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RETROGRADE AXONAL TRANSPORT AS AN INFORMATIONAL SYSTEM: A STUDY OF CHANGES IN THE TRANSPORT OF ENDOGENOUS MATERIALS FOLLOWING NERVE INJURY.

by

Catherine A. Leonard

A thesis submitted to the Faculty of Graduate Studies
in partial fulfillment of the requirements for the degree
of Master of Science.

Department of Medical Science.

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled, "Retrograde axonal transport as an informational system: A study of changes in the transport of endogenous materials following nerve injury" submitted by Catherine A. Leonard in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

Mammalian peripheral neurons respond to axotomy by initiating a series of morphological and biochemical changes which allow regeneration of the axon. The intracellular events which trigger this response are still unknown.

Experiments were performed to study the role of retrograde axonal transport in the events occurring after injury to the rat sciatic nerve. I have demonstrated quantitative changes in the retrograde transport of acetylcholinesterase, substance P and the muscarinic cholinergic receptor; the retrograde transport of these three molecules is affecte differently by axotomy, and the response of individual substances is likely to be a result of a combination of factors such as the amount of its anterograde transport and the organelle with which it is associated.

I have attempted to characterise the nature of the dignal for chromatolysis by looking for an early increase in the activity of transglutaminase in the dorsal root ganglion, as an indicator of the cell body response to axotomy. No such increase was detected up to 26h after nerve crush, and so it was not possible to give a conclusive answer to this question.

A more general analysis of axonally transported proteins in the rat sciatic nerve by two-dimensional gel electrophoresis was performed. Anterogradely transported proteins in the intact sciatic nerve are

moved along sensory axons within certain velocity ranges; molecules with a high charge variability such as glycoproteins appear to be preferentially transported at higher rates. These glycoproteins are not deposited in the axon or retrogradely transported. The components of anterograde and retrograde transport which travel more slowly in intact nerves are largely similar.

After an acute nerve injury, changes in the protein composition of both anterograde and retrograde transport fractions are induced. A very rapid increase in the anterograde transport of the growth-associated protein GAP43 was measured, occurring by 16h after axotomy. Further changes were seen after 7 days of regeneration.

Qualitative changes in retrograde transport were also induced by injury to the nerve. Three proteins were greatly reduced in the retrograde fraction after injury, and reappeared in small amounts after 7 days of regeneration. Three other proteins were seen to transiently increase their retrograde transport after injury.

It seems that peripheral neurons can respond to axotomy with changes in protein synthesis much more rapidly than has been previously documented. Injury to the nerve induces both transient and persistent changes in retrograde axonally transported proteins, and it is probable that such changes can be recognised by the cell body. The exact nature of the "signal for chromatolysis has not been determined, but changes in retrograde axonal transport probably play a role in monitoring of axonal status by the cell body.

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TABLE OF CONTENTS.

LIST OF FIGURES

INTRODUCTION

The neuron is a cell with unique morphology. Its processes extend over distances many times the diameter of the cell body; for example, a typical mammalian motoneuron cell body is 50 m in diameter, while the axon may be up to 1m long in large mammals. This means that intracellular components, which are made only in the cell soma, must be moved great distances from their site of synthesis into the axon and dendrites. Neurons have overcome this problem by extending the specialised transport system existing in all cells so that cellular components can be moved rapidly over relatively large distances. This is known as axoplasmic or axonal transport. Transport of a similar nature probably occurs in dendrites, but this is less well characterised (Lynch et al 1975). Axonal transport moves cellular material in both somatofugal (anterograde) and somatopetal (retrograde) directions.

Anterograde axonal transport was first demonstrated by Weiss and Hiscoe (1948), in experiments where a constriction was placed on a peripheral nerve. The nerve was seen to swell at the constriction, and when it was removed, accumulated material was seen to move along the axons at a rate of 1-2mm/day.

Transport was later studied by injection of various radiolabelled precursors into nerve cell bodies. Studies of 32P transport in the ventral roots of the cat showed movement of the label along the axons

at 4-5mm/day (Ochs and Burger 1958; Ochs et al 1962). Miani (1962) injected 32P and 14C amino acids into the fourth ventricle of the brain to study axonal transport in rabbit vagus and hypoglossal nerves. Radiolabelled amino acids were used by Droz and Leblond (1963) and by Koenig (1958) to demonstrate axonal transport of protein in rat sciatic and trigeminal nerves, and cat sciatic nerve. These early studies all reported transport velocities of between 1 and 5 mm/day, and it was not until several years later that faster transport velocities were measured (Grafstein 1967; Lasek 1968),and the different composition of the fast and slow transports were recognised (McEwen and Grafstein 1968; Karlsson and Sjostrand 1971). This delay was probably due to the relatively small amount of material travelling by fast axonal transport, and also to the lack of availability of isotopes with a sufficiently high specific activity to detect these small amounts. Fast and bidirectional transport of organelles (Burdwood 1965), noradrenaline (Dahlstrom 1967), acetylcholinesterase (Lubinska et al 1963) and protein (Lasek 1967) was demonstrated. Retrograde axonal transport was demonstrated using the exogenous protein horseradish peroxidase (HRP), which was shown to be taken up at nerve terminals and transported back to the cell body (Kristensson and Olsson 1971a & b).

Axonal transport is now well characterised with regard to the rates and components of slow anterograde, fast anterograde and fast retrograde transport (Grafstein and Forman 1980). There is some evidence for a slow retrograde transport (Mata et al 1986), but this is

not yet fully investigated.

Slow anterograde transport represents the movement of axonal cytoskeletal elements and the axoplasmic matrix. The components of slow transport have been characterised (Hoffman and Lasek 1975; Black and Lasek 1980), and consist of neurofilament, microtubule and actin filament proteins, along with microtubule-associated proteins and other proteins such as clathrin (Lasek 1986). In peripheral nerve, neurofilament proteins and most of the tubulin travel at a rate of 1-2mm/day in what is known as slow component a (SCa; Hoffman and Lasek 1975) or Group V (Willard and Hulebok 1977). Actin, some tubulin and about eighteen other proteins such as clathrin and fodrin, travel at 3-4mm/day in slow component b (SCb) or Group IV.

Fast anterograde transport conveys membranous axonal components and particulate material, including smooth endoplasmic reticulum (SER), vesicles and mitochondria. These contain a wide variety of proteins (Barker et al 1976; Bisby 1977; Cancalon et al 1976), phospholipid (Bisby 1985), amino acids (Ingoglia et al 1976; Sturman 1979) and calcium (Neale and Barker 1977). Also carried by fast axonal transport are gangliosides (Forman and Ledeen 1972), neurotransmitters and their enzymes (Brimijoin 1979; Laduron 1984), and nucleic acids (Por et al 1978). The maximum rate of fast anterograde transport is around 400mm/day (Grafstein and Forman 1980). Proposed mechanisms for the mediation of fast transport have included cytoplasmic streaming where organelles are carried by the movement of intracellular fluid (Gross

1975; Odell 1976), movement of SER along the axon (Rambourg and Droz 1980) and various other mechanisms involving actin filaments (Ochs 1971) or microtubules (Schmitt 1968). Recently, work on such a "ratchet mechanism" has characterised a protein named kinesin, which mediates fast transport of beads and organelles along microtubules of the squid giant axon in vitro, in a direction corresponding to anterograde transport (Vale et al 1985a,b,c,d). A separate molecule seems to be involved in mediating retrograde transport (Vale et al 1985d). However, other candidate proteins may be involved.

Retrograde axonal transport returns axonal constituents to the cell body. The maximum rate is similar to that for fast anterograde transport, but most retrogradely transported materials travel more slowly, at around 200mm/day (Grafstein and Forman 1980). Material undergoing retrograde transport arises either from components of anterograde transport which have reversed their direction or from endocytosis of substances at the terminal which enter the retrogradely transported organelles. Thus, retrograde transport carries materials which have both similarities to and differences from components of anterograde transport. The structure of transported membranous organelles has been studied in the mouse saphenous nerve (Tsukita and Ishikawa 1980), and in the squid giant axon (Fahim et al 1985). In the mouse, the anterogradely transported organelles were described as "axonal smooth endoplasmic reticulum and vesiculotubular structures", and differed from retrogradely transported material which appeared as

"large membranous bodies". Similar results were obtained in the squid. Fast transported proteins travelling in opposite directions have been found to be largely similar after one-dimensional electrophoresis (Abe et al 1974; Bisby 1981); this may be due to reversal of many anterograde components. Material which is endocytosed at the terminal may be derived from axonal components which have been previously released from the terminal, such as neurotransmitters (Streit 1980), substances derived from post-synaptic cells such as trophic factors (Hendry et al 1974), or components of the extracellular fluid such as albumin (Kristensson and Olsson 1971).

It is not known what determines the preferential binding of organelles to the proteins mediating anterograde or retrograde transport. Since some enzymes such as dopamine-B-hydroxylase (Nagatsu et al 1976) and receptors such as the cholinergic muscarinic receptor (Zarbin et al 1982) have different properties when travelling in opposite directions, it may be that this type of molecule can direct organelle transport by giving a certain "flavour" to the organelle membrane, allowing loading of the organelle onto the retrograde transport vector. The return of anterogradely transported substances to the cell body seems to be for the purpose of degradation and possible recycling of molecular constituents: evidence for the recycling of cellular glycoproteins in human erythroleukemia cells has been reported (Snider and Rogers 1986), and a similar proccess may operate in neurons. In addition, it is possible that retrograde

transport acts as an information-carrying system, allowing the cell body to monitor both intra- and extra-axonal events, such as neurotransmitter release or axonal injury.

In this thesis, I have made an attempt at further understanding retrograde transport as an informational system, with particular reference to the responses of the neuron to an acute axonal injury. It is relevant to review the literature on the response of the neuron to axotomy, the putative mechanisms for the initiation of this response, and to present evidence for the involvement of each of these mechanisms. I shall then present a rationale for the experimental protocol I have used.

Response of the neuron to injury

This subject has been extensively reviewed in the past (Lieberman 1971; Grafstein 1975), and so here I shall make only a brief synopsis of events occurring in the nerve cell body after axotomy. These events consist of a series of morphological and functional changes which seem to prepare a peripheral nerve cell for regeneration of its axon; suppression of the response in the frog by lowering the body temperature inhibits axonal regeneration (Carlsen et al 1982).

Studies of the cell body response to axotomy have been done in several systems, notably the hypoglossal nucleus (Kirkpatrick 1968; Sumner and Sutherland 1973) and the superior cervical ganglion of the rat (Matthews and Raisman 1972). In both these systems, the first

visible reponse of the cell to axotomy is dispersion of the Nissl substance, usually to the periphery of the cell. The nucleus becomes displaced from its usual position in the centre of the cell, and develops a dark rim and a series of indentations on its cytoplasmic side, which appear by six days post-axotomy. There is an accumulation of autophagic vacuoles and cytoplasmic dense bodies. In cases where the axons are prevented from regenerating by application of a permanent ligature to the nerve, chromatolytic changes persist for at least 55 days in the hypoglossal nucleus (Kirkpatrick 1968) and for 143 days in the rat superior cervical gangion (Matthews and Raisman 1972). In some cell types, a swelling of the cell body is seen in response to injury, for example, in goldfish retinal ganglion cells after optic nerve crush (Burmeister and Grafstein 1985). The chromatolytic reaction is generally seen only in cells whose axons have been damaged, although there is evidence of changes in the dorsal root ganglion cells after ventral rhizotomy (Rich and Johnson 1985), and in undamaged neurons which are sprouting (Watson 1973). In addition to changes in the neuronal soma, there is also a loosening of contact between the neuron and its surrounding glial cells (Kirkpatrick 1968), a decrease in the number of synaptic contacts on the soma (Sumner and Sutherland 1973), and associated changes in synaptic transmission (Purves 1976).

The morphological changes seen following axotomy are a reflection of metabolic changes occurring in the neuron and surrounding glial cells. The metabolic response is complex and variable, and differs between

cell types. In most cells, one of the earliest changes is an overall increase in the synthesis of RNA (Lieberman 1971). For example, the incorporation of 3H uridine into 18S and 28S RNA in the rat nodose ganglion is increased 3 days after vagotomy, followed by a second increase at 9-11 days (Austin et al 1983). It has been suggested that an increased RNA content is associated with the ability of the neuron to regenerate its axon, since goldfish retinal ganglion cells show an increase in total RNA content after axotomy, while those of the rat do not (Barron et al 1985). If the cell swells after axotomy, the concentration of RNA in the soma may remain constant or even decrease, and changes in the configuration of the RNA such as disaggregation of polyribosomes to single particles (Barron et al 1975; Murray and Forman 1971) results in disorganisation of the rough endoplasmic reticulum which is seen as one of the earliest morphological changes. In some cases, messenger RNAs for individual proteins have been measured; for example, a rise in the level of neurofilament mRNA has been reported during regeneration of the goldfish optic nerve (Tesser et al 1986). Decreased levels of the mRNA for the 68kD neurofilament protein has been measured in the rat superior cervical ganglion after post-ganglionic nerve crush (Koo et al 1986) and in the rat facial nucleus after axotomy (Tetzlaff et al 1986). Recently, studies on the expression of tubulin genes after facial nerve crush have shown that Ta1 mRNA, normally expressed only during development, is induced in axotomised neurons (Miller et al 1987). This increase in Ta1 mRNA can

be measured within 4h of nerve injury, and seems to be the earliest reliable indicator of the cell body response to axotomy so far discovered.

Alterations in protein sythesis seem to reflect those for mRNA. In general, studies with radiolabelled amino acids as precursors have shown an increase in their incorporation into protein after axotomy (eg Aldskogius et al 1984; but see Kung 1971, Cova and Barron 1981). The rat superior cervical ganglion shows continuous increases in both total protein content and the wet weight of the ganglion for 14 days (Ando et al 1984). The synthesis of enzymes associated with synaptic transmission has been shown to decrease in several systems following axotomy (Reis and Ross 1973; Cheah and Geffen 1973; Sinicropi et al 1982; Kreutzberg et al 1984); however, this is not a universal phenomenon, since AChE has been found to increase in the guinea-pig facial nucleus after facial nerve injury (Engel and Kreutzberg 1986).

Proteins of the neuronal cytoskeleton, which undergo slow axonal transport, (Hoffman and Lasek 1975) have also been studied. In the regenerating rat sciatic nerve, the axonal transport of tubulin is increased, while that of the neurofilament proteins is decreased (Hoffman and Lasek 1980); the SCa and SCb rates of transport are unaltered, but a larger proportion of the tubulin travels in SCb. In frog motoneurons, transsection of the ventral roots results in a decrease in tubulin content and a reduction in one of the neurofilament proteins (Sinicropi and McIlwain 1983).

Alteration in the synthesis of protein after axotomy is clearly a complex process, and crude measures of synthetic activity such as total protein content of ganglia are insufficient for the assessment of a response in which individual proteins are up- or down-regulated according to their function and possible role in the regeneration of the axon.

An increase in the overall synthetic activity of a cell would be expected to result in an increased "energy utilisation". Two studies have shown an increase in the utilisation of 14C 2-deoxyglucose after axotomy (Kreutzberg and Emmert 1980; Singer and Mehler 1980). Both studies used the hypoglossal nucleus and found an increase in glucose metabolism detectable 24h after transsection of the hypoglossal nerve. The increased glucose utilisation was recorded up to 28 days in one study (Kreutzberg and Emmert 1980). This increased glucose uptake does not seem to be reflected by energy use and lactate production (Harkonen and Kauffman 1973a); however, the activities of some pentose phosphate shunt enzymes were increased at 2-7 days post-axotomy in the rat superior cervical ganglion (Harkonen and Kauffman 1973b). Smith et al (1984) have measured both protein synthesis and glucose utilisation in the rat hypoglossal nucleus after axotomy, with similar results to those already described. The glucose utilisation returns to normal levels after nerve regeneration (Singer and Mehler 1986). The enzyme ornithine decarboxylase has been measured in both rat facial nucleus (Tetzlaff and Kreutzberg 1984 and 1985) and the rat SCG (Gilad et al

1985), since it catalyses the rate- determining step in the synthesis of polyamines, which seem to play key roles in development and growth (Seiler 1981). A very rapid rise in levels of the enzyme was measured in the SCG, while that in the facial nucleus took about 7 days to reach its peak.

The axotomised neuron also shows changes in its electrophysiological properties. While there are likely to be changes in the intrinsic properties of the nerve cell membrane, the decrease in excitatory post-synaptic potential amplitude which has been reported in the guinea-pig superior cervical ganglion (Purves 1975) and in goldfish Mauthner cells (Wood and Faber 1986) seems to be due to a decrease in the number or to retraction of synapses on the soma. A similar reactive deafferentation has been seen in the rat hypoglossal nucleus (Sumner and Sutherland 1973) and in the rat trigeminal system (Renehan and Munger 1986). In the cat, monosynaptic transmission in motoneurons after ventral root transsection is enhanced 7-20 days after injury (Kuno and Llinas 1970); excitatory post-synaptic potentials were induced in the motoneurons using afferent volleys from leg muscles. Bullfrog sympathetic neurons show a decreased amplitude and duration of the action potential after-hyperpolarisation after axotomy of the IXth and Xth spinal nerves, persisting up to 47 days (Kelly et al 1986). The change in duration returned to normal by six weeks post axotomy, when the axons should have reconnected with their targets (Kelly, personal communication).

One instance where a chromatolytic response is not elicited in the

cell after nerve injury is that of the rat dorsal root ganglion neurons after axotomy of the central axon branch (Hare and Hinsey 1940; Watson 1973). Since the central branch of the axon is smaller in diameter than the peripheral branch, it is possible that central axotomy is an insufficient stimulus to induce the cell to prepare for regeneration (Cragg 1970). Regeneration of the central branch can be enhanced by prior peripheral axotomy, presumably because this is a greater stimulus to the soma (Richardson and Issa 1984).

From this brief review, it is clear that there is a profound cell body response to axotomy, involving every aspect of metabolism and function. An obvious question to be asked is then: what is the mechanism which initiates these responses by signalling to the cell body that the axon is damaged?

Mechanisms for initiation of the cell body response.

There are several possible candidates for signals for chromatolysis, and most of these were critically assessed by Cragg (1970). Since then, some progress has been made towards defining the signal more clearly, but as yet, the mechanism has not been demonstrated. Possible signals fall into four main categories: i) "electrical"; ii) those involving Schwann cells iii) those concerned with the loss of trophic factors arising from target tissues; iv) those involving retrograde axonal transport in other ways.

i) When a nerve injury is made, there is a large injury discharge,

consisting of a wave of depolarisation passing along the axon in both directions from the injury site. Wall et al (1974) have studied the injury discharge in the sural nerve of the cat, damaging it by various means such as stretching, local pressure, teasing or sectioning. In all cases, they found that the injury discharge recorded in the sural nerve was brief, lasting only 2-4 seconds. A second type of potential is seen as a result of the inside of the axon being exposed at the cut end (Adrian 1930). This results in an injury potential, which can last up to 30 minutes until the end of the axon reseals (Lubinska 1956). No prolonged injury potential was recorded by Wall et al (1974) unless the cut end of the nerve was allowed to dehydrate, and this led them to suggest that the injury potential recorded by Adrian (1930) may have been due to some small branches of the nerve remaining intact. In any case, it is unlikely that such passive depolarisations would travel 100mm or more to the spinal cord of the cat: passive depolarisation falls to 1/27 of its original activity along 3mm of myelinated axon, and so could not constitute an "injury signal".

Attempts have been made to induce chromatolysis in spinal cord and dorsal root ganglia of the guinea-pig, and in cat hypoglossal neurons using a regimen of antidromic stimulation for varying time periods (Liu et al 1950). These authors found no changes in any of the guinea-pig neurons. Some changes which appeared to be the early stages of chromatolysis were seen in the cat hypoglossal nucleus; however, antidromic stimuli of 20V or more were used, and similar changes were

induced by the surgical preparation, without electrical stimulation. It seems from this that there is no relationship between injury discharges and cell reaction. As mentioned earlier, chromatolysis can also be induced in cells by proccesses which do not involve the production of injury potentials, such as collateral sprouting.

Edwards and Grafstein (1983) have shown that application of tetrodotoxin to the goldfish optic nerve will induce a cell body reaction in the retinal ganglion cells. However, this does not necessarily mean that the "signal for chromatolysis" is of an electrical nature; blockade of activity along the nerve may be accompanied by an associated reduction in the transport of molecular axonal components.

ii) Although Schwann cells undergo a large increase in mitotic and metabolic activity when a nerve is cut (Watson 1972; Salzer et al 1980; Oaklander et al 1987), it is unlikely that they are involved in initiation of the neuronal reaction, for example, by production and release of factors not produced in the intact nerve. The main evidence for this is that the chromatolytic reaction is largely confined to those neurons whose axons have been damaged: cutting a nerve such as the internal carotid nerve will produce selective chromatolysis in the superior cervical ganglion cells whose axons run in that nerve, and reacting cells can be seen in the ganglion adjacent to normal cells whose axons are undamaged (Matthews and Raisman 1973). There is a loosening of contact between glial cells and neurons after axotomy, but

as this occurs after the onset of chromatolysis, it is therefore a result, not a cause, of the cell reaction. In addition, chromatolytic changes can be seen in neurons which are surrounded by normal Schwann cells, such as cells which are producing collateral sprouts (Watson 1973). Chromatolysis can be produced by treating the nerve with agents such as colchicine, which block axonal transport by depolymerising microtubules (Pilar and Landmesser 1972; Purves 1976). This evidence points to a signal which is conveyed intra-axonally rather than one which arises from surrounding cells.

There is some evidence for trophic factors produced and released by peripheral Schwann cells. Richardson and Verge (1986) used rat dorsal root ganglion neurons to test the effects of peripheral axon lesion on the ability of the central axon branch to regenerate into a nerve graft. They found that sciatic nerve transection enhanced dorsal column axon regeneration. This effect was decreased with longer proximal stumps, but unaffected by crushing the proximal stump closer to the cell body than the transsection, or injecting the stump with colchicine or nerve growth factor. The authors suggest that "the central axons are stimulated to regenerate by peripheral axon injuries which reduce retrograde regulatory influences of Schwann cells". Trophic activity other than NGF has been measured in the chick embryo radial nerve after removal of the target muscle (Abrahamson et al 1986). The factor was retrogradely transported after target removal, but no evidence of its retrograde transport in intact nerves was

presented; this must occur if reduced transport of the factor after axotomy is to be proposed as an initiation signal for chromatolysis.

iii) Loss of a trophic factor arising from target tissues or endoneurial cells is also a candidate for the signal for chromatolysis. Such a factor would be released from target cells, taken up by the nerve terminals and conveyed to the cell body by retrograde axonal transport. Axotomy would therefore deprive the neuron of its supply of trophic factor. It may be useful to consider nerve growth factor (NGF) in this regard, since to date it is the best characterised of any such retrograde trophic factor.

Sympathetic and peripheral sensory neurons are dependent on NGF for correct development. Axotomy during development results in the death of 90% of rat superior cervical ganglion cells, and treatment with NGF increases the number of surviving cells (Hendry and Campbell 1975; Banks and Walter 1977; Hendry 1975). NGF acts via a specific, receptor-mediated uptake at the terminals of responsive neurons, and is retrogradely transported at a rate of 5-10mm per day (Hendry et al 1974; Claude et al 1982; Schwab et al 1983). Adult animals may be less dependent on NGF: axotomy in the adult rat produces very little cell death in the superior cervical ganglion unless the lesion is very close to the ganglion. However, it may be that adult neurons are supplied with NGF from sources other than their distal axons. NGF has been reported to decrease the chromatolytic reaction in the rat SCG (West and Bunge 1976) and to prevent synaptic depression in the guinea-pig

SCG after axotomy (Purves and Nja 1976; Nja and Purves 1978). Thoenen and associates have correlated the amount of NGF in target tissues with the degree of sympathetic innervation (Korsching and Thoenen 1983), demonstrating that the NGF needed by sympathetic axons is present in the innervated tissue. Densely innervated tissue such as the rat iris contained 1.9±0.3ng/g wet weight of tissue. More recent evidence suggests that NGF is produced by Schwann cells, especially after nerve injury (Heumann et al 1987).

It seems from the above data that NGF or other trophic factors could be involved in the initiation of chromatolysis in sympathetic and peripheral sensory neurons. Cutting off a neuron's supply of trophic factor by axotomy could account for the shorter reaction time which is seen if the lesion is made closer to the cell body (Watson 1968), since a shorter proximal stump would be 'drained' of its trophic factor by retrograde transport more quickly than a long stump. This signal could not account for the increased severity of reaction with a more proximal lesion, nor for the fact that the neuron will die if the injury is too close to the soma: once the trophic factor is 'drained' from the proximal stump, the cell reaction would be expected to be identical, since axotomy at any point eventually results in total loss of the factor. There are several other observations which are inconsistent with loss of trophic factors as the sole signals.

Hall and Wilson (1982) have tested NGF on the axotomised SCG of the adult rat to see if the protein changes induced by axotomy can be

reversed. They concluded from their 2-dimensional gel analyses of SCG protein that NGF does not reverse most of the changes in protein composition induced by axotomy, and that the loss of NGF alone could not be the signal for initiation of the cell reaction. It has been shown that repeated lesions to the hypoglossal nerve have a more profound effect in producing cell death in the hypoglossal nucleus than does a single lesion (Arvidsson and Aldskogius 1982). The two lesions were made at the same site, 24 hours apart. Only a positive signal arising from the injury site could result in a response to the second lesion, since the axons could not have reinnervated their targets so quickly, and therefore loss of a retrograde trophic factor could only invoke a single response in the cell. Singer et al (1982) have shown that application of colchicine to the hypoglossal nerve proximal to an axotomy delays the onset of the chromatolytic response, again suggesting a 'positive' signal. It seems that at early times after its application to a nerve, colchicine prevents the arrival of the chromatolytic signal at the cell body, presumably through its action in blocking axonal transport (Singer et al 1982); at later times, however, a reversal of anterograde transport may be induced at the site of application and the soma then receives a signal that its axon is damaged as a result of the action of colchicine (Pilar and Landmesser 1972).

The above data are more consistent with a signal which arises at the injury site than with loss of a trophic factor from the periphery or

Schwann cells. A likely candidate for this positive signal is alterations in the quantity or composition of material undergoing retrograde axonal transport. However, given the protective effect of NGF on cell death in developing animals, it is probable that neither change is the signal itself, but rather the cell responds to changes of both types.

iv) Alterations in retrograde transport could be involved in initiation of the cell body response. It has been shown that a reversal of anterograde axonal transport is induced at the site of an acute nerve injury (Bisby and Bulger 1977). This abnormal reversal could result in a premature and increased return to the cell body of axonal constituents previously destined for the distal parts of the axon. A lesion made closer to the cell body would then induce a more rapid return of abnormal constituents to the soma, and in increased amount relative to a distal lesion. This mechanism could therefore account for both the shortened reaction time and increased severity of the cell response with more proximal lesions, and cell death with lesions very close to the soma.

There are several possibilities for the nature of alterations in retrograde transport of individual components after axotomy. First, there could be a change in the quantity of a substance returning to the soma, depending on the amount transported in the intact axon. Second, the composition of the transported material could be different, since the reversal process occurring at the injury may be different from that

normally occurring at nerve terminals. Third, substances could pass across the axonal membrane at the injury site and be retrogradely transported. These possibilities are discussed further in the introduction to chapters 1 and 3.

A further question concerned with the involvement of retrograde transport in initiating chromatolysis relates to the time course of the signal reaching the soma and the first manifestation of the cell reaction. The rate of retrograde transport of most axonal components is 100-250 mm/day, giving an average of between 4 and 10mm/hour (Grafstein and Forman 1980). An injury made 2mm from the rat superior cervical ganglion produces chromatolysis visible by light microscopy by 6 hours (Matthews and Raisman 1972). Gilad et al (1985) have reported a rapid rise in transglutaminase activity 30 min after a lesion made 5mm from the ganglion, which is in accordance with the expected rate of travel of a signal conveyed by retrograde transport. Kristensson and Olsson (1975) have used an exogenous protein, horseradish peroxidase (HRP), to study the relationship between the time course of retrograde transport and the onset of the cell body reaction to axotomy. The rat sciatic nerve was transsected and HRP was applied to the cut end of the proximal stump, where it is taken up and enters the retrograde transport system. The HRP was always seen in the cell bodies before any manifestation of chromatolysis could be seen with the light microscope. Thus, the time course of arrival of the signal at the soma is consistent with it being conveyed by retrograde transport.

A suggestion has recently been made that the common factor associated with procedures which induce a chromatolytic response is a change in the status of microtubules at the lesion site (Grafstein 1987). Such a disruption could alter the tubulin monomer/polymer ratio in the axon and stimulate microtubule synthesis for regrowth of the axon. While there is clearly a disruption of microtubule structure caused by cutting or crushing the nerve, or applying colchicine, it is less clear how such a mechanism could induce chromatolytic changes in a neuron which is sprouting.

I have discussed the possibilities for signals initiating the cell reaction after axotomy. It seems fairly clear that the injury potential and injury discharge are not directly involved in this initiation, mainly from the work of Liu et al (1950); alterations in the synaptic inputs to the cell also seem to be a result rather than a cause of the cell reaction. The time course of arrival of the signal at the soma suggests that it is conveyed by retrograde axonal transport. There is some conflicting evidence regarding the nature of this retrograde signal, viz. whether it is a negative one due to the loss of a trophic factor from target or endoneurial cells, or a positive one arising from the injury site itself. Some observations cannot be explained by a negative signal, such as the work of Arvidsson and Aldskogius (1982) on the response of hypoglossal neurons to a second axotomy. However, given that NGF can reverse some of the changes in dorsal root ganglion cells after peripheral axotomy, it is

possible that cells respond to signals of both types, and that different cell types are more receptive to one kind of signal. Experiments which could distinguish between positive and negative signals are discussed in chapter 2.

Experiments

I have performed a series of experiments aimed at further understanding of changes in retrograde axonal transport after an acute nerve injury, in the hope that this would better characterise retrograde transport as an informational system. I have also addressed the question of the signal for chromatolysis.

The experimental system I have used is the rat sciatic nerve, for several reasons. First, it has been used as a model system in many previous studies on axonal transport (eg Bisby 1981; Gulya and Kasa 1984), and therefore a wealth of information is available on its characteristics. Secondly, the area is surgically accessible and has 5cm or more of nerve available for transport studies, which allows for easy manipulation and accurate calculation of transport velocities.

The experiments can be conveniently divided into three sections:

i) Changes in individual components of retrograde transport after injury.

ii) Studies on defining the signal for chromatolysis;

iii) More general examination of changes in transported protein after axotomy.

EFFECT OF NERVE INJURY ON INDIVIDUAL AXONALLY TRANPORTED SUBSTANCES

Changes in retrograde axonal transport may be involved in signalling to the neuron that its axon is damaged. In this chapter, I shall describe experiments designed to measure changes in the amount of specific molecules returning to the cell body after an acute axonal injury.

It has been demonstrated that a reversal of fast anterograde transport is induced at the site of an acute injury to the rat sciatic nerve (Bisby and Bulger 1977). There are three possible types of change in retrograde transport which could result from this:

i) Reduced retrograde transport of an intra-axonal substance due to cutting off the supply of material returning from the distal part of the axon. This may occur if there is a relatively large retrograde transport of the molecule in the intact axon, or if the reversal process developing at a site of injury is not as effective for this material as the reversal process in an intact axon.

ii) Increased retrograde transport of intra-axonal substances due to abnormal and increased reversal of anterograde transport at the injury site. This might occur for substances where the retrograde transport in the intact axon is small relative to the anterograde, due to the release or degradation of transported material in the distal axon or terminals.

iii) Arrival at the cell body of foreign substances. The integrity of

the axonal plasma membrane is compromised by making the injury (Olsson and Kristensson 1971), and extra-axonal substances which pass across at the crush site could be transported back to the cell body (Cragg 1970). Kristensson and Olsson (1971) have demonstrated that horse-radish peroxidase can arrive in the cell bodies of the rat dorsal root ganglion after application to a crush site on the sciatic nerve.

