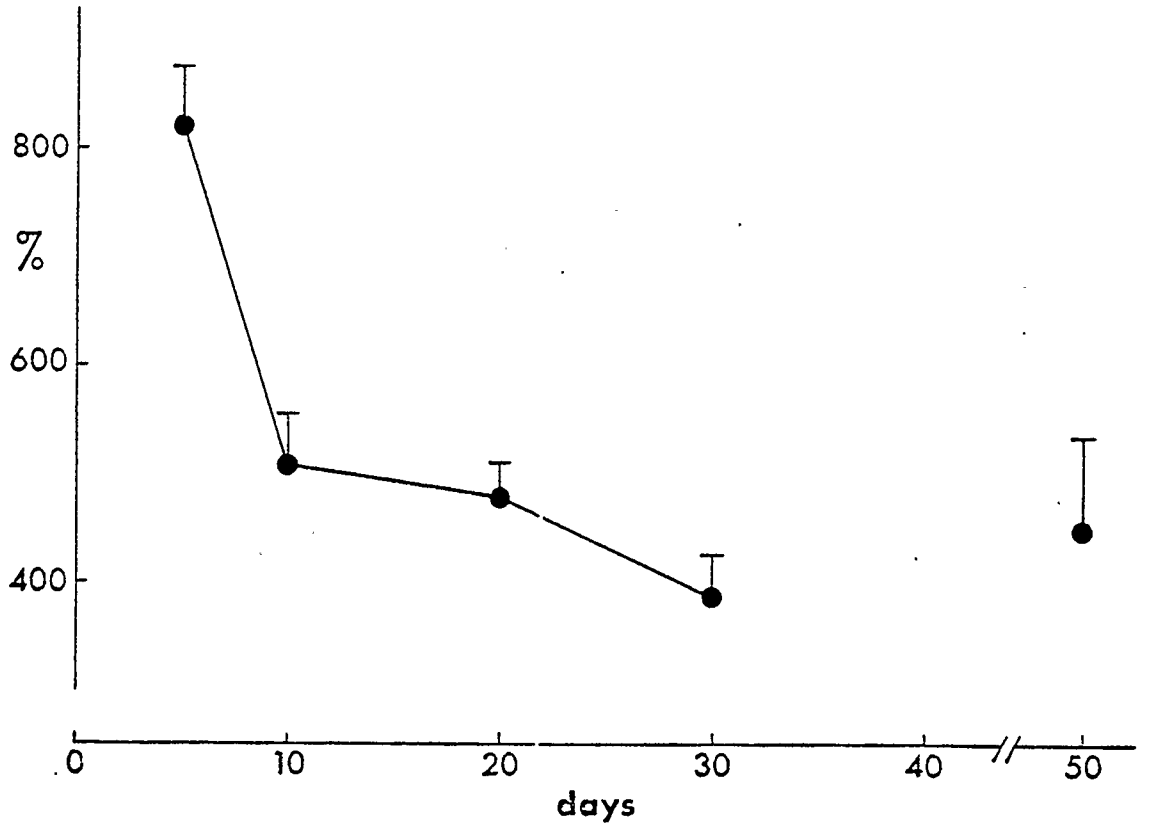

Figure 7.5 Relative incorporation of precursor into proteins of regenerated and intact portions of regenerating motor axons. Vertical axis: activity ratio of regenerated/intact portions of regenerating nerves expressed as a percentage of the distal/proximal activity ratio found in contralateral intact nerves (see text). Horizontal axis: time in days since axotomy.

in regenerating nerves Schwann cells increase in number (Thomas, 1970) and continue to elaborate myelin for several months (Reier & Webster, 1974), their uptake and synthesis of blood-borne precursor probably accounts for some of the noted difference in activity.

4. DISCUSSION

Fast-transported protein is incorporated into both terminal structures (Hendrickson, 1972; Droz *et al.*, 1973, 1975) and axolemmal membranes along the entire axon length in intact nerves (Droz *et al.*, 1973; Gross & Beidler, 1975) and has been proposed to contribute to the axolemma in growth cones of developing nerves (Bray, 1973; Bunge, 1973). Numerous investigators have studied changes in the axonal transport of materials synthesized by nerve cells responding to peripheral injury, but the many experimental models and techniques used to evaluate it have obscured its role in nerve regeneration.

