AXONAL INFLUENCES ON SCHWANN CELL

GENE EXPRESSION.

by

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DECLARATION:

The contents of this thesis are the work of the author with the exception of figure 5.12 which was produced by Dr. Lynn Mitchell. The thesis has not been previously submitted for the award of a degree to any other university.

Christine E. Thomson.

To mum and dad

Thank you for your love and support,
and for giving me an education

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SUMMARY:

The aim of these studies was to investigate some of the ways in which the axon influences Schwann cell gene expression.

One of the major parts of the project was attempting to establish an *in vitro* model of Schwann cell-axon interaction. Schwann cells cultured in isolation from neurons, down-regulate expression of molecules associated with the myelin sheath. It is proposed that the putative signal that stimulates myelin gene expression by the Schwann cell is located on the axolemma. Therefore, a model system was established whereby addition of an axolemmal-enriched fraction derived from neonatal rat brain, caused the upregulation of myelin-specific molecule expression by cultured Schwann cells. The ability of the Schwann cells to respond was influenced by the method of culture, and only those cells derived from explants of either neonatal rat sciatic nerve, or denervated adult rat sciatic nerve, were capable of responding. The role of basement membrane components in this system was also investigated.

Addition of axolemmal-enriched fractions of brain tissue did result in the upregulation of P_0 mRNA expression by the Schwann cells. This upregulation was documented both by *in situ* hybridisation and dot blotting. The degree of upregulation was variable and other, as yet, unknown factors appear to influence the ability of the Schwann cell to respond. By dot blotting, the cultures treated with these fractions expressed the P_0 message at a level 1.63 fold greater than non-treated cultures.

The role of the axon in influencing Schwann cell gene expression was also investigated by studying various aspects of Wallerian degeneration and primary demyelination.

Delayed Wallerian degeneration occurs in the C57Bl/Ola mouse mutant after axotomy. This delayed morphological breakdown was found to be associated with retarded down-regulation of the P_0 mRNA both on teased nerve fibres assessed by *in situ* hybridisation, and on pooled nerve samples evaluated by northern blotting.

Calcium is known to have a role in degeneration of nerve fibres both *in vivo* and *in vitro* and Wallerian degeneration of cultured explants can be delayed by chelating the calcium from the culture medium. The effect of calcium on degeneration was investigated morphologically, immunohistochemically and at the level of gene transcription. Calcium chelation retarded the morphologic aspects of

Wallerian degeneration and the upregulation of genes (NGF receptor, GFAP and N-CAM) which are associated with degeneration of myelin sheaths. However, the expression of P_0 mRNA was not maintained, thereby suggesting different mechanisms of control for myelin-specific genes and genes associated with the non-myelin-forming Schwann cell phenotype.

Tellurium causes primary demyelination when fed to weanling rats. Investigations into this neuropathy suggested that the expression of genes associated with the non-myelin-forming Schwann cell may be under very local, and focal control within a given Schwann cell. Partial demyelination of a single Schwann cell internode was associated with focal re-expression of NGF receptor protein and GFAP, and to a lesser extent, N-CAM in the demyelinated area. However, this same partially demyelinated internode could still be expressing P_0 mRNA quite strongly.

The combination of these studies have suggested that the putative myelinating signal is probably located on the axolemma, but have raised the possibility that expression of certain molecules (NGF receptor, GFAP) may be determined by factors other than the axon. Such factors may be of purely Schwann cell origin, and include the state of the myelin sheath, or the area of contact between the adaxonal Schwann cell plasmalemma and the axolemma. The possible effects of contact surface area on myelination are discussed in the final chapter.

"His wisdom is infinite, that of which we are ignorant is contained in him, as well as the little that we know" (Kepler, astronomer).

PREFACE

Development and regeneration within the peripheral nervous system are complex and begin with the outgrowth of axons from neurons. During development, this is closely followed by the migration of Schwann cells from the neural crest. Initially the Schwann cells lie outside large bundle of axons, but subsequently invade the fascicles, proliferate and begin to associate with the axons. During regeneration, pre-existing Schwann cells proliferate in response to contact with growing neurites, and then become intimately associated them. In both cases, this initial association is only the beginning of a very complex and fascinating interaction between the glial cell and the axon. How the Schwann cell ultimately relates to the axon appears to be dictated by the axon itself. Some Schwann cells just ensheath axons to form unmyelinated fibres, yet others will wrap the axon many times with a compacted extension of their plasmalemma to form a myelin sheath.

Without the myelin sheath, much of the mammalian nervous system would not function. The vital role of myelin can be observed directly from patients afflicted with primary dys/demyelinating diseases. The lack of myelin results in impaired nerve conduction which may either be slowed or completely blocked. Clinically this results in weakness, ataxia, sensory deficits, paresis and paralysis, and ultimately respiratory failure and death. The clinical signs depend on the location and severity of the lesion (Duncan and Griffiths, 1984). Despite good morphologic characterization of many of the diseases that affect myelin, much of the basic neurobiology underlying the process of ensheathment and myelination is not known. Likewise the trigger that initiates the production of the myelin sheath by the Schwann cell is a mystery.

The dependence of the Schwann cell myelin sheath on axonal contact is observed both during development and regeneration of the nerve. During development it is only after the Schwann cell adopts a 1:1 relationship with a single axon that the morphological features of incipient myelination appear i.e: the formation of the mesaxon and its subsequent elongation and spiralling around the axon to ultimately form the compact myelin sheath (Peters and Muir, 1959; Peters, 1976; Ranscht *et al.*, 1987) Pathologically, lack of axonal contact in the distal stump of transected sciatic

nerves has a negative effect on the expression of genes from which myelin sheath components are transcribed, and the cells fail to synthesize a myelin sheath and (Gupta *et al.*, 1988; Le Blanc *et al.*, 1987; Lemke and Chao, 1988b; Poduslo, 1984a; Trapp *et al.*, 1988b). Regeneration of axons into the area of these Schwann cells stimulates upregulation of myelin-specific molecule expression (Gupta *et al.*, 1988; Le Blanc *et al.*, 1987; Mitchell *et al.*, 1990; Politis *et al.*, 1982). These studies suggest that expression of these molecules by the Schwann cells is dependent upon axonal contact. This axonal dependence is directly reflected in the steady-state levels of the mRNAs encoding these proteins (Gupta *et al.*, 1988; Le Blanc *et al.*, 1987; Lemke and Chao, 1988b; Trapp *et al.*, 1988b).

In vitro, loss of myelin-specific molecule expression by Schwann cells is observed when Schwann cells are enzymatically dissociated from neonatal rat sciatic nerve. This dissociation causes loss of Schwann cell-axon contact which, in turn, causes down regulation of expression of myelin components within 5 days of culture (Brockes *et al*, 1981; Jessen *et al*, 1985; Lemke and Chao, 1988b; Mirsky *et al*, 1980; Morrison *et al*, 1991; Owens and Bunge, 1989; Winter *et al*, 1982). This low level of expression is maintained indefinitely in the absence of axonal contact. If axon-Schwann cell contact is re-established (by coculturing the Schwann cells with DRG neurites) then upregulation of myelin-specific molecules by the Schwann cells occurs (Winter *et al.*, 1982; Mirsky *et al.*, 1980).

Thus the axon is known to have a profound effect on the behaviour of the Schwann cell both with respect to the structural contortions it makes to envelop the axon and its molecular expression. The studies undertaken in this thesis were aimed at investigating the effect of the axon on Schwann cell gene expression, which, in turn, dictate the cell's behaviour and nature of ensheathment.

1.0 INTRODUCTION:

The nerve fibre of the peripheral nervous system (PNS) has developed to propogate nerve impulses from one point of the organism to another. In this thesis, the nerve fibre refers to the axon and its associated ensheathing Schwann cells. The axon is one of the processes conducting information to or from the nerve cell body. The satellite cells of the peripheral nerve fibre consist mostly of Schwann cells, some fibroblasts and a few immune cells (macrophages, mast cells).

Two types of mature nerve fibre exist in the normal animal, based on the type of axonal ensheathment - the fibre may be myelinated or unmyelinated (Thomas and Ochoa, 1984). Myelin is found in both the peripheral and central nervous systems (CNS); this thesis is concerned with that of the PNS. By forming a tubular sheath around certain axons within the PNS, peripheral myelin is the structural specialization that allows for much higher nerve conduction velocities. The sheath is formed by many Schwann cells that associate with the axon along its length from a point near the cell body, to within 1 to 2 μm from the axonal termination. Adjacent Schwann cells abut at the nodes of Ranvier, thus, the sheath is really a series of apposed myelin tubes. The myelin itself is a specialization of the Schwann cell membrane which has wrapped around the axon many times to form concentric lamellae. The intervening cytoplasm is lost from between the membranes as they compact to form the myelin. Morphologically, by electron microscopy, the myelin sheath appears as a set of concentrically arranged lamellae wrapping around the axon; its main constituents are lipid and protein.

The speed of nerve impulse conduction in the mammalian unmyelinated fibres is of the order of 1.0 m/sec. Without significantly increasing axonal diameter, higher conduction speeds cannot be achieved. The structural specialization of myelination allows for much greater conduction velocities without significantly increasing the axonal diameter. It is possible that increased transmission frequency also occurs (Thomas and Ochoa, 1984).

1.0.1 BASIC ANATOMY OF THE PERIPHERAL NERVE FIBRE:

Connective tissue sheaths subdivide nerves. The outermost covering is the epineurium. This gives the nerve its characteristic shape and strength and loosely separates bundles of nerve fibres (fascicles) from each other. The perineurium ensheathes the individual fascicles within the nerve and separates the nerve fibres and endoneurium from the extraneural environment. The perineurium is comprised of

flattened cells joined by tight junctions and forms the blood nerve barrier which effectively excludes many large molecules from the intraneural environment. The endoneurium is the innermost covering and invests the nerve fibre proper. It is mostly composed of collagen with occasional interspersed fibroblasts. Blood vessels course within the endoneurium and connective tissue sheaths (Bunge and Bunge, 1981; Jenkins, 1978) (fig 1.0.1).

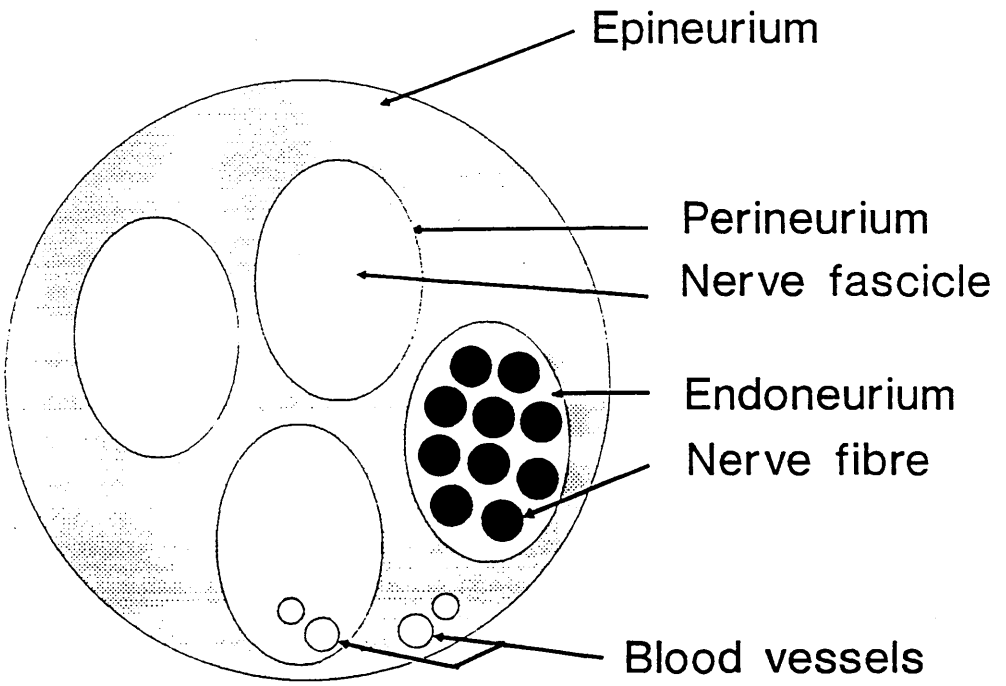


Figure 1.0.1: Diagram of a peripheral nerve. (Graphics by Mr. A. Miller, University of Glasgow).

1.1 AIM OF THESIS:

Contact of the Schwann cell with the appropriate axon is essential for the biosynthesis of myelin components by Schwann cell. However, little is known about the molecule(s) that initiate and maintain myelin-specific molecules (MSM) expression. The structure, dynamics and location of the putative myelinating signal remain an enigma. In this thesis, it is postulated that there is such a signal, it is produced within the neuronal soma, transported down the axon, and inserted in, or near, or possibly even secreted by, the axolemma. Here it interacts over a very short distance with a receptor on the Schwann cell membrane. Somehow, possibly via an intermediate carrier such as cyclic-AMP, the message is transduced from the Schwann cell plasmalemma to the Schwann cell nucleus. Here it interacts with the gene pool and causes upregulation of the genes that encode transcription of message for synthesis, and ultimately assembly, of the myelin sheath (fig. 1.1.1).

The aims of this thesis are to:

1) Investigate the location of the myelinating signal.

2) Investigate the mechanism of interaction of the myelinating signal with the myelin-forming Schwann cell.

3) Evaluate the lability of the myelinating signal during its interaction with the Schwann cell by studying situations of delayed Wallerian degeneration.

4) Investigate the effect of Wallerian degeneration on expression of other axonally-controlled molecules by Schwann cells.

5) Investigate the role of the extracellular matrix in MSM expression by Schwann cell.

6) Develop an *in vitro* model in which Schwann cells can be stimulated to express MSMs in the absence of contact with intact axons. Components of this model could then be manipulated to further study the composition of the myelinating signal, and mechanisms by which Schwann cells can be stimulated to upregulate MSM expression.

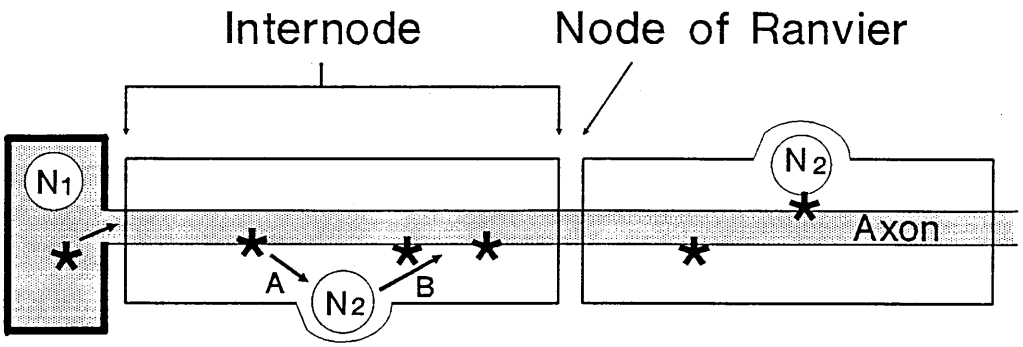


Figure 1.1.1: Possible mechanism of Schwann cell-axon interaction for upregulation of myelin-specific molecule expression. It is postulated that the putative signal (*) that stimulates myelin formation by the Schwann cell is produced in the neuronal soma, transported down the axon and becomes associated with the axolemma. It can then interact with the Schwann cell plasmalemma and the signal is then transduced to the Schwann cell nucleus (A), possibly via an intermediate carrier such as cyclic-AMP. The gene pool encoding the expression of myelin-specific molecules is stimulated and transcribed (B). N1 = neuronal nucleus; N2 = Schwann cell nuclei. (Graphics by Mr. A. Miller, University of Glasgow).