The mechanism for induction of transport reversal at the injury is unknown and it would be interesting to know whether this abnormal reversal is similar to that which normally occurs at the axonal terminals. There are differences in the properties of some molecules when they are travelling in opposite directions in the undamaged axon; for example, dopamine B-hydroxylase has only 20% as much enzymatic activity when undergoing retrograde transport as when travelling anterogradely (Nagatsu et al 1976). Muscarinic cholinergic receptors have a reduced affinity for agonist when returning to the cell body (Zarbin et al 1982), and the structure of the retrogradely transported organelles is different from the anterograde ones (Tsukita et al 1980; Fahim et al 1985).

I have conducted experiments to quantify the amount of individual transported materials undergoing retrograde transport in the intact and acutely injured rat sciatic nerve. The molecules studied were acetylcholinesterase (AChE), substance P (SP) and the muscarinic cholinergic receptor (MChR). All of these are both anterogradely and retrogradely transported in peripheral nerves (Lubinska and Niermierko

1971; Couraud and Di Giamberardino 1982; Keen et al 1982; Brimijoin et al 1980; Laduron 1980; Gulya and Kasa 1984), and were chosen as being representative of membrane-associated and vesicular transported material.

In addition, experiments using the muscarinic receptor as a model were designed to test whether the receptor has a decreased agonist affinity when returning from an injury site, in a manner similar to that seen for receptors undergoing retrograde transport in the intact sciatic nerve (Zarbin et al 1982).

METHODS

i) Surgery.

Male Sprague-Dawley rats weighing 250-350g were used. The sciatic nerve was surgically exposed under sodium pentobarbitol anesthaesia (50mg/kg) and ligated unilaterally at the level of the knee with 4-0 silk thread. The time of making this acute injury was taken to be 0 hours. After waiting 4, 10, 16, or 22h, two further ligatures were placed 10mm apart on the nerve in a proximal position, to act as collection crushes, trapping material travelling both anterogradely and retrogradely from the distal injury. The most distal of these collection crushes was 20-30mm proximal to the injury site. Collection ligatures were also applied to the contralateral, uninjured nerve in a similar proximal position at this time. The animals were allowed to

survive a further 6h, killed by sodium pentobarbitol overdose and cervical fracture, and the nerves were then removed and immediately frozen on ruled cards on dry ice.

The nerves were sectioned with a scalpel blade and segments were individually assayed. Separate groups of animals were used for assay of each of the molecules of interest. Figure 1 summarises the surgical and sectioning procedures; contralateral control nerves yielded only segments 1 to 5. In some nerves, segment 3 was subdivided into three parts to allow for calculation of mobile fractions of material moving along the axons: segment 3a (proximal 3mm), 3b (centre) and 3c (distal 3mm).

For MChR agonist affinity experiments, a 22hr post-injury time only was used, since the reversal of axonal transport at the distal injury should be well- established at this time (Bisby and Bulger 1977).

ii) AChE assay.

This method measures the formation of 14C acetate from 14C acetylcholine iodide by the action of AChE in the samples (Fonnum 1969).

Nerve samples were homogenised in 400µl ice-cold buffer using a Polytron homogeniser. The homogenisation medium contained:

40mM sodium phosphate buffer pH 7.4

0.2M sodium chloride

10mM magnesium chloride

0.5% Triton X-100

0.07M ethopropazine hydrochloride

AChE standards (Sigma) were diluted in sodium phosphate buffer as follows:

5.6mg AChE + 20ml buffer = 1U/ml (soln. A)

1ml soln A + 9ml buffer = 0.1U/ml

These stock solutions were then diluted to give final AChE concentrations of 1, 0.5, 0.2, 0.1, 0.05, 0.02 and 0.01U/ml.

1 Unit (U) of enzyme is defined as that amount which will hydrolyse 1umol of acetylcholine per minute at 37°C and pH 8.0.

2µl of standard or sample were mixed with 18ul of a reagent containing 20mM sodium phosphate buffer pH 7.4, 0.1% bovine serum albumin and 0.5mM 1-14C acetylcholine iodide (New England Nuclear, specific activity 5mCi/mmol). The standards and samples were incubated at 37°C for 30min. 200µl of sodium phosphate buffer (20mM, pH 7.4) was added to each tube, followed by 200µl sodium tetraphenylboron in 3-heptanone (50mg/ml). Tubes were centrifuged at 6000rpm for 2.5min, and the organic layer containing the unreacted 14C ACh was removed. The amount of 14C acetate in the aqueous layer was measured by taking 100µl aliquots, adding 3ml "Scintiverse" (Fisher) and counting the samples for 14C. A sample standard curve for the AChE assay is shown in Figure 2.

iii)SP radioimmunoassay

(Keen et al 1982).

Tissue preparation.

Each nerve segment was rapidly removed and immediately placed in a 1.5ml Eppendorf centrifuge tube with 250µl of ice-cold homogenising solution of the following composition:

11.5% glacial acetic acid (v/v)

0.1% concentrated HCl

1mM disodium ethylene diamine tetraacetic acid

1mM dithiothreitol

Samples were boiled for 10min in a water bath, then homogenised using a machined Teflon pestle. The tubes were centrifuged for 5min at 6000 rpm, the supernatents were decanted to fresh tubes and a further 250µl of homogenising medium was added to each sample. The samples were freeze-dried overnight to a "cobweb" appearance, then 200µl of sodium barbitone buffer (pH 8.6, 50mM, 0.1% bovine serum albumin) was added and the tubes were again centrifuged 5min at 6000rpm. 50ul aliquots of the samples were taken for assay.

Antiserum dilution.

Substance P antiserum was kindly donated by Dr. P Keen, Department of Pharmacology, University of Bristol. 10µl of stock solution (1:100)

was added to 6ml barbitone buffer (pH 8.6, 50mM) to give a final antiserum dilution of 1:60,000.

Tracer dilution.

125I substance P (Amersham, specific activity 2000Ci/mmol) was diluted in barbitone buffer such that a 50µl aliquot contained approximately 1000cpm.

SP standards

Standards were prepared from a stock solution of SP (Sigma) in barbitone buffer to give the following concentrations: 320, 160, 80, 40, 20, 10 and 5pg/50µl.

Assay method

Standards and samples were assayed in triplicate. Blanks contained only tracer and 100µl barbitone buffer to give the lowest expected reading. Controls contained tracer and antiserum plus 50µl buffer, to give maximum binding of tracer to the antibody. 50µl each of antiserum, standard or sample, and tracer were combined in test tubes, and the tubes were vortexed and then centrifuged briefly to ensure thorough mixing. A suspension of carbon decolorising neutral (Norit; 1mg/ml buffer) was prepared, stirred 30min and refrigerated overnight (4°C); assay tubes were also refrigerated overnight. 1ml of the charcoal suspension was added to each assay tube and left for 20min.

Tubes were then centrifuged for 30min at 4° C and 2000rpm, and the supernatants were decanted into fresh tubes. Samples and standards were counted in a gamma counter for 10min. Figure 3 shows a typical standard curve for the radioimmunoassay. Standard curves and calculations of the amount of SP in each sample were generated using a computer programme (Barlow 1983).

iv) Muscarinic receptor assay

(Yamamura and Snyder 1974; Laduron 1984)

Due to the low concentration of MChR in the sciatic nerve even adjacent to a collection crush, it was found necessary to pool nerve segments from four animals for use in this assay.

3mm nerve segments (Figure 1) were crushed in a metal mortar and pestle at -70°C, then homogenised in 1ml ice-cold sodium phosphate buffer (50mM, pH 7.4) using a Teflon pestle, to give a final tissue protein concentration of 0.2 - 0.4 mg/ml (Biorad protein assay kit; see below for method). 50µl of homogenate was added to:

a) 1ml buffer containing 0.5nM [3-H] quinuclidinyl benzylate (Amersham, specific activity 60Ci/mol; QNB), and

b) 1ml buffer containing 0.5nM [3-H] QNB plus 2µM atropine, to displace receptor-bound ligand and allow calculation of specific ligand binding. Details of these calculations are given in section (v).

0.5nM QNB was used since this concentration has previously been found to maximise specific ligand binding, while keeping non-specific

binding to a minimum (Yamamura and Snyder 1974).

Tubes were incubated for 20min at 37°C, then 3ml ice-cold phosphate buffer was added to each tube and the sample immediately filtered by vacuum through a Whatman GF/B filter. Filters were washed with 3x3ml ice-cold buffer and then placed in 12ml scintillation fluid (Scintiverse, Fisher). Vials were left in the dark overnight and next day counted for tritium. Blanks contained buffer in the assay tube instead of nerve homogenate. A standard curve for the receptor assay is shown in figure 4. Specific binding was found to be saturable with increasing QNB concentration, with half-maximal binding at a concentration of approximately 1nM. Non-specific binding increased linearly with QNB concentration and was not saturable.

Protein assay: 0.1ml of standards and appropriately diluted samples were placed in test tubes, with a 0.1ml aliquot of buffer as a control. 5ml of diluted Biorad dye reagent was added to each tube and vortexed. After 30 minutes, the optical density of each was measured at 595nm against the reagent blank, and protein concentrations in the samples were read from the standard curve, shown in figure 5.

(v) Agonist affinities of MChR.

For these experiments, proximal and distal nerve segments (segments 2 and 4 respectively) from control nerves and nerves injured 22h previously were used. Corresponding segments from four animals were pooled and homogenised in phosphate buffer. Each of the four

specimens (control proximal, control distal, injured proximal and injured distal) was used in the following protocol with 100µl homogenate per tube:

sample	3H QNB 0.5 nM	tissue homog.	atropine 2µM	carbachol 1µM
1	X	X	-	-
2	X	X	X	-
3	X	X	-	X

Samples containing only the radiolabelled ligand and tissue homogenate give total binding (specific plus non-specific) of the ligand to the tissue. Samples with ligand, tissue and atropine give non-specific binding of the ligand to the tissue, since the high concentration of atropine used will compete with the labelled QNB for specific receptor sites. Specific binding of QNB to receptors is then calculated from:

Specific binding = total binding - non specific binding

Addition of carbachol increases competition for the receptor sites; carbachol has a high affinity for the receptor and will displace QNB from specific sites. Sample 3 in the above table gives total binding in the presence of carbachol, which is lower than the total binding measured in the presence of atropine alone. If the receptor site has a

high agonist affinity, carbachol will displace more QNB from specific sites than in a sample with low agonist affinity receptors. The % specific binding in the presence of carbachol is calculated from:

$$\% \text{ SB} = \frac{\text{TBC} - \text{NSB}}{\text{TB} - \text{NSB}}$$

where TB = total binding

NSB = non-specific binding

SB = specific binding

TBC = total binding with carbachol.

If anterogradely transported receptors have a higher agonist affinity than retrogradely transported ones, the addition of carbachol will give a smaller calculated value of %SB for anterogradely transported receptors.

RESULTS.

All three molecules accumulated both proximal and distal to the collection crushes, demonstrating their anterograde and retrograde transport in both intact and injured nerves. The accumulations could not have been due to local synthesis at the crush sites, since the levels of activity in the isolated segment (3) were the same as in parts of the nerve far removed from the crushes (eg. segments 7 and 8 in injured nerves). The distal/proximal ratios given in the figures

were calculated by division of the recorded accumulation in nerve segment 4 (retrograde transport) by the accumulation in segment 2 (anterograde transport). This ratio was used because there was a high degree of variability in absolute levels of material between animals, but the proportion of retrograde to anterograde transport was fairly constant in groups of animals which were treated the same. Statistical inferences concerning the significance of changes in ratios were made using the non-parametric Mann-Whitney U-test.

Transport velocities of individual molecules along the sciatic nerve were calculated from the following formula:

$$\text{velocity} = \frac{(A - x)}{x} \cdot \frac{l}{F.t} \quad \frac{mm}{h}$$

where t = duration of collection (h)

x = concentration in isolated segment (3; units/mm)

A = concentration in segment 2 or 4 (units/mm)

F = fraction of total which is mobile in the nerve

l = length of segments 2 or 4 (mm)

The velocity formula is derived as follows:

Amount of material accumulating in segment 2 or 4 = $(A - x).l$

Length of nerve, L, which has "emptied"

into segment 2 or 4 during collection period $= \frac{(A - x).l}{x}$ mm,

assuming the mobile fraction, F = 1.

If F<1, then $L = \frac{(A - x)l}{x.F}$ mm

L has "emptied" over a collection period t,

therefore, velocity, $V = \frac{L}{t} = \frac{(A - x)l}{x.F.t}$ $\frac{mm}{h}$

The mobile fraction F is calculated as follows:

The assumption is made that during the collection period t, all mobile material moves from segment 3b to the end segments, 3a and 3c.

Then $F = 1 - \frac{\text{concentration in segment 3b}}{\text{mean concentration of entire isolated segment}}$

i)AChE

Figure 6 shows sample profiles of AChE activity along the sciatic nerve at a time 16h after acute nerve injury. Note the increase in activity in segment 4 relative to segment 2 in the injured nerve. Figure 7 shows a summary of distal/proximal AChE activity ratios for each time point post-injury, for control and injured nerves. No significant change in this ratio was seen at 4h and 10h after injury, compared to contralateral control nerves. By 16h after injury, the distal accumulation had increased significantly from 48% of proximal accumulation to 73%, but by 22h post-injury this increase was no longer

apparent.

The mobile fraction of AChE was calculated to be 0.12±0.02 (n=8), with anterograde and retrograde transport velocities of 255±40mm/day (n=8) and 100±16mm/day (n=8), respectively.

ii)SP

Figure 8 shows typical profiles of SP-like immunoreactivity levels measured along a control nerve and along a nerve injured 16h previously. Figure 9 shows distal/proximal ratios at the four post-injury times studied. No significant changes in the D/P ratio for SP were induced at any time measured up to 22h after making an acute nerve lesion. The mobile fraction of SP was found to be 0.45±0.04 (n=7), with an anterograde velocity of 86±12mm/day (n=7) and a retrograde velocity of 27±7mm/day (n=7).

iii)MChR

(Figures 10 and 11). The retrograde transport of MChR in injured nerves was not significantly different from that in control nerves until 16h post-injury, when a decrease in the distal accumulation from 71% to 32% of proximal was seen. This decrease was still apparent but no longer significant at 22h after injury.

A transport velocity of 82±12mm/day (n=8) was calculated for the anterograde direction, and a retrograde velocity of 25±4mm/day (n=8). The mobile fraction was found to be 0.39±0.06.

The anterograde transport of all three molecules studied was unaffected by injuring the nerve, for at least 22h following axotomy. Figure 12 shows proximal accumulations for AChE, SP and MChR at each time point post-injury. Thus, changes in the D/P ratio reflect changes in retrograde transport.

iv)Receptor affinity experiments

Using different carbachol concentrations, it was possible to reduce the specific binding of the ligand to the receptors in a concentration-dependent manner. This is shown for anterogradely transported receptors in the uninjured nerve (Figure 13). A similar experiment was performed for retrogradely transported receptors, and a trend showing reduced specific binding with higher carbachol concentrations was seen. However, the levels of specific binding measured in the presence of carbachol were greater than the total binding for all carbachol concentrations tested. Zarbin et al (1982) showed that the difference in agonist affinity between anterograde and retrogradely transported receptors was best demonstrated using a carbachol concentration of 1uM. However, our assay method proved too variable to detect the difference between these two receptor groups: in many of the assays, the binding of the ligand in the presence of carbachol was higher than the "total" binding with the ligand alone (eg Figure 13 at carbachol concentration 0.1uM). We were therefore unable to complete this study.

DISCUSSION.

These experiments have compared the retrograde transport of three individual materials in the rat sciatic nerve following an acute nerve injury. Three different types of response were seen: transport of AChE was increased 16h after injury, transport of SP was unchanged, and that of MChR was decreased.

Quantitative changes in the retrograde transport of a substance may be dependent on several factors: its normal retrograde transport in the intact nerve, including where it reverses direction along the axon, the organelle with which it is associated and the axonal type in which it is found. For example, the different responses of AChE and SP to nerve injury may be a result of both the different axons in which they are found, and their association with different organelles: SP is found in large, dense-cored vesicles (Brimijoin et al 1980) while AChE is associated with the smooth endoplasmic reticulum-like bodies (Brimijoin 1979).

AChE is found mainly in the motor axons of the sciatic nerve (Lewis and Shute 1966). It exists in several molecular forms, which may be distinguished by their sedimentation coefficients in sucrose density gradients (Brimijoin 1979). In the experiments described here, total AChE activity in the nerve was measured; butyrylcholinesterase activity was inhibited using ethopropazine. The transport rate of the enzyme was calculated to be 260mm/day for anterograde transport and 80mm/day for retrograde. These values fit well with previous measurements of

transport velocity, such as those reported by Lubinska and Niermierko (1970) of 260mm/day and 134mm/day for anterograde and retrograde transport, respectively.

In intact sciatic axons, 50% as much AChE is retrogradely transported as travels down the axons by anterograde transport (Figure 7). When a crush injury is made, this induces a reversal of anterogradely transported material. Since the retrograde transport is relatively small in intact axons, the major observed effect of the injury is the premature and increased return of anterogradely transported AChE.

No increase in retrograde transport was seen at 4h after injury. The time for establishing the turnaround process at the crush has been calculated at 1.9-2.4h (Bisby and Bulger 1977), and so a measurable effect of the injury would not be expected as early as 4h, since the point of observation (ie. the distal collection ligature) was 2-3cm proximal to the injury. At 10h after injury, an increased but statistically insignificant retrograde transport of AChE was measured. At 16h, the increase was significant ($p<0.01$), and at 22h, the amount of retrograde transport was returning to control levels. The transient nature of this increased retrograde transport may be due to changing conditions at the injury site with time.

In the intact axonal terminal, AChE is thought to be released into the synaptic cleft of the neuromuscular junction (Engel et al 1987), and this may explain why less AChE is retrogradely transported than is

anterogradely transported in intact axons. After crush injury, there is a delay of several hours before the motor axons begin to sprout (McQuarrie 1985), and during this period, AChE is unlikely to be released from the nerve endings. It is likely that enzyme which would normally enter the distal axon and be released from the terminal now reverses its direction at the injury site, increasing the measured retrograde transport. By 22h after injury, the crushed axons are developing growth cones, and the conditions in the "terminals" are such that AChE can now be released from the regenerating axons (Engel et al 1987). Thus, by 22h post-injury, retrograde transport of AChE in the sciatic nerve has almost returned to control levels. Such a relationship between the release of AChE and the amount of its retrograde transport could explain why the increased retrograde transport after nerve injury is transient.

SP is synthesised in the dorsal root ganglion neurons, in the form of a larger precursor (Harmar et al 1984). An anterograde transport rate of 240mm/day and a retrograde velocity of 216mm/day have been reported for the rat sciatic nerve (Bisby and Keen 1985); the rates calculated here were 86 ± 12mm/day and 27 ± 7mm/day, respectively. The discrepancy between the two sets of results is due to the higher value for the mobile fraction, F, calculated here, and also the assumption made in velocity calculation that an equal amount of SP is travelling anterogradely and retrogradely. Bisby and Keen (1985) calculated the retrograde velocity of SP transport based on a mobile fraction of 0.03,

compared to the value of 0.45 used here: this could easily account for the ten-fold difference in rate estimation. The antibody used for the SP radio-immunoassay is specific to the C-terminal region of the SP peptide, and will not detect the larger precursor peptide if it is present in the axons. Any changes in retrograde transport of SP-like immunoreactivity are therefore specific to SP itself.

The retrograde transport of SP was apparently unaffected by acute injury to the sciatic nerve, up to 22h post-injury. In the intact nerve, retrograde transport of SP is 40% of its anterograde transport. A crush injury cuts off the distal supply of SP returning from the axons, and induces turnaround of anterogradely transported SP at the crush; since there is a relatively low retrograde transport in control nerves, an increase in retrograde transport after injury similar to that seen for AChE might be expected. However, the location of the injury on the axon, relative to the soma will affect the amount of retrograde transport, since an injury closer to the cell body will reverse material originally destined for a longer length of distal axon. It may be that for substance P transport, cutting off the distal supply by making the injury balances the increased retrograde transport induced by premature reversal at the crush. In addition, AChE and SP are travelling in different axons, and in different organelles; the efficiency of the turnaround process developing at the crush may be less for the large dense-cored vesicles with which SP is associated (Brimijoin et al 1980) than for the smooth endoplasmic reticulum-like

vesicles containing AChE (Brimijoin 1979).

MChR is transported mainly in the motor axons of the sciatic nerve, as shown by a marked reduction in its anterograde transport after lesion of the ventral horn (Gulya and Kasa 1984). These receptors may be destined for the synaptic membrane, where they could act as feedback receptors for the amount of acetylcholine present in the syaptic cleft as has been previously suggested (Gulya and Kasa 1984). It has also been suggested that MChR are found in sympathetic neurons, and are transported in association with noradrenaline and dopamine-B-hydroxylase (Laduron 1984). The rate of anterograde transport of these receptors in sympathetic neurons has been previously reported as 65mm/day (Laduron 1984), and this agrees fairly well with the rate of 82$\pm$12mm/day calculated here.

A marked drop in the retrograde transport of MChR was measured at 16h after injury to the sciatic nerve. As explained previously, no changes were seen earlier than this because of the time taken to develop the turnaround process at the crush, and the distance of the point of measurement from the crush. The retrograde transport of MChR in intact nerves is about 80% of the amount anterogradely transported, and so the crush injury will cut off a substantial retrograde supply; this is likely to be the cause of the fall in retrograde transport at 16h post-injury. At 22h, there is some recovery towards control levels; by this time the turnaround process is well established and apparently capable of returning large amounts of MChR towards the soma.

The different responses of AChE and MChR to injury are probably a result of differential processing at the crush site of the organelles with which they are associated. However, it may also be due to the two substances being transported in different axonal types, which have different capabilities to evolve a reversal of axonal transport at a crush site: AChE is found in motor axons associated with SER-like vesicles (Brimijoin 1979), while MChR is probably in both motor (Gulya and Kasa 1984) and sympathetic axons in dense cored vesicles (Laduron 1984).

The properties of proteins such as AChE and MChR may be altered at the axonal terminal prior to or as a prerequisite for retrograde transport. This phenomenon is seen for another enzyme, dopamine B-hydroxylase, where the enzyme is only 20% as active when returning to the cell body (Tsukita et al 1980). Zarbin et al (1982) have described a decreased agonist affinity of MChR when it is undergoing retrograde transport. It is possible that changes such as these are related to the recycling of these proteins, in that a protein which has performed its function at the axonal terminal becomes "worn out" and this is a signal which permits its association with the retrograde transport system. It would be interesting to know whether the MChR returning from a distal injury also has decreased agonist affinity, which would indicate that similar reversal processes operated in the intact nerve and at a site of injury, and experiments aimed at determining this were performed. The assay we used to test this worked fairly well for

anterograde transported receptors, and the specific binding of the [3H]- QNB was progressively decreased by increasing the carbachol concentration. However, in all of the assays for retrograde transported receptors, the addition of carbachol to the incubation medium caused an increase in ligand binding which in many cases gave readings higher than the "total binding" determined in the presence of ligand alone. This is highly unusual, and difficult to explain except in terms of an interaction between the QNB and carbachol which increases the binding affinity of the ligand. As a result of this problem, it was not possible to measure the agonist affinity of retrogradely transported receptors in the injured nerve, and so no comparison with the anterograde transported ones could be made.

Reversal of axonal transport at an injury has been previously described (Bisby and Bulger 1977). In these experiments, rat dorsal root ganglion cell were injected with 3H leucine, and "waves" of radioactive proteins were detected travelling along the sciatic nerve. A retrograde "wave" was seen at earlier times in a nerve with a distal crush injury, indicating the reversal of axonal transport at the crush site. The time taken for the turnaround process to be established was calculated at 1.9-2.4h. The time course of changes measured here for individual proteins fits quite well with these calculations, in that several hours were required for anterograde transport, turnaround, retrograde transport and accumulation at the collection sites. 16h is somewhat longer than would seem necessary from theoretical

calculations, but it may be that the specific components studied here behave differently from the groups of proteins which are detected by injection of radioactive precursors into the cell bodies. Also, the changes recorded at 16h post-injury may have been detectable earlier, but levels of retrogradely transported materials were not measured at times between 10 and 16h after axotomy.

The collection crush technique used in these experiments provides a simple and useful way of assessing amounts of axonally transported materials, and of concentrating it for assay procedures. The measured concentrations of material in the isolated segment (segment 3) can be used to calculate transport velocities. These velocities agree fairly well with those calculated by other workers, although the ligation technique cannot distinguish a spectrum of velocities either in a single axon, or in different axonal types within the nerve. The possibility that material accumulated adjacent to the ligatures was locally synthesised was eliminated in these experiments by the use of the isolated segment of nerve between the collection crushes. The levels of AChE, SP and MChR in the isolated segment were similar to those found in parts of the nerve far distal to the collection area. For all three molecules studied, it must be remembered that it is their activity and binding properties which are being measured; for AChE, enzymatic activity, for SP, immunoreactivity, and for MChR, ligand binding properties. The possibility that such properties may be altered either by loading onto the intact retrograde transport system,

or by the turnaround process at a crush site cannot be excluded. However, the methods used give an accurate representation of the amount of material present, as shown by the consistent measurements of the amount of anterograde transport over time, as shown by the data plotted in figure 12.

The experiments described here have shown that the nerve cell body may detect quantitative changes in retrograde axonal transport after axotomy. It is likely that the individual substances studied here are representative of a larger class of membrane- and vesicle-associated proteins, whose processing will also be affected by axonal injury. Thus, the relative amounts of proteins returning to the soma after injury is probably quite different from those retrogradely transported in the intact axon, and the neuron may respond to such changes by inititating a regenerative response, first observable as chromatolysis. Qualitative changes in transport after injury are also possible, and these are discussed in chapter 3.

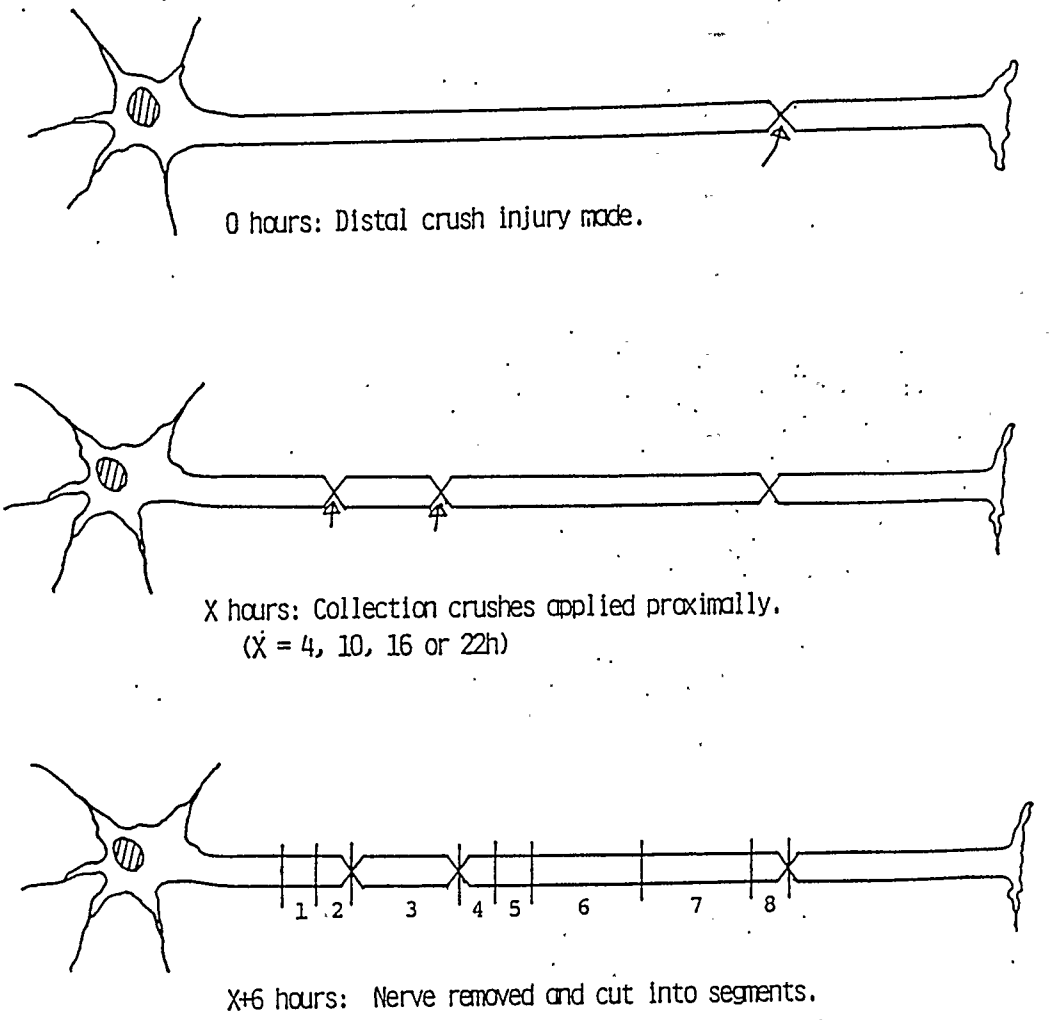


Figure 1. Summary of surgical and sectioning procedures for AChE, SP and MChR transport experiments. In some cases segment 3 was subdivided into three smaller segments to allow calculation of mobile fractions of materials in the axons.

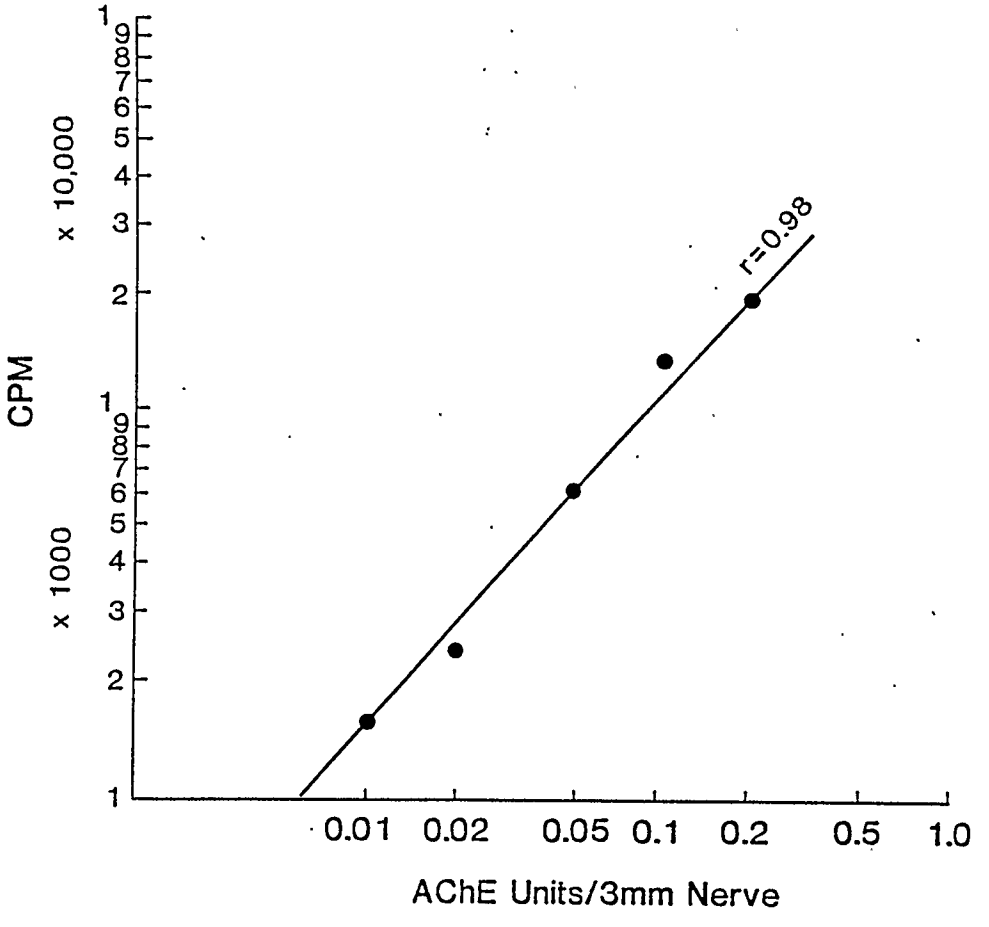


Figure 2. Typical standard curve for the AChE assay.