Axotomy is followed after several days by a regenerative metabolic response characterized by increased cell body synthesis of RNA (Watson, 1965a, 1968a; Lambert & Daneholt, 1968; Kaye *et al.*, 1977) and protein (Miani *et al.*, 1961; Francoeur & Olszewski, 1968; Murray & Grafstein, 1969). Within a few days of axotomy the increase in protein content (Brattgard *et al.*, 1957; Harkonen & Kauffman, 1973) is followed by an increased rate of protein loss from the cell body (Murray & Grafstein, 1969; Koenig & Droz, 1971). This is presumably due to alterations in the orthograde transport of newly-synthesized protein and could be achieved by increases in the velocity of transport and/or in the amount transported per unit length of the transport system. The latter

could occur since it is likely that transport of some materials is reduced during regeneration, thus making the transport mechanism capable of carrying increased amounts of other material. For example, the magnitude of accumulated noradrenaline proximal to a ligature decreases in regenerating sympathetic axons of the sciatic nerve (Karlstrom & Dahlstrom, 1973). This is likely to be characteristic of all transported materials related to neurotransmission, given evidence of reduced cell body levels of enzymes involved in catecholamine (Eranko & Harkonen, 1965; Cheah & Geffen, 1973; Ross *et al.*, 1975) and acetylcholine (Eranko & Harkonen, 1965; Koenig & Droz, 1971) synthesis after axotomy.

Changes found in axonal transport during regeneration may include alterations both in amounts transported and in velocity of transport, although most investigations do not evaluate both types of alteration. Jablecki & Brimijoin (1975) found that a constant choline acetyltransferase (ChAc) accumulation rate was maintained in regenerating mouse sciatic nerve by an increase in transport velocity simultaneous with reduced axonal ChAc content. These factors were not considered by Frizell & Sjostrand (1974a), who found reduced acetylcholinesterase and ChAc accumulation after rabbit hypoglossal nerve ligation.

Labelled protein transport has also been extensively studied in regenerating nerves. Regenerating goldfish optic nerve axons transport increased amounts of protein at increased velocity in both fast and slow phases of transport (Grafstein & Murray, 1969). Frizell and Sjostrand (1974a) have found, in regenerating rabbit hypoglossal nerve, increased accumulation at a ligature of fast-transported ^{3}H-fucose labelled glycoproteins accompanied by decreased ^{3}H-leucine

labelled protein accumulation 1 week after axotomy. At 4 weeks accumulation of both types of protein was greater than normal. This was interpreted to represent the reordering of axonal transport priorities during regeneration, although it has not been demonstrated that glycoproteins are preferentially synthesized during the initial stages of regeneration. In the same model Frizell & Sjostrand (1974c) found an increase in the velocity of slow-transported protein. More recently Griffin *et al.* (1976) have shown increased amounts of fast-transported protein in regenerating motoneurone axons of the rat sciatic nerve. Conversely, using regenerating sensory axons of rat sciatic nerve, Bisby (unpublished observations) has found a decrease in the amount of fast-transported protein carried by the orthograde wavefront in the first 7 days following axotomy. Ochs (1976) has found no change either in the velocity of the orthograde wavefront in regenerating sensory axons of the cat sciatic nerve, in agreement with the previous two studies, or in the amount of protein transported. These last contradictions may even suggest that fast-transported protein has no role in nerve regeneration, this process instead of being mediated by more slowly transported materials. A number of major axonal constituents are delivered at slow transport velocities (Hoffman & Lasek, 1975).