1.0 LITERATURE REVIEW

1.2 AXON

1.2.0 Introduction

The PNS is comprised mainly of the axon, its surrounding glial cell (Schwann cell), with or without an attendent myelin sheath, fibroblasts and occasional leucocytes such as mast cells or macrophages. The axon is the outgrowth of the neuron and is composed of a plasma membrane, called the axolemma, and the axoplasmic matrix with enclosed cytoskeleton and organelles. The Schwann cell either just ensheathes the axon to form an unmyelinated nerve or spirals around the axon to form a myelin sheath and a myelinated fibre. The length of axon surrounded by the myelin sheath of one Schwann cell is the internode, the node of Ranvier is the area where adjacent Schwann cells abut and the paranode is the specialized area just lateral to the node bordering the margins of the sheath. The structure of the axon, especially the axolemma, depends upon the nature of Schwann cell ensheathment. In the unmyelinated fibre the axon is more uniform, but in the myelinated fibre the structure varies between the node, paranode and internodal regions.

In mammals, axonal diameter varies between 0.1 and 16 μm with unmyelinated axons ranging between 1 to 2 μm and the larger diameter fibres being myelinated. The length varies between 1.0 cm and several meters depending upon the size of the animal. Axons tend not to branch except near the sensory or effector fields (Berthold, 1978).

1.2.1 Axoplasm

In most nerves greater than 90% of the neuronal cell volume is axonal (Berthold, 1978). Studies have shown that the axonal calibre is directly proportional to the ratio of target size to the number of innervating axons. This suggests that axonal calibre is influenced by retrogradely transported signals (Voyvodic, 1989). The axonal diameter at the node is reduced to 1/3 to 1/6 of the internodal diameter (Berthold 1978).

1.2.1.1 Myelinated fibres

The axonal cytoplasm has a viscosity approximately five times that of water. It contains a variety of organelles, including the longitudinally oriented microtubules and neurofilaments (Thomas and Ochoa, 1984). The cytoskeletal proteins include neurofilaments, microtubules, actin, clathrin, actin-binding proteins and fodrin (brain spectrin). Actin and fodrin are predominantly subaxolemmal in location, whilst the microtubules and neurofilaments are fairly regularly distributed throughout the axon (Papasozomenos *et al.*, 1982).

The microtubules are non-branching structures about 25 nm diameter comprised of a 5 nm thick wall surrounding a hollow core (Thomas and Ochoa, 1984). They are composed of tubulin dimers consisting of an α and ß subunits (Thomas and Ochoa, 1984; Wallin 1988) which are synthesized in the neuronal somata and transported down the axon by slow axonal transport (Pleasure, 1984). The average microtubule length in cultures of sensory neurones is about 100 μm. Fine processes, 20 to 100 nm long, extend into the axoplasm from the tubules (Thomas and Ochoa, 1984). Other polypeptides have been found in association with microtubules. Microtubule associated proteins (MAPs) such as MAP1, MAP2, and tau factors, facilitate microtubule assembly. MAP1 and 2 are high molecular weight proteins (350 and 270 kDa respectively) which have been observed as projections extending from the microtubule surface, whereas the tau proetins (55 to 70 kDa) form a fuzzy coat on the microtubule surface (Wallin, 1988). Tau factors may form the side arms of microtubules connecting them with neurofilaments and other organelles (Thomas and Ochoa, 1984; Papasozomenos *et al.*, 1982)

The density of the microtubules is generally 10 to 20/μm^2, however, this varies depending on whether the nerve is sensory or motor, or a dorsal or ventral root. The number of microtubules also varies within the axon; there may be more in the branch of an axon than in the parent axon, which raises the possibility of local polymerization within the branch axoplasm from pre-existing tubulin units. A state of labile equilibrium exists between microtubules and monomeric tubulin. Tubules may be disassembled into units by calcium, colchicine, vincristine and vinblastine, increased hydrostatic pressure and cold (Ochs, 1984; Pleasure, 1984; Wallin 1988). Microtubules are important in providing a supportive cytoskeleton for elongated cell processes, such as axons, and are a major component of the fast anterograde and retrograde axonal transport systems (Pleasure, 1984; Wallin, 1988). They also have roles in cell division and cell movement/shape changes (Wallin, 1988).

Neurofilaments are the major component of the neuronal cytoskeleton and are composed of three proteins; the 68 kDa protein appears to form the core; the 145 kDa protein wraps around it; and the 200 kDa protein contributes to the side arms which link the neurofilaments to each other and to the other components of the cytoskeleton. The latter two proteins are rich in phosphorylation sites which may mediate interactions of neurofilaments with each other and with other cellular components (reviewed Rosenfeld *et al.*, 1987). Neurofilaments are of undetermined length and are about 10 nm in diameter. They are unbranched, but at 50 nm intervals give rise to 1 to 8 lateral filaments which radiate 30 to 60 nm out to interconnect with neighbouring neurofilaments. A regular network of neurofilament groups are formed and these probably pursue a spiral course down the length of the axon. The neurofilament density is 100 to 300/μm^2 (Thomas and Ochoa, 1984). Neurofilaments are synthesized in the neuronal soma and carried by slow axonal transport (Pleasure, 1984). They play a major role in determining axonal calibre as well as neuronal size and shape. The degree of phosphorylation varies within different regions of individual neurons. Normally the phosphorylated form is present in the axons and the nonphosphorylated form within the perikarya, dendrites and axons. Phosphorylated forms are found in perikarya in some pathologic states, but the factors leading to their distribution here are not known (reviewed Rosenfeld *et al.*, 1987). Neurofilaments appear to have an important role in determining axonal calibre (Friede and Samorajski, 1970; Hoffman *et al.*, 1984). In the constricted portion of the axon at the node of Ranvier, the numbers of neurofilaments are reduced correlating with the decreased axonal calibre, but their density remains constant with the internodal axon (Hoffman *et al.*, 1984). Axonal calibre is more closely related to the neurofilament content than to the thickness of the myelin sheath (Friede and Samorajski, 1970).

A monoclonal antibody, A60, has been produced from bovine brain by immunizing mice. The antibody binds to a 60 kDa subaxolemmal protein which is thought to be a cytoskeleton associated peripheral membrane protein. Its *in vivo* role may be linkage to neurofilaments or other membrane or cytoskeleton components (Rayner and Baines, 1989)

Axonal mitochondria are 0.1 to 0.3 μm in diameter and 0.5 to 8.0 μm in length. They are found in higher densities in smaller axons (2 to 5/μm^2) and lower densities in larger axons (0.1/μm^2). They are thought to be formed within the neuronal somata and transported at an intermediate rate down the axon where they are longitudinally oriented. The smooth endoplasmic reticulum (sER) forms a tubular or flattened cisternal network that extends from the axonal hillock to the nerve terminals occupying less than 2.5% of the axonal area. Within the axon proper, a primary

system of sER is formed which is composed of thicker tubes located in the sub-axolemmal position. The secondary system consists of thinner tubules located more centrally in the pre-terminal axonal branches. Ribosomal particles are rarely observed distal to the axon hillock, but may occasionally be encountered free in the axoplasm or related to the sER or mitochondria. Other structures observed in the axon include vesicles, vesiculo-tubular bodies, multivesicular bodies and lamellated osmiophilic bodies which may be lysosomes. Glycogen particles are observed in some axon terminals and membrane bound glycogen particles (glycogenosomes) are also occasionally observed within nerve trunks (reviewed by Thomas and Ochoa, 1984; reviewed by Berthold 1978).

1.2.1.2 Unmyelinated fibres

The axoplasm of the unmyelinated fibre differs very little from the myelinated fibre except that it has a less conspicuous neurofilament network and sER and more prominent microtubules (reviewed by Berthold, 1978; Friede and Samorajski, 1970). A diffuse electron-dense coating 5 to 20 nm wide is observed beneath the axolemma of unmyelinated fibres apposing each other in the same Schwann cell trough (reviewed by Berthold, 1978).

1.2.2 Axolemma

The axolemma is an excitable trilaminar membrane 7 to 8 nm thick bounding the axon. The inner leaflet is slightly thicker and more osmiophilic than the outer from which it is separated by a clear zone (reviewed by Thomas and Ochoa, 1984). Freeze fracture studies have identified the presence of rounded particles 7 to 12 nm in diameter distributed throughout the P and the E faces of the axolemma. In the nodal region there is particle pleomorphism and a much greater density of particles on the axolemmal E face than on the E face in the internode, suggesting nodal specialization (Rosenbluth, 1976; Stolinski *et al.*, 1981; Stolinski and Breathnach, 1982). Specializations of the paranodal and juxta-paranodal regions were first detected by freeze fracture studies when the Schwann cell had completed 3 to 6 turns around the axon. On the axolemma these specializations included parallel rows of diagonally oriented particles on the P face and rows of particles and pits on the E face. Rows of particles specifically positioned with respect to the axolemmal particles were noted on the opposing Schwann cell membranes. The juxta-paranodal region on the axolemma and the adaxonal Schwann cell membrane also had specific particulate patterns

(Stolinski *et al.*, 1981; Stolinski and Breathnach, 1982). Suggested roles for these axon-glia membrane specializations include ionic channels used during the propogation of nerve impulse, or that of biomechanical interaction such as axon-glia adhesion (Rosenbluth, 1976; Stolinski *et al.*, 1981; Stolinski and Breathnach, 1982). The concentration of particles in this area may be due to their physical trapping by the structures of the nodal area or they may be anchored there by a submembranous axonal filamentous network (Rosenbluth *et al.*, 1976).

On the axolemmal faces of unmyelinated fibres much fewer particles and no specialized areas were noted in the regions of adjacent Schwann cell overlap (Stolinski *et al.*, 1981; Stolinski and Breathnach, 1981). Particle aggregates were observed on the adaxonal Schwann cell plasmalemma in the regions of the mesaxon and Schmidt-Lanterman incisures. These corresponded to axolemmal particle accumulation suggesting a potential means of axon-glia communication. Other than these accumulations, internodal intramembranous particles were rare and did not occur in regions of compact myelin. The nature of these particles was not known (Stolinski *et al.*, 1985).

1.2.3 Nodal specialization

Apart from the axolemmal specializations just described, the node has some other specializations. The nodal axolemma is corrugated and may be increased by a series of spines that run across the long axis of the node (reviewed by Thomas and Ochoa, 1984). It is usually 20 to 30 nm thick but may be up to 80 nm thick and has an electron dense layer of undefined wispy material on the cytoplasmic side of the membrane. The nodal axolemma is loosely connected to other axoplasmic elements especially neurofilaments and sER. This part of the axolemma coincides with an immunohistochemically ATPase positive layer (reviewed by Berthold, 1978). An isoform of ankyrin has been localized to the subaxolemmal site at the node of Ranvier. It was also observed scattered in the subaxolemmal position along unmyelinated fibres, but not along the internodal axolemma. It may have a role in linking specialized proteins at the node to the underlying axonal cytoskeleton (Kordeli *et al.*, 1990). The density of microtubules relative to neurofilaments is increased at the node which is the converse of the internodal axon. The proximal paranodal axoplasm contains numberous vesiculo-tubular bodies whilst the more distal paranode contains more dense bodies. The axoplasmic elements, such as neurofilaments, mitochondria and sER, are increased throughout the constricted nodal axon. Electron dense granular material of unknown composition is also observed in the nodal axon

and the nodal axoplasm stains intensely with heavy metal salt (reviewed by Berthold, 1978). Nodal specialization is also illustrated by the markedly increased sodium channel density in this area compared with the internodal area. In the unmyelinated fibre there is no obvious difference in sodium channel density along the axolemma. The clustering of sodium channels in the developing nerve at the future nodes occurs prior to myelin formation (Waxman and Ritchie, 1985). The nodal specialization of the axolemma is partially determined by interaction with a myelin-forming Schwann cell (Rosenbluth, 1979; Tao-Cheng and Rosenbluth, 1983). Particle concentration is observed in the nodal area, but not in the internodal axolemma or the axolemma of an unmyelinataed axon. The differentiation of the axolemma is disturbed in cases of abnormal myelination (Rosenbluth, 1979). After demyelination, remyelination produces short internodes. However, these nerves conduct at near normal velocities, which reflects the synthesis of new channels. Similarly, conduction velocities are near normal on regenerated axons (reviewed by Waxman and Ritchie, 1985). Saxitoxin binding studies have indicated that the density of sodium channels is of about $12{,}000/\mu m^2$. Therefore, the increase in nerve impulse conductance at the node is thought to be due to a concentration of channels and the internodal axolemma is thought to be inexcitable due to lack of channels (Ritchie and Rogart, 1977).

1.2.4 Periaxonal space

The internodal periaxonal space is located between the axolemma and the adaxonal Schwann cell membrane. It was reported to be about 20 to 30 nm wide (Berthold, 1978) although more recent studies suggest that it is only 12 to 14 nm wide (Trapp *et al.*, 1984b). At the paranode the space is reduced to 3 nm (Thomas and Ochoa, 1984; Rosenbluth, 1983), and at the node Schwann cell processes project to within 5 nm of the axolemma (Peters, 1976; Thomas and Ochoa, 1984). It is either virtually empty or contains diffuse, finely granular, poorly electron dense material. This space is sealed from the intramyelinic extracellular space of the intraperiod line by a tight junction. The nodal gap is the ring-like space surrounding the axolemma and situated between the terminations of the myelin sheaths of the adjacent internodes. This space is continuous with the perinodal space. The axolemma-adaxonal Schwann cell plasmalemma association appears to be fairly resistant to damage during pathologic states. This includes changes that result in axonal shrinkage or swelling, or swelling of the periaxonal cytoplasmic collar of the Schwann cell (cited in Trapp *et al.*, 1984b). Spaces between the axon and the myelin sheath are due to disruption of the adaxonal Schwann cell cytoplasm and not because of expansion of the periaxonal

space (reviewed by Bernholdt, 1978; reviewed by Thomas and Ochoa, 1984).

1.2.5 Axonal Transport

Degeneration of the nerve distal to an axotomy site implies the dependence of the axon on the cell body for its maintenance. Materials are transported both in an anterograde (somatofugal) and retrograde fashion along the axon. Anterograde transport is fast (410 ± 50 nm/day) or slow (0.1 to 2mm/day) (Grafstein and Forman, 1980; Ochs, 1984; Schnapp and Reese, 1986).

Fast axonal transport is closely dependent on oxidative metabolism and the supply of energy in the form of ATP. Calcium is also thought to have a role. Compressive ischaemia and compounds which block oxidative phosphorylation (2,4-dinitrophenol, sodium cyanide or azide) result in blockade of fast axonal transport within 10 to 15 minutes. Fast axonal transport is also blocked by compounds which interfere with glycolysis and the citric acid cycle, temperatures less than 11^{o}C and substances which disrupt microtubules (Grafstein and Forman, 1980; Ochs, 1984). Fast axonal transport is used to transport particulate matter including cellular membranous structures such as sER, plasma membrane, vesicles and mitochondria or their constituent materials. Glycolipids, lipids, cholesterol, and glycoproteins, along with acetylcholinesterase and norepinephrine, are also carried by this mechanism. The rate of transport does not appear to be affected by the age of the individual, nor whether the fibre is sensory or motor, myelinated or unmyelinated (Grafstein and Forman, 1980; Ochs, 1984).

Slow axonal transport is in the anterograde direction only and is thought to carry cytoskeletal components (microtubules and neurofilaments) and soluble proteins (Ochs, 1984; Schnapp and Reese, 1986). The neurofilaments and microtubules are carried as assembled units, but they are not cross-linked (reviewed by Hollenbeck, 1989) Cytoskeletal elements can thus be continually turned over and replaced (Schnapp and Reese, 1986).