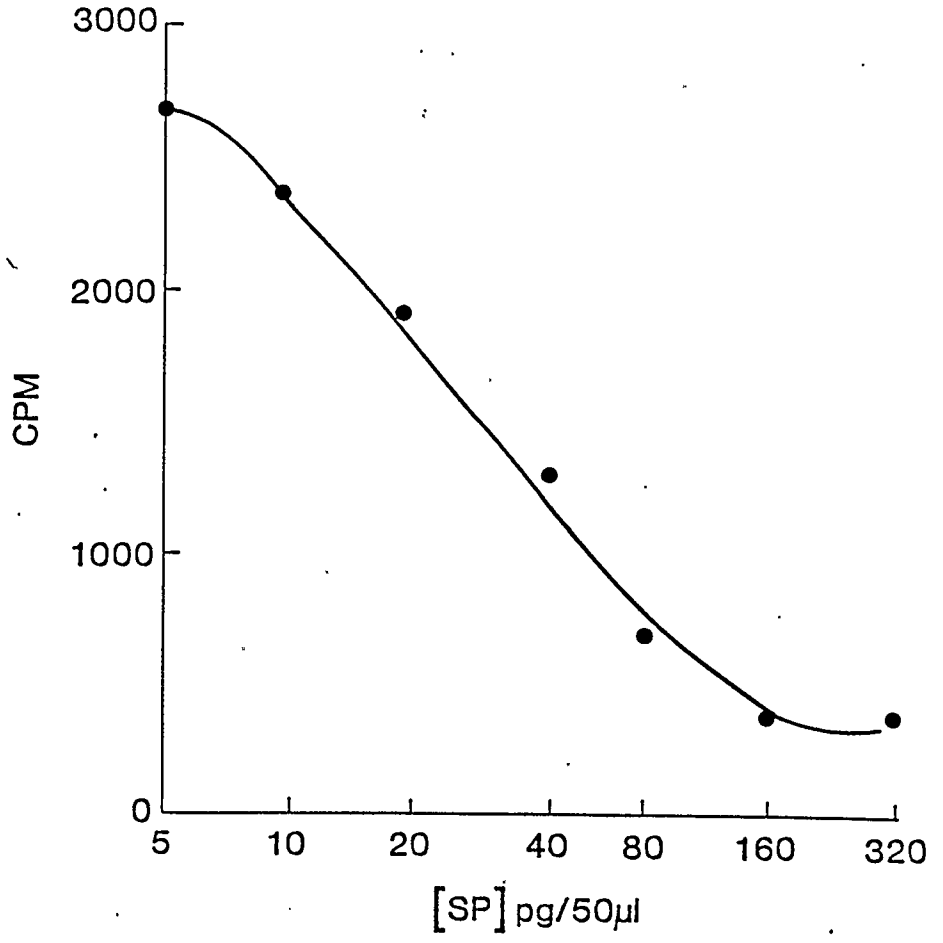


Figure 3. Typical standard curve for the SP radioimmunoassay, generated by the computer programme "Inhibition" (Barlow 1983).

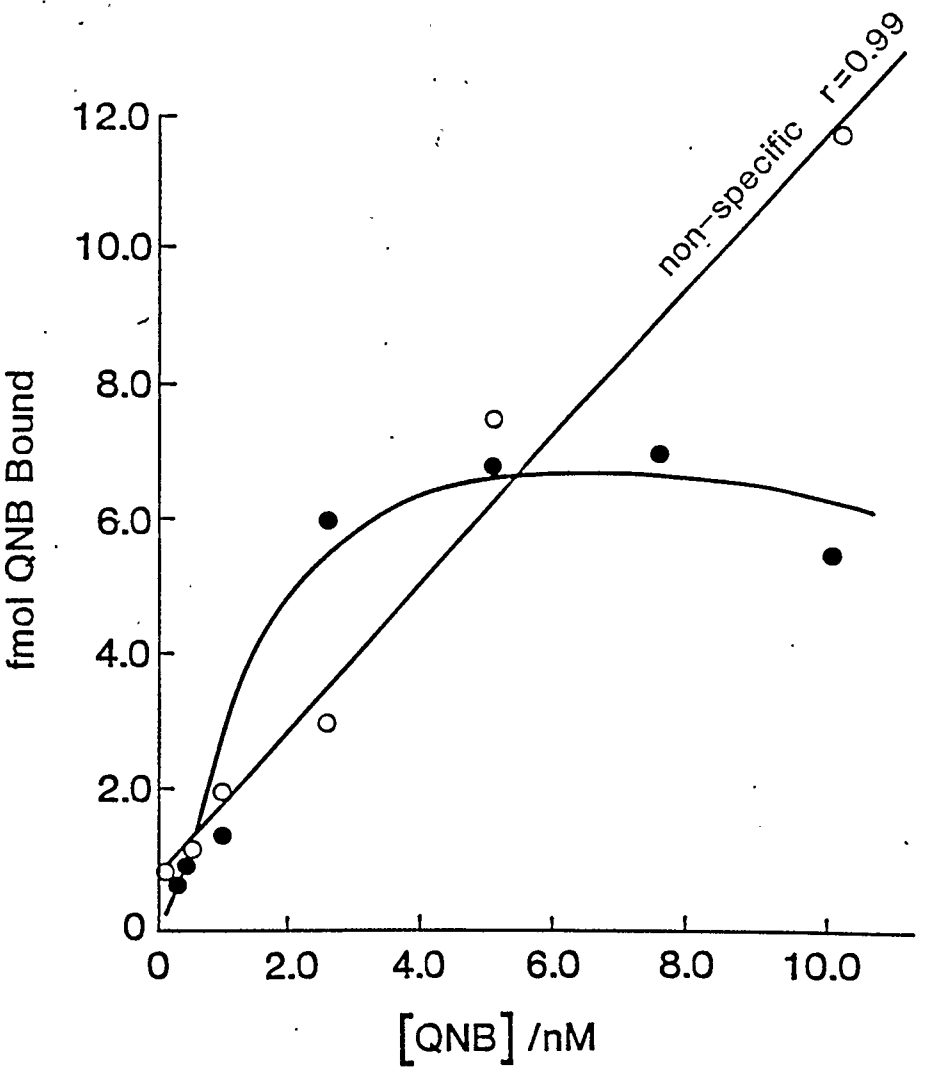


Figure 4. Curves for binding of 3H-QNB to rat sciatic nerve homogenate. ○ Non-specific. ● Specific.

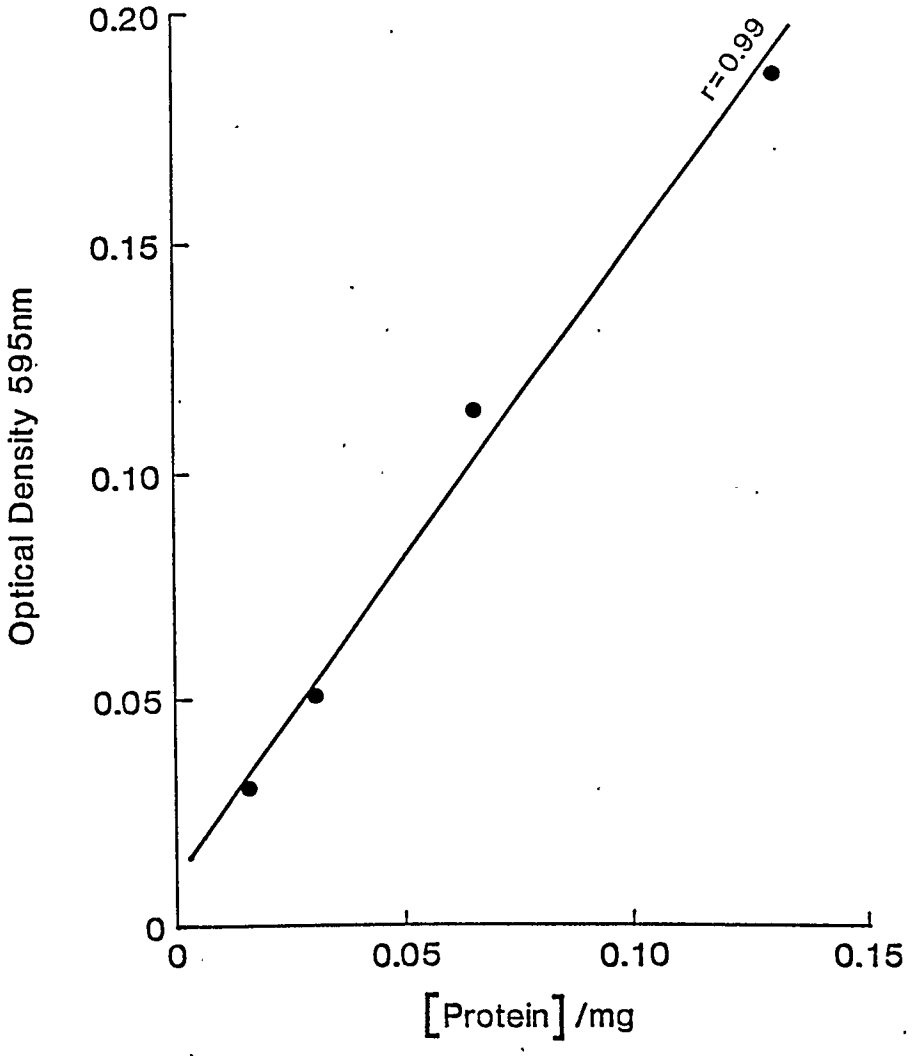


Figure 5. Standard curve for the BioRad protein assay.

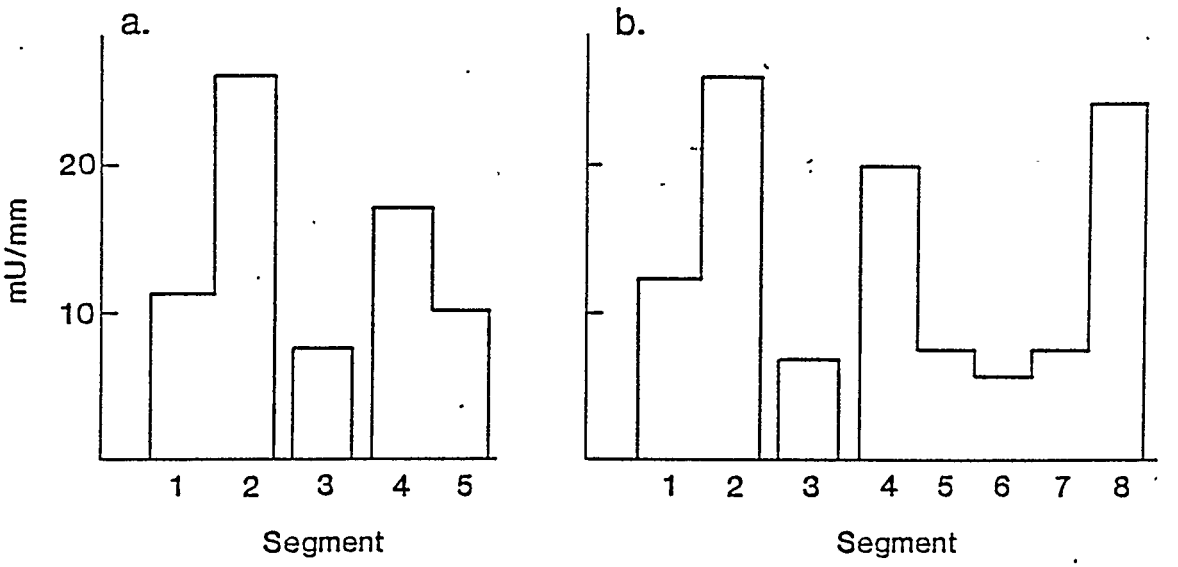


Figure 6. Comparison of the axonal transport of AChE in a) control and b) acutely injured rat sciatic nerves. Segment numbers refer to Figure 1.

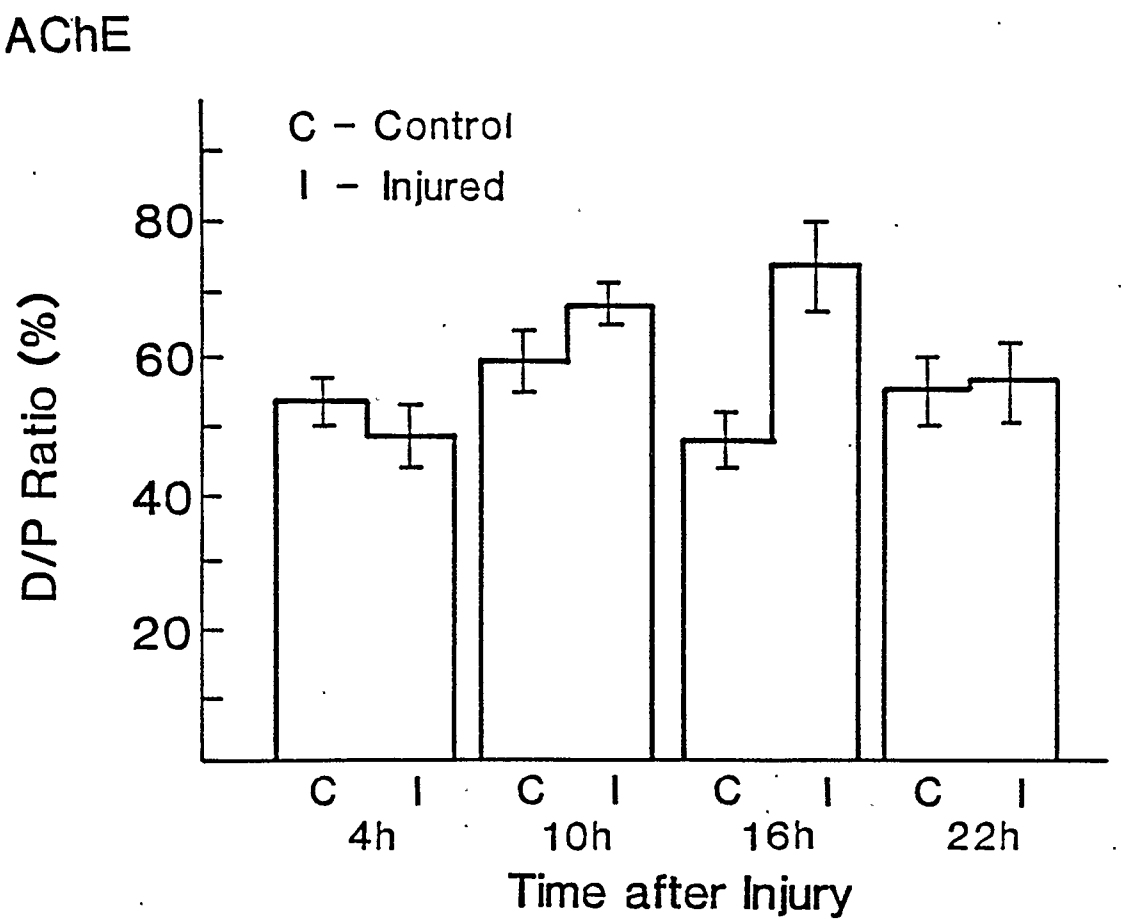


Figure 7. Summary of changes in axonal transport of AChE in rat sciatic nerve following distal injury. Bars are standard error of means (n=8).

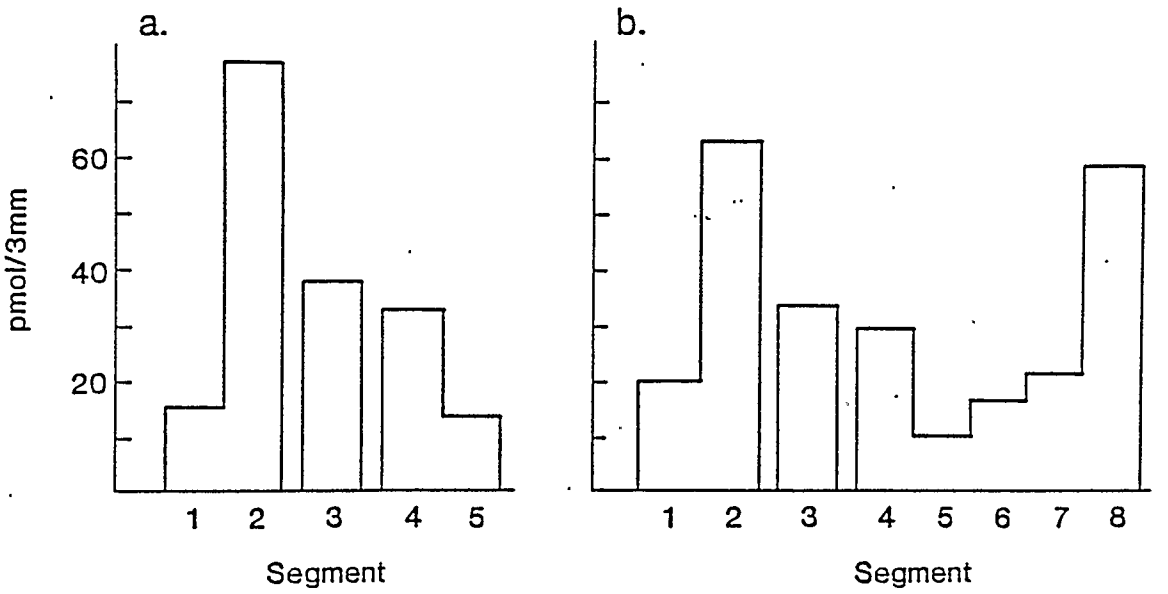


Figure 8. Comparison of the axonal transport of substance P in a) control and b) acutely injured rat sciatic nerves. Segment numbers refer to Figure 1.

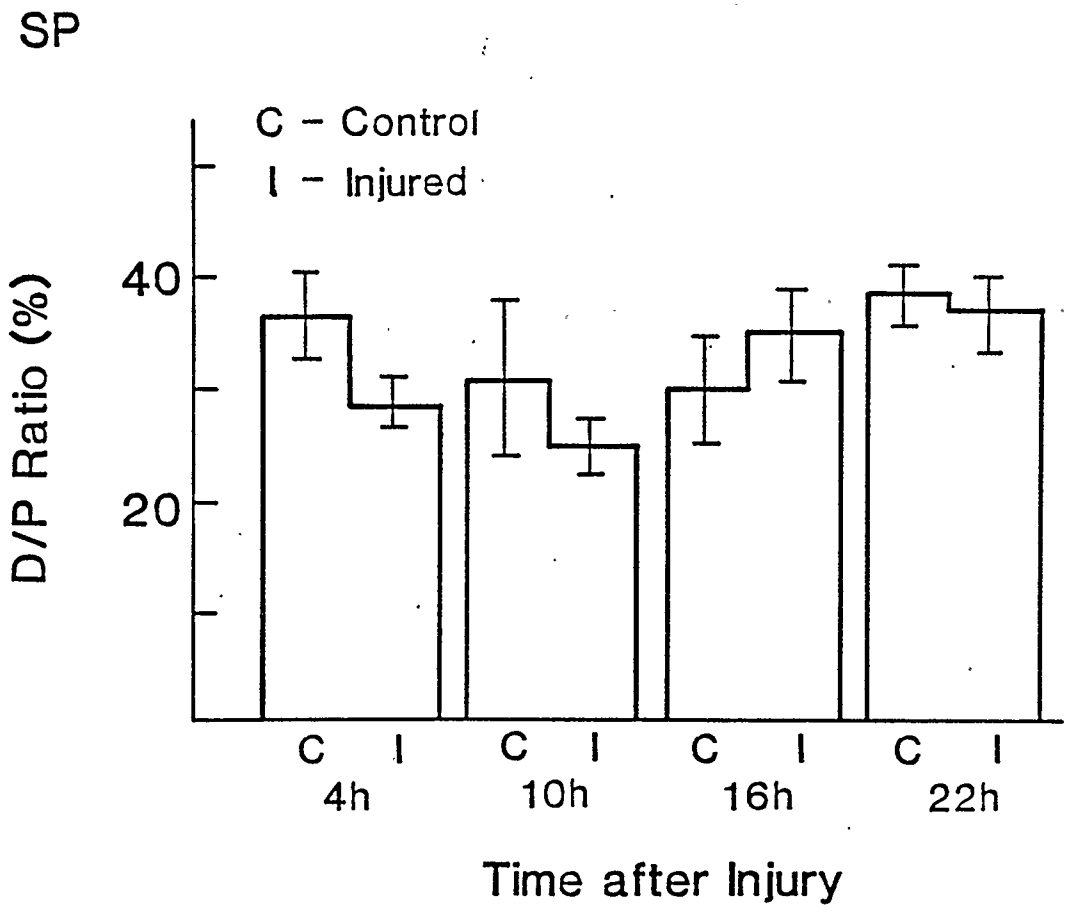


Figure 9. Summary of changes in axonal transport of SP in rat sciatic nerve following distal injury. Bars are standard error of means (n=8).

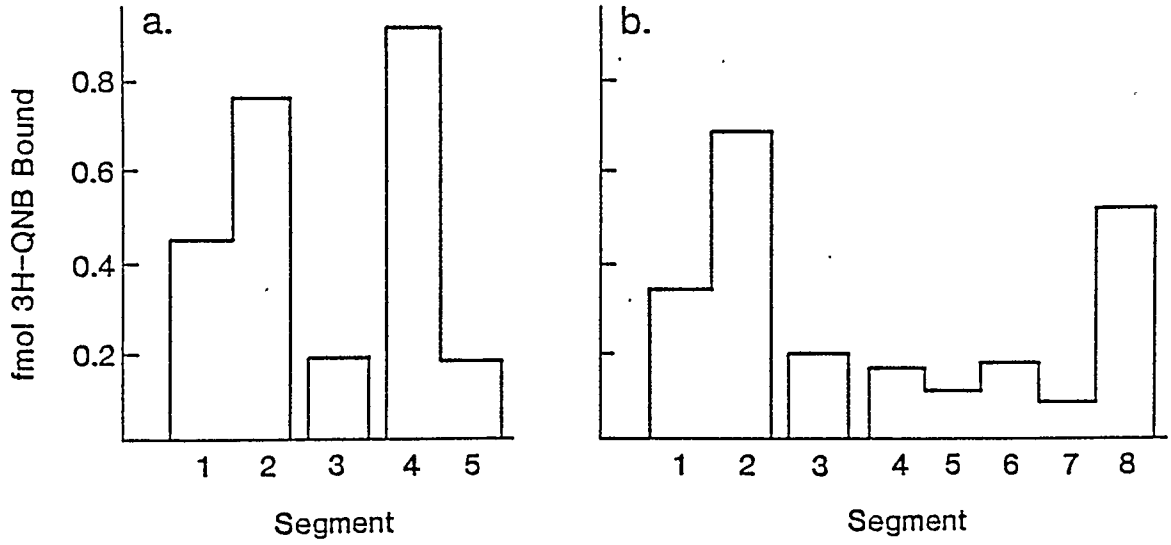


Figure 10. Comparison of the axonal transport of MChR in a) control and b) acutely injured rat sciatic nerves. Segment numbers refer to Figure 1.

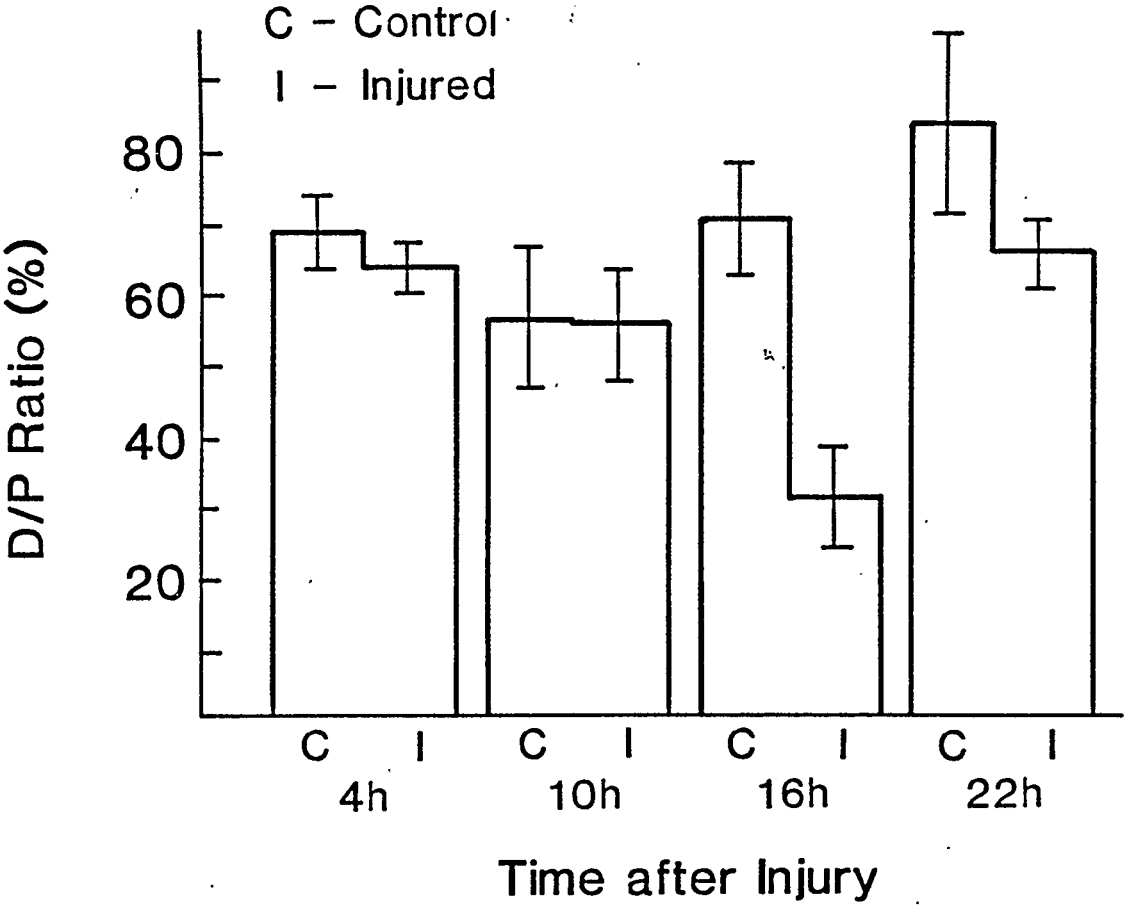


Figure 11. Summary of changes in the axonal transport of MChR in rat sciatic nerve following distal injury. Bars are standard error of means (n=8).

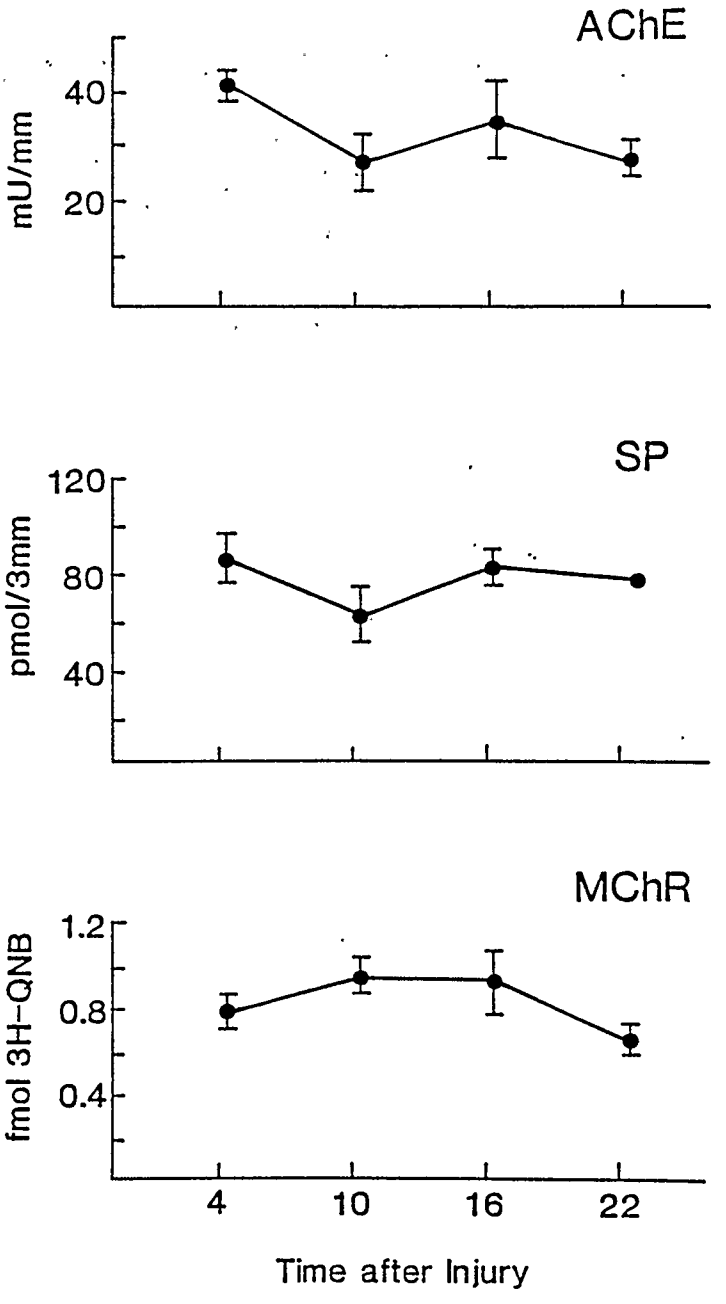


Figure 12. Anterograde axonal transport of AChE, SP and MChR in rat sciatic nerve at times up to 22h after distal nerve injury. Bars are standard error of means (n=8).

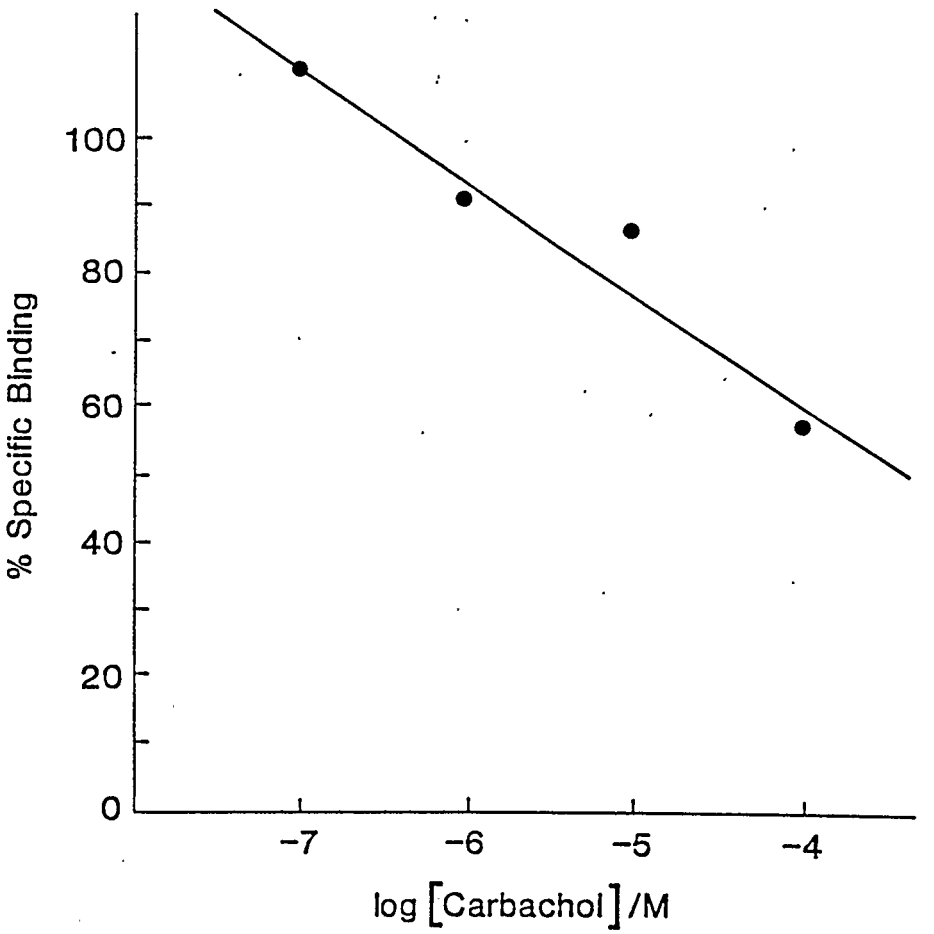


Figure 13. Reduction of the specific binding of 3H-QNB to anterogradely transported muscarinic receptors by the addition of carbachol.