The results presented here extend the study of the response of the axonal transport system to nerve injury. Whereas earlier chapters predicted an effect of protein transport on the nerve cell body the present one reveals how regenerative metabolic changes in the cell body are manifested in the axonal transport of newly-synthesized protein. Transport was determined by short term accumulation of

labelled protein adjacent to ligatures tied on the sciatic nerve. Calculating accumulation relative to a measure of background activity in the nerve controls for variations in protein transport from nerve to nerve. In injured nerves this variability would be produced not only by inconsistencies of precursor injection, but also by changes in incorporation rate (i.e. higher specific activity of transported activity, as found by Murray & Grafstein (1969)), and variability in the numbers of neurones surviving axotomy (Lieberman, 1971). Quantification of transport in accumulation units reveals axonal transport in terms of the proportion of fast-transported protein that is in transit at a specific location on the nerve. Thus the changes in labelled protein accumulation at 9-11 hours p.i. seen after acute or chronic nerve ligation represent alterations in the "mobile fraction" of fast-transported protein in the axons. This assumes that transport velocity does not change, as found by Griffin *et al.*, (1976) and Ochs (1976).

By determining protein accumulation over the same time interval after precursor injection in nerves allowed to regenerate for increasing lengths of time, changes in transport could be related to the process of nerve elongation, the characteristics of which are well established (Lubinska, 1964).

A biphasic change in retrograde transport was observed in injured nerves (Fig. 7.3). Initially there is an increase in the amount of returning material, simultaneous with the early stages of regeneration. This increase is also seen in Figure 7.4 as an increase in the peak magnitude of early-returning protein in 5 day regenerating nerves. Changes in retrograde transport at 5 days could result from the maturation of the transport reversal process at the nerve ending.

During the first few days after axotomy there is a transformation in the regenerating stump of nerve from a seemingly random aggregation of an abnormally high concentration of organelles to the more organized state characteristic of a regenerating growth cone (Blumke *et al.*, 1966; Morris *et al.*, 1972). This could demonstrate the development of a more efficient reversal mechanism at the proximal nerve stump.

Retrograde transport at 9-11 hours p.i. decreased after 5 days and in regenerating nerves reached intact levels by 30 days after axotomy. This was due to a shift in the time of peak return as regeneration proceeded, and thus illustrates an important point. Firstly, comparison of the magnitude of retrograde transport in nerves allowed to regenerate for different intervals must take into account differences in distance travelled by transported materials. Frizell *et al.* (1976) noted differences in retrograde-transported accumulation of labelled proteins and glycoproteins 16-22 hours after precursor injection in rabbit hypoglossal nerves which had regenerated for 1 and 6 weeks without considering the effect of axon length on transit times. Implicit in this is the recognition that the advancing growth cone functions as a major point of protein reversal in regenerating nerves. Clearly, although in an intact nerve much of the fast-transported protein is incorporated into the axon itself (Cancalon & Beidler, 1975; Ochs, 1975), the time of peak protein return in a regenerating nerve appears to depend upon the length of the regenerated segment. By 30 days the peak of the retrograde transport curve is somewhat flattened, similar to that found in intact nerves (Fig. 3.2). This could demonstrate a return to a situation in which returning protein may reverse at points all along the length of the axon, after disappearance of the

nerve growth cones.

The changes in orthograde transport at 9-11 hours p.i. in injured nerves are as sudden and drastic as they are in retrograde transport (Fig. 7.2). There is a greater than 30% decrease in the amount of accumulating protein 1 day after axotomy. This immediacy of response has previously been found in rat sciatic nerve motoneurones with respect to phospholipids (Dziegliewski *et al.*, 1976), but not labelled protein. Also, as found for retrograde transport, later changes are dependent upon the length of regenerated nerve. Transport into non-regenerating nerves remains at a depressed level for at least 30 days. In regenerating nerves the amount of protein accumulating at 9-11 hours p.i. increased as the nerve lengthened, reflecting progressive increases in the proportion of mobile protein. Changes in the axons length-dependent metabolic requirements due to axotomy and regeneration therefore appear to be answered by an appropriate alteration in the mobile fraction of fast-transported protein. Less protein is thus transported beyond the collection crushes in shorter, recently-injured nerves then in regenerating or intact nerves. As mentioned before these changes probably do not result from transport velocity changes (Griffin *et al.*, 1976; Ochs, 1976).