The rate of retrograde axonal transport is approximately half the rate of anterograde transport. Proteins, glycoproteins, phospholipids, enzymes such as acetylcholinesterase, nerve growth factor, various markers, toxins and viruses are transported by this mechanism (Ochs, 1982; Ochs, 1984). The phenomenon of axonal reaction (see section 1.5.2.4) is thought to be linked to retrograde axonal transport (Ochs, 1984).

The mechanism and regulation of axonal transport is complex. It is a microtubule-based motility system utilizing accessory factors and the enzymes kinesin

and dynein in fast and retrograde axonal transport respectively (Sheetz *et al.*, 1989). Transport may be achieved by binding of the compounds to be carried to a common carrier (transport filaments) which are moved down the microtubules by means of their sidearms. ATP is required for the conformational changes of the sidearms to pull the transport filaments along (Ochs, 1982). The unitary hypothesis suggests that both slow and fast transport may occur via the same mechanism. Compounds may drop off the transport mechanism; this probably occurs with the slowly transported compounds. Thus compounds are distributed along the axon, whereas elements transported along the fast mechanism drop off less readily and are more likely to be transported to the nerve terminal (Ochs, 1982; Ochs, 1984).

1.0 LITERATURE REVIEW

1.3 SCHWANN CELLS

1.3.1. *In vivo*

1.3.1.1 Types of Schwann cells

The Schwann cell is widely distributed throughout the PNS where it forms a satellite cell to the axons. Its two main roles in the PNS are ensheathment and myelination of the axon. Schwann cells may either just envelop the axon by embedding it in a superficial trough to form an unmyelinated fibre or they may undergo structural specialization to form the myelin sheath. Hence the Schwann cell may be myelin-forming or non-myelin-forming (Peters, 1976).

1.3.1.2 Morphology of the Schwann cell

Numerous Schwann cells are required for ensheathment of the the full length of the axon. In human peripheral nerve, individual Schwann cells range in length from 100 to 1800 μm for myelinated fibres and 20 to 300 μm for unmyelinated fibres. The diameter range of myelinated fibres is 2 to 22 μm and of unmyelinated fibres is 0.5 to 3.5 μm. The internodal length is approximately 100 to 200 times the axonal diameter (Thomas and Ochoa, 1984; Hildebrand, 1989). There is some interdigitation between adjacent Schwann cells (Peters, 1976).

The myelin-forming Schwann cell

The myelin-forming Schwann cell is like a Swiss roll wrapped around the axon. The cytoplasm has been squeezed out of the majority of the "roll" by compaction of the cell's membranes. Significant amounts of cytoplasm are found in the outer and inner portions of the "roll" (abaxonal and adaxonal cytoplasm respectively). Veins of cytoplasm also run, fully or partially, from the abaxonal to the adaxonal cytoplasm across the sheath (Schmidt-Lanterman incisures) and in rims at either end (paranodal cytoplasmic pockets). In cross section, the abaxonal and adaxonal cytoplasm execute just over one turn around the myelin sheath or axon respectively. The outer mesaxon is the area of apposition formed by overlap of the outer cytoplasmic membrane. The inner mesaxon is similarly formed by the overlap of the inner cytoplasmic membranes. Tight junctions are present in both these

mesaxons to exclude extracellular material (Thomas and Ochoa, 1984; Peters, 1976).

The quantity of the adaxonal cytoplasm is relatively greater around smaller axons than larger axons, yet very few organelles are located within it. The abaxonal cytoplasm contains the nucleus and is very limited except in the perinuclear area. The nucleus, situated in the mid-internode, is elongated along the length of the axon and contains peripherally clumped chromatin. The majority of organelles, such as rough endoplasmic reticulum, Golgi apparatus, mitochondria, centrioles, lipid vacuoles and Reich granules, are located within the perinuclear cytoplasm. The Reich granules are thought to be lysosomes and are membrane bound, lamellated, rod shaped bodies approximately 1 μm in length (Peters, 1976; cited in Thomas and Ochoa, 1984). Schwann cell intermediate filaments are composed mainly of polymers of vimentin which is a 52 to 58 kDa polypeptide (Pleasure, 1984). The plasmalemma is a triple layered membrane and contains caveolae, especially in the perinuclear region and adjacent to the incisures. These may increase the surface area of the Schwann cell membrane and therefore compensate during stretching of the nerve fibre subsequent to movement. The row of Schwann cells aligned along an axon is invested by a tube of basal lamina. The basal lamina is continuous across the nodes of Ranvier (Peters, 1976; cited in Thomas and Ochoa, 1984).

Schmidt-Lanterman Incisures

Between the Schwann cell nucleus and the node of Ranvier is a series of oblique clefts which cross the sheath at an average angle of 9 degrees (Peters, 1976). The number of clefts is related to myelin thickness. In the largest fibres of the sciatic nerve of the rat, for example, each internode has approximately 25 incisures. They have been found to be more numerous in developing and regenerating fibres (cited in Thomas and Ochoa, 1984). The clefts are formed by cytoplasmic bulges within the sheath and may traverse the sheath fully or involve only a few lamellae. If these incisures fully traverse the sheath, they provide a means of cytoplasmic continuity across the sheath from the outer mesaxon to the inner mesaxon (Peters, 1976). The amount of cytoplasm enclosed within the incisures is variable. The incisures are rich in actin, frequently contain a single helical microtubule and occasionally contain electron dense granules and multivesicular bodies. The exact function of these incisures is not known. It has been postulated that they allow for elongation and expansion of the nerve fibre; or longitudinal growth of the fibre; or that they are involved in the passage of metabolites across the myelin sheath and the metabolic maintenance of myelin (cited in Thomas and Ochoa, 1984).

The node of Ranvier

The node of Ranvier is the structure where adjacent myelinating Schwann cells abut (Peters, 1976). Over this region the Schwann cell cytoplasm external to the myelin sheath forms a cytoplasmic collar which irregularly overlaps with the cytoplasmic collar from the adjacent Schwann cell. These collars are bordered by the basal lamina externally, the paranodal bulbs laterally and internally by the axolemma. Nodal villi originate from these collars and form many finger like processes 70 to 100 nm wide which project to within 5 nm of the axolemma. It is possible that specialized Schwann cell-axon interaction occurs at these points. Metabolic exchange may occur between the mitochondria-rich processes and the mitochondria-poor axon (Peters, 1976; Thomas and Ochoa, 1984).

"Gap substance" is moderately electron dense, polyanionic material found in the space between the villi and the external basal lamina. It is known to bind ions and has been postulated to have a role in replenishing sodium or recapturing potassium during impulse transmission across the node (Berthold, 1978; Rosenbluth, 1983; Thomas and Ochoa, 1984;).

The paranode

Adjacent to the nodes of Ranvier the myelin sheath is modified to form the paranode. Here, the myelin lamellae terminate by forming a series of finger like projections. The innermost one terminates first and successive lamellae overlap and project towards the axolemma. The major dense line is the fused apposing cytoplasmic membranes of the Schwann cell (see section 1.5.1.1). It opens up at these terminations which are filled with cytoplasm to create cytoplasmic pockets known as paranodal or lateral loops. Tight junctions, and possibly desmosomal junctions, are found between adjacent loops (Rosenbluth, 1983; Thomas and Ochoa, 1984). Within the cytoplasm of these loops are found microtubules and, occasionally, membrane bound vesicles and electron dense inclusions (Rosenbluth, 1983). The alignment of these paranodal loops is much more orderly for small nerve fibres than for large fibres. In the latter, not all of these "fingers" contact the axon. Via the paranodal specialization, continuity is established between the adaxonal and abaxonal Schwann cell cytoplasm. Microtubules, vesicles and some electron dense particles are observed within paranodal loops. The axolemma is closely apposed to the plasmalemma of the Schwann cell at these terminal cytoplasmic rims. The exterior surface of the axolemma has a series of ridges called tranverse bands, which come into close contact with the Schwann cell membrane reducing the extracellular space in this region to 3 nm. This distance is fixed in the paranodes so that, in longitudinal section, the

axolemma's appearance is scalloped due to the paranodal pockets bulging into it. Within this space is a series of 1.5 nm densities which are separated from each other by regular spaces. Apart from the mechanical stability provided by this area of contact, it is thought that the paranodal area could be involved in other interactions between the Schwann cell and the axon such as limiting diffusion into the internodal periaxonal space (Rosenbluth, 1983; Thomas and Ochoa, 1984). Freeze fracture studies have identified the presence of intramembranous particles in the plasma membrane of the perinodal Schwann cell processes. These particles may be related to voltage-sensitive sodium channels (Stolinski *et al.*, 1981; Stolinski and Breathnach, 1982; Waxman and Black, 1987).

Examination of the paranodal region in cross section reveals that the myelin has a corrugated appearance which forms a series of 3 to 6 ridges running longitudinally along the length of the paranode. The axon is fluted in appearance as it follows the contour of the myelin. External to the myelin sheath, the troughs between the myelin corrugations are filled with organelle rich cytoplasm (Peters, 1976; Thomas and Ochoa, 1984).

The non-myelin-forming Schwann cell

Unmyelinated fibres tend to be the small diameter, slow conducting nerves (Thomas and Ochoa, 1984). Their axons embed in troughs on the Schwann cell surface with one Schwann cell ensheathing between 1 and 15 axons (cited in Peters, 1976; Thomas and Ochoa, 1984) although up to 100 axons/Schwann cell have been suggested (Berthold 1978). These Schwann cells just ensheath the axons and do not form myelin (Peters, 1976). A single Schwann cell may not ensheath the same group of axons throughout its entire length (cited in Berthold 1978). The extent of axonal envelopment is variable. The axon may only be partially ensheathed by the Schwann cell such that the enveloping folds do not meet on the outer aspect of the axon leaving part of the axolemma covered only by basement membrane. More commonly, the Schwann cell cytoplasmic folds come together so that their apposed plasma membranes run parallel to each other to form a mesaxon. A different situation exists in the autonomic nervous system plexi; here some axons may be individually enclosed, but groups of axons may also be enclosed within the same trough (cited in Peters, 1976).

1.3.1.3 Functions of the Schwann cell

Development, maintenance, degeneration and regeneration

The function of the Schwann cell depends on the age of the animal and the ongoing developmental or repairative events within the PNS. The cell is very active in the ensheathment and myelination of the perinatal PNS during its development (see section 1.5.1.1). The axolemma is affected by the nature of Schwann cell ensheathment as evidenced by the freeze fracture studies mentioned in section 1.2.2. Hence the Schwann cell affects the differentiation of the axolemma. Nerve growth factor protein and receptor are expressed by Schwann cells in the distal stump of an axotomized nerve and play a role in haptotactic guidance of the regenerating neuron (see section 1.5.2.7). The function of the Schwann cell during degeneration and regeneration of peripheral nerve is described under section 1.5.2.

Other Schwann cell functions

Rippling, undulating movements of Schwann cell and the myelin sheaths have been noted in developing and mature tadpole nerve fibres. The rippling movements were observed to squeeze closed the Schmidt-Lantermann clefts. It has been postulated that these movements could facilitate exchange between axonal and extracellular compartments and possibly also affect axoplasmic flow. Substances could thereby enter the Schwann cell, cross the myelin sheath and penetrate the axon (cited in Peters, 1976). However, similar movements have not been observed in the mammalian system and it seems unlikely that axoplasmic flow or movement into the axon is effected via this method (Peters, 1976).

Schwann cells and the endoneurial collagen help provide support and elasticity for axons during movment of body parts (Peters, 1976). They also have a role in synthesizing extracellular matrix and basement membrane components (see section 1.3.3.3).

A variety of ionic channels have been identified on the Schwann cell including the type of sodium and potassium voltage-gated channels which are usually associated with excitable membranes. The functions of these are not clear, but it has been speculated that these channels on the Schwann cell could support the channels used by the axon (reviewed by Gray and Ritchie, 1985).

It has been postulated that the ensheathing cells of the central or peripheral nervous system can actively sequester and phagocytose organelles from both normal and diseased axons. This is achieved by infolding and invagination of the glial cell

cytoplasm to sequester and subsequently phagocytose the axoplasmic organelles. In this way the glial cell can "police" the adjacent axon and selectively remove effete organelles (Spencer and Thomas, 1974).

1.3.1.4 Schwann cell Proliferation

The increase in Schwann cell numbers in developing rat nerve is very large between embryonic day 16.5 and birth; this appears to be due to proliferation of Schwann cells within the nerve rather than cell migration from the neural crest (Peters, 1976). Experiments in mice identified that Schwann cell proliferation began on about embryonic day 12 and continued after birth. But, by 2 days postnatal, less than 25% of Schwann cells were still dividing and myelination was well advanced by the end of the first week (Asbury, 1967).

Schwann cell division occurs *in vivo* during development and regeneration but, in the mature animal, they are relatively quiescent (Brockes *et al.*, 1981). Much of the current information on Schwann cell proliferation has been derived from *in vitro* studies. Cultures of Schwann cells and bare spinal ganglia (i.e. spinal ganglia free of Schwann cells) have been grown separately to investigate the nature of the Schwann cell mitogen (Wood and Bunge, 1975). The basal doubling time is 7 to 8 days for pure Schwann cells cultured in growth medium with 10% foetal calf serum after antimitotic treatment. Growth kinetics suggest that the population divides homogeneously rather than as a mixture of rapidly-dividing and non-dividing cells (Raff *et al.*, 1978b).

A number of different mitogens are known to be effective in inducing Schwann cell proliferation. The growing neurite acts as a potent stimulus to Schwann cell proliferation both *in vivo* and *in vitro* (Wood and Bunge, 1975; Pellegrino and Spencer, 1985). Schwann cell proliferation is marked during active neuritic outgrowth, but the number of dividing Schwann cells decreases as the outgrowth slows. Induction of the synthesis phase in the Schwann cell occurs in the proximity of the growing tips of the axons (Salzer *et al.*, 1980b). Less than 5% of isolated Schwann cells incorporated [^{3}H] thymidine, but this incorporation increased to 90% within 42 hours of unensheathed neurites growing into these cultures (Wood and Bunge, 1975). In a similar study, the first dividing Schwann cell was observed 14 hours after axonal contact was made (Ratner *et al.*, 1987). Schwann cell proliferation was also stimulated by sympathetic neurons, embryonic spinal cord and rat retinal ganglion neurons but less vigorously than spinal ganglia neurons (Ratner *et al.*, 1987; Wood, 1976). Isolated neuritic membrane fractions, but not cytoplasmic preparations,

also stimulated Schwann cell proliferation *in vitro* (Salzer *et al.*, 1980b; Salzer *et al.*, 1980c). This implies that the Schwann cell mitogen is located on the plasma membrane (Salzer *et al.*, 1980b). This signal is very labile and disappears from the neurite rapidly after axotomy. For example, excision of the spinal ganglia from a coculture results in neuritic degeneration and within 48 hours there is a rapid decline in Schwann cell proliferation (Salzer and Bunge, 1980a). Very close contact of the Schwann cell with the axon is required to stimulate cell division. Schwann cells are not stimulated to proliferate if they just share the same medium as the neurites nor if they are only separated from the neurites by a very small distance or a 6 μm strip of collagen (Wood and Bunge 1975; Salzer *et al.*, 1980c; Ratner *et al.*, 1987). However, none of these experiments ruled out a very short-lived protein secreted from the neuron and active at only minute distances (Ratner *et al.*, 1987).