THE RESPONSE OF SENSORY NEURONS TO AXONAL INJURY

If the signal initiating the cell reaction after axotomy is conveyed by retrograde axonal transport, it is prerequisite that such a signal would arrive at the soma before any signs of a cell body response can be detected. The exact nature of the signal is unimportant in this regard, since the rate of travel of a message conveyed by a change in retrograde transport will be similar, whether the cell is responding to a "positive" feedback from the injury site, or a "negative" change due to loss of a peripheral trophic factor.

Morphological changes in the cell body are unlikely to give a true indication of the time of onset of the cell reaction, since they occur as a result of metabolic changes which have begun earlier. In addition, in some cell types it is difficult to detect early morphological changes, since they are similar in appearance to morphological profiles seen in the intact cell; for example, Matthews and Raisman (1972) reported that normal rat superior cervical ganglion cells contain irregular clumps of endoplasmic reticulum which can be seen with the electron microscope, and these are indistinguishable from clumping of the endoplasmic reticulum induced during the first 24h after axotomy. Early metabolic changes seem to be the best indicators of a cell reaction, since they can be shown to begin soon after axotomy and are more easily detectable using biochemical assays.

The earliest changes induced by axotomy of peripheral neurons which

have been detected to date are alterations in the expression of RNA. Austin et al (1983) have studied 18S and 28S RNA in the rat nodose ganglion after vagotomy, and measured an increase in its synthesis peaking at 3 days post- axotomy. Miller et al (1987) have recently shown an increase in the expression of the tubulin gene Ta1 in the axotomised rat facial nucleus, beginning only 4h after nerve lesion. This is now the earliest reliable marker for the cell body response so far discovered. Changes in mRNA are followed by complex and varying alterations in protein synthesis, depending on the cell type studied. These changes have been discussed in the Introduction.

At the time the experiments in this chapter were performed, the earliest indicator of a cell body response was an increase in enzyme activity. Gilad et al (1985) studied the rat superior cervical ganglion after axotomy and reported a very rapid increase in the measurable activity of the enzyme transglutaminase, which incorporates polyamines into proteins. The increase in activity was seen as a 150% rise, measurable at 30min after axotomy of the post-ganglionic nerve trunk at a distance of 5mm from the ganglion. By one hour after axotomy, the increase was no longer measurable. It is not clear whether this is due to an increased synthesis of the enzyme, or an activation as a result of altered conditions in the cell such as increased calcium levels. Transglutaminase was chosen for study because it is likely to respond rapidly to changes in calcium levels induced by axotomy.

The experiments described in this chapter were designed to

investigate two aspects of the signal for chromatolysis. Firstly, to test whether the retrograde tranport system in an acutely injured nerve is capable of returning material from the injury site to the cell body, and the time course of any such observations. Snyder (1986) has reported that radiolabelled proteins in the frog sciatic nerve do not appear to return to the soma from a crush site on the nerve, but become deposited in the proximal axon. For retrograde transport to carry a chromatolytic signal, it is necessary that such material does return to the soma, and this question was addressed here by the injection of rhodamine labelled latex beads into the sciatic nerve, and demonstration of their presence in the cell bodies of the dorsal root ganglion at later times. A similar experiment has been performed previously (Kristensson and Olsson 1971b; 1975), applying horseradish peroxidase to a crush site on the rat sciatic nerve; the HRP was retrogradely transported to the cell bodies. Albumin (Kristensson and Olsson 1971b) and NGF (Schwab et al 1981) can also be retrogradely transported to nerve cell bodies.

Secondly, experiments were performed in the rat dorsal root ganglion to measure the activity of transglutaminase, since changes in the levels of this enzyme seem to be a very early indicator of a cell reaction. If a similar response to that measured by Gilad et al (1985) in the rat superior cervical ganglion could be detected in the dorsal root ganglion, then the much longer length of nerve available in the DRG-sciatic nerve system could be manipulated in several ways to

distinguish the nature of the signal for chromatolysis. These are explained further in the discussion.

METHODS.

i)Injection of rhodamine-linked beads.

Male Sprague-Dawley rats (250-350g) were anaesthetised with sodium pentobarbitol (i.p., 50mg/kg). The right sciatic nerve was surgically exposed and an acute injury was made by ligating it with 4-0 silk thread at a distance of 4-5cm from the spinal cord. 1µl of a solution containing rhodamine-linked latex microspheres 0.1µm in diameter (Tracer Technology, NY) was injected 1mm proximal to the ligation injury. This was done using a 30g stainless steel needle attached to a Hamilton syringe; following expulsion of the liquid into the nerve, the needle was left in place for 10min to prevent leakage. The muscles of the leg were sutured and the skin was closed with wound clips. The animals were allowed to survive 4 days and then killed by sodium pentobarbitol overdose and cervical fracture. The L4 and L5 dorsal root ganglia on the injected side were removed and placed on a microscope slide in several drops of Locke's solution. The ganglia were desheathed using Dumont fine forceps, flattened beneath a coverslip and viewed under rhodamine fluorescence on a Zeiss microscope.

ii)Transglutaminase assay.

This assay measures the addition of radiolabelled putrescine to the protein dimethylcasein by the endogenous transglutaminase enzyme of the dorsal root ganglion. The radiolabelled protein is collected by centrifugation and can then be quantitated.

Surgery

Male Sprague-Dawley rats (250-350g) were anaesthetised with sodium pentobarbitol (i.p., 50mg/kg) and the right sciatic nerve surgically exposed. The nerve was injured by tying a 4-0 silk thread tightly around it at a distance 30mm from the spinal cord. After various times from 4 to 26h, the rats were killed by pentobarbitol overdose and the L4 and L5 dorsal root ganglia were removed for assay. Corresponding contralateral ganglia were also removed and assayed as controls.

Preparation of tissue and assay reagent

The assay reagent was prepared as follows:

Sodium chloride	30mM
TRIS HCl	10mM
Calcium chloride	5mM
Dithiothreitol	10mM

Sodium hydroxide was added to the solution to give a pH of 7.5, in a

total volume of 50ml. 0.2g of dimethylcasein was added to give a large amount of external protein for the addition of radiolabelled putrescine, and stirred to dissolve. This assay reagent was kept up to 1 week at 4°C as stock solution. Immmediately prior to the assay, 10μCi of [1,4(n)-3H] putrescine dihydrochloride (Amersham, specific activity 30Ci/mmol) was added to 1.2ml of the reagent.

Dorsal root ganglia were homogenised in ice-cold Tris-HCl buffer (5mM, pH7.5), 200ul of buffer per ganglion, using a glass/glass homogeniser.

Assay method.

50μl of ganglion homogenate and 50μl of assay reagent containing [3-H] putrescine were mixed for each tube; samples were assayed in triplicate. Blanks for the assay contained 50μl of assay reagent and 50μl of Tris-HCl buffer. Samples were incubated for 20min at 37°C in a water bath, then the reaction was stopped by the addition of 500μl of ice-cold 10% trichloracetic acid (TCA) and allowed to stand for a further 20min. Tubes were centrifuged at 6000g for 5min, and the supernatant was aspirated without disturbing the pellet. The pellets were washed three times more with 500μl ice-cold 5% TCA, centrifuging each time. After discarding the final TCA supernatent, 500μl of tissue solubiliser (Protosol, New England Nuclear) was added and the tubes were left overnight at 50°C. The dissolved samples were then counted for tritium using toluene phosphor scintillation fluid. Figure 1 shows

a standard curve obtained for the transglutaminase assay by increasing the volume of rat sciatic nerve homogenate in each assay tube and measuring the corresponding increase in the radioactivity of the protein pellet. There is apparently some uncatalysed addition of 3H putrescine to dimethylcasein, since the blanks containing no nerve homogenate still contain several hundred cpm in the pellet, as shown in the figure.

RESULTS.

i) Rhodamine-linked beads.

When the dorsal root ganglia ipsilateral to the injected sciatic nerve were examined, several interesting features were noted. Firstly, cell bodies which clearly contained fluorescent beads were seen in several areas of the ganglion; one such group of cells is seen in figure 2. Since the site of injection of the beads into the nerve was approximately 4cm from these cell bodies, these beads must have arrived in the dorsal root ganglia via retrograde axonal transport. This demonstrates that the retrograde transport system in the injured axons remains viable, and is capable of transporting material from the lesion site to the soma; it is thus very likely that anterogradely transported material which reverses its direction at the crush can also be returned to the cell body. The labelling pattern of groups of cells in the ganglion is probably due to the selective exposure of certain axons to

the rhodamine-linked beads at the injection site; the sciatic nerve contains many thousands of axons, and the injection of only 0.1ul of solution is unlikely to expose the damaged portion of all the axons to the beads. This also demonstrates the high affinity which the transport system must have for the beads. Other labelling patterns were also seen in the dorsal root ganglia. Filamentous networks and labelled proccesses such as those seen in Figure 3 were observed, and appear to be axons containing labelled beads, giving the axons a reticulated appearance.

ii) Transglutaminase assay.

Figure 4 shows transglutaminase activity in the L4 and L5 dorsal root ganglia plotted against time after axotomy of the sciatic nerve 4cm from the spinal cord. Each point on the graph represents the mean ratio of activity $\pm$ SEM between the contralateral and ipsilateral DRG in a single animal; a greater number of animals were used for times between 0-4h after axotomy, since this is the time period in which a signal conveyed by retrograde axonal transport would be expected to reach the cell bodies after an injury 4cm away. Injury to the sciatic nerve did not induce any detectable rise in transglutaminase activity in the DRG at any time measured up to 26h after axotomy.

DISCUSSION.

The presence of rhodamine-labelled beads in the cell bodies of the

dorsal root ganglia indicates that the retrograde transport system in the injured sensory axons is capable of transporting material from the crush site to the soma. Since the diameter of the beads is greater than that of the endogenous retrogradely transported components, as shown by the reticulation of the axons produced by the presence of beads, it is probable that smaller diameter anterogradely transported material arriving at the crush site can return to the cell bodies in the dorsal root ganglion following its directional reversal. It has previously been reported that such material may not return to the cell bodies of the frog DRG (Snyder 1986). These experiments used an apparatus which acts as a series of radiation detectors and which can follow radiolabelled material in the axon following a pulse-label of tritiated amino acid to the cell bodies. A wave of labelled material was measured travelling down the axons, and also a wave which had reversed direction at an acute lesion site and was travelling back towards the ganglion. However, the retrogradely-transported wave dispersed as it travelled, and could no longer be detected as a peak of activity at a distance 10mm from the ganglion. These experiments suggested that turnaround-transported material did not reach the soma, although a retrograde wave could be sufficiently dispersed along the axon that it was no longer detectable as a peak of activity above the background radioactivity in the nerve. The experiments performed here have shown that material can be transported from a site of injury to the cell body several centimetres away.

The experiments to measure the activity of the enzyme transglutaminase in the dorsal root ganglion were undertaken to see if an early increase in enzyme activity could be detected after an axotomy. A rapid, transient rise in transglutaminase activity has been detected in the rat superior cervical ganglion following a post-ganglionic axotomy 5mm from the ganglion (Gilad et al 1985). This system has only a short length of post-ganglionic nerve, and surgical access is difficult. We attempted to reproduce the results of Gilad et al (1985) in the rat dorsal root ganglion, since the sciatic nerve has a 5-6cm length available for experimental manipulation. An early, transient indication of the cell body response to axotomy could be used in such a system to distinguish between a "positive" signal arising from the injury site, and a "negative" one due to the loss of a peripheral trophic factor. This distinction could be achieved in several ways:

a) A second lesion made at the same injury site several hours after the early response to the first lesion would only induce a second response in the cell body if the signal were arising from the injury site (a "positive" signal);

b) The application of the microtubule-disruptor and transport blocker colchicine to the nerve between the lesion site and the cell body would accelerate the cell body response if the signal for chromatolysis were a "negative" one, and would delay the response by preventing the signal reaching the soma if it were a "positive" one;

c) An early response to axotomy such as an increase in transglutaminase should be preventable if the signal were "negative" by application of nerve growth factor to the cut end of the nerve, assuming that this is the peripheral trophic factor required for the maintainance of sensory neurons, as suggested by Miyata et al (1986).

These experiments are all dependent on the discovery of an early, transient, reliable and easy to measure component of the cell body response in the dorsal root ganglion-sciatic nerve system. We were unable to detect any early changes in the levels of transglutaminase activity in the dorsal root ganglion on the lesioned side up to 26h after axotomy. Other workers have tried similar experiments in rat nodose ganglion (Gilad, personal communication) and rat facial nucleus (Tetzlaff, personal communication), and also measured no early increase in activity. Thus, it seems that either the superior cervical ganglion has a unique, rapid response to axotomy, or that the axonal enzyme is activated by increased calcium concentrations only at the lesion site, and that this was construed by Gilad et al (1985) as being a cell body response because of the proximity of the lesion to the soma in their experiments.

These experiments indicate that retrograde axonal transport could be involved in the signal for axotomy, since material from a lesion site on the sciatic nerve can return to the sensory neuron cell bodies in the dorsal root ganglion. It was not possible to further characterise the nature of the chromatolytic signal because no early rise in

transglutaminase activity as an indicator of chromatolysis could be detected in the sensory neurons. It would still be possible to perform the experiments outlined here if a more reliable indicator were to be discovered in this peripheral system. Recently, it has been reported that the expression of a-tubulin genes in facial motoneurons is altered by axotomy (Miller et al 1987). The change is detectable as early as 4h after facial nerve crush, and is a possible candidate for further experiments on the nature of the signal for chromatolysis.

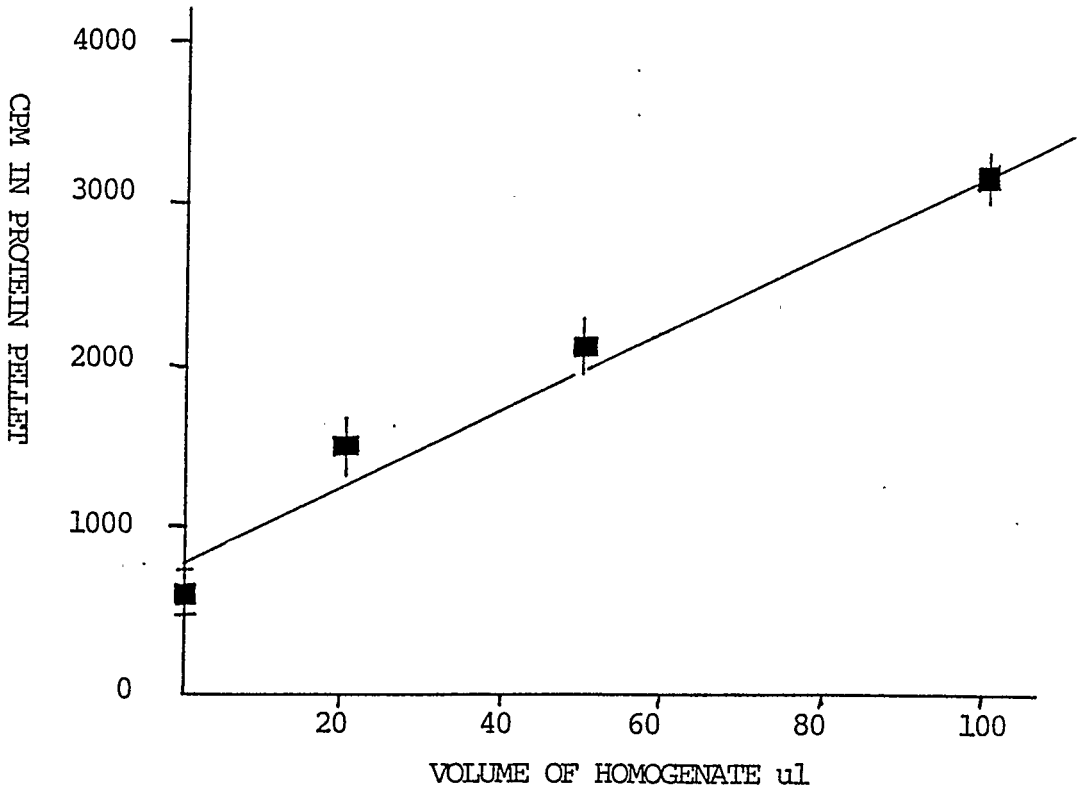


Figure 1. Increase in transglutaminase activity with increasing volume of rat sciatic nerve homogenate. Bars are S.E.M., (n=3)

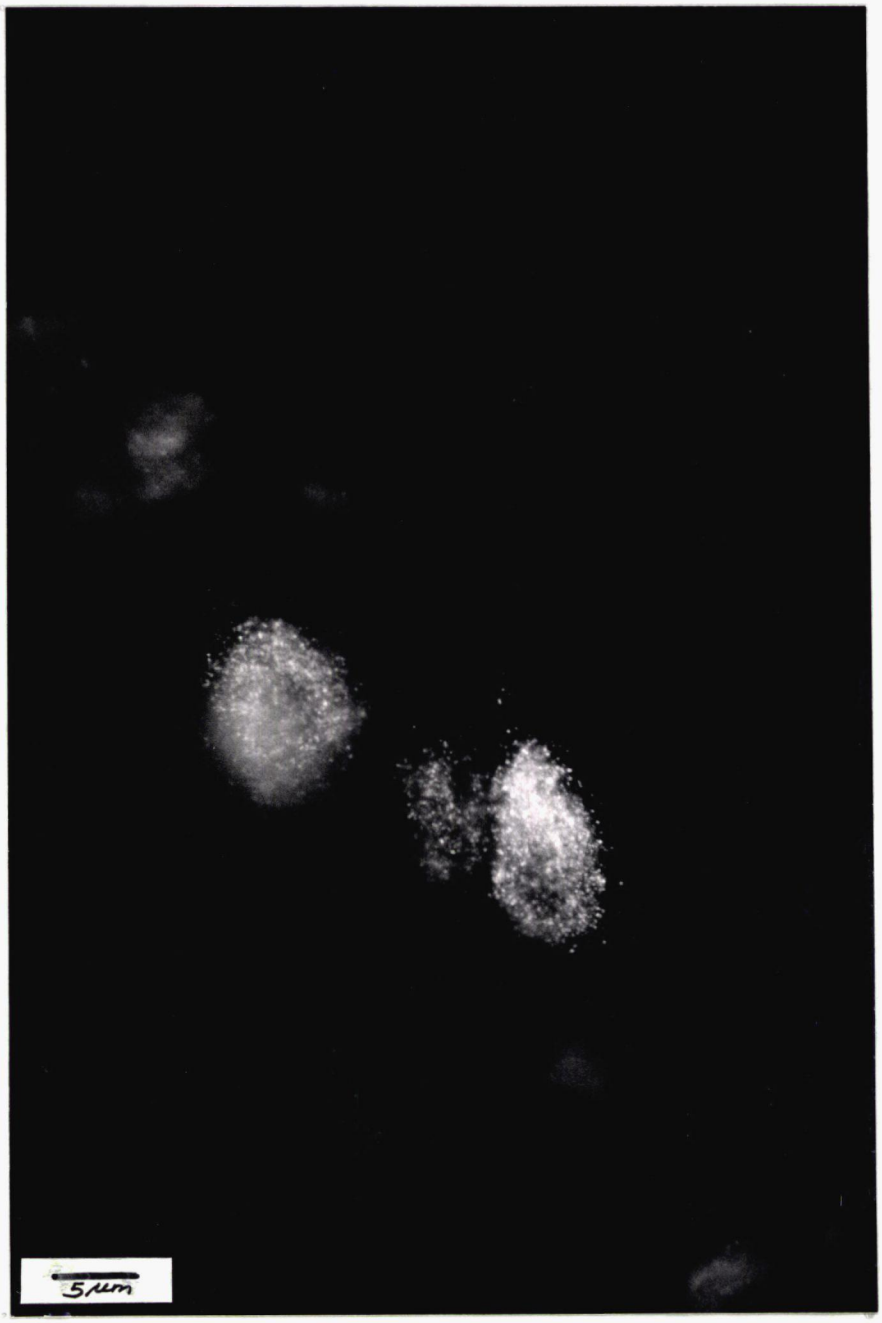


Figure 2. Cell bodies of rat L5 dorsal root ganglion labelled with rhodamine-linked microspheres. The beads were injected proximal to a sciatic nerve crush, 4 days earlier.

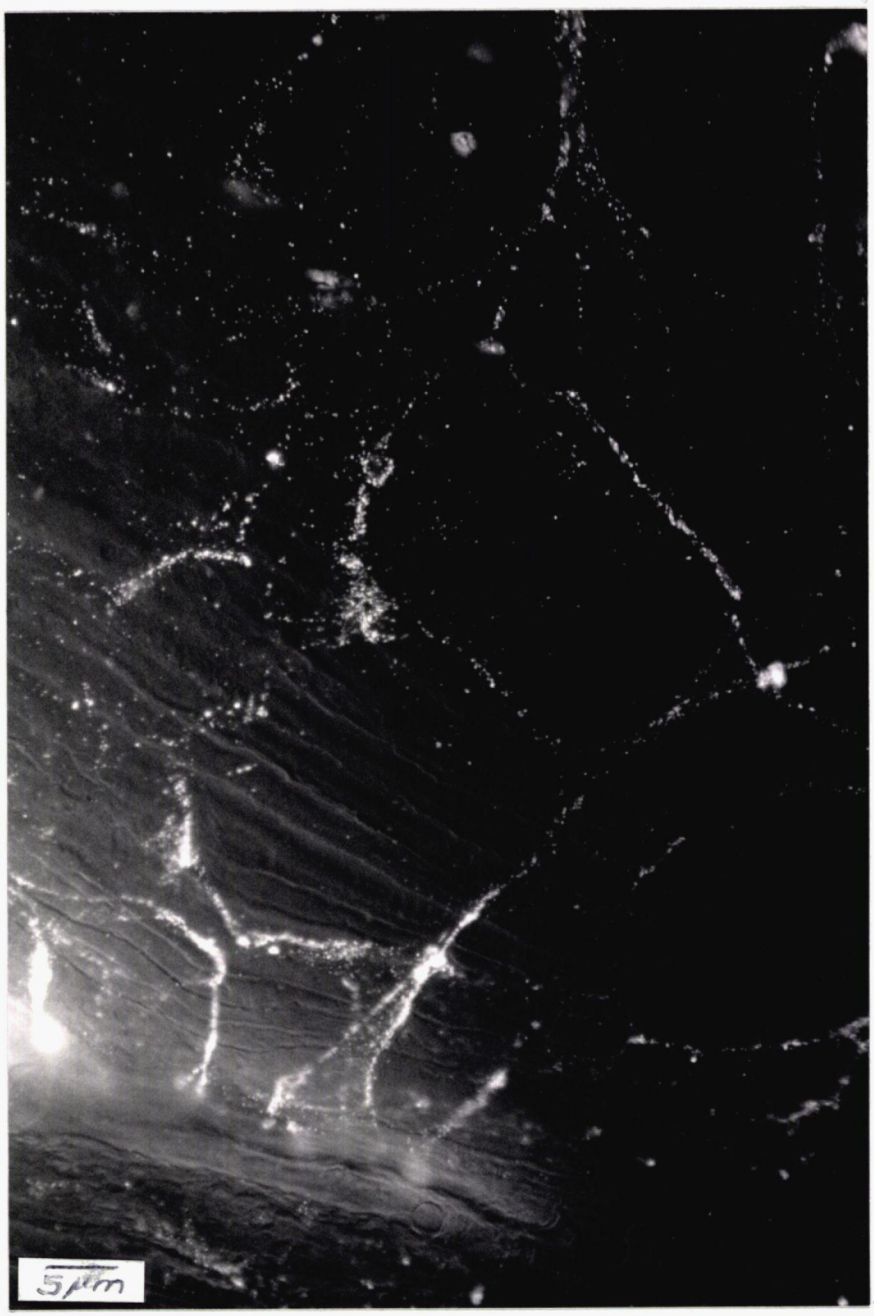


Figure 3. Processes in rat dorsal root ganglion labelled with rhodamine-linked microspheres, 4 days after injection of the beads into the sciatic nerve. The processes are probably axons, given a reticulated appearance by the presence of the beads.

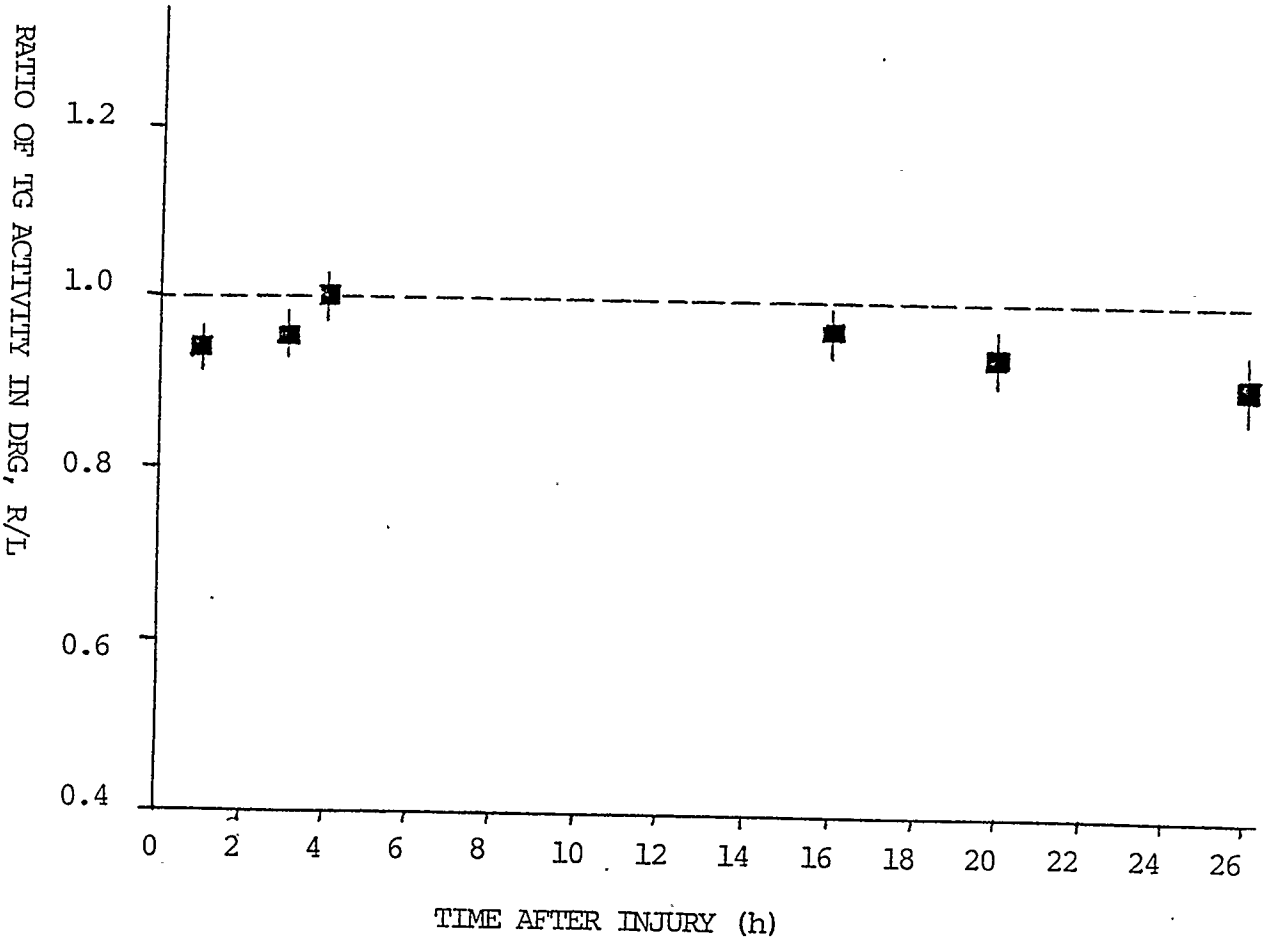


Figure 4. Summary of results from transglutaminase assays at various post-injury times. Bars are S.E.M., (n=8).

2D GEL ANALYSIS OF AXONALLY TRANSPORTED PROTEINS

The experiments described in this chapter are concerned with the analysis of fast axonally transported proteins in the rat sciatic nerve under several different conditions. These experiments were designed to make a more general analysis of axonally transported components after an acute nerve injury, since changes in individual proteins such as AChE and MChR had already been made, and it was thought that these may have been representative of a wider spectrum of alterations in transport induced by injury.

Electrophoresis was chosen as a method of protein separation because the proteins of fast axonal transport are associated with intra-axonal membranes (Willard et al 1974), and a detergent such as sodium dodecyl sulphate is needed to solubilise them. The technique of protein separation by two- dimensional gel electrophoresis was developed by O'Farrell (1975). Previous work on the electrophoretic analysis of fast axonally transported proteins has concentrated on changes seen in anterograde transport during regeneration, in a variety of systems; for example, studies have been made in regenerating goldfish optic nerve (Freeman et al 1986; Schwartz et al 1985; Perry et al 1987; Yoon et al 1986), in regenerating rat sciatic and hypoglossal nerves (Skene and Shooter 1983; Redshaw and Bisby 1984), and in frog and toad sciatic nerves (Perry et al 1983; Skene and Willard 1981a). Particular attention has focussed on the growth-associated protein GAP43, in the

systems mentioned above and in mammalian central and peripheral nervous systems during development and regeneration (Jacobson et al 1986; Skene and Willard 1981b; Reh et al 1987). Two dimensional gel electrophoresis has been done on fast anterogradely transported proteins in intact and regenerating frog sciatic nerve (Stone et al 1978; Perry et al 1983).

In the experiments described in this chapter, fast anterograde and retrograde transported proteins have been studied and compared in intact, acutely injured and 7day regenerating rat sciatic nerves. Axonally transported proteins were separated by two-dimensional, sodium dodecyl sulphate, polyacrylamide gel electrophoresis (2D SDS PAGE), with the following aims:

a) To study changes with time in protein composition of fast anterogradely transported material, following the injection of 35S methionine into the vicinity of the cell bodies of intact dorsal root ganglion neurons. Studies of the proteins transported in rabbit and toad optic nerves have been reported, classifying proteins transported within certain velocity ranges into five groups (Willard et al 1974; Willard and Hulebok 1977). However, a similar study has not been performed for the rat peripheral nervous system. Anterogradely transported proteins were also compared with those undergoing retrograde transport in intact nerves, to look for differences in composition which may result from protein degradation or secretion (Stone et al 1984) at the axonal terminals.

b) To look for changes in protein patterns which may be induced by acutely injuring the nerve, and to examine further changes during nerve regeneration. Alterations in the composition of anterogradely transported material may result from altered synthesis of proteins necessary for the regeneration process, such as the growth associated protein, GAP43 seen by Skene and Willard (1981a and b). Changes in retrograde transport may result from differential processing of proteins in the distal axon regions in intact, acutely injured and regenerating nerves.

The time course of reversal of anterograde transport in intact and injured nerves has been previously calculated, measuring "waves" of radiolabelled protein in the rat sciatic nerve (Bisby and Bulger 1977). A crush injury to the nerve was found to induce a premature and increased return of labelled protein towards the cell bodies; the times for maximum transport of labelled proteins was used here to determine appropriate times for the analysis of the various protein fractions studied.

Such experiments may reveal novel changes, particularly in retrograde transport, since this has not previously been studied by 2D SDS PAGE. Changes in the composition of retrogradely transported proteins induced by acute nerve injury are of particular relevance to this thesis, since they may be detected by the soma and constitute part of an informational system which allows the cell to monitor axonal status.

METHODS

i) Surgery and injection of radiolabelled amino acid.