It is impossible to tell from the reported data whether changes observed in the mobile fraction of transported protein represent alterations in the magnitude of protein transported in regenerating nerves. In view of the biochemical and autoradiographic evidence of altered protein synthesis (Miani *et al.*, 1961; Murray and Grafstein, 1969) and export (Murray & Grafstein, 1969; Koenig & Droz, 1971) during regeneration, however, this relationship is strongly suggested.

The demonstration here of high levels of protein incorporation into regenerated nerve segments (Fig. 7.5) adds to the evidence asserting a role for fast-transported material in the process of nerve regeneration (Frizell & Sjostrand, 1974a; Griffin *et al.*, 1976). Although increases in the amount and velocity of slow-transported protein have been found in regenerating nerves (Grafstein & Murray, 1969; Frizell & Sjostrand, 1974c) and axonal constituents are known to be slow-transported (Hoffman & Lasek, 1975), integral axonal components must also be delivered to the nerve in the fast phase of transport. Such materials no doubt serve in the process of increasing axon diameter at up to 50 days after axotomy, and may continue to do so for many months (Gutmann & Sanders, 1943).

Immediately after axotomy, then, the nerve cell body probably decreases the amount of orthograde-transported protein, perhaps in response to the abnormal high return from the injury site. Increased protein synthesis begins in a matter of days and resultant increases in protein transport produce the gradual recovery of orthograde accumulation back to normal levels in regenerating nerves. The latter finding, along with the contrasting maintained low level of orthograde transport in non-regenerating nerves, implies that the synthetic machinery of the cell body is aware of and responsive to changes in axonal length: a non-regenerating nerve is sustained by a constant level of protein transport, but increasing amounts of transported material are required by elongating nerves. As argued in previous chapters the cell body may be continuously informed of the metabolic requirements of its axon by signals transmitted along the retrograde transport system. In regenerating nerves such signals could be the changing intervals

between transport out of the cell body of newly-synthesized protein and peak return as axon length increases, the decrease in amounts of material returning as more is incorporated into regenerated axon, or, as Chapter IV suggested, changes in the composition of returning protein. In any case it is to intracellular cues that a regenerating neurone seems to respond.

Although it has been suggested that nerve regeneration is a programmed response (Grafstein, 1975), it was shown that after probably triggering the regenerative response (Chapter III), retrograde-transport mediated feedback may also regulate the course of regenerative metabolic alterations.

CHAPTER VIII

CONCLUSIONS

The preceeding research tested the hypothesis that retrograde axonal transport functions as part of a feedback loop, signalling the metabolic requirements of the axon to the synthetic apparatus in the cell body. Experiments were divided into two groups: i) those investigating the acute response of labelled protein transport following nerve interruption, and ii) those investigating long term changes in transport after axotomy.

Acute experiments revealed that retrograde transport of labelled protein differs in ligated and intact nerves, with a premature return of fast orthograde-transported protein occurring in the former. The changes in peripheral metabolic requirements after axotomy therefore are reflected in alterations of retrograde transport. Prematurely-returning protein is similar in electrophoretic characterization to fast orthograde-transported protein. The metabolic response of the neurone to axotomy may result from a negative effect on protein synthesis of the returning newly-synthesized protein. Premature return of labelled protein was also demonstrated after colchicine block of orthograde transport. Colchicine effects have been found to mimic the effects of axotomy (Pilar & Landmesser, 1972; Cull, 1975; Purves, 1976). In addition, a greater amount of labelled protein was returned after reversal at a more proximal injury ligature. If returning protein regulates neuronal synthesis, a greater metabolic effect would probably be produced by greater amounts of returning protein. This could account

for the severity of the chromatolytic response being dependent upon the distance between an injury and the nerve cell body. The attempt made to relate the latency of chromatolysis after axotomy to the return of protein reversed at a ligature proved inconclusive.