The proliferative response of Schwann cells to growing neurites is very rapid whilst the response to membrane fractions is slower (Ratner *et al.*, 1987). The longer the cells have been in culture and the higher the cell density, the less responsive they are to the neuritic membranes (Cassel *et al.*, 1982; Salzer *et al.*, 1980b).

It is possible that the mitogen is a peripherally associated with the lipid bilayer as mitogenic activity can be removed from crude plasma membrane preparations derived from central nervous system tissue (Ratner *et al.*, 1988). The mitogen can be enriched in the membrane by base extraction to remove non-mitogenic protein; by enzymatic degradation of the heparan sulphate proteoglycan; or by displacement using heparin (DeCoster and DeVries, 1989).

An axolemmal-enriched fraction has been prepared from the myelinated axons of both the central and peripheral nervous systems from murine and bovine sources. These fractions will stimulate quiescent Schwann cells to divide. Hence the signal is not species-restricted, system-restricted (that is, both peripheral and central nervous system-derived signals are stimulatory) or age-restricted as both young and mature sources of nervous system were used (DeVries *et al.*, 1982). However, preparations derived from whole spinal cord had no mitogenic activity, whereas whole brain preparations had a weak effect (Salzer *et al.*, 1980b). Axolemmal-enriched fractions from both myelinated and unmyelinated nerve were found to be stimulatory for Schwann cell proliferation (Mason *et al.*, 1989). Sympathetic neuron-like PC12 tumour cell membranes were also mitogenic for Schwann cells and caused an increase in intracellular cyclic-AMP of the Schwann cells (Ratner *et al.*, 1984).

Axolemmal-enriched fractions were sensitive to sonication, homogenization, trypsin and heat, although the heat sensitivity varied between preparations (DeVries *et*

al., 1982; DeVries *et al.*, 1983b; Sobue *et al.*, 1983). The neuritic fraction was rendered ineffective by treatment with heat, aldehyde fixatives and trypsin (Salzer *et al.*, 1980c). Using various metabolic inhibitors and enzymes, studies have suggested that a heparan sulphate proteoglycan and a glycoprotein compound are required on the neuronal surface for stimulation of Schwann cell division and that a proteoglycan-associated protein may be the mitogenic signal (DeCoster and DeVries, 1989, Mason *et al.*, 1987; Ratner *et al.*, 1987; Salzer *et al.*, 1980c). The neuritic mitogen and the Schwann cell mitogen receptor are both trypsin sensitive (Ratner *et al.*, 1987; Salzer *et al.*, 1980c). The mitogenic molecule on the growing neurite did recover from trypsin treatment but this took 30 hours. Schwann cells plated onto trypsin-treated neurites lacked the longitudinal orientation of cells plated onto control neurites and assumed a random orientation. After 10 to 15 hours, they began to orient longitudinally (Salzer *et al.*, 1980c).

Trypsin sensitivity, freeze fracture and neurotoxin binding studies of the molecule suggests that the signal may reside on the outer surface of the axonal membrane *in situ* (DeVries *et al.*, 1982). The preparations made from the central nervous system contained a substantial amount of gangliosides, however, various gangliosides were tested and were not found to be mitogenic for Schwann cells (DeVries *et al.*, 1983b). Serum was required by axolemmal-enriched fractions, but not neuritic membranes, to stimulate Schwann cell mitosis. Similarly, the mitogenic responses of Schwann cells to pituitary extract, dibutryl cyclic-AMP and cholera toxin (see section 1.3.2.1 on growth factors) were lower in serum-free medium (Cassell *et al.*, 1982). The rate of Schwann cell proliferation was not altered by changing the serum concentration from 5 to 20% in the presence of a constant amount of axolemmal-enriched fraction (DeVries *et al.*, 1983b).

The effect of axolemmal fragments on Schwann cells is specific. Fractions prepared from rat sarcolemma, fibroblasts, erythrocytes, or mitochondrial membranes had minimal stimulatory effect on the Schwann cells. This indicated that axolemmal-induced mitosis was not a general response to exogenous membranes (DeVries *et al.*, 1983b; Salzer *et al.*, 1980b; Sobue *et al.*, 1983). Liver microsomes, fibroblastic and heat treated neuritic membranes were phagocytosed by Schwann cells but were not mitogenic for them, so phagocytosis, *per se*, is not the mitotic stimulus (Meador-Woodruff *et al.*, 1985). Conversely, the axolemmal-enriched fraction did not stimulate fibroblasts to divide (Sobue *et al.*, 1983).

The amount of proliferation stimulated is directly proportional to the amount of axolemmal-enriched fraction added to the culture. The interaction appears to be a

surface based phenomenon as brief trypsinization after 24 hours incubation of the fraction with the Schwann cells released 94 to 97% of bound fraction. Maximum adhesion is achieved within 4 hours of addition of the axolemmal-enriched fraction to the Schwann cell cultures, but contact must be sustained for at least 6 hours and optimally for greater than 24 hours to induce DNA replication (Sobue and Pleasure, 1985).

Schwann cell proliferation may occur in response to mitogens other than the axolemmal associated mitogen. *In vivo* it occurs in peripheral nerves undergoing Wallerian degeneration without direct influence from intact axons. In the distal stump, Schwann cell proliferation was found to increase 7 fold at 3 days, peak sharply at 4 days (43 x control level) and decline to approximately 1/2 of the 3 day level by the fifth day. The main proliferating cell was the Schwann cell (Pellegrino *et al.*, 1986). It continues over a 2 week period in the absence of contact with intact axons.

The proliferative stimulus is thought to involve the breakdown products of myelin (Bunge *et al.*, 1978b). In considering this fact, it is interesting to note that less Schwann cell proliferation occured after axonal degeneration in an unmyelinated or thinly myelinated nerve (cited in Ohara and Ikuta, 1988). Proliferation by non-myelin-forming Schwann cells does occur after axotomy, but the degree depends upon the nerve. In the sciatic nerve, just over 1/3 of non-myelin-forming Schwann cells were labelled with [^{3}H] thymidine by the second day. However, labelling in Schwann cells obtained from the transected cervico-sympathetic trunk was less than half that seen in the mixed peripheral nerve. Thus the non-myelin-forming Schwann cell does divide in response to axotomy, but the magnitude of the response was greater in cells obtained from a mixed peripheral nerve.

The timing of the division corresponded with the peak macrophage invasion into the distal stump and may be due to the presence of myelin-phagocytosing macrophages which are thought to have a role in Schwann proliferation. Myelin-phagocytosing macrophages would not be present in the distal stump of the cervico-sympathetic trunk, possibly accounting for its lower division rate (Clemence *et al.*, 1989). Similarly, *in vitro*, after neuritic degeneration in a myelinating culture, the myelin-related Schwann cells proliferated, whilst those Schwann cells which had not formed myelin remained quiescent. These studies suggested that axonal degeneration was not the mitogen, as only myelin-forming Schwann cells were stimulated to divide. It would seem that the mitogenic stimuli during development and degeneration are distinct and that breakdown of myelin within the Schwann cells or turnover of Schwann cell membranes is the stimulus operative during degeneration (Salzer and

Bunge, 1980a). The role of the macrophage in Schwann cell proliferation is considered later in this section. Another suggested reason for Schwann proliferation was loss of supply of an axolemmal component transported from the neuronal somata by fast axonal transport (Pellegrino *et al.*, 1986).

Like the axolemmal-enriched fraction, a myelin-enriched fraction prepared from rat brainstem stimulates the incorporation of [^{3}H] thymidine by cultured Schwann cells in a dose dependent manner. The maximum stimulation produced by the myelin fraction was approximately half that observed with the axolemmal fraction (Yoshino *et al.*, 1984). However, a myelin fraction prepared from the PNS did not stimulate Schwann cell proliferation (DeVries *et al.*, 1982). Schwann cells presented with a myelin-enriched fraction actively and rapidly phagocytosed it and cell division was noted within 9 hours. Phagocytically active Schwann cells had elongated mitochondria, increased amounts of microtubules and 4 to 6 nm microfilaments. Interwoven filaments formed dense accumulations especially in the perinuclear area. Polysome rosettes tended to accumulate in the region of the cell processes and the Golgi apparatus appeared active. Prominent rough endoplasmic reticulum was located mainly in the perinuclear area and often had widely dilated cisterns filled with homogeneous granular material. Phagolysosomes were observed. Ingested myelin was still lamellar in structure and was variably bounded by a membrane (Bigbee *et al.*, 1987).

The shape of the dose response curves for the myelin- and the axolemmal-enriched fractions was different. Also the method of cellular response was different in that the myelin-enriched fraction required lysosomal processing, whilst the axolemmal-enriched fraction did not (Meador-Woodruff *et al.*, 1985; Yoshino *et al*, 1984). Addition of lysosomal inhibitors such as ammonium chloride (Yoshino *et al.*, 1984) or chloroquinone (Meador-Woodruff *et al.*, 1985) to the system significantly decreased the potency of the myelin-enriched fraction, but did not affect the axolemmal-enriched fraction. These results suggest that axolemma and myelin stimulate Schwann cell mitosis by different methods; with the axolemmal-enriched fraction acting at the Schwann cell surface, whereas the myelin-enriched fraction appears to require phagocytosis and lysosomal processing (Meador-Woodruff *et al.*, 1985).

Myelin basic protein failed to stimulate the Schwann cells, suggesting that Schwann cell mitosis stimulated subsequent to demyelination must be due to factors other than just this protein (DeVries *et al.*, 1982).

The rate of Schwann cell proliferation was different for the two fractions. It increased with myelin-enriched fraction in the 48 to 72 hour period after addition of the fraction, whereas there was no change for the axolemmal-enriched fraction. This increase coincides temporally with the increase in proliferation observed *in vivo* during Wallerian degeneration. Therefore two distinct mitogenic signals for cultured Schwann cells can be distinguished using fractionated nervous tissue (Meador-Woodruff *et al.*, 1985; Yoshino *et al.*, 1984).

Macrophages also seem to have a role in permitting myelin-enriched fractions to be mitogenic for Schwann cells. Conditioned medium derived from cultured macrophages which have phagocytosed myelin-enriched fractions is mitogenic for cultured Schwann cells, but conditioned media from macrophages exposed to axolemma, liver microsomes, polystyrene beads or lipopolysaccharide was not stimulatory. Lysosomal processing of the myelin-enriched fraction by the macrophages is required for activity (Baichwal *et al.*, 1988).

Agents, such as cholera toxin, dibutryl cyclic-AMP, isobutylmethylxanthine and forskolin, which increase the intracellular level of cyclic-AMP in Schwann cells, are successful in stimulating Schwann cell division (see section 1.3.2.1) (Porter *et al.*, 1986; Raff *et al.*, 1978a; Raff *et al.*, 1978b). These results suggest that one of the intracellular signals for Schwann cell division may be sustained elevation of intracellular cyclic-AMP (Raff *et al.*, 1978b). However, cyclic-GMP and heparin (which inhibits membrane bound adenylate cyclase) were not found to be stimulatory for Schwann cell division (DeVries *et al.*, 1983b).

The role of cyclic-AMP as a second messenger in stimulating Schwann cell division is controversial because myelin- and axolemmal-enriched fractions do not appreciably alter the level of cyclic-AMP in Schwann cells (Meador-Woodruff *et al.*, 1984). Cholera toxin had no synergistic effect on the mitogenic activity of the axolemmal-enriched fraction, unlike its synergistic effect with bovine pituitary extract (Sobue *et al.*, 1983; see section 1.3.2.1). Isobutylmethylxanthine did not effect the mitotic response of Schwann cells to neuritic membranes, also suggesting that these membranes do no act via the cyclic-AMP second messenger system either (Salzer *et al.*, 1980c).

Stimulation of Schwann cells by axolemmal- and myelin-enriched fractions may be mediated by calcium ions as the mitogenicity of the fractions is proportional to the calcium concentration of the culture environment (Meador-Woodruff *et al.*, 1984; Saunders and DeVries, 1988). It is possible that the mitogenic expression of different compounds involves the activation of several classes of protein kinase.

Calcium may act as the second messenger for axolemmal and myelin fractions. These calcium dependent and cyclic-AMP dependent protein kinases may then act upon the same substrate resulting in stimulation of mitosis of Schwann cell. Supraphysiologic doses of cyclic-AMP may be able to overwhelm the system and activate the calcium dependent protein kinases (Meador-Woodruff *et al.*, 1984). It has been suggested that activation of protein kinase C by either the axolemmal or myelin fractions may result in activation of the Na^+/H^+ exchanger and cause the observed increase in intracellular pH of Schwann cells during their mitosis (Saunders *et al.*, 1989). Agents which raise intracellular cyclic-AMP levels alter the morphology of the Schwann cells, causing them to lose their bipolar shape and assume a more flattened appearance (DeVries *et al.*, 1982; DeVries *et al.*, 1983b). Non-cyclic-AMP mediated mitogens such as pituitary extract, neuritic membranes and axolemmal derived fractions do not alter the morphology of the Schwann cells. This also suggests that the two groups of agents act via a different mechanism (DeVries *et al.*, 1982; DeVries *et al.*, 1983b).

Termination of Schwann cell division is not fully understood, however, it is known that type I collagen is inhibitory to cultured Schwann cell proliferation. This collagen preparation was not entirely pure, so the inhibition may not be due just to the collagen. Denatured type I collagen, other types of collagen and other basement membrane components were not found to be inhibitory to Schwann cell proliferation (Eccleston *et al.*, 1989).

1.0 LITERATURE REVIEW

1.3 THE SCHWANN CELL

1.3.2. Schwann cells *in vitro*

1.3.2.1. Techniques of Schwann cell culture

There are two basic ways of culturing Schwann cells. They may either be dissociated from each other and the other endoneurial elements to form single cell preparations, or, perhaps less commonly, they may be cultured from explants of nerve tissue from which they are encouraged to grow out.

Dissociated Schwann cells

The two most commonly employed methods of producing dissociated Schwann cells are from either spinal ganglia (dorsal root ganglia) of foetal rats or from sciatic nerve of neonatal rats.

1) Spinal ganglia

Spinal ganglia cultured *in vitro* recapitulate many of the developmental stages of the nervous system; i.e. nerve fibres grow out initially, Schwann cells migrate and proliferate along the fibres, followed by a slowing of proliferation, ensheathment and myelination (Salzer *et al.*, 1980a). Spinal ganglia harvested from 19 to 21 day rat foetuses are decapsulated and cultured on collagen-coated coverslips. The cultures are maintained in 5% CO_2, at 35^{o}C. The three major types of cells that can be identified in these cultures are the centrally located ganglionic mass of neurons, Schwann cells and fibroblasts. The Schwann cells remain closely associated with the axons, unlike fibroblasts which wander freely over the culture surface. The number of Schwann cells usually increases during the active outgrowth of axons in the culture, whilst the number of fibroblasts increases throughout much of the life of the culture (Wood, 1976)

Cultures of spinal ganglia can be denuded of Schwann cells by extensive treatment with antimitotics. Quiescent Schwann cells can subsequently be induced to settle upon, ensheath and myelinate bare neurite within 2 weeks (Wood, 1976).

Dissociated Schwann cells have also been produced from adult cat spinal ganglia. This tissue source yielded both neuronal and Schwann cells. Like Schwann cells obtained by other methods, these cells tended to align in a staggered fashion next

to each other and a lacework of cells was ultimately formed, rather than a true monolayer. Electron microscopic studies identified the presence of the usual subcellular organelles such as Golgi apparatus, mitochondria, many free ribosomes, rough endoplasmic reticulum and membrane bound dense and lamellar bodies. The perinuclear cytoplasm or proximal portions of the cell process were rich in these ultrastructural elements. No basal lamina was observed. Scanning EM identified that the perinuclear portion of the Schwann cell was quite 3-dimensional and raised above the surface of the dish. This would account for the refractile appearance of these cells under phase microscopy. The authors felt that the dissociation method yielded better Schwann cell lines than the explant method (see next section) (Wrathall *et al.*, 1981a).