Male Sprague-Dawley rats (250-350g) were anaesthetised with sodium pentobarbitol (50mg/kg, i.p.). The L4 and L5 dorsal root ganglia were unilaterally exposed by dorsal laminectomy. The radiolabelled amino acid precursor was L-35S methionine (Amersham, specific activity 1120 Ci/mmol), which was concentrated by freeze drying and dissolved in Locke's solution to give 50μCi/ul. Each exposed dorsal root ganglion was injected with 1ul of precursor solution, giving a total of 100μCi per animal. The glass injection pipette was left in place for 5min following the injection, to reduce leakage of the precursor from the ganglion into the surrounding tissue. The exposed spinal cord and ganglia were covered with Gelfoam, the muscles of the back were sutured, and the skin closed with wound clips. The body temperature of the rats was maintained by heating until recovery from anesthetic. The animals were allowed to survive up to 30h, with the time of precursor injection being 0h.

Intact nerves. Animals were injected with precursor at time 0h and left 0, 10 or 24h. At this time, collection crushes were applied to the ipsilateral sciatic nerve, consisting of 4-0 silk thread ligations. For the short time period animals (0-6h collection), a single collection crush was applied to the nerve 3cm distal to the ganglia at the time of injection, and left in place for 6h to trap labelled, anterogradely transported material. For the later time periods (10-16

and 24-30h), two collection crushes were applied to the sciatic nerve, the proximal one being 3cm from the dorsal root ganglia, and the second being 1cm distal to the first. These crushes were also left in place for 6h, to collect both anterogradely and retrogradely transported material, and isolate a segment of nerve between the collection crushes to assess the amount of radiolabelled protein which is stationary in the nerve.

Injured nerves. The procedure was the same as for intact nerves, except that the sciatic nerve was injured 6cm from the dorsal root ganglia at the time of injection, 0h, by tightly ligating it with 4-0 silk thread. Anterograde and retrograde transport was studied in injured nerves at 10-16h after injection of labelled amino acid into the dorsal root ganglia.

Regenerating nerves. The procedure was the same as for injured nerves, except that the sciatic nerve was crushed 7 days before injection. This time was used because the rate of sensory axon regeneration has previously been calculated at 4mm/day with an initial delay of 37h (Bisby 1978), and so at this time, most axons will have regenerated approximately 22mm beyond the crush site. The crush site was the same as the acute injury site; the crush was performed by holding the nerve tightly in Dumont No. 5 forceps for 30s. Anterograde and retrograde transport was studied in regenerating nerves at 0-6 and 10-16h after injection of labelled amino acid.

At the end of the collection period, the rats were killed by sodium

pentobarbitol overdose. The collection crush region of the sciatic nerve was removed, giving a total length of 3cm, which was cleaned, placed on a card and rapidly frozen. Three, 3mm segments of the nerve were taken for 2D SDS PAGE; one proximal to the proximal ligature, one distal to the distal ligature and a third from between the two collection crushes. The 0-6h anterograde transport experiments in intact nerves yielded only the proximal 3mm segment, because this time point is too soon after precursor injection for any labelled protein to be returning along the axon.

The various transport fractions examined are summarised thus:

a) Intact nerves:

-anterograde transport at 0-6, 10-16 and 24-30h after injection;

-retrograde transport at 10-16 and 24-30h after injection.

b) Injured nerves:

-anterograde and retrograde transport at 10-16h after injection.

c) 7 day regenerating nerves:

-anterograde transport at 0-6h after injection;

-anterograde and retrograde transport at 10-16h after injection.

ii) Preparation of samples for 2D SDS PAGE.

(O'Farrell 1975; Wilson et al 1977).

Each 3mm segment of sciatic nerve was placed frozen in a stainless steel mortar at -70 °C, crushed with a cooled metal pestle and then placed in 60µl of lysis buffer (9.5M urea; 5% v/v 2-mercaptoethanol;

2% v/v NP40; 2% v/v carrier ampholytes with pH ranges 3.5-10, 4-6 and 6-8 from LKB, in the ratio 1:2:2) in a 1.5ml Eppendorf centrifuge tube. Samples were homogenised for 30s using a machined Teflon pestle, and centrifuged for 5min at 6000rpm in an Eppendorf centrifuge. A 5µl aliquot of each supernatant was removed and 10ml of scintillation fluid (Scintiverse, Fisher) was added; the aliquots were counted in a scintillation counter for 10min and the total radioactivity in the whole supernatant sample was calculated.

iii) 2D SDS PAGE.

(Adapted by J.D. Redshaw from O'Farrell 1975)

Protein samples were separated in the first dimension on 3mm diameter 10% urea isoelectric focussing gels. The remaining supernatant samples were focussed for 15h at a constant voltage of 400V, and a further 1h at a constant voltage of 800V. For pH calibration, an IEF gel with lysis buffer only was cut into 3mm pieces following the focussing run. Each piece was placed in 10ml of distilled water, left for 1hr, shaken, and the pH of the solution taken with a pH meter. Second dimension resolving gels were 5-15% polyacrylamide gradient gels with a 5% stacking gel. First dimension gels were sealed to the top of the stacking gels with a 1% solution of agarose in equilibration buffer (10% v/v glycerol; 5% v/v mercaptoethanol; 0.0625M TRIS; 3.6% w/v SDS). 3ul each of high and low molecular weight protein standards in 3.6% SDS (BioRad) were dissolved

in 4ml of agarose solution, and 0.5ml of this mixture was applied to the top right hand corner of each slab gel in a band approximately 2cm wide. Second dimension gels were run at an initial current of 30mA per gel, with a maximum voltage of 350V, for approximately 5h.

iv) Staining and fluorography.

Gels were placed in a stain solution of 0.1% w/v Coomassie Brilliant Blue R250 in 5% v/v acetic acid and 47.5% v/v ethanol. Gels were stained overnight and next day destained in 40% v/v methanol/ 5% v/v acetic acid, changing the solution as necessary to give a clear background. Staining was performed to check the resolution of proteins on the gels before further processing, and to visualise the protein standards. The gels were processed for fluorography according to the method of Laskey and Mills (1975) and Bonner and Laskey (1974). Gels were placed in dimethyl sulphoxide (DMSO) to remove water, then placed in a solution of PPO in DMSO (20% w/v) for 2h. The PPO was then precipitated by immersing the gels in distilled water. Gels were dried under heat and vacuum onto filter paper, placed in contact with a sheet of Kodak XAR film, and kept in the dark at -70°C. The film was preflashed for 7 seconds prior to exposure, as this has been shown to increase the linearity of the film-darkening process (Bonner and Laskey 1974). Fluorographs were developed after 6 weeks.

v) Scanning of fluorographs.

Scanning and quantitation of 2D fluorographs was done using an LKB Ultroscan XL laser densitometer connected to an IBM PCXT computer with LKB 2400 Gelscan software. A sample scan of a 2D fluorograph is given in Figure 1a, showing anterogradely transported proteins 24-30h after injection of precursor, in an intact sciatic nerve. Figure 1b shows a sample integration report generated by the analysis of the five proteins marked with boxes on the scan.

vi) Quantitation of proteins and comparison of fluorographs.

Individual protein spots were selected for quantitation on the basis of their prominence on the fluorograph, and observable differences between the fluorographs being compared. Proteins were identified on the corresponding gel scan, and a "volume" parameter was produced for each protein by the GelScan XL programme, which analyses an area defined by the user as a spot, on a pixel by pixel basis. The "volume" parameter is the scanned area of the spot multiplied by its density in absorbance units. For each spot scanned, a "density index" was calculated from:

$$\text{Density Index, D.I.} = \frac{\text{"volume"}}{\text{total cpm on gel}} \times 10{,}000$$

Since all the fluorographs had been exposed for six weeks, it was not necessary to adjust the D.I value to correct for exposure time.

A calibration curve for the densitometer programme was made to check for linearity of the "volume" parameter with increasing cpm. Using a fluorograph of a gel strip containing known amounts of activity from 1000 to 200,000 cpm, equal areas of each portion of the strip were scanned and the volume parameter plotted against cpm, as shown in Figure 2. This demonstrates that the scanning process is linear with increasing cpm over the range of activity obtained on 2D gels, which was 5000 - 70,000cpm in these experiments.

To check independently of the computer analysis that the D.I. value was representative of the amount of 35S activity associated with a protein, five prominent protein spots were cut out from a series of three gels, taken from the 0-6h transport fraction on regenerating nerves, using fluorographs of the gels as templates. Excised spots were rehydrated with a drop of water, solubilised in Protosol (New England Nuclear), 10ml of scintillation fluid was added, and the samples counted for 35S activity in a scintillation counter. A plot of D.I versus cpm in individual spots is shown in Figure 3; there is a good correlation between these two parameters, demonstrating that the density of spots as measured on fluorographs by the Gelscan programme gives a true representation of the activity in each spot.

For each experimental situation or time period post-injection studied, the D.I. values for up to 23 proteins were calculated from six to eight fluorographs of different nerve samples, and a mean D.I. value for each spot of interest obtained.

Comparisons between series' of fluorographs were made as follows; times are the post-injection period over which transported material was collected.

a) anterograde transport in intact nerves at 0-6, 10-16 and 24-30h, to examine changes with time after injection;

b) anterograde transport in intact, injured and 7 day regenerating nerves, all at 10-16h, to look for changes in protein composition which may be induced by injury and regeneration;

c) retrograde transport in intact nerves (24-30h) and in injured and 7 day regenerating nerves (10-16h), to see if injury to the nerve induces any changes in retrogradely transported proteins, and if any of these changes persist through regeneration;

d) anterograde versus retrograde transport in intact nerves (24-30h);

e) anterograde versus retrograde transport in injured nerves (10-16h);

f) anterograde versus retrograde transport in 7 day regenerating nerves, (10-16h);

Comparisons d, e and f were performed to look for differences in the composition of anterogradely and retrogradely transported proteins, which may give evidence of breakdown of proteins in the nerve terminals, or of secretion of some proteins, as has been suggested (Stone et al 1984).

g) anterograde transport (0-6h) versus retrograde transport (24-30h), in intact nerves;

h) anterograde transport in intact nerves (0-6h) versus retrograde

transport in injured nerves, (10-16h).

Comparisons g and h were made to test whether proteins anterogradely transported soon after precursor injection are later returned to the cell body by retrograde transport (Bisby 1981). In h, the 10-16h time point was used because retrogradely transported material returns towards the soma sooner in injured nerves than in intact nerves (Bisby and Bulger 1977).

i) anterograde transport in intact and regenerating nerves (0-6h), to examine changes in very rapidly transported material during regeneration.

In comparison c, retrograde transport in intact nerves at 24-30h after injection was studied, since the amount of labelled protein undergoing retrograde transport at 10-16h after injection is too small to give useful fluorographs after an exposure time of 6 weeks.

For comparisons a, b and c, one-way analysis of variance was used to test for statistically significant differences between the D.I. values of individual proteins; where necessary, pairwise comparison of the samples was performed using the least significant difference, Student's t test method. Significance of observed differences in the remaining comparisons (d-i) were assessed using the unpaired, two- tailed Student's t test.

vii) Half-time for free amino acid in ganglia

An experiment was performed to determine the length of time after

injection that the amino acid precursor remains as such in the dorsal root ganglia, since this is a factor in interpretation of the time course of protein synthesis changes and the rate of axonal transport of labelled proteins. L4 and L5 DRG were exposed as before, and injected bilaterally with 4-5μCi of 35S methionine per ganglion. Rats were allowed to survive 10min, 30min, 1, 2, 4, or 10h. At this time, DRG were rapidly removed, rinsed in cold saline to remove any precursor from the outside of the ganglion, and frozen on cards. Each ganglion was crushed in a stainless steel mortar and pestle at -70°C, placed in 1ml trichloracetic acid (TCA) in a 1.5ml Eppendorf centrifuge tube, and left overnight. Samples were then centrifuged at 6000rpm for 10min, and the supernatent separated from the pellet. The pellet was rinsed with a further 1ml of 10% TCA and this was added to the supernatent, giving a total volume of 2ml TCA containing free, labelled amino acid from each ganglion. A 500μl aliquot of this was added to 3ml Scintiverse (Fisher) and counted in a scintillation counter. The pellet containing radiolabelled protein was dissolved in 500μl Protosol (New England Nuclear), 3ml scintillation fluid was added and the samples counted. A plot of percentage total activity in the ganglion which is TCA soluble, against time is shown in Figure 4.

RESULTS.

In general, it was possible to achieve a level of labelling of fast

axonally transported proteins equivalent to around 30,000cpm in the 3mm segments of nerve adjacent to the collection crushes (range 5000 - 75,000). The radioactivity in the distal (retrograde transport) segment was approximately 60% of that in the proximal segment, with the isolated segment containing around 10% as much. Sample profiles of the levels of radioactivity in the various nerve segments are given in Figures 5 and 6. In nerves with less than 15,000cpm in the proximal segment, it was difficult to obtain good fluorographs for quantitation of retrogradely transported, due to the low levels of activity in the distal segment.

In Figure 5 it can be seen that the radioactivity associated with anterogradely transported material in the proximal accumulation segment varies from animal to animal over approximately a ten-fold range. This is not due to any difference in the activity of the 35S methionine, as animals 1-4 and 5-12 comprised two separate series', injected on separate days, and it can be seen that animal 5 and 6 have activity equally as high as 1-4. The variation must therefore arise from variability in the injection technique; if different numbers of cell bodies were exposed to the precursor in different animals, this would result in the variation in activity associated with anterogradely transported material in the sciatic nerve.

In Figure 6, some activity profiles show quite high activity in the isolated segment between the collection crushes, relative to the accumulated activity in the proximal and distal collection segments.

Part of the activity in the isolated segment is associated with mobile material which will move towards the ends of the segment, but part will also be associated with non-mobile material. Since this stationary material has presumably been deposited all along the axon, it will produce similarities in the protein composition of material in the proximal and distal collection segments. Observable differences between the anterograde and retrograde transport fractions are therefore more remarkable than if all the radiolabel in the nerve were mobile.

In the comparisons which follow, the numbering of protein spots follows a general trend of numbering higher molecular weight proteins first. It should be noted however, that the numbering of proteins in separate comparisons is not completely consistent, although an attempt was made to give prominent proteins the same number throughout.

i) Comparison a: anterograde transport in intact nerves.

A summary of the quantitative analysis is given in Figure 7a, and the proteins assessed are indicated in the fluorographs of anterogradely transported proteins in intact sciatic nerve at times 0-6, 10-16 and 24-30h after precursor injection shown in Figure 7b. The pattern of protein separation seen at 0-6h after injection represents material anterogradely transported at a minimum rate of 120mm/day. Rates of transport in this comparison were calculated by assuming that the amino acid precursor is immediately incorporated into protein,

which is then rapidly exported into the axon. Assuming this, for the 0-6h time period, labelled proteins have a maximum of 6h to reach the collection crush, 3cm from the soma. The slowest transported material must therefore travel 30mm in 6h, or 5mm/h (120mm/day). Any delay in uptake or processing of the precursor, or in the export of labelled protein would increase this estimate of the minimum transport velocity. The 0-6h fraction appears to contain a large number of glycoproteins. These are seen on the fluorograph as a series of spots or streaks with the same molecular weight but different isoelectric points (O'Farrell 1975). This is known as "stuttering", and is due to the highly charged nature of glycoproteins; the series of spots represents forms of the same molecule with different net charges. Most of the proteins seen in the 0-6h fluorograph are found only at this early time point, and therefore must travel exclusively at rates above 120mm/day in the axon; proteins unique to the 0-6h time period are numbers 1, 8-14, 16 and 17, in Figure 7. Proteins 3-7 can be seen at all three times studied, but these make up a small fraction of the total radioactive protein in the nerve.

The pattern of proteins seen 10-16h after injection is very different from the earlier time period. Apart from the small proteins present at all three times (number 3-7), only three others were found to be common to both 0-6 and 10-16h times; these are numbers 2, 15 and 23. The 10-16h material travels at rates between 45 and 72mm/day, again assuming instantaneous synthesis and loading.

Proteins travelling between 25 and 30mm/day appear in the 24-30h time period. The pattern here is almost the same as the 10-16h time point, except numbers 2, 15 and 23 are almost absent. Other differences between the 10-16 and 24-30h fractions are quantitative; proteins 18, 19, 21 and 22 are already being transported in small amounts at 10-16h, but are more heavily labelled in the 24-30h fraction. Very few glycoproteins appear to be present in the more slowly transported fractions, as shown by the lack of "stuttering" on these gels. The identity of any of the proteins is difficult to determine, since they are characterised only by their apparent molecular weight on the gel, their isoelectric point, and the shape of the spot they produce. In addition, many of the proteins which appear on a fluorograph compromise a very small fraction of the total protein in the sample, and are not seen when the gel is stained.

ii) Comparison b: anterograde transport in intact, injured and 7 day regenerating nerves, 10-16h.

Figures 8a and b show anterogradely transported proteins from intact, injured and regenerating nerves, 10-16h after injection. The overall pattern of separation was similar in all three fluorographs, and only a few quantitative differences were observed. Non-significant changes were measured in proteins 2, 3, 4, 6 and 12. Number 12 is the protein known as S1, and its transport in sensory neurons was observed to decrease during regeneration, confirming the finding of Reh et al

(1987). GAP43 (protein number 1) was identified by its apparent molecular weight and high acidity, and by the characteristic shape of the spot (Skene and Willard 1981b). It could be seen in all three situations; however, greatly increased amounts were present in injured and regenerating nerves. Small increases in the levels of a few proteins (5, 7-9 and 11) were seen in injured nerves. No novel proteins were transported along the sensory axons at this time as a result of injuring the nerve.

iii) Comparison c: retrograde transport in intact, injured and 7 day regenerating nerves, 10-16h.

Many of the same proteins are found in the retrograde transport fraction regardless of the state of the axon; a summary of the mean D.I. values for this comparison, and fluorographs of the retrogradely transported proteins are shown in Figure 9a and b. Quantitated proteins which were consistently present in the retrograde transport fraction are numbers 9, 10 and 12. Injuring the sciatic nerve induces changes in the retrogradely transported proteins, some of which persist through the regeneration process, and some of which appear to be transient, lasting only a short time after injury. Comparison of proteins retrogradely transported in intact nerves at 24-30h with those in injured and regenerating nerves at earlier times after injection (10-16h) was necessary, since very little radiolabelled protein is returning towards the cell body in intact nerves at 10-16h. It is

possible that some of the differences in retrograde transport between intact and injured or regenerating nerves are due to this difference in the time of sampling; however, since the peaks of retrograde transport have been previously calculated to occur at the times examined here (Bisby and Bulger 1977), it is likely that the same retrograde transport fraction is being examined in all three situations, and that differences in protein composition are the result of injury and regeneration, not the different times of protein analysis.

One protein of molecular weight 55kD is indicated (number 1), and is detectable only in the fluorograph from intact nerve. This may be an example of a change in protein transport which is induced by injury and persists through regeneration; the protein can be seen anterogradely transported in intact nerves at 10-16h, and in greater amounts at 24-30h (protein number 18, Figure 7). Proteins 5 and 7 are increased in amount after injury and during regeneration, Number 5 is GAP43, and the greatest amount is seen in injured nerves, although this is not significantly different from the amount in regenerating nerves. Number 7 has molecular weight 20kD and is also found in the greatest amounts in injured nerve. Thus, both increases and decreases in the retrograde transport of certain proteins are seen after nerve injury, and persist at least 7 days into regeneration.

Transient changes in retrograde transport also occur after injury. Proteins 2, 3 and 4 are retrogradely transported in intact sciatic nerve. After injury, material returning to the cell body from the

injury site contains very low levels of these proteins. When the nerve has been regenerating for 7 days, they are present in increased amounts, but still less than the intact nerve. They are anterogradely transported in amounts greater than their retrograde transport throughout injury and regeneration (see protein numbers 2, 3 and 4, Figure 8). Proteins 6, 8 and 11 are increased in injured, but not regenerating nerves, and thus also represents a transient change in the composition of retrogradely transported proteins after injury.

iv) Comparison d: anterograde versus retrograde transport in intact nerves, 24-30h.

Figures 10a and b. The overall pattern of protein separation seen in the anterograde and retrograde transport fractions is very similar 24-30h after precursor injection. As mentioned previously, this is probably due in part to the contribution of non-mobile proteins to both fractions. Major differences are shown in the bar graph in Figure 10a. Protein numbers 1-4 were detected in significantly greater amounts in the anterograde transport fraction, while two proteins of molecular weight around 16kD (numbers 8 and 9) were seen to travel only in the retrograde direction. Proteins 5-7 did show differential transport between the two fractions, but these were statistically non-significant.

v) Comparison e: anterograde versus retrograde transport in injured nerves, 10-16h.

Figure 11a and b. There were many differences, both qualitative and quantitative in the patterns of proteins undergoing anterograde and retrograde transport in acutely injured nerves. Proteins 2, 3 and 4 travel almost exclusively in the anterograde direction; these three proteins are normally seen in the retrograde transport fraction of intact nerves (numbers 2, 3 and 4, Figure 9), and the large decrease in their transport is induced by injuring the nerve. Proteins 5, 8, 10, 12, 16, 17 and 19 also travel predominantly in the anterograde direction. Proteins 1 (GAP43), 7, 9, 14 and 15 travel bidirectionally with non-significant differences between the anterograde and retrograde fractions. Numbers 6, 11 and 13 move mainly in the retrograde direction.

vi) Comparison f: anterograde versus retrograde transport in 7 day regenerating nerves 10-16h.

Figures 12a and b. Four prominent proteins (number 2, 3, 4 and 12) were seen to travel predominantly in the anterograde direction. Quantitative differences were also seen in proteins 1 (GAP43) and 5-11 but these were statistically non-significant.

vii) Comparison g: anterograde transport 0-6h versus retrograde transport 24-30h, in intact nerves.

Figures 13a and b. This comparison was made because it has previously been calculated from axonal transport velocities in the rat sciatic nerve that proteins anterogradely transport at early times (here, 0-6 after precursor injection) should be returning along the axons by 24-30h (Bisby and Bulger 1977). It was thought that comparison of these two components might show more similarity in protein composition than a comparison of proteins anterogradely and retrogradely transported at the same post injection time. However, this did not appear to be the case, as shown in Figure 13. Most of the proteins were confined to one fraction (for example, number 1-4 and 10, retrograde; numbers 12 and 15-18, anterograde). Proteins 5-9 and 11, 13 and 14 travel in both directions.

viii) Comparison h: anterograde transport in intact nerves, 0-6h versus retrograde transport in injured nerves, 10-16h.

Figures 14a and b. When axons are shortened by crush injury, anterogradely transported material reverses its direction earlier than in the intact axon (Bisby and Bulger 1977), and so this comparison was made by the rationale as for comparison g. More similarities in the two transport fractions were seen here than for comparison g: for example, proteins 1-4 disappear from the retrograde transport fraction after injury, and are also absent from the 0-6h anterograde fraction.

Numbers 15-18 were still found exclusively in the anterograde fraction.

ix) Comparison i: anterograde transport in intact versus regenerating nerves, 0-6h.

Figures 15a and b. Several changes were induced in the 0-6h proteins during nerve regeneration. Number 1 is GAP43, showing a significant increase in transport in the regenerating nerve as expected. Other significant increases in transport during regeneration were seen in numbers 3 and 6. Number 6 may correspond to a protein described by Skene and Willard (1981a and b), which they designated GAP23 and was seen in toad and rabbit regenerating systems. The increase in protein 3 appears to be due to the different resolution of the large complex of molecular weight around 30 kD into several components in the regenerating nerve. Four small proteins marked with an asterisk also appeared in the regenerating nerve.

SUMMARY OF SIGNIFICANT CHANGES IN PROTEIN COMPOSITION.

ANTEROGRADE TRANSPORT.

a) Increased 10-16h after injury.

i) Increase persisting through regeneration:

-GAP43 (No.1, Fig. 8).

ii) Transient increase after injury:

-MW 42kD, pI 5.6 (No. 5, Fig. 8).

-MW 42kD, pI 6.3 (No. 9, Fig. 8).

b) Decreased 10-16h after injury:

-No significant decreases.

c) Increased during regeneration 0-6h:

-GAP 43 (No. 1, Fig. 15).

-MW 24kD, pI 6.5 (No. 3, Fig. 15).

-MW 25kD, pI 5.8 (No. 6, Fig. 15).

RETROGRADE TRANSPORT.

a) Increased 10-16h after injury.

i) Increase persisting through regeneration:

-GAP 43 (No. 5, Fig 9).

ii) Transient increase after injury:

-MW 40kD, pI 5.8 (No. 6, Fig. 9).

-MW 28kD, pI 6.5 (No. 7, Fig. 9).

-MW 26kD, pI 6.2 (No. 8, Fig. 9).

b) Decreased 10-16h after injury.

i) Decrease persisting through regeneration:

-MW 55kD, pI 6.3 (No. 1, Fig. 9).

ii) Transient decrease after injury:

-No significant decreases.

x) Half-time of [35-S] methionine in dorsal root ganglia.

Figure 4 shows a plot of the percentage of total radioactivity in the ganglion which is TCA soluble, against time. The initial component

of the curve, a, gives a half time for the [35-S] methionine of 22min, and probably represents uptake of the precursor into the blood. The second component, b, has a half time of 1h, and is likely to be incorporation of amino acid into protein. After 4h, the levels of unincorporated activity in the ganglion appear to reach a "baseline" level of around 3% of the total radioactivity in the ganglion; this may be a result of breakdown of the methionine into other TCA soluble components, or of recycling of labelled amino acid.

DISCUSSION.

The experiments described here have studied fast axonally transported proteins in intact, acutely injured and 7 day regenerating rat sciatic nerves, by 2D SDS PAGE.

Anterograde transport in intact nerves.

Fast anterograde transport in intact nerves was studied at 0-6, 10-16 and 24-30h after injecting the radiolabelled amino acid precursor into the vicinity of the cell bodies in the dorsal root ganglia. The fluorographs obtained from these three time periods indicate that there may be a wide range of transport velocities included under the general title of "fast axonal transport", and that proteins appear to be preferentially carried within a certain range of velocities. Most of the proteins appearing in the axon within 6h of precursor injection are unique to this time period; many of them are glycoproteins, suggesting

that highly charged proteins such as this are very rapidly transported in sensory axons. Proteins travelling more slowly in the axons have less charge variability, since they appear on the fluorographs as discrete single spots. It is possible that this "selectivity" of proteins for a certain velocity range in the axons is related to their final destination, in that there is evidence for the secretion of glycoproteins from the axonal terminals, as discussed later.

A progressive change in protein separation patterns was seen when more slowly transported material was examined. Proteins appearing at the 10-16 and 24-30h intervals are commonly seen at both these times, probably because the difference in the transport velocities of material collected in these intervals is much smaller than the difference between 0-6 and 10-16h. The progressive change in protein composition with time suggests that the labelled amino acid precursor is present in the ganglion for only a short time, and is therefore essentially a pulse label. If free amino acid remained in the ganglion for a long time, we would expect to see all the proteins in the 0-6h time period present at later times also, which is not the case. An experiment performed to determine the half time of 35S methionine in the dorsal root ganglia confirmed this, and the precursor concentration was calculated to fall to half its original concentration in 20 minutes (Figure 4). From this, it seems that all proteins which are detected subsequently in the sciatic nerve were synthesised within the first hour following injection, by which time free precursor has fallen to

approximately 10% of its original level. Since the TCA-soluble fraction also includes breakdown products of 35S methionine, any significant breakdown would lengthen the apparent half time of the amino acid, and so the actual half life may be even less than 22min.

In the velocity calculations for the three anterograde transport fractions in comparison a, it was assumed that the synthesis of labelled proteins and initiation of its transport along the axon followed immediately after injection of the precursor. It is possible that some proteins do not enter the axon immediately, but enter a storage or processing pool for some time before being axonally transported, thus giving an apparently slower transport rate. However, this does not seem to fit with the observation that the most rapidly transported proteins are glycoproteins, because they should require more processing in the cell body before their synthesis is complete.

A recent study of the transport of radiolabelled protein in the bullfrog sciatic nerve has distinguished proteins which are preferentially routed to the axonal terminals of sensory neurons (Rulli and Wilson 1987). A wave of radiolabelled proteins was allowed to pass along the nerve in an anterograde direction in vitro, and the "wavefront" region of the nerve, and the plateau of activity left behind were examined by 2D SDS PAGE. Many of the proteins observed on these 2D gels appear to be preferentially routed to the terminals,and very few of the proteins found in the plateau region of the nerve are glycoproteins. This suggests that the restriction of glycoproteins to

the most rapidly transported fraction in rat sensory axons may be related to their final destination in the axon.

Bisby and Buchan (1981) have reported that a wave of radiolabelled, fast axonally transported proteins will travel along sensory axons at a similar rate after release from a cold block on the nerve, regardless of the interval between injection of the precursor amino acid and application of the cold block, up to 24 after injection. This observation is difficult to reconcile with the observation of changing protein patterns with time seen here by 2D SDS PAGE, unless the later proteins are in fact released after a period of storage in the cell body, because a cold block applied to the nerve at 10h after the injection of precursor would be expected to trap proteins travelling more slowly than 400mm/day. However, application of a cold block to the nerve wil allow anterogradely transported proteins to "drain" from the distal portion of the nerve towards the terminals, and this may vacate binding sites on the microtubules which are normally occupied with very rapidly moving organelles. When the cold block is removed after 2h, the accumulated proteins could occupy these sites and result in a wave of transport moving much more rapidly than these proteins would travel in the intact nerve.

Radiolabelling of transported proteins and gel analysis.

Radiolabelling axonally transported proteins by the injection of radiolabelled amino acids into the vicinity of the neuronal cell bodies

is a widely employed and useful technique. In order to interpret results obtained from experiments such as those described in this chapter, it is necessary to consider possible fates of the labelled amino acid following its injection into the ganglion. Some leakage of the precursor may occur, although this is minimised by leaving the injection pipette in the ganglion for several minutes after the injection. Some of the precursor will also be taken up into the blood, and this may cause the rapid, initial drop in free amino acid in the ganglion with a half time of 22min (Figure 4). Once the amino acid has been taken up into the neuronal cell bodies, it may be degraded, stored, or synthesised into protein. Any storage of the methionine must be short-lived, considering its short half time in the ganglion; much of the precursor must therefore be synthesised into protein, and this is probably the second component of the curve in figure 4, with a half time of 1h. Once the radiolabelled protein has been synthesised, there is again the possibility that it enters a storage pool, thereby increasing the time between its labelling and detection at the collection crush, and resulting in an apparently slower transport rate. It seems unlikely that a protein which is undergoing continuous synthesis in an intact nerve for export into the axon would be stored for more than a few minutes.

As the labelled protein is transported along the axons, a plateau of radioactivity is left behind in the nerve (Rulli and Wilson 1987). This protein contributes to the activity measured in the isolated

segment of nerve between the collection crushes, and is also present in both the proximal and distal accumulation segments. The rapidly transported glycoproteins do not seem to be deposited in the axon, since thay are not present in the accumulation segments when transported proteins are examined at 10-16 and 24-30h. This is further evidence for the preferential routing of rapidly transported glycoproteins to the axonal terminals.

In this thesis, experiments have been described which demonstrate quantitative changes in the retrograde axonal transport of AChE and MChR in the rat sciatic nerve following an acute distal injury. It was suggested that these changes might be representative of more general alterations in retrograde transport after injury, and this seems to be the case, as confirmed by this work with 2D SDS PAGE, as is discussed later. The identity and function of fast axonally transported proteins separated by 2D SDS PAGE is unknown, since they are recognisable only by their isoelectric point, apparent molecular weight and the characteristic shape of the spot they produce on the gel. It is therefore not possible at present to correlate the changes in protein composition induced by injury and regeneration to the changing functional needs of the axon, except in general terms. One exception to this is the growth associated protein GAP43, which has been described in association with regeneration in several neuronal systems (Skene and Willard 1981 a and b; Benowitz and Lewis 1983; Reh et al 1987). It has been shown that GAP43 is a component of growth cones

(Skene et al 1986; Meiri at al 1986), and Jacobson et al (1986) have shown that it is identical to the rat brain synaptic phosphoprotein, B50, characterised by Zwiers et al (1978, 1980). It has been proposed that GAP43 is associated with the induction of synaptic plasticity (Jacobson et al 1986), and so changes in the axonal transport of the protein during regeneration may be correlated with the amount required at the growth cones.