Chronic experiments afforded a demonstration of the metabolic response of motoneurones to temporary and permanent changes in axonal length. Labelled protein transport was determined at 9-11 hours p.i. in regenerating and non-regenerating nerves in order to estimate the duration of the premature return of protein, the postulated axotomy signal. Orthograde transport was therefore also determined in that interval. Changes in the mobile fraction of orthograde-transported protein after axotomy were interpreted to reflect changes in the amount of orthograde-transported protein at that time interval. Initial decreases therefore appeared to demonstrate decreased synthesis of orthograde-transported protein in response to nerve shortening. A reduced magnitude of orthograde transport was maintained in non-regenerating nerves, while it gradually recovered in regenerating nerves, and equalled that found in intact nerves at the time of functional reinnervation.

Orthograde transport's length-dependent magnitude, if it reflects cell body synthesis levels, supports the hypothesis that retrograde transport mediates the regulation of nerve cell body metabolism by the axon. This is further suggested by observing the reciprocal changes in orthograde and retrograde transport during regeneration (Figs. 7.2 and 7.3).

At present the problem exists of not knowing the distribution within the fast phase of orthograde transport of labelled macromolecules

with different axonal destinations. This problem is partly due to the inadequacies of electrophoretic resolution. Studies of orthograde-transported proteins in regenerating nerves are therefore unable to relate changes in magnitude to altered transport of specific macromolecules. It might therefore prove worthwhile to investigate protein transport over a range of accumulation intervals after precursor injection in regenerating nerves. That is, to reproduce Figure 7.2 for intervals before and after 9-11 hours p.i. This could more precisely demonstrate major changes in orthograde transport magnitude during regeneration, and point to intervals when electrophoresis could reveal altered levels of specific polypeptides.

The preceeding discussion related observed dynamic properties of labelled protein transport to the hypothesis that retrograde transport functions as part of a feedback loop for metabolic regulation. Other observations were made which revealed characteristics of the mechanism of bidirectional axonal transport.

In nerves in which orthograde transport was blocked by ligation or colchicine the premature return of labelled protein was considered to result from random reversal following disruption of the normal axonal transport systems. A bulk turnaround of orthograde-transported material at the block was suggested by the similarity in return from ligated sensory axons and from motor axons ligated at different distances from the spinal cord, and by the similarity in electrophoretic profiles of prematurely-returned and fast orthograde-transported proteins. It would be logical to assume that "returning" material and organelles are somehow recognized as such, and incorporated by the retrograde transport system in the intact axon. How this is accomplished is

not known. The seemingly random reversal at an injury perhaps results from transformations of orthograde-transported material, causing it to be recognized as "returning". The presence of large numbers of lysosomal bodies proximal to an axonal injury (Holtzman and Novikoff, 1965) may be involved in this transformation. Obviously a closer morphological analysis of retrograde-transported organelles in intact and injured axons is called for.

Axonal transport's mysterious connotations derive in part from its being considered an exclusively neuronal phenomenon. An attempt to integrate axonal transport with other intracellular transport phenomena might dispel some of the mystery, and as well prove useful in suggesting new ways of investigating its mechanism. For instance, it has been proposed (DeDuve, 1969) that all cellular organelles may be classified into two groups: i) those having as their origin cellular synthetic mechanisms (eg. endoplasmic reticulum), and ii) those originating in phagocytic mechanisms (eg. pinocytotic vesicles). Axonal transport might therefore be simply an adaptation for maintaining normal cellular synthetic and phagocytic functions across the length of an axon. Conceptually, then orthograde-transported material is seen to originate from cell body synthetic activity, and retrograde-transported material results from phagocytic or recycling functions. Yamamoto (1963) has found that differences in the thickness of organelle membranes in frog sympathetic ganglion cells permit their separation into groups corresponding to a division into synthetic and phagocytic organelles. Further experiments using electron microscopic techniques could attempt to relate the direction of transport of axonal organelles to their membrane thickness. Optimistically, cell fractionation

techniques might then separate orthograde- and retrograde-transported organelles, and perhaps isolate membrane components involved in determination of the direction of transport.

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