2) Neonatal sciatic nerve

In 1979, Brockes *et al.*, devised a method for enzymatic dissociation of Schwann cells from sciatic nerve of neonatal rats. The sciatic nerve was dissected from freshly killed rats and incubated with trypsin and collagenase. The softened nerves were dissociated by trituration through a hypodermic needle and the suspension passed through fine nylon gauze to remove debris. After centrifugation, the pellet was resuspended in Dulbecco's Modification of Eagle's Medium (DMEM) with 10% foetal calf serum (FCS) and plated out. The cells were kept at 37^{o}C and cultured in the same medium. Within 24 hours the cells assumed the spindle shaped appearance and oval nucleus characteristic of cultured Schwann cells. A few flat fibroblasts were also noted. These latter cells proliferated rapidly in the absence of any inhibitors and within 7 days formed a monolayer under the Schwann cells. Because of this, a method for obtaining pure cultures of Schwann cells was devised using antimitotics (see next section on purification of cultures) (Brockes *et al.*, 1979).

A similar method of enzymatic and mechanical dissociation has been used to obtain Schwann cells from neonatal mice. Likewise, after 5 to 6 days *in vitro*, bipolar Schwann cells were observed overlying fibroblast like cells. The bipolar cells aligned and formed chains in areas free of fibroblasts (White *et al.*, 1983).

3) Adult peripheral nerve

Dissociated Schwann cells have also been produced from adult cat sciatic and ulnar nerves using several cycles of exposure to trypsin or collagenase. Nerve dissociated in this fashion consisted primarily of myelin sheath segments which had a thin rim of Schwann cell cytoplasm. A few free cells were also noted which were Schwann cells associated with unmyelinated axons. Fibroblasts and perineurial cell types were also observed. After 24 to 48 hours the predominant cell type was the

sheet like fibroblast, but a few bipolar spindle shaped cells were observed growing superficially. The morphology of these adult Schwann cells was similar to that of the neonatal dissociated cell, in that they were small bipolar and spindle shaped and tended to grow in chains and fascicles. They had a refractile appearance under phase microscopy. If the nerve tissue fragments attached between 1 and 8 days after dissociation, then the percentage of Schwann cells in culture was enriched compared with allowing Schwann cell attachment to occur within the first 24 hours (Wrathall *et al.*, 1981b).

Non-dissociated Schwann cells

Schwann cells may also be cultured from tissue without subjecting them to mechanical and/or enzymatic dissociation. A fragment of tissue is cultured as an explant on a culture surface covered with a substrate such as rat tail collagen.

1) Cat spinal ganglia

Explant cultures have been prepared from adult cat spinal ganglia and within 4 days an outgrowth of non-neuronal cells was produced. Initially the cells were pleomorphic and multipolar, but by the second week a monolayer of flattened sheet like cells were observed surrounding the explant. After 14 days the explants were excised and transferred to a new culture dish and could, thus, be maintained over at least four subcultures. The secondary outgrowth from the explant after transplantation was enriched in spindle shaped cells. Fibroblastic cells were mainly eliminated by transplantation of the explants and production of secondary or tertiary outgrowth. The explant was ultimately removed leaving behind a culture of cells (Wrathall *et al.*, 1981a).

2) Peripheral nerve explants

Endoneurial slices have been prepared from the sciatic nerves of adult rats which had been transected and allowed to degenerate *in vivo* for 7 weeks. The distal stump was desheathed and sliced into 1.0 mm segments and cultured on rat tail collagen coated coverslips. Explants were also prepared from 4 day-old rat pups without prior transection (Poduslo and Windebank, 1985b).

Explants of peripheral nerve (brachial and sciatic) have been prepared from human embryos. Initial cell outgrowth occurred within 2 days and was primarily fibroblastic. Spindle shaped Schwann cell like cells were observed in the outgrowth within 4 to 5 days. Schwann cells were also cultured from sympathetic ganglia and nerves, with outgrowth beginning within 3 to 5 days (Murray and Stout, 1942).

Askanas and colleagues (1980), developed a method of culturing human Schwann cells from mature peripheral nerve using explant/re-explant technique to enrich the culture with Schwann cells. Palisades of Schwann cells were obtained in dense cultures grown on gelatin. Electron microscopy identified elongated nuclei with few invaginations of nuclear membrane and 1 to 2 nucleoli/nucleus. A basement membrane was not observed exterior to the 12 nm thick plasmalemma. Many focal invaginations of thickened (27 nm) plasmalemma were observed with fuzzy unidentified material observed on the cytoplasmic face of these invaginations. The cytoplasm was rich in free and bound ribosomes. Golgi apparatus and mitochondria were concentrated around the nucleus. Longitudinally oriented microtubules and a few subplasmalemmal microfilaments were noted. Centrioles, occasional dark osmiophilic and laminated bodies were visualized in some cells. The long spindle shaped morphology, cytoplasm enriched with microfilaments which are presumed to be organelles of motility, and lack of basement membrane suggests that cultured Schwann cells were in the migratory phase (Askanas *et al.*, 1980).

However, generating Schwann cells from normal nerve is difficult because there is very little outwandering of Schwann cells from the uncut (unpredegenerated nerve). Rabbit sciatic nerve was cut and allowed to degenerate for various times *in vivo* before explant cultures were produced. Outwandering activity of the Schwann cells was first noted in nerves cut 4 days before culture initiation and peaked at 19 to 25 days. At this time the level of activity was 40 fold that at 4 days. Activity declined until the 60th day, but there was still some present 1 year after cutting. The rise to peak of outwandering activity coincided with the total duration of mitotic activity observed *in vivo*. There was variability in the degree of outwandering depending on where the explant was taken from in the distal stump. The terminal bulb region adjacent to the transection of the distal stump was not found to be a good source of Schwann cells due to extensive fibroblast proliferation. It was thought that the wandering activity of the Schwann cells and fibroblasts was due to the degeneration of nerve fibres and, in the region close to the cut, a superimposed traumatic effect of cutting the nerve (Abercrombie and Johnson, 1942).

Purification of cultures

Fibroblasts are a common cell contaminant of Schwann cell culture. The fibroblasts are larger, more sheet like cells, with extensive cytoplasm, bigger nuclei often with prominent nucleoli (Brockes *et al.*, 1979). These cells divide much more rapidly than the Schwann cells and if their proliferation is not controlled they will overgrow the rest of the culture forming a monolayer under the other cells within 7 days (Salzer *et al.*, 1980a; Brockes *et al*, 1979). Mesenchymal cells are generally required for support of epithelial growth and differentiation (Rheinwald and Green, 1975). But Schwann cells are ectodermal cells which do not appear to require this support for serial cultivation up to at least 6 passages (Brockes *et al.*, 1979).

Because of their high mitotic index, fibroblasts cultured with tritiated thymidine become heavily labelled (Wood, 1976). Double labelling studies performed by Brockes *et al.*, (1979), have shown that 80 to 90% of Thy-1 positive cells (fibroblasts) take up tritiated thymidine, but only 15 to 30% of Ran-1 positive cells (Schwann cells) were labelled. Thus, *in vitro*, the fibroblasts are dividing rapidly, whereas the Schwann cells are either proliferating slowly, or a minority of them are dividing at a faster rate (Brockes *et al.*, 1979). This difference in the mitotic index permits the use of antimitotic agents such as cytosine arabinoside or fluorodeoxyuridine to selectively eliminate the fibroblasts. However, these drugs are not specific for fibroblasts, but also reversibly inhibit Schwann cell proliferation. Although Schwann cells are relatively resistant to antimitotic agents, during brief exposure to these agents they may become susceptible as they enter the mitotic cycle during prolonged drug usage. The complete elimination of the Schwann cells from cocultures with spinal ganglia requires extensive treatment with antimitotic agents for several weeks (Brockes *et al.*, 1979; Wood, 1976). Schwann and neural cell characteristics of ensheathment and myelination are not irreversibly affected by these drugs as these events will subsequently occur in culture (Wood, 1976). However, the addition of antimitotic agents to explant cultures from adult cat spinal ganglia was thought to slow cellular outgrowth and decrease the proportion of Schwann cells in the final culture (Wrathall *et al.*, 1981a).

A single pulse application of an antimitotic only controls the number of fibroblasts for a period of time; it does not eliminate them. Within 7 days of the pulse the fibroblasts were again observed in significant numbers, so an immune-based, complement-mediated kill of the fibroblasts was developed. This was based on the expression of a surface antigen Thy-1.1 by fibroblasts, but not Schwann cells. After

detachment with trypsin the mixed cell culture was incubated with murine antiserum against Thy-1.1 and then rabbit complement, causing lysis of the fibroblasts. This technique resulted in Schwann cell populations that were 99.5% pure at confluence. The cultures were able to be passaged further with additional anti-Thy-1.1/complement-mediated kills if necessary. Viable Schwann cell cultures could be maintained for 6 passages or 150 days *in vitro*. After passaging, the morphology of the Schwann cells altered so that the cells were larger, with a more triangular or polygonal cell body tapering into 2 to 5 processes. Both before and after passaging the Schwann cells showed a strong tendency to line up side-to-side and end-to-end, extending parallel processes (Brockes *et al.*, 1979).

Two other methods of enriching cultures with Schwann cell have been described. Short trypsin treatments of cultures containing fibroblast and Schwann cells has been used in neonatal mouse and adult cat cultures. Selective detachment of the Schwann cells occurred, leaving behind the fibroblasts and resulting in Schwann cell enriched secondary cultures (White *et al.*, 1983; Wrathall *et al.*, 1981a; Wrathall *et al.*, 1981b). The other method is based on the different adhesion properties exhibited by Schwann cells and fibroblasts after dissociation. The latter are more likely to attach during the first 30 minutes if the cultures are vibrated during this time. This resulted in a Schwann cell rich suspension which was subsequently plated out in fresh culture dishes. With time *in vitro*, the proportion of Schwann cells in these cultures decreased but even at 21 days *in vitro*, the percentage of Schwann cells was still 80%. This proportion was affected by the composition of the culture medium with increased fibroblast growth in media enriched with serum, glucose, pyruvate or sodium bicarbonate (Kreider *et al.*, 1981).

Growth factors

Glial cell division occurs *in vivo* during development and subsequent to axotomy, otherwise these cells are relatively quiescent in the mature animal (Brockes *et al.*, 1981). As stated in the last section, the doubling time of cultured neonatal rat Schwann cells, after antifibroblastic treatment, is approximately 8 days (Brockes *et al.*, 1979). *In vitro*, the growth rate appears to be species dependent (White *et al.*, 1983). It is faster in mouse and cat cultures than in rat (cited White *et al.*, 1983). The growth rate in some cultures also appears dependent upon the length of time in culture. Dissociated Schwann cells from neonatal mouse sciatic nerve doubled in number every 22 hours at 5 days *in vitro*; at 12 days 60 to 70% of cells were labelled with [^{3}H] thymidine after a 24 hour pulse; but the cells failed to divide after 20 days *in vitro* (White *et al.*, 1983). Similarly, the proliferation rate of adult cat Schwann

cells slowed with time, and by the fifth passage further subculture was not possible (Wrathall *et al.*, 1981b). In human foetal (8 to 10 week gestation) Schwann cell cultures, 34% of Schwann cells divided over a 48 hour period as assessed by bromodeoxyuridine incorporation (Kim *et al.*, 1989).

The results of cell growth kinetic curves on Schwann cell proliferation have suggested that these cultures consist of a homogenous population of infrequently dividing cells rather that a mixture of rapidly-dividing and non-dividing cells (Raff *et al.*, 1978b). However, these studies were performed on cultures that had previously been treated with cytosine arabinoside and anti-thy 1.1 complement-mediated kill of fibroblasts. No information was given about cell growth kinetics for cultures untreated with antimitotics. If Schwann cells are dividing at different rates within a culture, then use of antimitotics to purify cultures may result in selection of slower dividing or quiescent Schwann cell populations (White *et al.*, 1983). The doubling time of Schwann cells was markedly decreased to 20 hours by transfecting them with Simian virus 40 in an attempt to immortalize the cell line. However, these cells have different morphologic and biologic characteristics to untransfected Schwann cells (Chen *et al.*, 1987; Tennekoon *et al.*, 1987). Therefore, antimitotic usage may not be without problems. For example, White and colleagues (1983) found that use of antimitotics resulted in a legacy of Schwann cells which could not proliferate and be maintained. It may also be that different subpopulations of Schwann cells exist as Schwann cells derived from adult cat spinal ganglia and peripheral nerve have different proliferation characteristics (Wrathall *et al.*, 1981a; Wrathall *et al.*, 1981b).

A number of different compounds have been tried as Schwann cell mitogens. An extract has been prepared from bovine pituitary that induces a 12 to 15 fold increase in the uptake of [^{3}H] thymidine and a doubling time of less than 3 days (Brockes *et al.*, 1979; Raff *et al.*, 1978a). The stimulatory effect of the extract was greater at 48 and 72 hours compared with 24 hours and was detectable over a wide range of concentrations. Proteolytic digestion and moderate heating reduced its activity suggesting a proteinaceous nature for the compound (Raff *et al.*, 1978a). Extracts of bovine nerve roots, liver and kidney were inhibitory for mitosis, whereas extracts of bovine brain were stimulatory, but much less so than pituitary extracts. Pituitary hormones, per se, did not stimulate Schwann cell mitosis. Some foetal calf serum was required in the medium for the action of the pituitary extract (Raff *et al.*, 1978a). An extract from bovine caudate nucleus was found to have higher specific activity than the pituitary gland (Brockes *et al*, 1981). The activity of the pituitary extract has been greatly enhanced by purification and this extract is referred to as glial growth factor (GGF). The activity of GGF resides in a basic protein with a native

molecular weight on gel filtration of 60 kDa (cited in Brockes *et al*, 1981).

Schwann cell proliferation is stimulated by compounds that raise intracellular cyclic-AMP concentration. Cholera toxin decreases the doubling time of Schwann cells to every 48 hours (Raff *et al.*, 1978b). It interacted synergistically with GGF and reduced the doubling time to less than 2 days. Dibutryl cyclic-AMP was only moderately stimulatory (Raff *et al.*, 1978a; Salzer *et al.*, 1980a). Similarly, forskolin interacted synergistically with GGF resulting in a 60 fold increase in [^{3}H] thymidine uptake (Porter *et al.*, 1986). Pituitary extract does not raise the intracellular cyclic-AMP levels in the Schwann cells and will interact synergistically with agents that do raise it. This suggests that cyclic-AMP elevators and pituitary extract act by different mechanisms (Raff *et al.*, 1978a). This mechanistic difference was accompanied by a difference in cell morphology. Whilst no appreciable change in Schwann cell morphology was noted with GGF, cholera toxin and forskolin caused the Schwann cells to adopt a more flattened fibroblastic appearance (Raff *et al.*, 1978a; Raff *et al.*, 1978b; Porter *et al.*, 1986).

The use of mitogens did not seem to affect the Schwann cells' functional capability. When compared with Schwann cells derived from spinal ganglia, they secreted the same proteins, expressed the same cell surface antigens and could ensheath and myelinate bare neurites. They also maintained their surface markers to Ran-1, laminin, N-CAM, L1 and L2 and J1 antigens. Cell proliferation decreased with confluence (Porter *et al.*, 1986).