Throughout the analyses of radiolabelled proteins presented here, the assumption is made that an increase in the amount of a particular protein is reflected by a proportional increase in the degree of blackening of the x-ray film used for fluorography, and that quenching of radioactivity does not increase due to a greater concentration of protein in that area of the gel.

Anterograde versus retrograde transport.

When comparing anterograde and retrograde transport in intact nerves at 24-30h (Figure 10), several differences in protein composition were observed which have not previously been detected. A similar experiment has been reported, separating proteins using one-dimensional electrophoresis (Bisby 1981); this technique detected very few differences in the anterograde and retrograde protein compositions in intact motor neurons; the only major difference detected was a reduced amount of the polypeptide known as S1 in the retrograde fraction. S1 is seen on 2D gels as a large prominent spot (number 10, Figure 10b).

A smaller amount of S1 was retrogradely transported as measured on 2D gels, although this was not significantly less than the amount anterogradely transported. Using 2D analysis, it is possible to detect at least 6 highly significant differences in protein composition between the anterograde and retrograde transport fractions at 24-30h, including two (numbers 8 and 9, figure 10) which travel only in the retrograde direction and therefore are possibly breakdown products of larger anterogradely transported proteins.

It is clear from the different results obtained using 1D and 2D analysis that each technique has advantages. 2D analysis gives a much greater resolution and therefore reveals many more proteins ; however, comparison of different nerve samples is easier using the 1D technique, as they can be compared directly on the same gel. In 2D analysis, only one nerve sample can be run per slab gel, and so the quantitation of results is more complex.

Comparisons g and h were performed to test the hypothesis that protein which have been anterogradely transported are later returned to the cell body. There were few similarities in the anterograde fraction at 0-6h and the retrograde fraction at 24-30h in intact nerves. This suggests that either the very rapidly transported proteins collected at 0-6h are degraded in the nerve terminals, secreted, or that they have already passed through the collection crush region before the ligature was placed. It seems unlikely that degradation is the reason for the many differences observed, because

a) the molecular weight range of the 24-30h retrograde fraction is similar to that seen in the anterograde fraction, and not largely confined to lower molecular weight breakdown products; b) there is a much greater homology between anterograde transport at 24-30h and retrograde transport at the 10-16h and 24-30h times (comparison d), in a parallel situation to that previously reported by Bisby (1981).

It is possible that rapidly transported glycoproteins are destined for secretion at the nerve terminals (Rulli and Wilson 1987), and it has also been reported that a group of sulphoproteins are anterogradely transported in the bullfrog sciatic nerve, and not retrogradely transported (Stone et al 1984). These sulphoproteins can be seen in rat sensory axons as proteins 13, 14 and 15 in Figure 7b. In comparison g, they were not observed in the retrograde transport fraction of intact nerves, which supports the hypothesis of Stone et al (1984) that these proteins are secreted. If this is the reason for their absence from the retrograde transport fraction, (rather than their having traversed the collection crush region prior to application of the ligature), then many glycoproteins must also be secreted, since they are also absent from the retrograde transport fraction.

Changes in transport during injury and regeneration.

Injury and subsequent regeneration of sensory axons does not cause the synthesis of any novel proteins which are then anterogradely transported; changes observed in anterograde transport were

quantitative only. Some proteins (numbers 5, 8, 9 and 11, Figure 8) appear to be transiently elevated in the period between injury and sprouting of the axons, ie at 10-16h after injury. These proteins may be involved in the initiation of sprouting, and are no longer needed in such large amounts when the regeneration process has begun. The only protein anterogradely transported whcih increased soon after injury and remained elevated through regeneration was GAP43. This protein has been previously observed to be elevated during the regeneration of a variety of systems, including the toad optic and rabbit sciatic nerves (Skene and Willard 1981a and b). The increase in GAP43 observed here for rat DRG occurred very rapidly, and it is significantly elevated in nerves which have been injured only 16h earlier. This is much sooner than has previously been seen in other mammalian systems, mainly because most other workers have examined axonally transported proteins several days after regeneration has begun (Skene and Willard 1981b). In light of recent evidence that GAP43 is found in large amounts in growth cones (Skene et al 1986; Meiri et al 1986), it is perhaps not surprising to find that it is supplied to the axon stumps in increased amounts both before and during the formation of sprouts. The response of the neuron to axonal injury seems to be extraordinarily rapid, considering that the half time of [35-S] methionine in the ganglion is only 20min, and that the injury was made at the time of precursor injection. The increase in GAP43 synthesis probably occurs within 2h of precursor injection and axotomy, taking into account the apparent

second rate constant for the disappearance of the amino acid.

When the most rapidly transported protein fraction was examined in regenerating nerves was compared to the same fraction in intact nerves, several differences were note (comparison i, Figure 15). The transport of the low molecular weight protein marked number 6 was seen to increase considerably, and this may correspond to a protein of similar molecular weight described by Skene and Willard (1981a and b), designated GAP23. The increased synthesis of the protein seen here (number 6, Figure 15) has not been obvious in previous studies of axonally transported proteins in regenerating rat nerves (Reh et al 1987; Redshaw and Bisby 1984). The four proteins which are marked with an asterisk on the fluorograph from regenerating nerve in Figure 15 may correspond to the protein designated S2b by Redshaw and Bisby (1984).

Changes in retrograde axonal transport were induced during injury and regeneration, some of which are transient and some of which last through the regeneration process. In particular, three proteins of molecular weights 42, 50 and 75kD (numbers 2, 3, and 4, figure 9) disappear from the retrograde transport fraction in acutely injured nerves, but can be seen in reduced amounts in nerves which have been regenerating for several days. Anterograde transport of these proteins continues during injury and regeneration. Such a change may be explained by considering the state of the axon endings at the times when retrograde transport was measured.

In the intact nerve, proteins are transported down the axon and then

reverse their direction and return to the soma after fulfilling their function. Much of this reversal occurs at the axonal terminals, as shown by Bray et al (1974), who demonstrated that a distal ligature on the chicken sciatic nerve reduced the retrograde accumulation of radiolabelled protein at a collection crush by around 80%. Some protein is also degraded in the terminal, since the amount of retrograde transport is about 50% of the anterograde (see Figure 6). When the nerve is injured, the terminals are cut off from the cell body and reversal of axonal transport now occurs at the nerve crush (Bisby and Bulger 1977), where conditions such as calcium levels have been greatly altered by damaging the axonal membrane. This could result in an increased breakdown of proteins by calcium-activated proteases, and these proteins would then be absent from the retrograde transport fraction. Once the axons form growth cones, they may be considered to have reduced terminals with improved calcium homeostasis, and less breakdown of proteins may occur, although still more than in the intact nerve; thus, a small amount of these proteins is now seen being retrogradely transported.

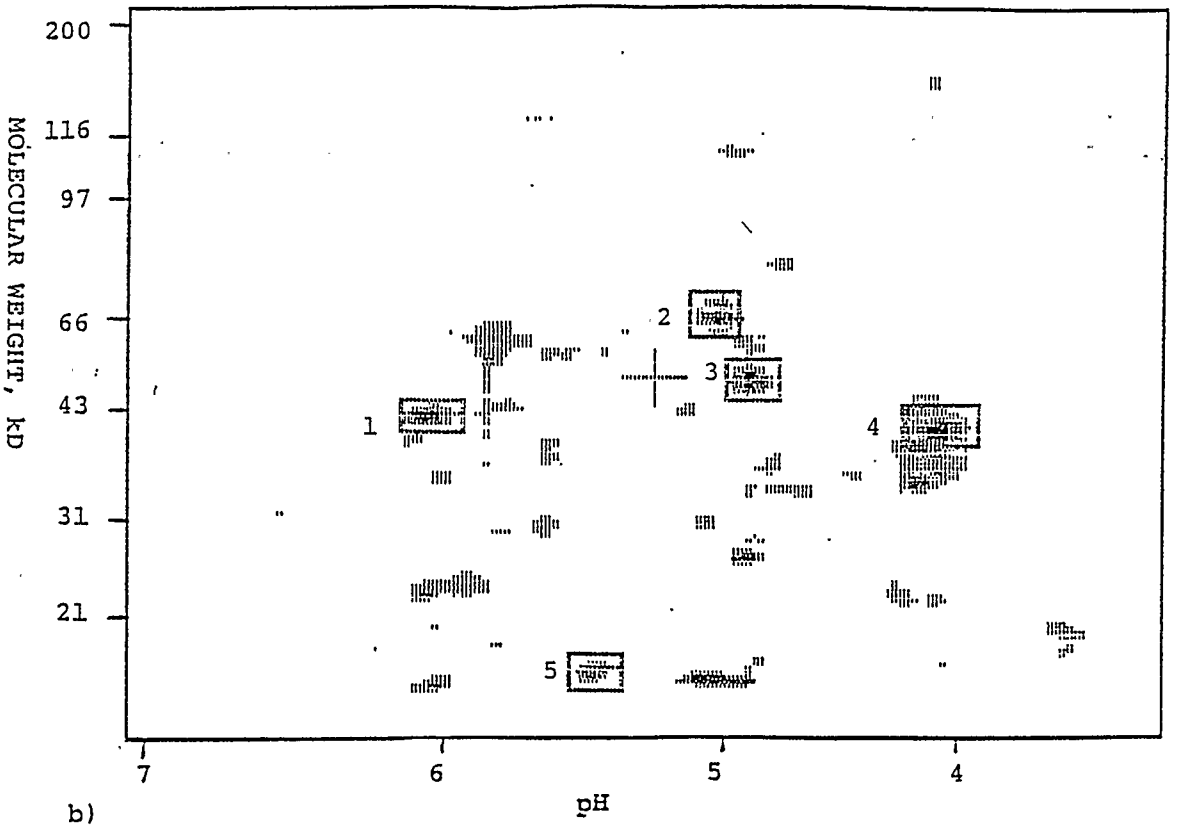
Proteins 6, 8 and 11, Figure 9, increase transiently after acute injury; such a change may be explained by the increased return of protein induced by nerve crush (Bisby and Bulger 1977), followed by a down-regulation of synthesis during regeneration. Many proteins are detectable undergoing retrograde transport under all conditions; these are probably proteins, or the breakdown product thereof, which are

essential for the function of the cell no matter what its state, such as ion pump molecules, mitochondrial proteins, etc.

Conclusion.

The objectives of the 2D gel electrophoresis experiments outlined in the introduction to this chapter were largely achieved. Progressive changes with time after injection in anterogradely transported proteins were observed in intact nerve, with the most rapidly transported material being glycoproteins which appear to be destined mainly for the axonal terminal and possibly secreted. Changes in the composition of retrogradely transported proteins after injury were discovered, along with a much earlier than expected increase in the synthesis and anterograde transport of GAP43.

a)



b)

nr	Positions mm A/B x-min	x-max	y-min	y-max	Area mm*mm	Height AU	Volume AU*mm*mm
1	99.55	104.2	87.82	91.92	22.88	0.31	7.13
2	124.24	127.91	99.26	103.9	19.20	0.26	5.00
3	126.57	130.57	91.46	95.62	21.12	0.32	6.76
4	140.92	146.59	86.26	90.94	31.20	0.27	8.41
5	113.56	117.56	58.72	61.34	15.34	0.30	4.76

Figure 1. Typical gel scan from a fluorograph of labelled, fast anterogradely transported proteins in intact rat sciatic sensory axons, 24-30h after precursor injection into DRG. White = background absorbance; progressively darker greys indicate increasing density in absorbance units. Spots 1 - 5 were integrated to generate the table shown in b).

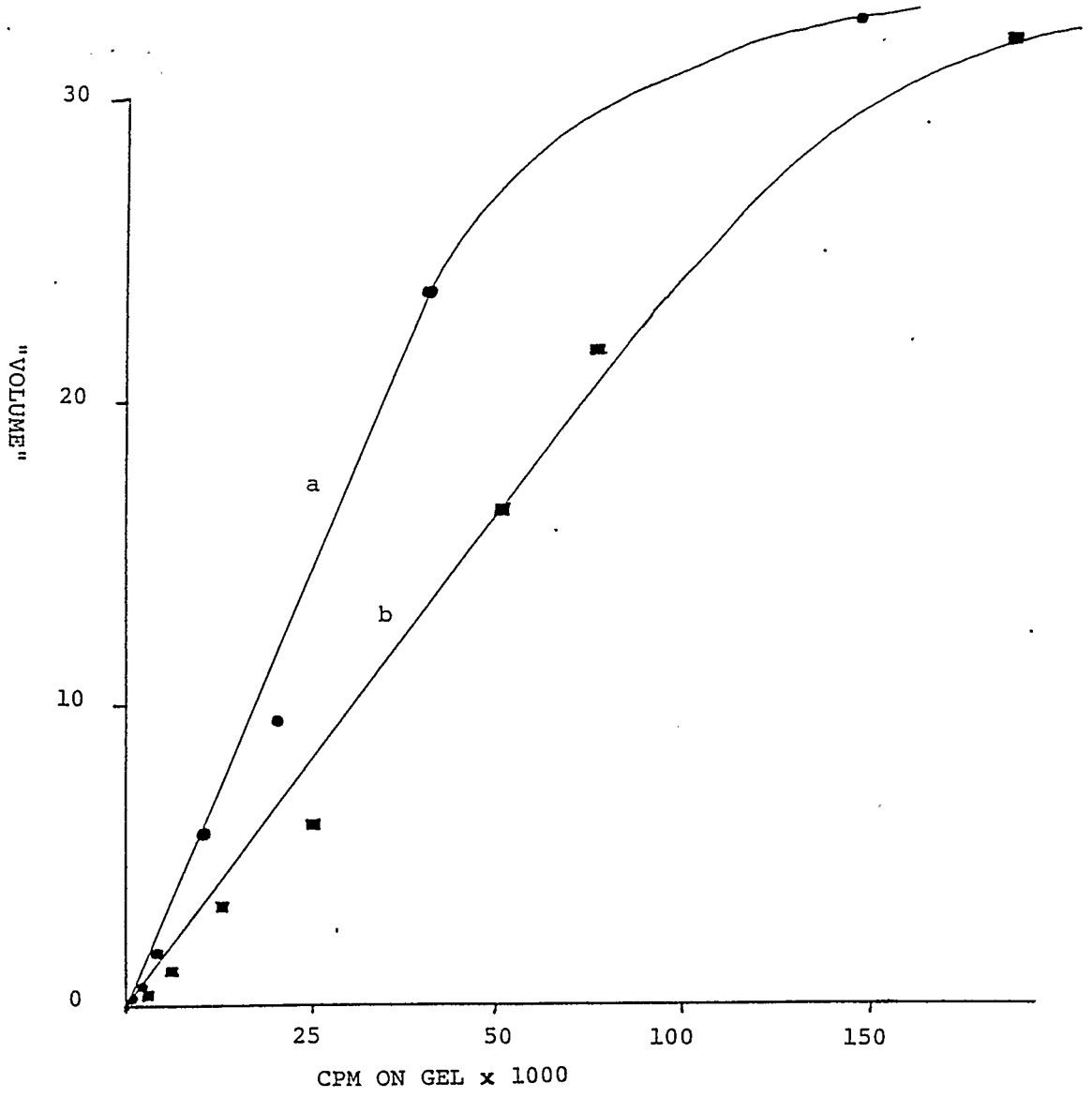


Figure 2. Calibration curves for densitometry showing linearity of densitometer scan "volume" values with respect to increasing cpm applied to slab gel. a = 4 week exposure; b = 1 week.

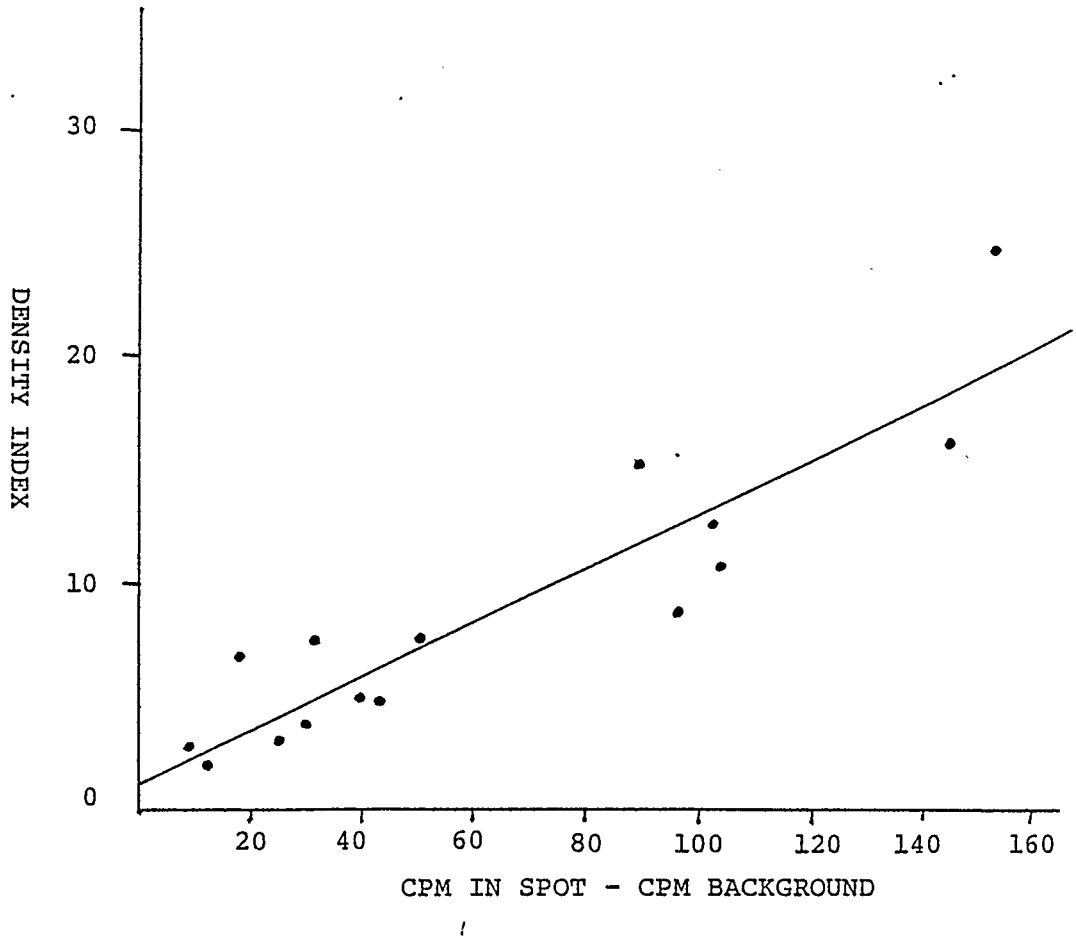


Figure 3. Plot of Density Index versus cpm in protein spot for fifteen individual proteins. The line was fitted by simple linear regression, correlation coefficient = 0.91.

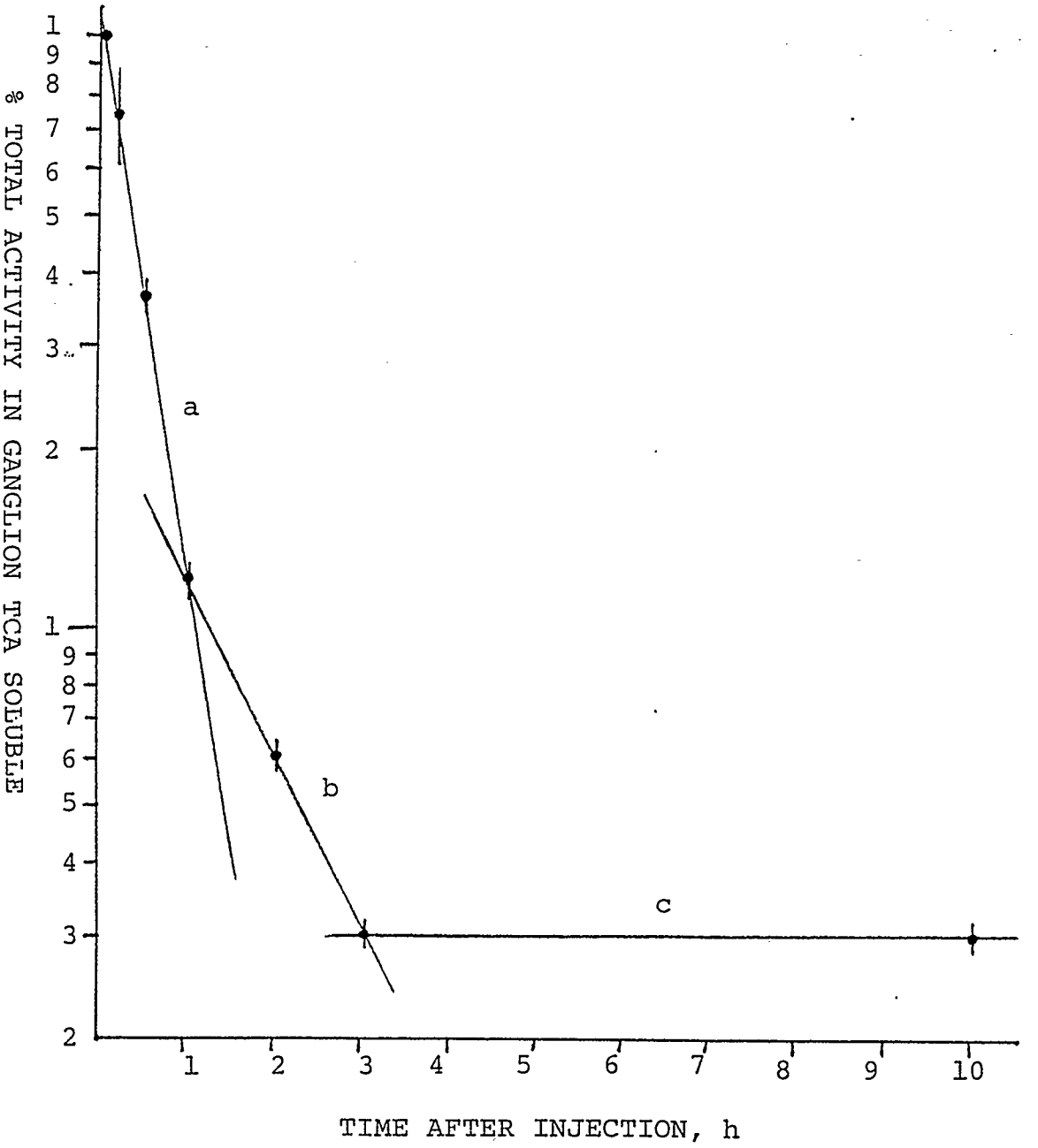


Figure 4. Disappearance of 35S methionine following injection into DRG. There are three phases of removal: a) half-time 22min, probably uptake into blood; b) half-time 1h, probably protein synthesis; c) long-lasting "baseline" level.

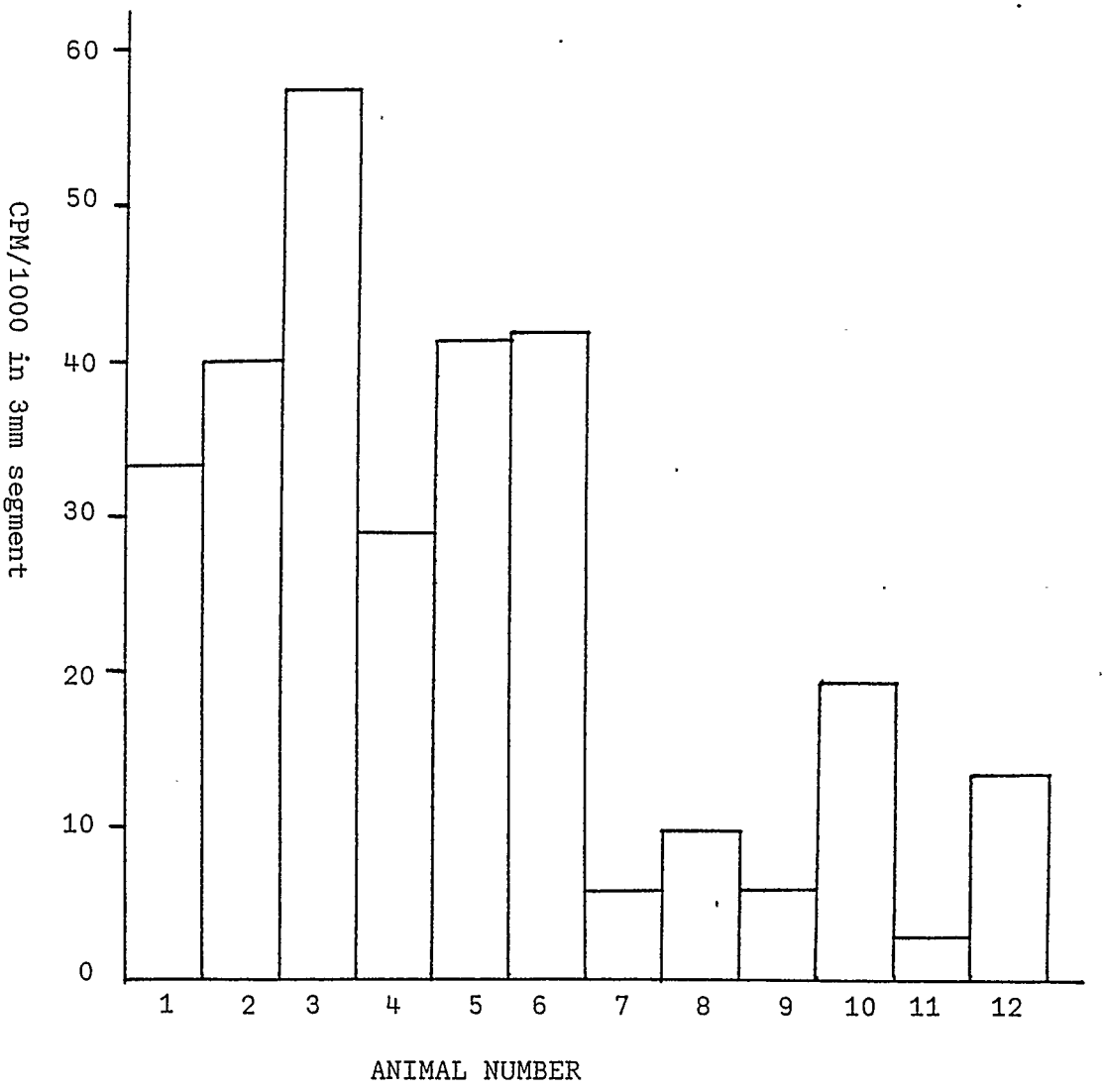


Figure 5. Histogram showing variability of labelling of proteins anterogradely transported in intact sciatic nerve, 0-6h after injection of [35-S]-methionine into ipsilateral dorsal root ganglia.

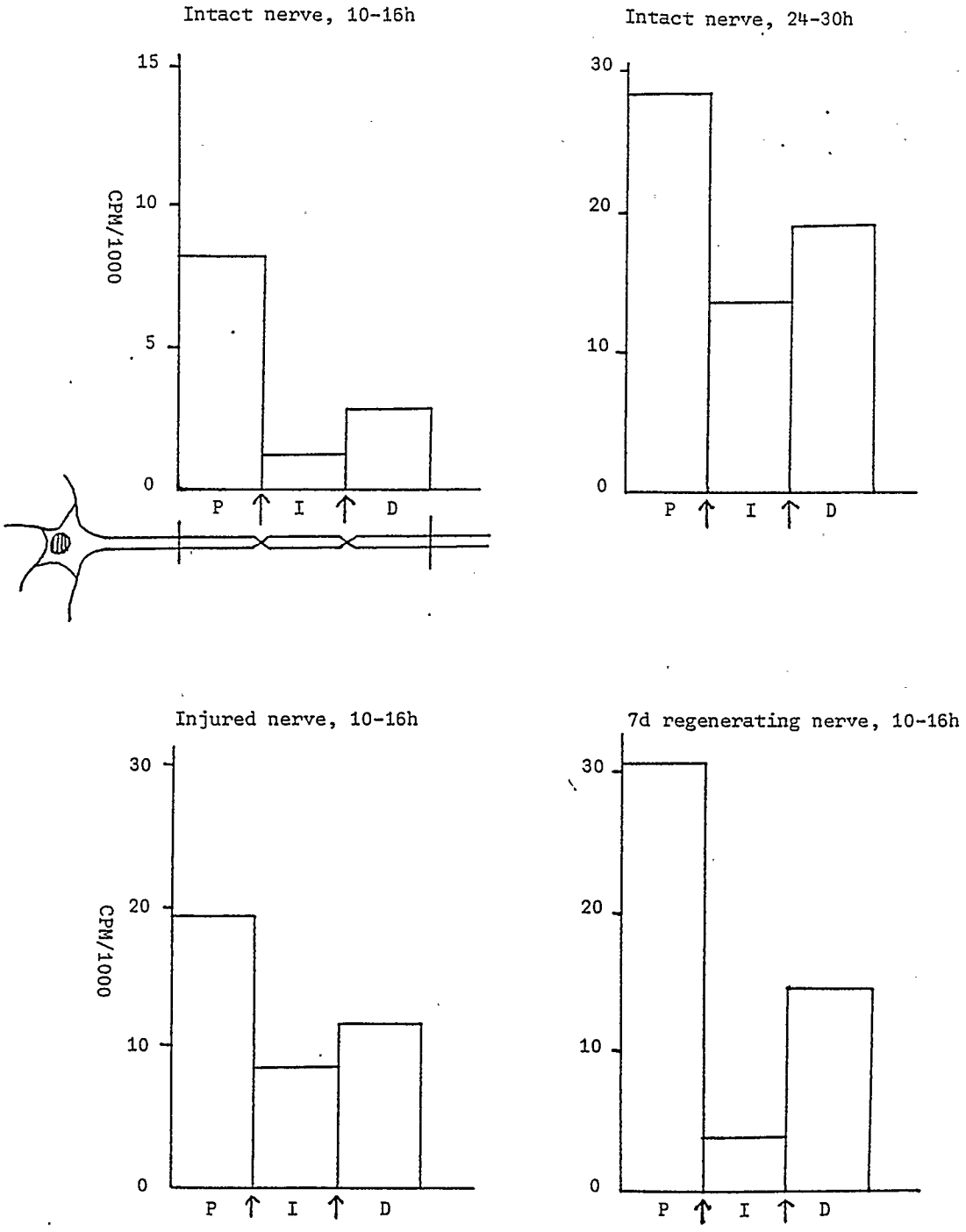


Figure 6. Typical profiles of labelled axonally transported proteins in sciatic sensory axons. Arrows indicate location of collection crushes.

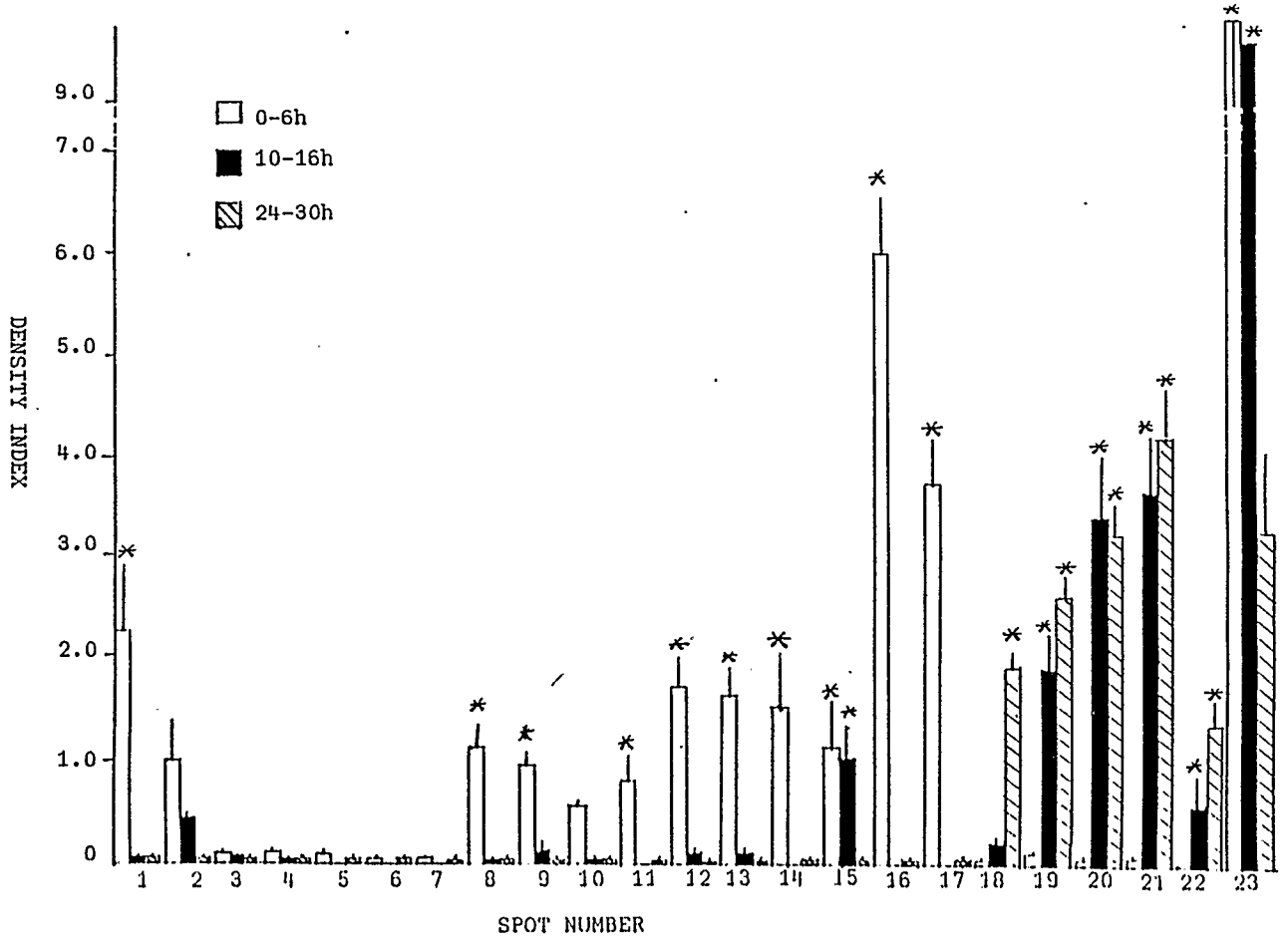
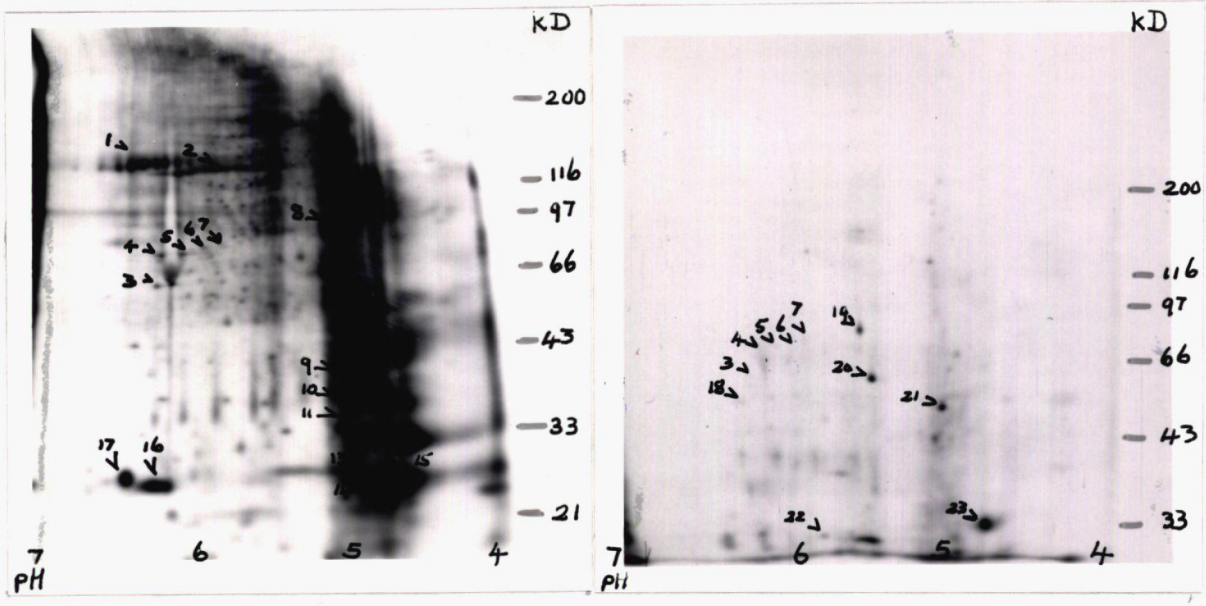
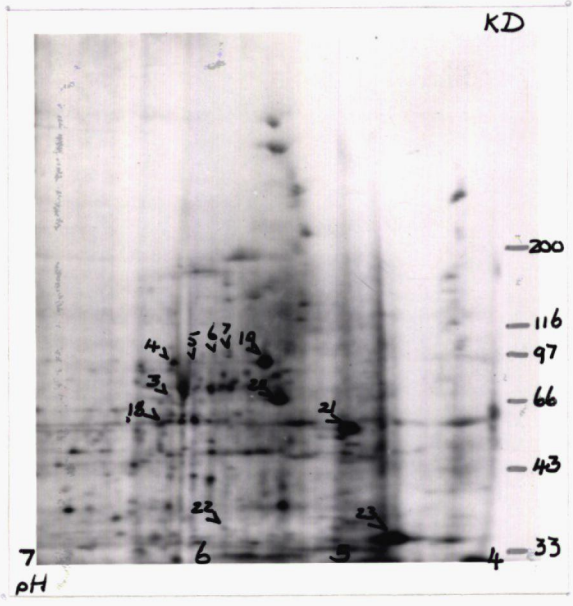


Figure 7a. Bar graph of density indices for anterogradely transported proteins in intact sciatic nerves, 0-6, 10-16 and 24-30h after injection. Proteins quantitated are marked in Figure 7b. Bars = SEM (n = 6 or 7); * = significant difference.



0-6h

10-16h



24-30h

Figure 7b. Comparison a. Fast anterogradely transported proteins in intact sciatic nerves at 0-6, 10-16 and 24-30h after injection of 35S methionine into DRG. Numbers refer to Figure 7a.

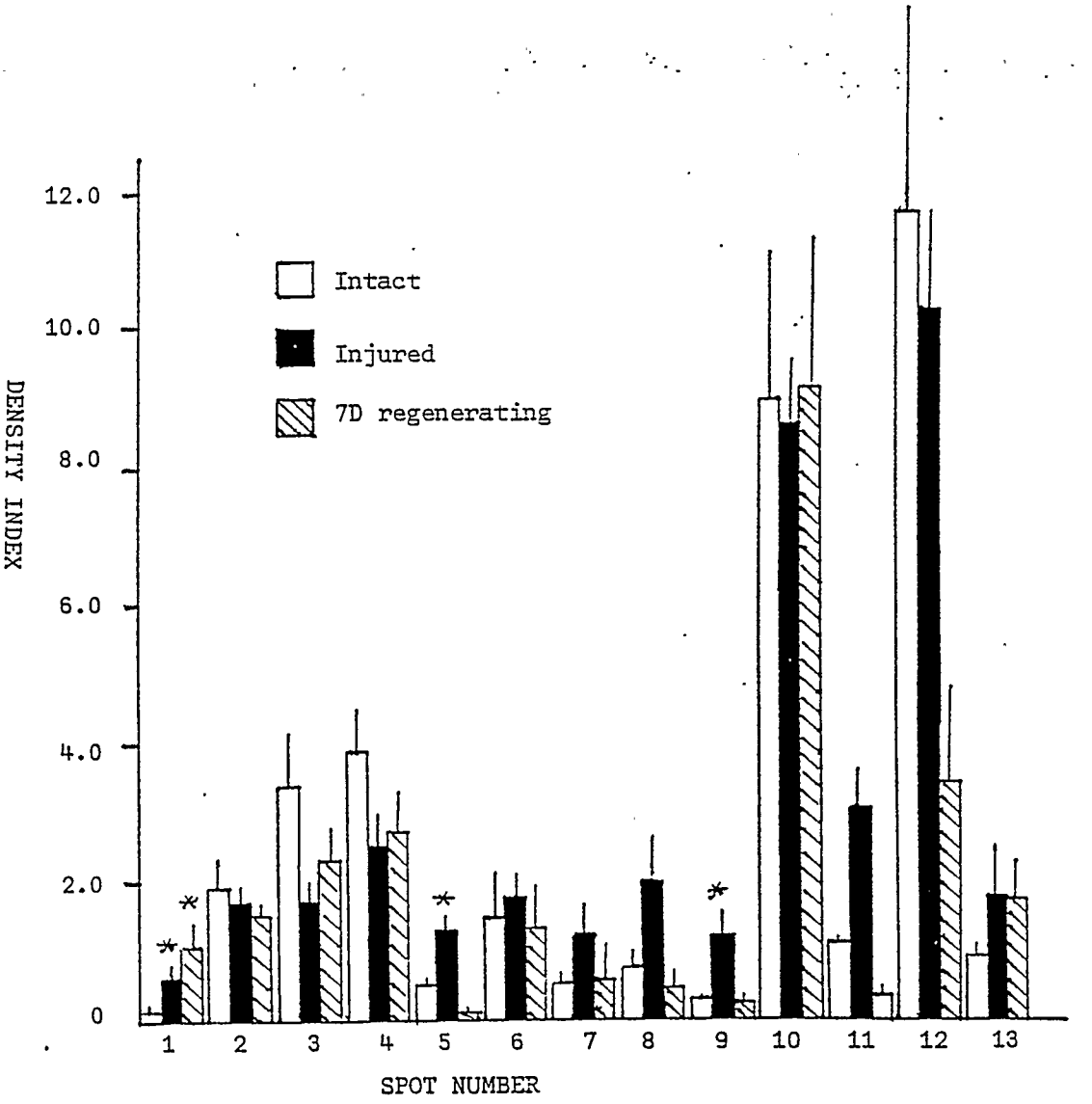


Figure 8a. Bar graph of density indices for anterogradely transported proteins in intact, injured and 7 day regenerating sciatic nerves, 10-16h after precursor injection. Proteins quantitated are marked in Figure 8b. * = significant change; bars are SEM, (n = 6 or 7).

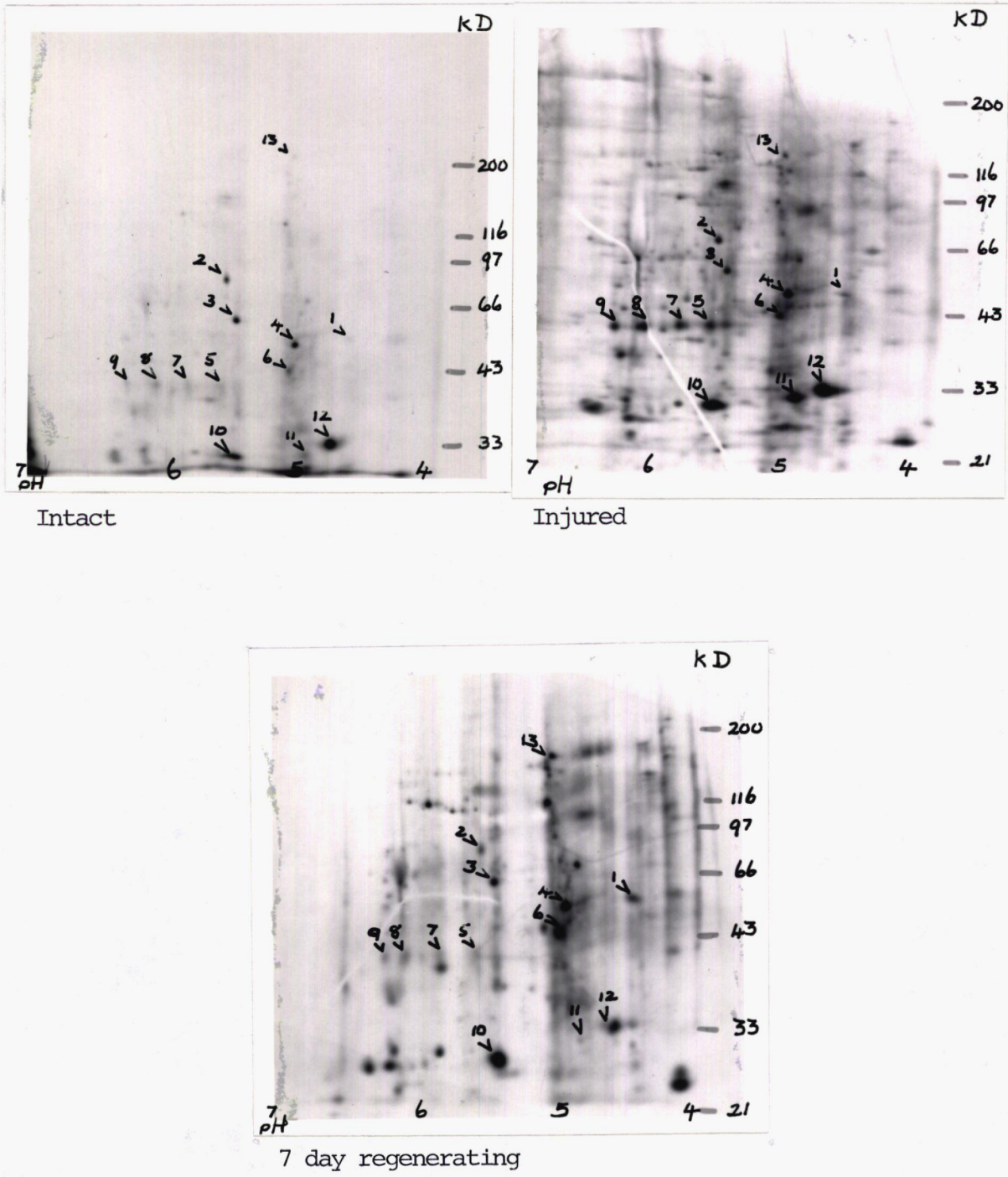


Figure 8b. Comparison b. Anterogradely transported proteins in intact, injured and 7 day regenerating sciatic nerves, 10-16h after precursor injection. Numbers refer to figure 8a.

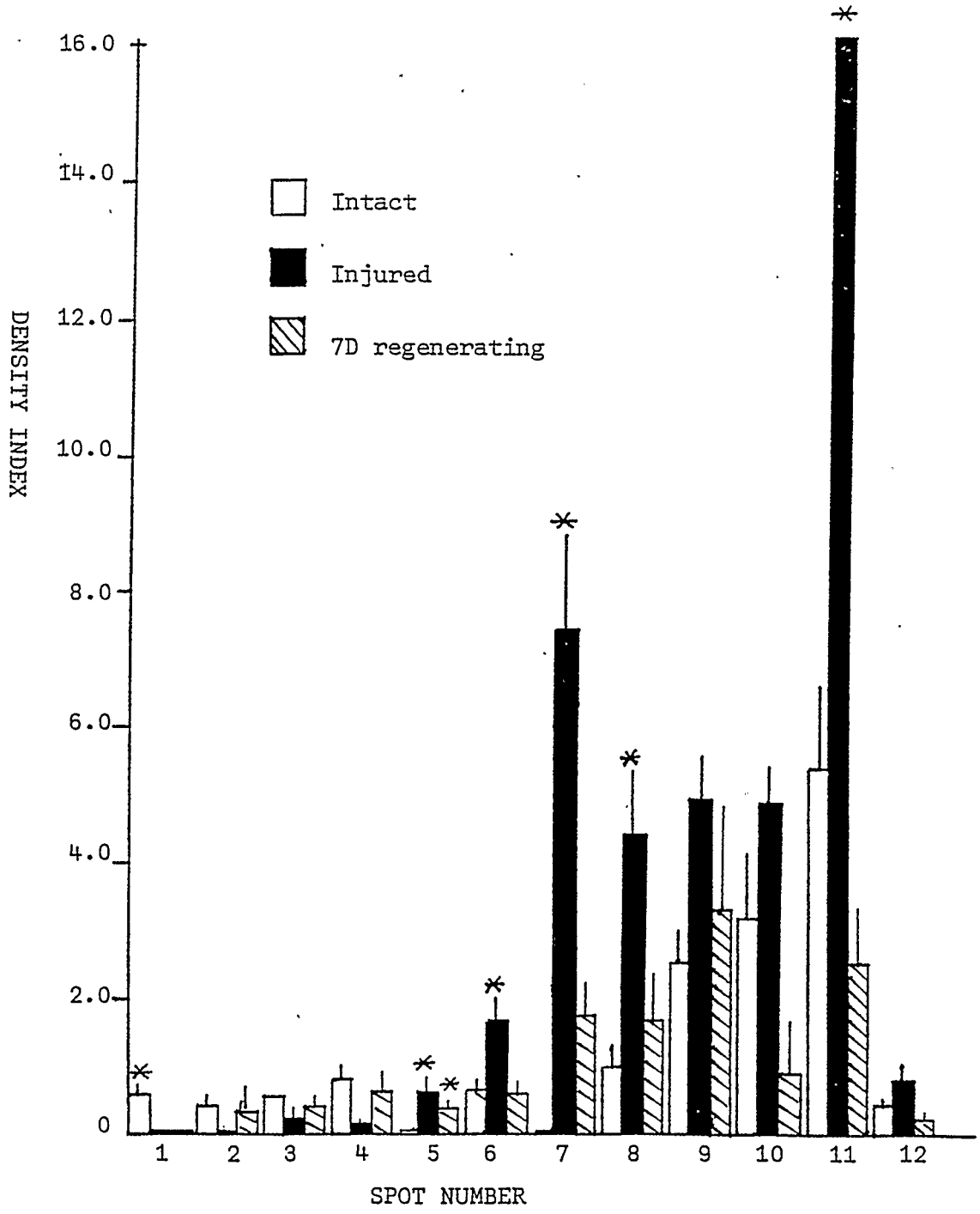
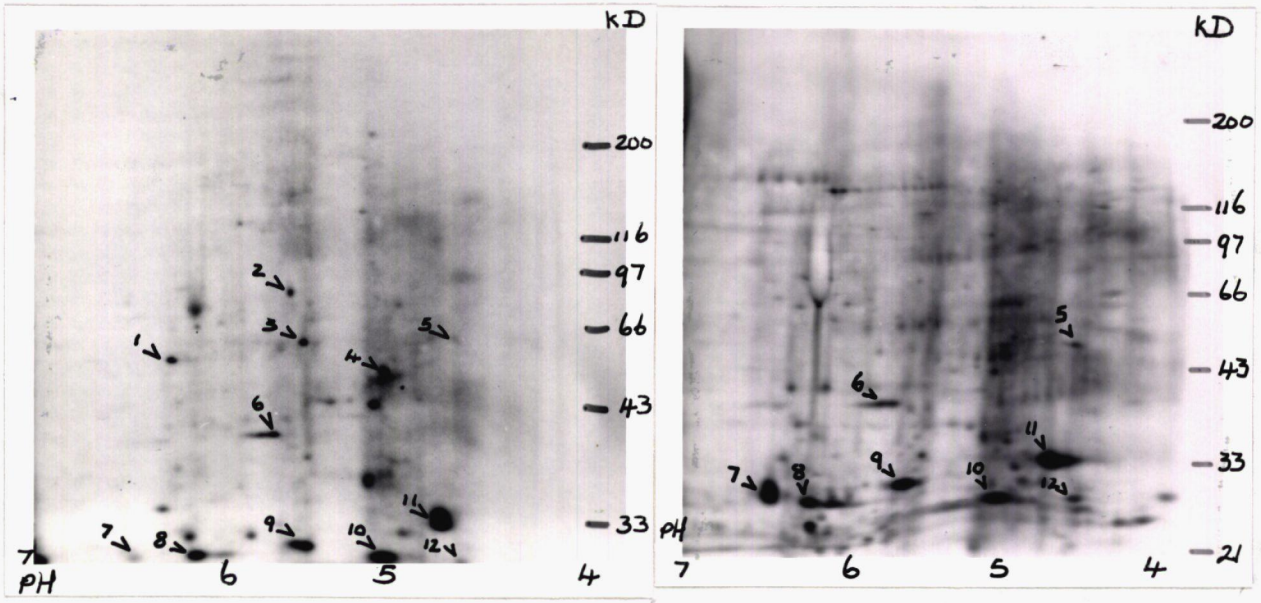
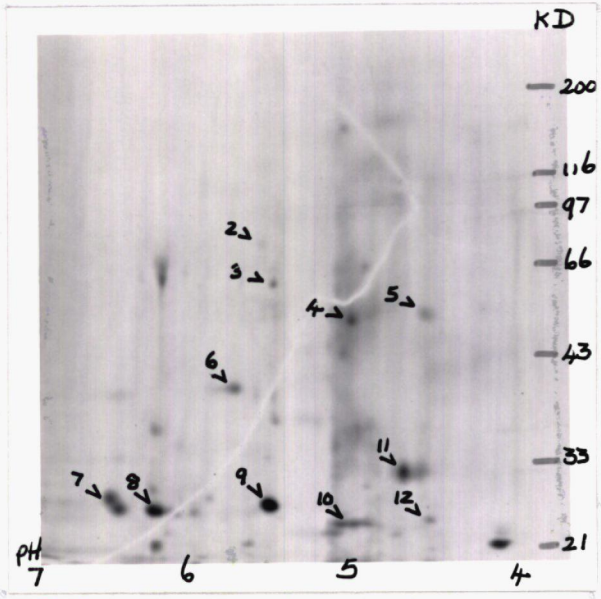


Figure 9a. Bar graph of density indices for retrogradely transported proteins in intact (24-30h), injured and 7 day regenerating (10-16h) sciatic nerves. Proteins quantitated are marked in Figure 9b. Bars are SEM (n = 7); * = significant change.



Intact, 24-30h

Injured, 10-16h



7 day regenerating, 10-16h

Figure 9b. Comparison c. Retrogradely transported proteins in intact, injured and 7 day regenerating sciatic nerves. Numbers refer to Figure 9a.

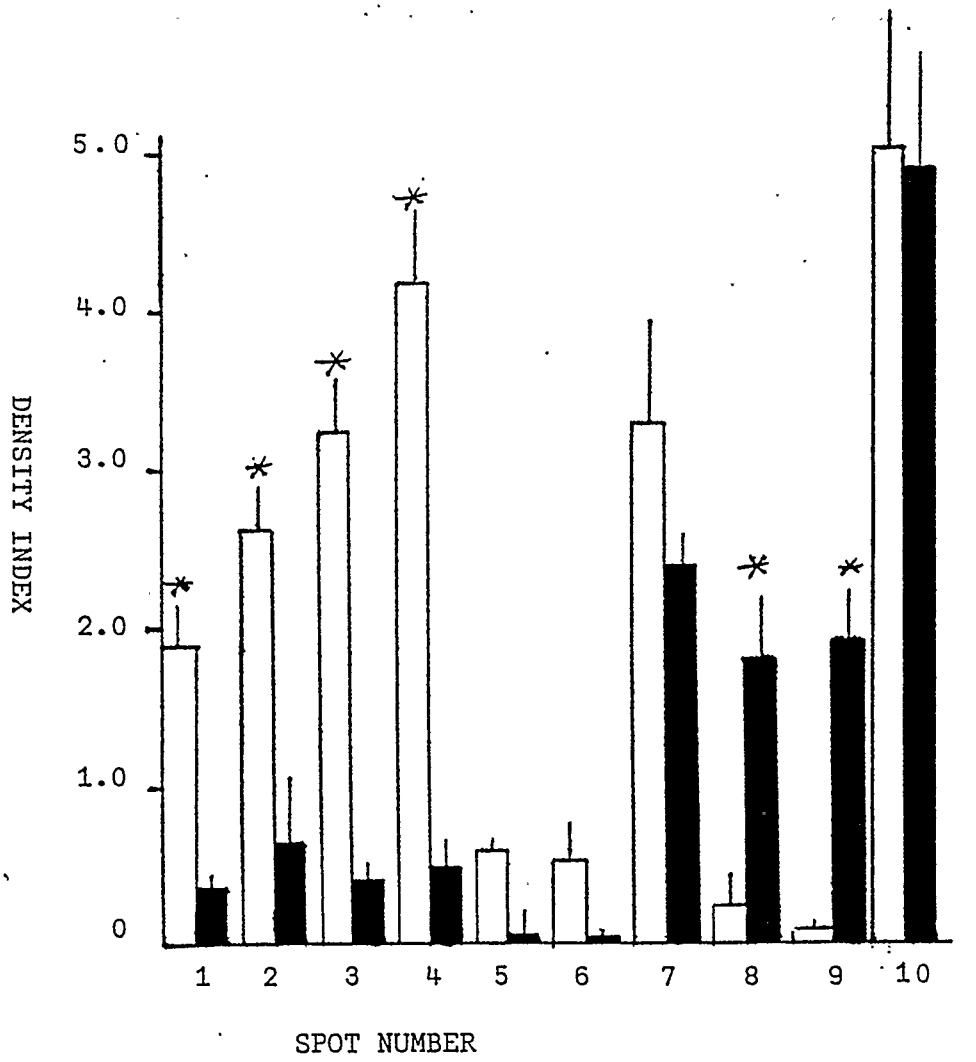
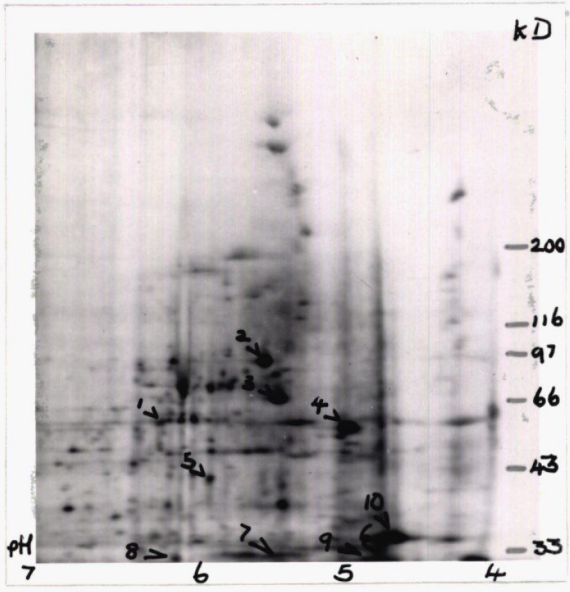
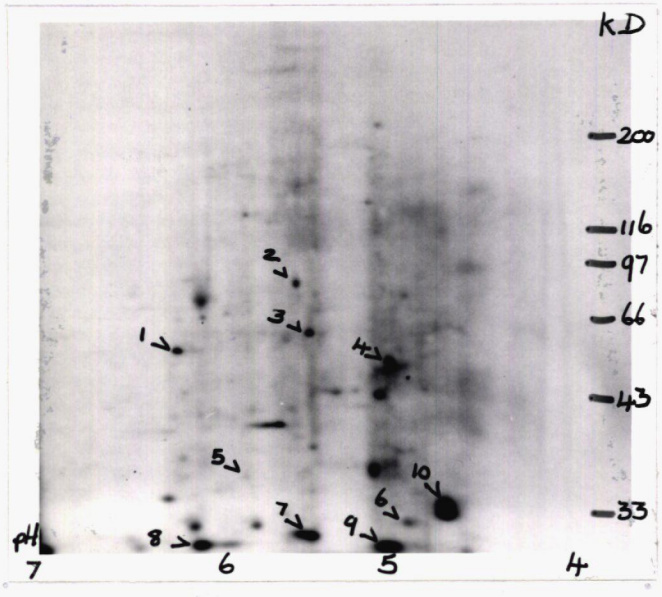


Figure 10a. Bar graph of density indices for anterograde (open bars) and retrograde (shaded bars) transported protein in intact sciatic nerve, 24-30h after precursor injection. Proteins quantitated are marked in Figure 10b. SEM is indicated (n = 7); * = significant change.



Anterograde



Retrograde

Figure 10b. Comparison d. Fast axonally transported proteins in intact rat sciatic nerve, 24-30h after precursor injection. Numbers refer to Figure 10a.

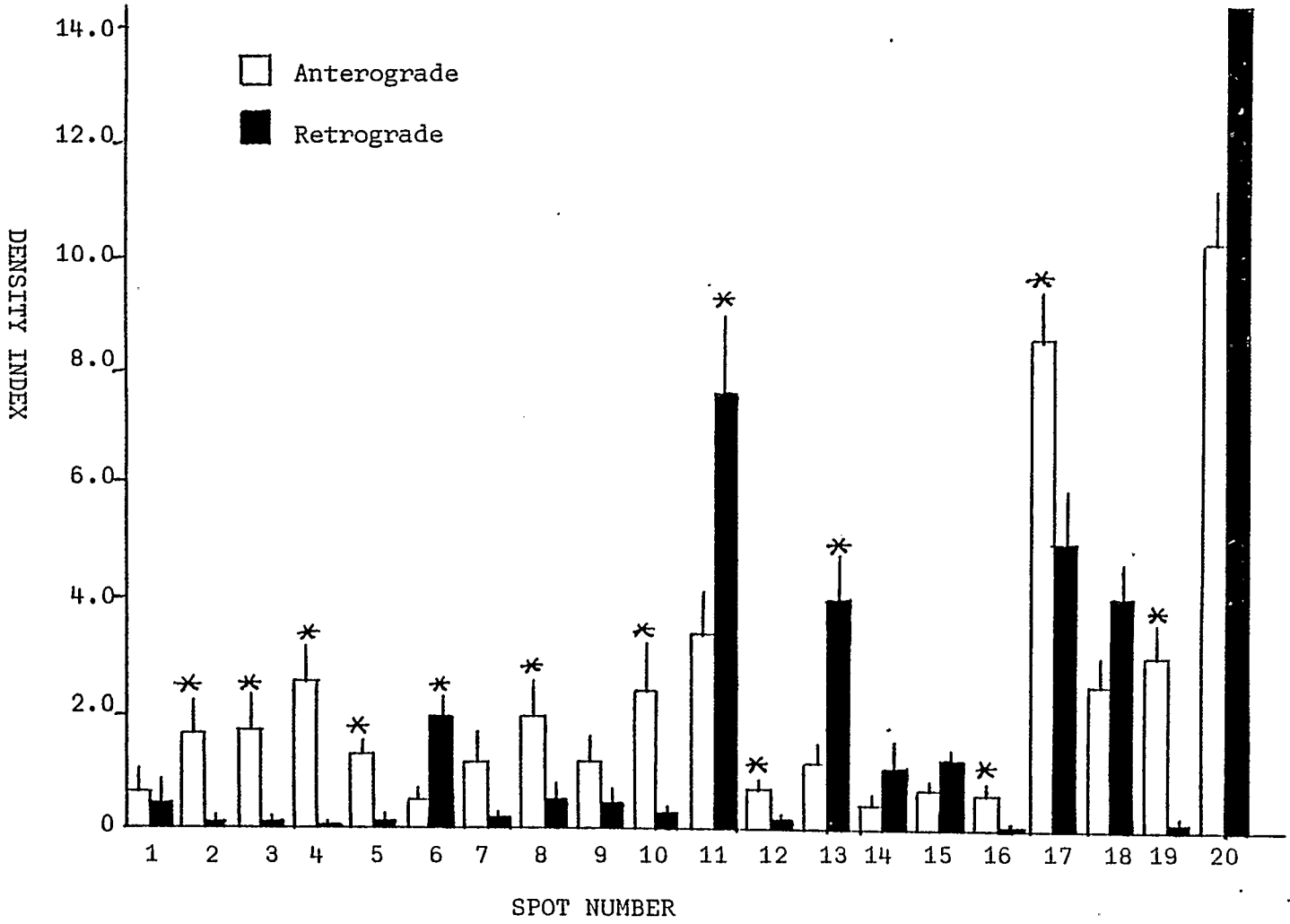
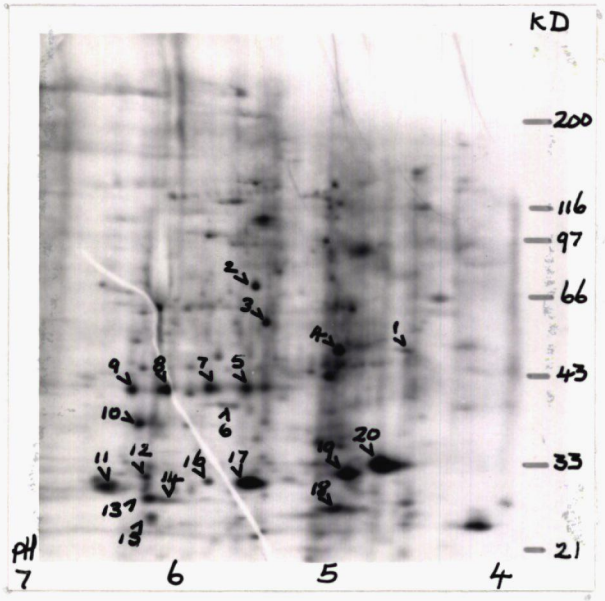
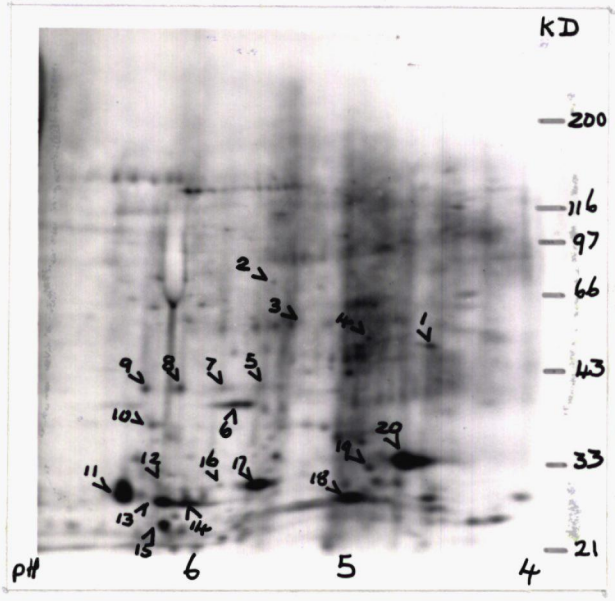


Figure 11a. Bar graph of density indices for anterogradely and retrogradely transported proteins in injured sciatic nerves, 10-16h after precursor injection. SEM is indicated (n = 8); * = significant change.