Many other agents have been tested as Schwann cell mitogens, including neurotransmitters and known mitogens for lymphocytes or fibroblasts, but have failed to stimulate Schwann cell division. Increases in intracellular calcium have been indirectly implicated in cell division, but addition of the calcium ionophore A23187 had no effect on Schwann cell proliferation (Salzer and Bunge, 1980a). Several growth factors have been shown to stimulate Schwann cell mitosis to varying degrees in the presence of serum and forskolin or other cyclic-AMP activators. These include growth factors of platelet and fibroblast origin. Transforming growth factors and the carboxymethyl cellulose fraction of glial growth factor were found to be mitogenic with or without forskolin (Davis and Stroobant, 1990). In animals, some of these growth factors have been implicated in tissue repair. Activated macrophages release some of these factors after injury and may thus have a role in Schwann cell mitogenic stimulation via this mechanism (Davis and Stroobant, 1990)

1.3.2.2 Schwann Cell Markers

As the morphology of Schwann cells and fibroblasts may overlap, unambiguous identification of cell types is achieved using cell surface and cytoplasmic antigens specific to the cell in question (Brockes *et al.*, 1979; Brockes *et al.*, 1981). Time-lapse cinematography has shown that bipolar Schwann cells may undergo a morphological transition to a fibroblast-like form (Ernyei and Young, 1966) supporting the identity of these intermediate shaped cells as Schwann cells (Brockes *et al.*, 1977). Cell surface antigens can also be used to select for a population of cells (Brockes *et al.*, 1981). The antigens expressed by Schwann cells depend upon whether the cell is forming myelin and other factors. Non-myelin-forming Schwann cells express GFAP-like intermediate filament protein; A5E3 antigen, Ran-1 and the adhesion molecules N-CAM, L1/Ng-CAM. These antigens are not present, or are found at much lower levels, in myelin-forming Schwann cells. The latter cells express myelin basic protein (MBP), myelin associated glycoprotein (MAG) and P_0 proteins. Both Schwann cells express galactocerebroside (GalC), sulphatide, O4, O8 and O9 lipid antigens (Eccleston *et al.*, 1982; Jessen *et al.*, 1985; Jessen *et al.*, 1989; Jessen *et al.*, 1990; Mirsky *et al.*, 1990a). Expression of all of these molecules appears to be driven by axonal contact except GFAP (Jessen *et al.*, 1989). Schwann cells do not express receptors for tetanus toxin, LETS or Thy-1. However, 30 to 50% of Schwann cells in dissociated neonatal culture were positively labelled by cholera toxin suggesting that they expressed the GM-1 ganglioside (Brockes *et al.*, 1979) Neurons demonstrate the thy-1 antigen and also express receptors for tetanus toxin, whilst fibroblasts are positive for Thy-1 and LETS (large external transformation sensitive protein) (Fields *et al.*, 1978; Brockes *et al.*, 1979).

Ran-1

Ran-1 antigen has been detected only on Schwann cells and on a minor population of cells in the leptomeninges (Brockes *et al.*, 1979). It is a rat specific antigen raised against a chemically induced rat neural tumour (Fields *et al.*, 1978). It is not found on fibroblasts, spinal ganglia neurons or enteric neurons. Cranial (superior) cervical ganglia neurons are positive for Ran-1 (Fields 1985). The Ran-1 cell antigen is the NGF receptor and is related to a protein identified by the 217c antibody (Ferrari *et al.*, 1991; Jessen *et al.*, 1990; Mirsky and Jessen, 1990b). Ran-1/NGF receptor is expressed by non-myelin-forming Schwann cells *in vivo*, but is suppressed on myelin-forming Schwann cells. This suppression is dependent upon

dissociated cell culture of neonatal rat sciatic nerve with time *in vitro* (Jessen *et al.*, 1990). However, Ran-1 is a trypsin sensitive molecule and therefore may not be observed in freshly dissociated cultures (Mirsky *et al.*, 1980).

Ran-2

This is a 140 kDa cell surface protein and is only expressed on non-myelin-forming Schwann cells of the PNS. Ran-2 immunoreactivity was also found on enteric glia, CNS astrocytes, some perineurial cells and fibroblasts (Jessen and Mirsky, 1984a; Mirsky and Jessen, 1984).

S-100

S100 is an acidic protein dimer of varying molecular masses that exists in soluble and membrane bound forms within the mammalian CNS (reviewed Stefannson *et al.*, 1982). It is found within astrocytes, Schwann cells, and also melanocytes, chondrocytes and adipocytes (Dunn *et al.*, 1987). Schwann cells and the outermost part of the myelin sheath stain intensively for S100, but the sheath itself was negative in human and rat peripheral nerve (Neuberger and Cornbrooks, 1989; Stefansson *et al.*, 1982). Axons were found to be negative for it in one study, (Stefansson *et al.*, 1982) but some were positive in another study. (Neuberger and Cornbrooks, 1989). S100 was also detected in the satellite cells, but not neurons, of spinal, myenteric and sympathetic ganglia. Some cells within the adrenal medulla were also positive and these cells may be satellite cells of sympathetic nerves and ganglia. The stain was both nuclear and cytoplasmic in location. (Stefansson *et al.*, 1982). It is found in cultured Schwann cells, and all Ran-1+ Schwann cells expressed it in their nuclei and cytoplasm. (Brockes *et al.*, 1981; Kim *et al.*, 1989).

The mRNA encoding the ß subunit is 1.6 kb when obtained from rat and beef brain but was 600 kb shorter from rabbit. The loss of these 600 bases suggested that this portion of the sequence is non-essential. Experiments using colchicine or vinblastine affected the organization of microtubules within the cell, which decreased the levels of S100 mRNA. It is a calcium-binding protein, but its functional role and relationship to calcium regulation are not known (Dunn *et al.*, 1987). The overall function of this protein is also not known, but it may have a role in regulating Schwann cell functions and possibly in axonal regeneration (Neuberger and Cornbrooks, 1989).

O series of antigens

The O series of monoclonal antibodies have been raised against surface lipids on developing oligodendroglia. The antigens may also be expressed on Schwann cells. These antigens appear on myelin-forming Schwann cells around the time they become competent to make myelin. O7, O8, and O9, but not O11, were also expressed on non-myelin-forming Schwann cells. It is thought that the appearance of the antigens detected by the monoclonal antibodies, O7, O8 and O9, may be related to ensheathment of axons by Schwann cells. O7 antibody binds to GalC. Non-myelin-forming Schwann cells mature more slowly than myelin-forming Schwann cells as suggested by continuing lipid changes occurring up to 4 weeks postnatally. However, by postnatal day 20, dissociated cultured Schwann cells derived from the cervico-sympathetic trunk had a high percentage of cells labelled with these antibodies. The intensity of staining varied between the different antibodies. O antigen expression is lost with time in culture and is no longer expressed after 7 days *in vitro* (Eccleston *et al.*, 1982).

The O4 monoclonal antibody was produced by immunizing mice with whole white matter and recognizes a developmentally-regulated sulphatide antigen (cited in Fields 1985; Mirsky *et al.*, 1990a). It is specific to Schwann cells in rat peripheral nerve and was not detected on any S100 negative cells. Although less than 0.5% of S100 stained Schwann cells were positive for it at embryonic day 15 to 16, by birth 99% of cells were positive. It was also detected in the cervico-sympathetic trunk with 93% of these Schwann cells being positive for the antigen at embryonic day 20 (Mirsky *et al.*, 1990a).

The AOO7 antibody has been raised against sulphatide and its developmental appearance is almost identical to that of the O4 molecule. The appearance of both these molecules at embryonic day 16 is not intrinsically programmed within the cell but requires extrinsic factors. They are manifested 2 days before the appearance of GalC. O4 and AOO7 binding occurs prior to the commitment of the Schwann cell to the myelin-forming or non-myelin-forming pathway and is not specifically related to myelination. It is possible that the sulphatide to which these antibodies bind appears in the Schwann cell membrane associated with the stabilization and formation of the basal lamina. Both non-myelin-forming and myelin-forming Schwann cells were positively labelled with O4 and AOO7 in adult rat sciatic nerve. The expression of these molecules is dependent upon extrinsic factors such as axons or perineurial contents, as cells dissociated from 5 day old rat pups became negative for O4 and AOO7 surface binding within 5 days of culture. Similarly the binding of these antibodies to Schwann cells obtained from distal stump 4 weeks after axotomy is

markedly down-regulated. It is not thought to be totally lost because sulphatide synthesis continues at low levels in the absence of axonal contact. Expression of these antigens was induced on significant numbers of cells by treatment of cultures with cyclic-AMP or cyclic-AMP analogues (Mirsky *et al.*, 1990a).

Laminin

Laminin has been detected both as a patchy cell surface marker and intracytoplasmically on cultured Schwann cells. The intracytoplasmic location is consistent with the ability of the Schwann cell to synthesise laminin. It was not detected on neuronal somata or their processes in culture (Cornbrooks *et al.*, 1983; Kim *et al.*, 1989; McGarvey *et al.*, 1984). It has been suggested that it could be used to distinguish Schwann cells from fibroblasts or neurons (Cornbrooks *et al.*, 1983). However, McGarvey *et al*, (1984) have also detected laminin on the surface of fibroblasts, albeit at a lower level.

Thy-1 (AKR antigen)

This is a surface glycoprotein which is found on murine T lymphocytes, myoblasts, epithelial cells and fibroblasts. It is also a prominent cell surface glycoprotein on rodent neurons, but not glia, of both the peripheral and central nervous systems (Brockes *et al.*, 1981; Fields *et al.*, 1978; cited in Terkelsen *et al.*, 1989). It is useful for identifying fibroblasts in Schwann cell cultures (Brockes *et al.*, 1977).

The functional significance of Thy-1 is unknown, but has been proposed to be involved in cell surface recognition. Although Thy-1 is not a cell adhesion molecule, it is distributed on dendrites and axons where it might serve as a recognition molecule leading to more specific interaction between neuronal processes (Terkelsen *et al.*, 1989).

C4

This is a monoclonal antibody that recognizes a surface associated protein on Schwann cells. It is transiently expressed on ensheathing Schwann cells prior to myelin formation (Cornbrooks and Bunge, 1982; Neuberger and Cornbrooks, 1989).

Glial fibrillary acidic like-protein (GFAP)

GFAP is a 49 kDa protein located in the perinuclear area of the Schwann cell and extending out into the cell processes (Jessen *et al.*, 1984b; Neuberger and Cornbrooks, 1989). GFAP is found in peripheral nerves and some satellite cells of

ganglia and enteric glia, as well as astrocytes. It is thought that CNS GFAP and PNS GFAP are not identical as they do not react with the same antibodies (Jessen *et al.*, 1984b). It is restricted to non-myelin-forming Schwann cells (Jessen *et al.*, 1984a; Jessen *et al.*, 1984b; Jessen *et al.*, 1990) and is quickly re-expressed by cells which had been forming myelin after loss of axonal contact. It was first detected on embryonic day 18 cells dissociated from rat sciatic nerve, was present on less than half of the cells at birth and was down-regulated during myelination. Staining was filamentous or non-filamentous and was not observed on cells with fibroblastic morphology (Jessen *et al.*, 1990).

A5E3

A monoclonal antibody A5E3 recognizes a 130 kDa surface protein found in rat neural tissue, myoblasts and perineurium. Seventy-five percent of sciatic Schwann cells were positive for this marker by embryonic day 15 to 16 and virtually all cells are positive for it at birth. A5E3 is expressed only on non-myelin-forming Schwann cells and like GFAP is quickly re-expressed by hitherto myelin-forming cells that have lost axonal contact (Jessen *et al.*, 1984b; Jessen *et al.*, 1990; Mirsky *et al.*, 1985).

Cytoskeletal elements of Schwann cells

Schwann cells are known to contain the proteins vimentin, actin and spectrin (Carey and Todd, 1986b). Vimentin is an intermediate filament protein found not only in the abaxonal cytoplasm of myelin-forming Schwann cells, but also in enteric glia, fibroblasts and endothelial cells (cited in Neuberger and Cornbrooks, 1989). In transverse sections of sciatic nerve, vimentin was observed as thin rings due to its location within the outer Schwann cell cytoplasm of myelin-forming cells (Neuberger and Cornbrooks, 1989).

2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase)

Cultured Schwann cells, but not fibroblasts, prepared from mouse and human tissue, possess CNPase activity. As the expression of CNPase by Schwann cells is independent of axonal contact, it may be used as a Schwann cell marker in long term culture. Schwann cells which were maintained in culture for 8 weeks were still positive for CNPase activity (Yoshino *et al.*, 1985).

L2/HNK-1

The monoclonal antibodies L2 and HNK-1 recognize a carbohydrate epitope

found on a number of cell surface glycoproteins including N-CAM, L1/NgCAM and MAG (Gennarini *et al.*, 1989). Human foetal Schwann cells were also recognized by HNK-1 antibody although the particular antigen was not known (Kim *et al.*, 1989).

1.3.2.3 Molecule expression in culture

A precursor of the Schwann cell has been molecularly identified on embryonic day 15 as Ran-1 and nerve growth factor (NGF) receptor positive, but S100 negative. On embryonic day 16 it irreversibly becomes S100 positive which is thought to distinguish it from the precursor cell. With axonal contact it rapidly becomes O4 positive and from there may develop along the myelin-forming or non-myelin-forming Schwann cell line (Jessen *et al.*, 1989).

Myelin-specific molecules (MSM)

Because of the temporal development of expression of the various myelin components, the age at which foetal or neonatal tissue is obtained for culture determines which MSMs will be being expressed. Thus more Schwann cells express MSMs in cultures obtained from 5 to 6 day old rats than from neonatal rats. The level of MSMs expressed in embryonic cultures is much lower. Schwann cells cocultured with embryonic day 16 spinal ganglia were only weakly positive for P_0 mRNA, but similar cultures assessed 4 to 5 days later had obvious expression of the mRNA (Morrison *et al.*, 1991). Galactocerebroside (GalC) and sulphatide were first detected in frozen sections and Schwann cells isolated from embryonic day 19 rat sciatic nerves before the onset of myelination (Mirsky *et al.*, 1980). The myelin proteins P_0, P_1 and P_2 were first seen in neonatal cells and did not occur significantly before compact myelin formation was observed with EM (Winter *et al.*, 1982). During the first 5 days after birth, there is a rapid increase in the proportion of Schwann cells expressing Gal-C, P_1 and P_0 in culture coincident with the increase in myelination occurring during this period (Eccleston *et al.*, 1982; Winter *et al.*, 1982). For example, the percent of Schwann cells positive for P_0 after 2.5 to 3 hours in culture at birth was 2%; at day 2 it was 7%; whereas from 5 to 10 day old rats, 46 to 56% of cells were positive. However, in 3 hour dissociated Schwann cell cultures from the cervico-sympathetic trunk of rats on postnatal days 1 to 40, less than 1% of Schwann cells expressed the P_0 protein. (Eccleston *et al.*, 1982; Winter *et al.*, 1982). P_0 may be expressed slightly earlier than MBP as a higher proportion of Schwann cells expressed P_0 than MBP in neonatal sciatic nerve and spinal ganglia (Mirsky *et al.*, 1980). The fact that the glycolipids appear before the myelin proteins suggests that

expression of GalC and sulphatide may be an important step preceding compact myelin formation in the peripheral nerve (Winter *et al.*, 1982).

Loss of axonal contact results in loss of MSM expression over the ensuing 5 days. The proportion of Ran-1+ Schwann cells in sciatic nerve or spinal ganglia cultures that were expressing Gal-C and sulphatide progressively decreased with time in culture, so that by 72 hours only rare cells expressed these glycolipids and no such cells were found at 96 hours. Similarly, P_0 and MBP, and MAG disappeared although at a slightly slower rate than the glycolipids and, by 5 to 6 days in culture, only rare cells expressed these proteins (Mirsky *et al.*, 1980; Owens and Bunge, 1989; Sobue and Pleasure 1984; Winter *et al.*, 1982). However, sulphatide was still formed in the absence of axonal contact after more than 2 weeks in culture (Fryxell, 1980). Unlike rat Schwann cells, cultured mouse Schwann cells continue to express GalC, P_0, P_1 and P_2 at low levels in the absence of any myelin stimulating factor, for up to 21 days. The GalC was only expressed intracytoplasmically and required prefixing of the cells with paraformaldehyde for visualization (Burroni *et al.*, 1988; White *et al.*, 1983). Cultured Schwann cells can start remanufacturing MSMs, like P_0, GalC and MAG when subsequently cocultured with neurites (Owens and Bunge, 1989; Winter *et al.*, 1982).

Cultured Schwann cells transfected with, and expressing SV-40 large T antigen, expressed P_0 and GalC for the first two passages in culture. The markers 217c/Ran-1, Ran-2 and A5E3 were then expressed on the plasma membrane, whilst S100, GFAP and CNPase were expressed as cytoplasmic markers. When zinc was absent from these cultures, then the SV-40 large T antigen was not expressed in the Schwann nucleus and the Ran-2 plasma membrane marker was not detected. The loss of this marker may represent a more differentiated state of the Schwann cells as myelin forming-Schwann cells are also negative for it (Tennekoon *et al.*, 1987).

Studies on neonatal Schwann cell cultures expanded by addition of cholera toxin have shown that the cells continue to express various markers. Ran-1/217c, S100 and laminin were expressed by primary (cytosine arabinoside treated) cells and by secondary (cholera toxin expanded) cells. Primary cells also stained for P_0 protein, CNPase and GalC, but not A5E3, GFAP or Ran-2. Secondary cells only reacted weakly to P_0 protein and CNPase whilst GalC staining was lost. These secondary cells also became positive to A5E3; approximately 20% became positive to GFAP, but Ran-2 was not detected. These immunofluorescence stains were performed on fixed cells. The authors thought that these cells expressed a phenotype intermediate between myelin-forming and non-myelin forming Schwann cells, but not that the cells had dedifferentiated. Although GalC was not detected in secondary cultures by

immunostaining, it was still being synthesised as detected by lipid synthesis, and P_0 protein was detected in secondary Schwann cell cultures by western blotting. Therefore it was considered that these cells were not dedifferentiating in culture, but just down regulating the expression of myelin components. Once Schwann cells differentiate, they continue to incorporate small amounts of various myelin-specific molecules into their plasmalemma in the absence of an axonal signal (Rutkowski *et al.*, 1990).

The expression of cell adhesion molecules by Schwann cells is covered in section 1.5.1.

1.0 LITERATURE REVIEW

1.3 SCHWANN CELLS

1.3.3 Basal lamina

The basal lamina forms a fuzzy coat on the exterior of the cell surface. It consists of a relatively electron dense layer about 10 nm thick called the lamina densa, and the lamina rara which is more electron lucent. The lamina rara is about 2 to 5 nm thick and separates the lamina densa from the cell surface. External to the lamina densa may be a layer of fibrils which is referred to as the reticular lamina and, in conjunction with the basal lamina, forms the basement membrane. These laminae are composed of glycosylated proteins to which are attached carbohydrates. Collagenous and non-collagenous glycoproteins and glycosaminoglycans comprise the extracellular matrix. Laminin and heparan sulphate proteoglycan are concentrated in the lamina rara and type IV collagen is concentrated in the lamina densa (reviewed by Sanes, 1983).

1.3.3.1 Role of basal lamina within the peripheral nervous system.

> "*The Schwann cell is unable to function normally in its role in myelination and ensheathment unless it is able to produce and relate to extracellular matrix components*" (Bunge *et al.*, 1986).

The exact time of appearance of basal lamina during development is not known. Migrating Schwann cells do not possess basal lamina. However it appears when the Schwann cell begins to associate with axons and initiate myelin formation (Billings-Gagliardi *et al.*, 1974; Carey *et al.*, 1983). *In vitro* studies have indicated that the basal lamina is not produced until Schwann cell migration has ceased and the Schwann cell has contacted an axon or another Schwann cell. Collagen fibrils were not observed in regions in which the Schwann cells lacked basal lamina (Armati-Gulson, 1980). Therefore both *in vivo* and *in vitro*, the formation of basal lamina is characteristic of Schwann cell maturation. It has been suggested that the acquisition of basal lamina by the Schwann cell initiates a whole new series of axon-glia interactions. Axonal sorting, ensheathment and myelination are events associated with Schwann cell differentiation and occur at this time. Basal lamina is initially patchy around the Schwann cell but then becomes a complete covering surrounding the

exterior of the Schwann cell. It is not found on the Schwann cell plasmalemma apposed to the axon, nor over the mesaxon (Billings-Gagliardi, *et al.*, 1974; Bunge *et al.*, 1982; Eldridge *et al.*, 1987; Gould and Matsumoto, 1982). The presence of basal lamina surrounding Schwann cells is thought to be characteristic of the non-proliferative state of these cells and may be an inhibitor of proliferation (Gould and Matsumoto, 1982; Ratner *et al.*, 1987). However, it cannot be the only inhibitor because Schwann cells will still proliferate in its presence, both *in vitro* and *in vivo* (Carey *et al.*, 1986a; Ratner *et al.*, 1987).

It appears that basal lamina formation is a prerequisite for myelination *in vitro.* If Schwann cells are cocultured with neurones in defined/non-myelinating medium (N2), then the Schwann cells fail to ensheath and myelinate axons, or produce basal lamina (Carey *et al.*, 1986a; Eldridge *et al.*, 1987; Moya *et al.*, 1980). Schwann cells grown in isolation on type I collagen were mostly bipolar and extended extremely long, thin processes from a small, central, nuclear region of cytoplasm. These cells tended to form long, parallel chains apparently by associating with each other along their processes. In contrast, Schwann cells grown on basement membrane had broader, more sheet-like processes. Schwann cells grown in coculture in N2 medium on type I collagen only proliferated and migrated along the neurites. When grown on basement membrane they also formed myelin. The basement membrane was thought to have induced terminal differentiation in the Schwann cells (Carey *et al.*, 1986a).

However, Schwann cells cocultured with spinal ganglia neurons do express readily detectable levels of P_0 gene when cultured in defined medium in which basement membrane is not produced (Brunden *et al.*, 1990a; Morrison, *et al.*, 1991). This suggests that basement membrane may have a role in the assembly of myelin rather than synthesis of its components. The P_0 protein thus formed is cleared from the cell probably via lysosomal degradation within 2 to 3 hours (Brunden *et al.*, 1990a). Similarly Schwann cells of cocultures grown in defined medium will strongly express MAG and GalC despite failure of normal ensheathment and basal lamina formation. It has been suggested that MAG cannot function in ensheathment without basal lamina formation. Basal lamina formation polarizes the Schwann cell so that its exterior is in contact with basal lamina and the interior contacts axon. It may be that the basal lamina induced polarization is necessary to allow a critical number of MAG molecules to interact with axonal surface receptors and thus ensheathment can ensue (Owens and Bunge, 1989).

It is possible that laminin component of the basal lamina has a role in axon-glia interactions such as adhesion and alignment (Kuecherer-ehret *et al.*, 1990; McGarvey *et al.*, 1984). Anti-laminin staining was more intense on the developing neonatal sciatic nerve than on the adult nerve. The Schwann cell membranes, axonal surfaces and the interstitium were positively stained (Kuecherer-ehret *et al.*, 1990). The presence of laminin increased Schwann cell attachment onto collagen coated dishes suggesting it is an important attachment protein. The cell processes were also much longer and finer when grown on laminin (McGarvey *et al.*, 1984).

Basal lamina appears to initiate of a whole new cascade of events (cessation of division, ensheathment and myelination) in the Schwann cell. It is possible that this is mediated through a membrane spanning molecule in the Schwann cell plasmalemma. Heparan sulphate proteoglycan may have a role in this message transduction (Carey and Todd, 1986b).

Dystrophic mice

Abnormalities of the basement membrane have a significant effect on the peripheral nerve, as is seen with the autosomal recessive abnormality in dystrophic mice. This abnormality consists of a patchy deficiency of the basement membrane and undifferentiated Schwann cells are associated with bundles of naked axons. Bare axons up to 6 μm in diameter have been noted in these mice (Bray *et al.*, 1983; Madrid *et al.*, 1975). The amyelinated areas were lacking in basement membrane (Madrid *et al.*, 1975). Schwann cells formed loose connections with these amyelinated axons. The total number of myelinated fibres was reduced to 1/10 normal in the dorsal and ventral lumbosacral nerve roots and to 1/3 normal in the proximal sciatic nerve. The distal portion of the sciatic nerve did not appear to be affected. Some cranial and cervical nerve roots were also affected. Some motor and some sensory axons were variably myelinated along their course through the roots and into the peripheral nerve (Bradley and Jenkison, 1975). A Schwann cell based abnormality of the basal lamina may be responsible for the pathogenesis of the dystrophic mouse neuropathy (Bray *et al.*, 1983; Madrid *et al.*, 1975). It was considered that failure of basement membrane synthesis prevented termination of Schwann cell migration and ensheathment of axons (Madrid *et al.*, 1975). Inadequate interaction of Schwann cells with connective tissue components in the nerve roots of dystrophic mice may also be involved in their failure to differentiate beyond an intermediate embryonic level. Thus ensheathment and myelination fail to occur. An analogous situation happens *in vitro* when Schwann cells become associated with neurite fascicles suspended above collagen substratum (Bunge and Bunge 1978a; Bunge *et al.*, 1978b). Similar

morphological abnormalities were observed when collagen formation was made abnormal by administration of cis-4-hydroxyproline to cocultures of neurons and Schwann cells (Copio and Bunge, 1980), or if the cocultures were grown in defined medium in which Schwann cell protein secretion was abnormal (Carey and Bunge, 1981). This data supports the theory that the pathogenesis of this disease is due to abnormal extracellular matrix (Copio and Bunge, 1980).

After nerve crush, the regenerating fibres in the dystrophic mouse resembled their counterparts in normal mouse crushed nerve with myelin debris, and axons ensheathed to form myelinated or unmyelinated fibres. The basal lamina coverage of the Schwann cell was also increased from 80% to 97.2%, although it was not complete on almost 2/3 of cells. Therefore, after crush, there were fewer discontinuities of the basal lamina, but it was still not complete. Nerve regeneration after crush is accompanied by an increase in the extracellular collagen, therefore changes in the extracellular environment influence the defect (see also section 1.4.4.2) (Bray *et al.*, 1983).

1.3.3.2 Composition of basal lamina

Basement membranes are thin continuous sheets containing laminin, types IV and V collagen, and heparan sulphate proteoglycan in a 1:1:0.1 ratio. A small amount of nidogen is also present. Individually each of these components is soluble, whereas they precipitate when incubated in combination. These components are specific and other collagens or glycoproteins cannot substitute for them. Type IV collagen or heparan sulphate proteoglycan are important for the deposition and gelling of the complex (Carey *et al.*, 1986a; Pleasure, 1984). The basement membrane obtained from the murine tumour, Engelbreth-Holm Swarm sarcoma, produces a basement membrane which contains laminin, nidogen, entactin, type IV collagen and heparan sulphate proteoglycan. Ultrastructurally, it resembles the lamina densa zone of the basal lamina (Kleinman *et al.*, 1986). Immunostaining for laminin identifies the presence of a thin network of fibrils on reconstituted basal lamina gel (Carey *et al.*, 1986a). This complex affects a number of cells with respect to differentiation. In general, epithelial cells assume a much greater polarity on this substrate than on plastic, collagen, laminin or fibronectin substrates. It was also noted to encourage the growth and differentiation of cultured Schwann cells (Kleinman *et al.*, 1986).

Peripheral nerve contains collagen types I, III, IV and V. Types I and III form the interstitial collagen fibrils, whereas the other two are components of the basement

membrane (reviewed by Pleasure, 1984). The Schwann cells appear capable of synthesising all the endoneurial types of collagen but in cocultures of neurons and Schwann cells there is increased collagen production (Bunge *et al.*, 1977; Bunge *et al.*, 1980; Carey *et al.*, 1983; Mc Garvey *et al.*, 1984). Neurons are required to maintain a high level of collagen production by the Schwann cells, but do not themselves directly contribute to production. The mechanism of the regulation of collagen secretion by neurons is unknown (Carey *et al.*, 1983). Larger extracellular fibrils are formed in cultures also containing fibroblasts, suggesting that these cells also contribute to collagen synthesis (Bunge *et al.*, 1977; Bunge *et al.*, 1980). Thus, the extracellular matrix probably derives from both Schwann cells and fibroblasts (Bunge *et al.*, 1977).

Laminin is a large glycoprotein of 900 kDa composed of three subunits - an α and two ß chains bonded with disulphide bonds. It is a cross shaped structure with each ß subunit forming one of the short arms and the longer arm formed by the three subunits combined (Akiyama *et al.*, 1990; Ohno *et al.*, 1991). It is a major and ubiquitous consitituent of basement membranes (Akiyama *et al.*, 1990; Cornbrooks *et al.*, 1983; Paulsson *et al.*, 1987) with the roles of modulating various cellular activities and interacting with other matrix components. Laminin may act as a multi-binding agent between extracellular matrix components and the cell membrane and this may involve divalent cations such as calcium (Ohno *et al.*, 1991; Paulsson *et al.*, 1987). It forms stable complexes with nidogen, entactin and collagen (Ohno *et al.*, 1991; Paulsson *et al.*, 1987) and it may be that nidogen modulates certain activities of laminin (Paulsson *et al.*, 1987). Laminin is found throughout the basement membranes of Schwann cell-neuron units, perineurium and blood vessels in sciatic nerve (Cornbrooks *et al.*, 1983). It was also detected on the plasma membranes of the Schwann cell and the unmyelinated axon, whereas endoneurial connective tissue was only faintly labelled by antilaminin (Kuecherer-ehret *et al.*, 1990). It was not detected around neuronal somata of spinal or cranial cervical ganglia, or their processes (Cornbrooks *et al.*, 1983). During regeneration after nerve crush, laminin was observed on the regrowing axonal membrane and in the basal lamina tube in the distal stump. It has been speculated that laminin may play a role in the interaction of axons and Schwann cells via specific laminin receptors (Kuecherer-ehret *et al.*, 1990).

Cultured Schwann cells can synthesise laminin (Cornbrooks *et al.*, 1983; McGarvey *et al.*, 1984). Dissociated rat sciatic Schwann cells have patchy surface staining for laminin and granular intracytoplasmic staining for laminin. Even after

prolonged culture laminin was still found on the cell surface and intracytoplasmically. Only small amounts of laminin have been detected on Thy 1-1 positive fibroblasts. Laminin increases Schwann cell adhesion and proliferation and changes cultured Schwann cell morphology so that the cells become very long and thin. This appearance was thought to be similar to the long processes the Schwann cells extend to wrap around neurons (McGarvey *et al.*, 1984).

Heparan sulphate proteoglycan is found in basal laminae throughout the body. In the PNS it is present in basal laminae of Schwann cell-axon units of both myelinated and unmyelinated fibres, in synaptic and extrasynaptic portions of muscle fibre basal laminae, and in the basal laminae of satellite cells in sympathetic and sensory ganglia. Neurons, Schwann cells and fibroblasts cultured alone do not assemble a basal lamina or accumulate immunocytochemically detectable amounts of heparan sulphate proteoglycans (Eldridge *et al.*, 1986).