Anterograde



Retrograde

Figure 11b. Comparison e. Fast axonally transported proteins in injured rat sciatic nerve, 10-16h after precursor injection. Numbers refer to Figure 11a.

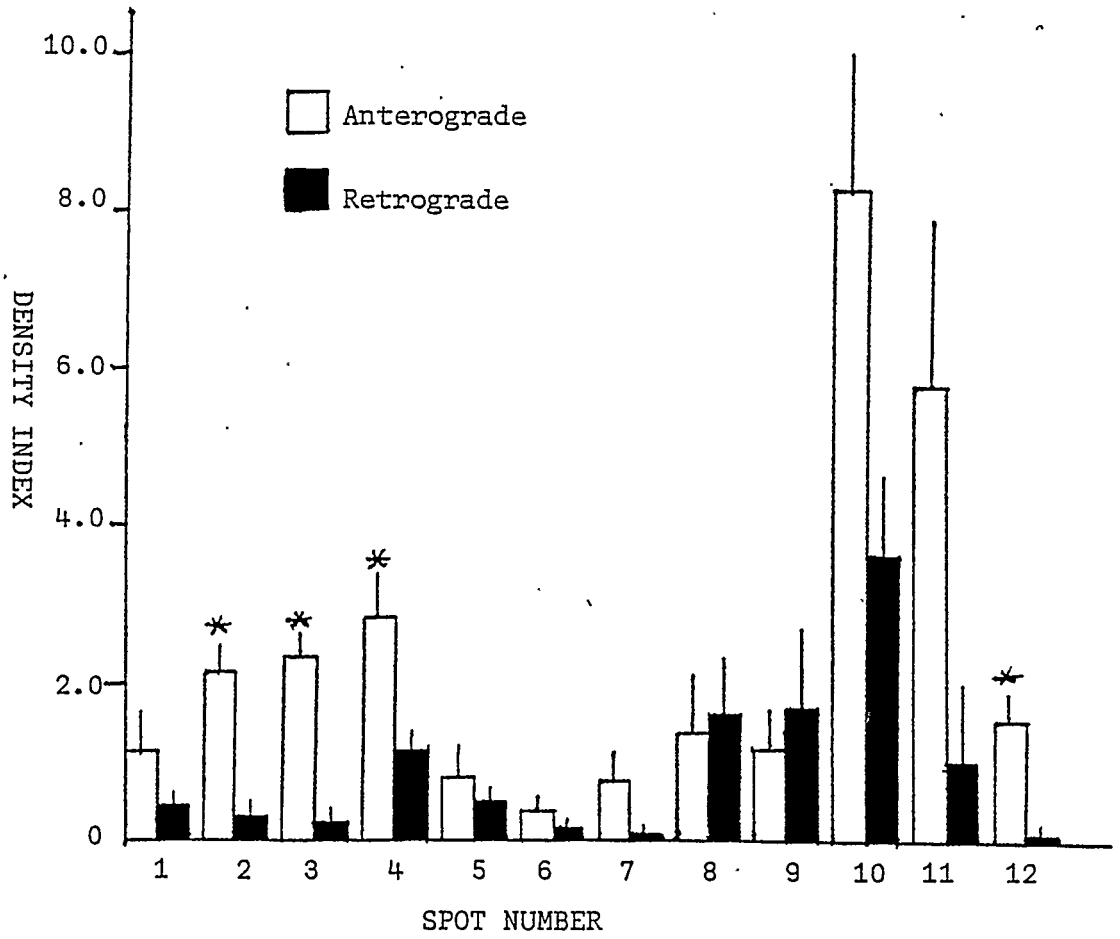
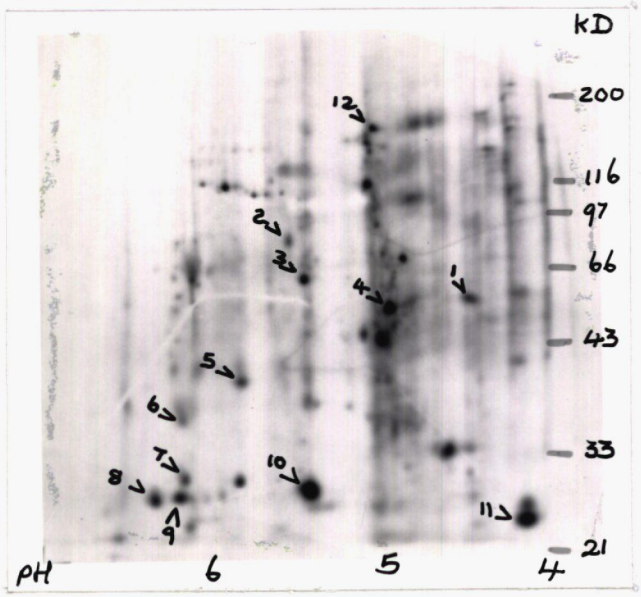
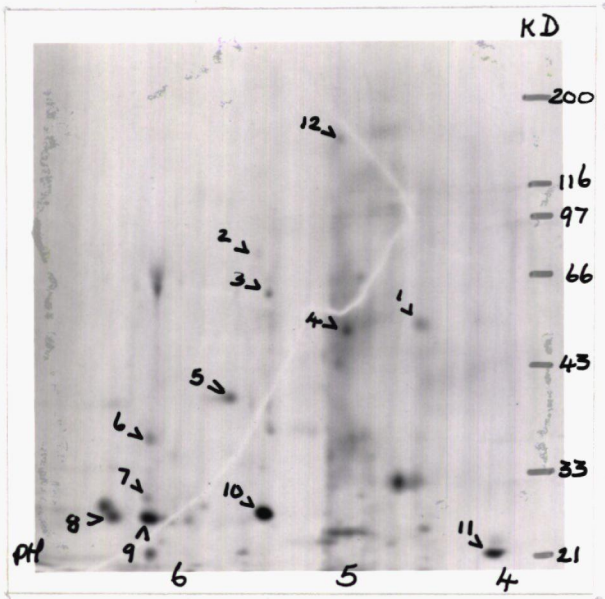


Figure 12a. Bar graph of density indices for anterogradely and retrogradely transported proteins in 7 day regenerating sciatic nerves, 10-16h after precursor injection. Proteins quantitated are marked in Figure 12b. SEM is indicated (n = 8); * = significant change.



Anterograde



Retrograde

Figure 12b. Comparison f. Fast axonally transported proteins in 7 day regenerating rat sciatic nerve, 10-16h after precursor injection. Numbers refer to Figure 12a.

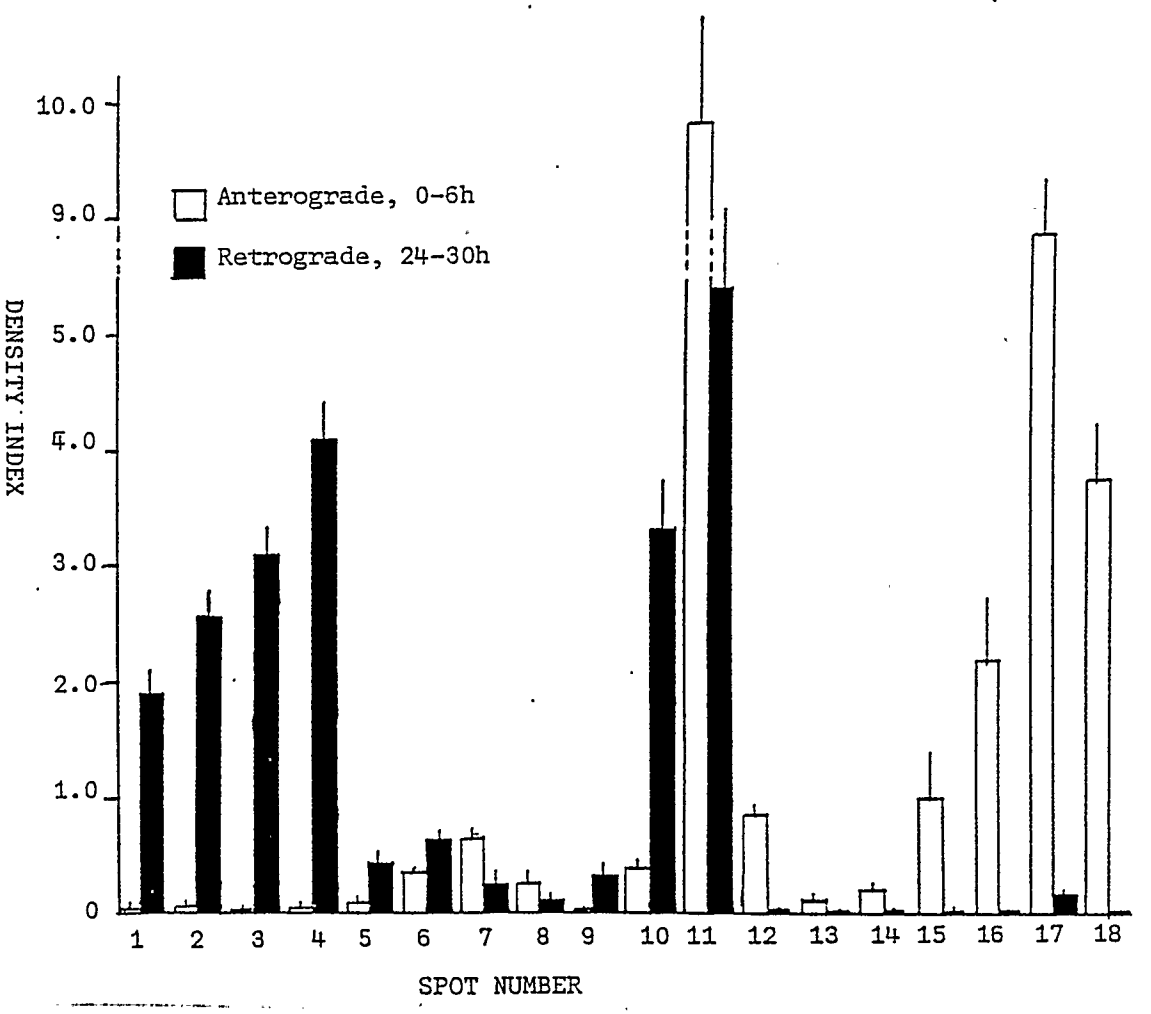
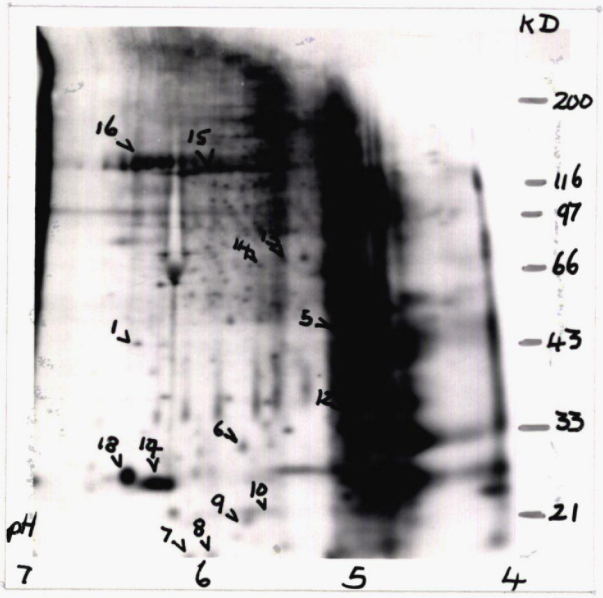
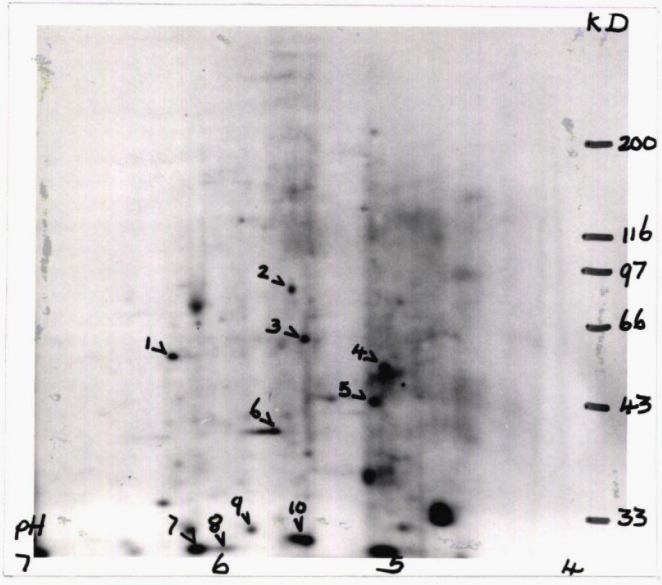


Figure 13a. Bar graph of density indices for anterogradely and retrogradely transported proteins in intact sciatic nerves, 0-6 and 24-30h after precursor injection, respectively. Proteins quantitated are marked in Figure 13b. SEM is indicated (n = 7); *= significant change.



Anterograde 0-6h



Retrograde 24-30h

Figure 13b. Comparison g. Anterogradely (0-6h) and retrogradely (24-30h) transported proteins in intact sciatic nerve. Numbers refer to Figure 13a.

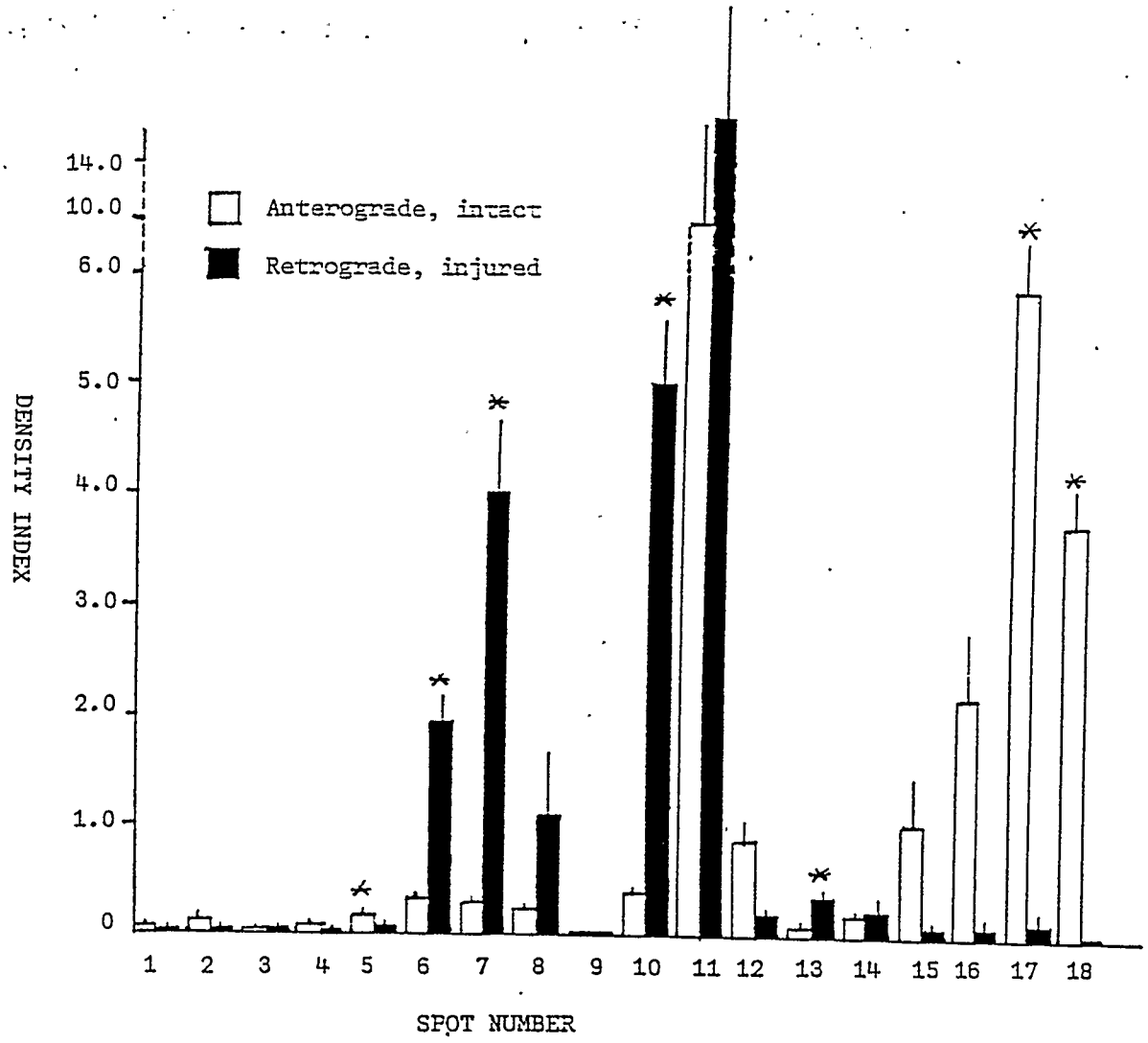
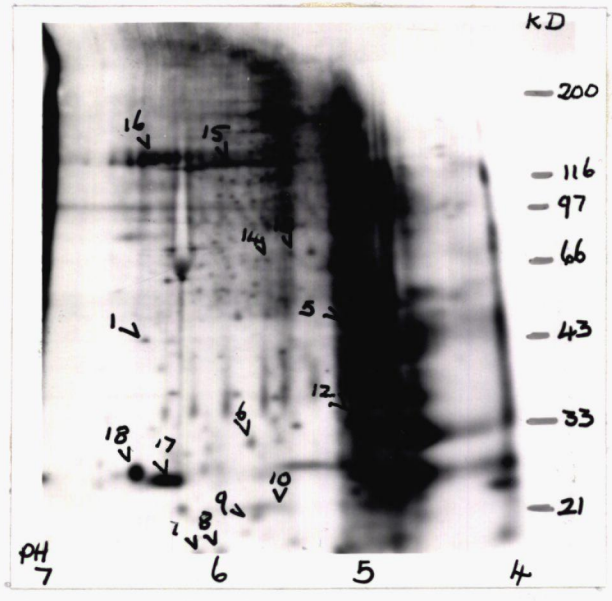
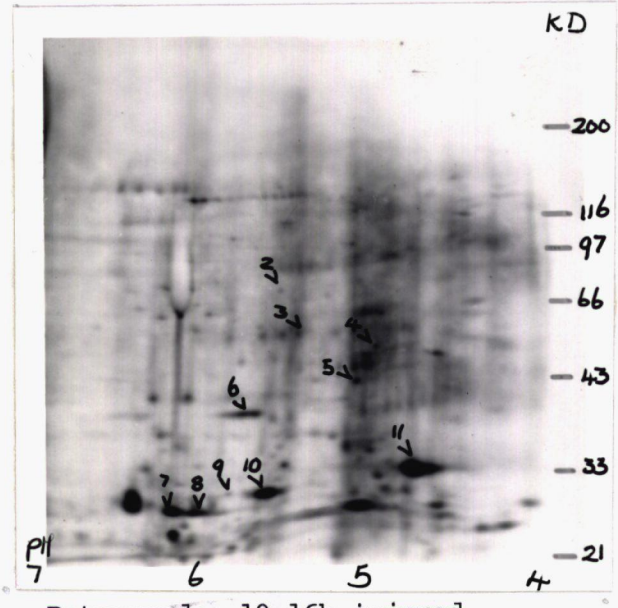


Figure 14a. Bar graph of density indices for anterograde transport in intact nerves (0-6h) and retrograde transport in injured nerves (10-16h). Proteins quantitated are marked in Figure 14b. SEM is indicated (n =7); * = significant change.



Anterograde, 0-6h intact



Retrograde, 10-16h injured

Figure 14b. Comparison h. Anterogradely transported proteins in intact nerve (0-6h) and retrogradely transported proteins in injured nerves (10-16h). Numbers refer to Figure 14a.

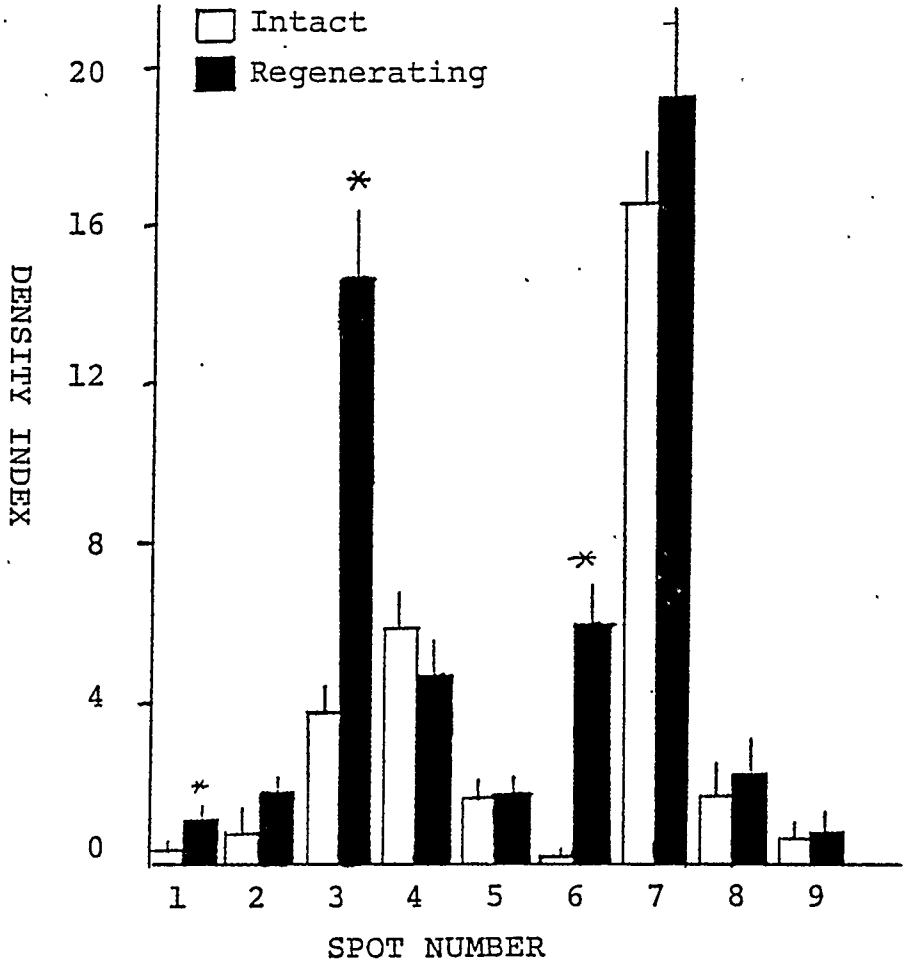
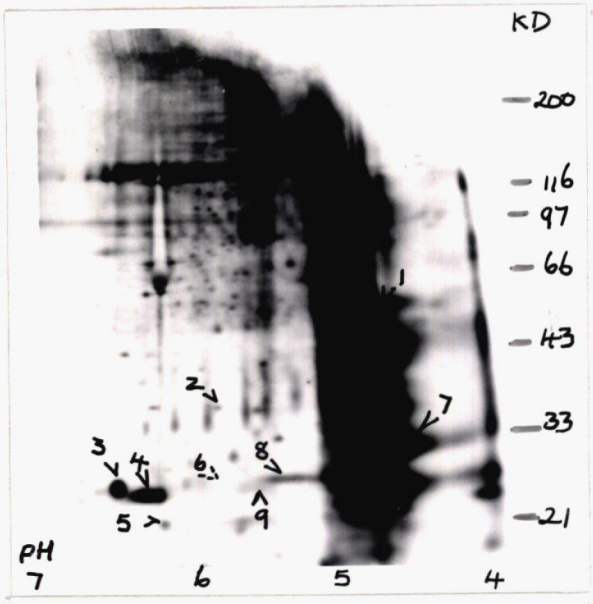
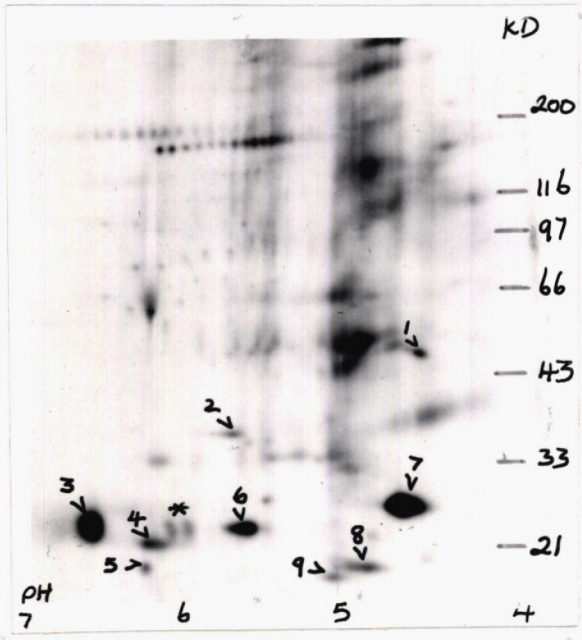


Figure 15a. Bar graph of density indices for anterograde transport in intact and 7 day regenerating nerves, 0-6h after precursor injection. SEM is indicated (n = 8); * = significant change.



Intact



7 day regenerating

Figure 15b. Comparison i. Fast anterogradely transported proteins in intact and 7 day regenerating sciatic nerves, 0-6h after precursor injection. Numbers refer to Figure 15a.

CONCLUSIONS.

What conclusions can be drawn from these experiments about the nature and velocity of the "signal for chromatolysis"? In the Introduction to this thesis, I suggested four possible mechanisms for the initiation of the cell body response to axotomy: i) "electrical"; ii) those in which Schwann cells release substances after axotomy which are detected by neurons; iii) those concerned with loss of trophic factors retrogradely transported from the periphery in the intact axon; iv) those involving retrograde transport in other ways such as the uptake of extra-axonal substances at the crush, or increased return of retrogradely transported material.

In "The effect of nerve injury on individual axonally transported substances", quantitative changes in the retrograde transport of acetylcholinesterase, substance P and the muscarinic cholinergic receptor were measured; a larger number of changes in retrograde transport after injury were seen when proteins were analysed by 2-dimensional gel electrophoresis. From these results, it is clear that injury to the axon induces many changes in the retrograde transport of proteins; from the demonstration in "Response of sensory neurons to axonal injury" that rhodamine linked beads are detectable in the soma after injection into the sciatic nerve at a crush site, it is probable that endogenous axonally transported materials which reverse their direction at a crush return to the cell bodies, because the beads must

be associated with endogenous vesicular structures following uptake into the axon. Alterations in retrograde transport after injury are thus likely to be detectable in the soma. It is not known whether the cell body can respond to such changes.

Could such a change in retrograde transport constitute a "signal for chromatolysis? In "Response of sensory neurons to axonal injury", I have attempted to differentiate between possible mechanisms iii) and iv), listed above. To do this it was necessary to discover a rapid, transient, reliable and easy-to-measure indicator of the cell body response in a system with a long length of nerve; the dorsal root ganglion-sciatic nerve system in the rat was used, and the activity of the enzyme transglutaminase was measured to test for such an indicator. The activity of the enzyme did not increase up to 26h after injury, and so experiments designed to conclude whether the "signal for chromatolysis" arises at the injury site, or is due to a loss of peripheral, retrogradely transported trophic factor could not be completed.

In the Introduction (pages 24-27), I have presented evidence that many observations made by other workers are consistent with a "signal" arising from the injury site, rather than with loss of a peripheral trophic factor, and that the time course of observations such as the onset of a cell body reaction in superior cervical ganglion cells (Mathews and Raisman 1972) is also consistent with the velocity of retrograde axonal transport. In the chapter "2D gel analysis of

axonally transported proteins", I have demonstrated many changes in the composition of retrogradely transported proteins after nerve injury, and these could be interpreted by the soma as an indication of axonal damage. However, I have also measured an increase in the synthesis and anterograde transport of the growth-associated protein GAP43, occurring by 10-16h after injury. In these nerves, the crush was made 6cm from the cell bodies in the dorsal root ganglia. Assuming a maximum retrograde transport rate of 17mm/h, a "signal" arising at the injury site would then take 60/17 = 3.5h to arrive at the cell body. In the chapter, I have shown from the short half life of [35-S] methionine in the ganglia that changes in protein synthesis probably occur within 2h of precursor injection. Considering that the time of injection is coincident with the time of nerve injury, these changes in protein synthesis seem to occur far too rapidly for them to be initiated by any kind of retrogradely transported signal, whether diversion of transported proteins or loss of retrograde trophic material.

There is some evidence for the involvement of each of the four possible mechanisms I have suggested for initiation of the cell body reaction:

i) Given the calculation above that the "signal for chromatolysis" travels faster than retrograde axonal transport, "electrical" mechanisms may need to be reevaluated;

ii) I have not addressed the question of new factors being released from Schwann cells after nerve injury, although there is some evidence

for this (see Introduction, pages 20-22);

iii) Loss of peripheral trophic factors is likely to be involved in some aspect of the cell body response to axotomy, as discussed in the Introduction, pages 22-25;

iv) I have shown that there are many changes in the composition of retrogradely transported materials after injury, and these may be detected by the soma. There is also a large amount of other evidence showing that the time course of many aspects of the cell body response is consistent with a retrogradely transported "signal for chromatolysis".

It is possible that there are multiple signals to which the cell responds after axotomy. Recent evidence (Bisby et al 1987) has shown that growth-associated proteins are expressed in increased amounts by axotomised frog sciatic neurons, even when the frogs were kept at 15°C and showed no classical cell body response to axotomy. The separation of responses to axotomy in this way suggests that different components of the reaction may be a result of different signals from the axon and Schwann cells. For example, a rapid electrical signal could be the event which initially "primes" the cell body for axonal regeneration, and that this is then followed by changes in other aspects of cell function such as release of new factors from Schwann cells and changes in retrograde axonal transport. Each of these subsequent events may trigger a specific set of metabolic responses in the soma, which together are seen as the classical changes observed in the cell bodies of axotomised neurons.

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