Schwann cells synthesize two major heparan sulphate proteoglycans which differ in size. The larger one (PG1) is probably a component of the Schwann cell basal lamina; the smaller one (PG2) is present in significant amounts in cultures lacking basal lamina and is thought to be associated with cell membranes. The functions of these proteoglycans are not known (Mehta *et al.*, 1985).

Addition of the proteoglycan inhibitor (4-methylumbelliferyl-ß-D-xyloside) to cocultures inhibited up to 90% of Schwann cell proteoglycan synthesis and also blocked deposition of type IV collegen into the basement membrane. Schwann cell surface laminin was sparse and patchy in these cultures compared with non-treated controls. The drug affected synthesis of both basal lamina and plasma membrane associated heparan sulphate proteoglycans. This precluded basement membrane synthesis and thus myelination. These results suggested that heparan sulphate proteoglycans play an essential role in basement membrane assembly (Carey and Todd, 1987a). The proposed structure for basement membrane is of a lattice of type IV collagen molecules onto which other basement membrane molecules are attached by non-covalent interactions (cited in Carey and Todd, 1987a). It is possible that heparan sulphate proteoglycans provide a mechanism for cross-linking laminin and type IV collagen to form a stable lattice. It is also conceivable that the membrane associated heparan sulphate proteoglycans provide receptors for the attachment of the basement membranes to the cell surface (Carey and Todd, 1987a).

Fibronectin, a 220,000 dalton glycoprotein, is found throughout the endoneurium and on cultured fibroblasts, but not on Schwann cells or Schwann cell-

neuron units in culture (Cornbrooks *et al.*, 1983; Baron-van Evercooren *et al.*, 1982). Exogenous fibronectin markedly promoted Schwann cell proliferation, moderately stimulated Schwann cell chemotaxis and had a small positive effect on Schwann cell adhesion (Baron-van Evercooren *et al.*, 1982).

1.3.3.3 Factors required for basal lamina formation *in vitro*.

In vitro, the formation of Schwann cell basal lamina, but not its persistence, requires the presence of neurons, but not fibroblasts (Bunge *et al.*, 1980; Bunge *et al.*, 1982). Schwann cells grown in isolation from neurons do not form basement membrane components (Dubois-Dalcq *et al.*, 1981). Basal lamina is formed on Schwann cell surfaces in the presence of nerve cells in culture. The collagenous fibrils in the extracellular space were usually associated with the lamina and situated parallel to the longitudinal axis of the fascicles. Basal lamina and collagen fibrils are not formed in cultures of sensory neurons only (Bunge *et al.*, 1982). When nerve cells are removed from 3 to 5 week old cultures the basal lamina disappears almost completely from the Schwann cells within 7 to 8 days. If nerve cells are added back to the glial cell cultures, the Schwann cell basal lamina reappears. Removal of nerve cells from older (3 to 4 months) cultures was associated with a patchy deterioration, but not loss, of the basal lamina suggesting that basal lamina persists but is not reformed. Trypsin or collagenase treatment causes partial or complete disruption of the basal lamina. If basal lamina is treated with trypsin, it is reformed in neuron plus Schwann cell cultures, but not in Schwann cell populations alone. It reforms faster on myelinated compared with unmyelinated fibres. This is possibly because trypsin disrupts the ensheathment in the latter (Bunge *et al.*, 1980; Bunge *et al.*, 1982). Schwann cells have been identified as the source of the collagens and glycoproteins (mainly laminin) in the basal lamina. Although the neurons themselves do not contribute directly to type IV collagen production, the Schwann cells require their presence to maintain the high levels of type IV collagen production (Bunge *et al.*, 1980; Carey *et al.*, 1983; Cornbrooks *et al.*, 1983).

As previously identified, Schwann cell basal lamina formation appears to be an essential prerequisite for the final stages of Schwann cell differentiation and hence myelination. Certain factors are required *in vitro* for the production of basal lamina. In non-myelinating/defined medium (N2), fasciculation, formation of basal lamina and extracellular fibrils, ensheathment and myelination failed to occur (Carey *et al.*, 1986a; Eldridge *et al.*, 1987; Moya *et al.*, 1980; Owens and Bunge, 1989). Schwann

cells proliferated and formed initial associations with axons in this medium but all stages after that were blocked. The Schwann cells were morphologically abnormal, appearing as round cells perched upon nerve fibre bundles or as thin, flat cells with round nuclei (Carey *et al.*, 1986a; Eldridge *et al.*, 1987; Owens and Bunge, 1989). The Schwann cells also failed to form an ultrastructurally detectable basal lamina, although they did accumulate small amounts of laminin and heparan sulphate proteoglycan on their surfaces. They also failed to express or accumulate type IV collagen whereas cocultures grown in myelinating medium had strong positive staining for laminin and type IV collagen (Eldridge *et al.*, 1987). Replacement of the defined medium with standard myelinating medium resulted in rapid Schwann cell differentiation with detectable morphological changes in 24 hours, the occurrence of myelin formation and production of basal lamina within one week. (Eldridge *et al.*, 1987; Moya *et al.*, 1980). In defined medium, it is likely that all the axonal signals are present for ensheathment and myelination, but failure of Schwann cell protein secretion underlies the arrest of differentiation (Carey and Todd, 1987a; Moya *et al.*, 1980). This was suggested by the presence of distended Schwann cell endoplasmic reticulum and the rapid correction of these abnormalities by switching to myelinating medium (Moya *et al.*, 1980).

Chick embryo extract and human placental serum are components of standard myelinating medium. Both were thought to be essential for normal basal lamina and myelin formation (Eldridge *et al.*, 1987). However, chick embryo extract is adequately replaced by ascorbic acid and foetal calf serum by human placental serum. Treatment of the supplemented medium with ascorbic acid oxidase blocked basal lamina formation and myelination. Ascorbic acid and non-dialysable serum components are essential for promotion of both basal lamina assembly and myelination. The serum component(s) alone has little detectable effect but is essential in allowing ascorbic acid to work (Eldridge *et al.*, 1987; Eldridge *et al.*, 1989).

An ascorbate like factor has been derived from foetal brain of a number of different species and, like ascorbic acid, has been found to increase the deposition of laminin and various types of collagen on myotubes (Vogel *et al.*,1987).

Schwann cell basement membrane production was assessed by determining what radiolabelled proteins are released in the presence of different media. Ascorbic acid alone enhanced release of some, but not all, polypeptides especially the collagenous ones. The presence of fetuin, the major protein in foetal bovine serum, significantly increased (15 to 30 fold) the accumulation of all normally released Schwann cell. However, the addition of just fetuin to defined medium lead to poor deposition of surface laminin (Carey and Todd, 1987a); addition of ascorbic acid to

N2 resulted in punctate surface staining (Carey and Todd, 1987a; Eldridge *et al.*, 1989); whereas the addition of both components to N2 produced nearly continuous surface staining for laminin on the Schwann cells (Carey and Todd, 1987a). The addition of ascorbic acid to cultured Schwann cells also increased the staining for type IV collagen on the cell surface (McGarvey *et al.*, 1984). The effect of fetuin was non-specific and could be reproduced by substituting bovine serum albumin. The best myelination under these conditions was observed in fully supplemented medium. Without ascorbic acid, no myelination occurred; when it was added, myelination occurred within one week (Carey and Todd, 1987a). Without exception, media which promoted myelin formation also promoted basal lamina formation and vice versa (Eldridge *et al.*, 1987). These results support the hypothesis that Schwann cells cannot form myelin without the extracellular matrix first being formed (Carey and Todd, 1987a). Thus treatments that prevent basal lamina formation block the later stages of Schwann cell differentiation (Moya *et al.*, 1980). Ascorbic acid is required for the formation of triple helical collagens (type IV basement membrane collagen) (Dean *et al.*, 1986; Eldridge *et al.*, 1987). Because of its effect on collagen hydroxylation it seems that collagen molecules are required for matrix assembly (Carey *et al.*, 1987). The pepsin resistant, triple-helical collagens are synthesized and released only in myelinating medium and not in defined medium. The presence of ascorbate was thought to account for this (Eldridge *et al.*, 1989). Thus, ascorbate appears necessary for basal lamina formation and Schwann cell differentiation, rather than for myelination. Basal lamina formation may be necessary for the creation of Schwann cell plasma membrane polarity and the organization of the cytoskeleton necessary for the production of myelin sheaths (Eldridge *et al.*, 1987).

The addition of ascorbate or dibutryl-cyclic-AMP to dissociated neonatal Schwann cells enhanced the deposition of basement membrane components such as fibronectin, collagen type IV, laminin and heparan sulphate proteoglycan. By immunostaining and EM these deposited compounds were found to be similar, but not identical to authentic basement membrane (Baron-van Evercooren *et al.*, 1986).

Coculture of Schwann cells with neurons substantially increased the expression of the mRNA encoding laminin, but had no effect on the mRNA for type IV collagen. Therefore axonal contact is also one of the requirements for basal lamina production and it is mediated at the transcriptional level for laminin (Dean *et al.*, 1986).

Schwann cells released 2.5 to 4 times less protein and polypeptides into culture medium if they were grown in defined medium compared with fully supplemented

medium. Schwann cells also released other compounds into the medium. These may have been other basal lamina components, trophic factors, enzymes, and ligands used in Schwann cell-axon interaction. Neurons were not required for this release of proteins, but were required for basal lamina formation. This suggested that the neurons have a more subtle role in dictating basal lamina formation than just controlling gross secretion of some component. Another possibility is that the neuron affects the interaction of the Schwann cell surface with the basal lamina (Carey and Bunge, 1981).

1.3.3.4 Effect of addition of exogenous basal lamina to cultures.

An alternative to having the Schwann cells form their own basement membrane is to add an exogenous basement membrane, such as EHS tumour extract, to Schwann cells growing in defined medium.Schwann cells grown on rat tail tendon collagen in N2 medium with nerve fibres did not ensheath or form myelin. Beginning 18 hours after polymerization of exogenous basement membrane onto the Schwann cells, they elongated and migrated along the nerve fibre bundles ultimately ensheathing and myelinating the axons (Carey *et al.*, 1986a; Eldridge *et al.*, 1989). However, rat tail collagen gel overlay did not result in myelination or even the required preceding morphological changes in the Schwann cells (Eldridge *et al.*, 1989). This effect required direct contact between the Schwann cells and basement membrane and did not occur in areas where there was no basement membrane (Carey *et al.*, 1986a). The morphology of Schwann cells grown on basement membrane complex compared with type I collagen or poly-L lysine suggests that the effect of basement membrane was direct upon the Schwann cells and not mediated through the neurons (Yoshino *et al.*, 1990; Carey *et al.*, 1986a). Therefore contact of the cells with a basement membrane induced terminal differentiation of the cell - i.e. changed morphology, behaviour and production of myelin. It was postulated that the 3-dimensional nature of the matrix was important in the polarization and terminal differentiation of the Schwann cell. However, during active neuritic outgrowth the matrix supported active Schwann cell migration and proliferation (Carey *et al.*, 1986a).

Matrigel $^{(R)}$ is a reconstituted basement membrane gel derived from the EHS tumour (Collaborative Research Inc., 1991; Yoshino *et al.*, 1990). This compound could be diluted at 1:50 to 1:100 and still have the same effect resulting in deposition on Schwann cell surfaces of laminin, type IV collagen and heparan sulphate

proteoglycan, and myelination (Eldridge *et al.*, 1989). Myelin formation was not stimulated by addition of laminin in one study (Carey *et al.*, 1986a), but it was efficacious in another study (Eldridge *et al.*, 1989). This difference was not discussed by the latter workers. Type IV collagen and heparan sulphate proteoglycan had no effect in stimulating myelination (Carey *et al.*, 1986a; Eldridge *et al.*, 1989). These data demonstrated that Schwann cells do not require either ascorbate or serum to myelinate axons if they are given basement membrane components from exogenous sources. Therefore the role of ascorbic acid is to enable Schwann cells to form basal lamina and this then enables Schwann cells to myelinate axons. The authors raise the possibility that neurons may control basal lamina formation by acting as "purveyors" of ascorbic acid to the cells they contact (Eldridge *et al.*, 1989).

1.3.3.5 Requirement for extracellular matrix components

Some connective tissue components appear to be necessary for normal differentiation of Schwann cells. *In vivo*, the requirement for collagen was detected when Schwann cells were injected into demyelinated areas of central nervous system. Remyelination occurred only in those areas in which local collagen provided a stable base from which the cells could radiate outwards. Cells deposited on axons in collagen free areas failed to establish a normal relationship with the axon (Blakemore and Crang, 1985). *In vitro*, cultured spinal ganglia may have fascicles suspended above the substrate and not contacting collagen. In these "guy rope" like fascicles, the number of Schwann cells were low, their alignment and ensheathment of nerve fibres incomplete and no myelination occurred. This was despite the presence of basal lamina around the cells. Within hours of overlaying collagen on the cells, alignment began and ensheathment and myelination of the nerve fibres was initiated within several days. Therefore the Schwann cells required contact with connective tissue matrix for correct interaction with the axon. Without contact, only axonal adhesion and production of basal lamina occurred. The normal Schwann cell number was not obtained, nor was correct ensheathment or myelination. Extracellular matrix appears necessary to permit full expression of differentiation in the Schwann cell (Bunge *et al.*, 1978a, Bunge *et al.*, 1978b).

Disruption of collagen synthesis was caused by addition of cis-4-hydroxyproline to cocultures of Schwann cells and neurons. As a result, there was loss of collagen fibrils, patchy basement membrane and improper ensheathment of the axons. These effects were dose dependent and also suggested that collagen formation is a necessary prerequisite for the proper differentiation of Schwann cells (Copio and

Bunge, 1980).

To produce basement membrane *in vitro* that resembles that *in vivo* may require the presence of fibroblasts in cultures. Schwann cells cultured in the presence of neurons and the absence of fibroblasts ensheathed axons in the same manner as that seen *in vivo*. However, the larger diameter collagen fibrils observed *in vivo* were not seen in cocultures without fibroblasts. When fibroblasts were added to the cultures these larger diameter fibrils, and also a far greater number of banded fibrils, were produced. Fibroblasts may play a role in the development of the typical array of endoneurial collagen fibrils, possibly by polymerizing collagen or contributing enzymes for the conversion of procollagen to tropocollagen or for fibril assembly. In the absence of fibroblasts Schwann cells produced basal laminae, but these may be incomplete as the fibroblasts possibly secrete substances which contribute to the basement membrane (Bunge *et al.*, 1980). Could this requirement for fibroblasts explain why fibroblast-free cocultures appear to require contact with a collagenous substratum for normal functional development?

1.3.3.6 Effect of basal lamina on Schwann cell proliferation.

Basal laminae glycoproteins, laminin and fibronectin have been shown to stimulate Schwann cell division by unknown mechanisms (cited in Bunge *et al.*, 1989).

As previously stated, the presence of basal lamina surrounding Schwann cells *in vivo* may be an inhibitor of proliferation (Ratner *et al.*, 1987; Gould *et al.*, 1982). *In vitro*, the rate of proliferation of SV-40 transfected Schwann cells was greater on poly-L lysine then on collagen, then on Matrigel (Yoshino *et al.*, 1990). Very little [^{3}H] thymidine was incorporated by transfected Schwann cells plated onto basement membrane complex from EHS tumour. This decrease in labelling was not observed on cells cultured on pure fibronectin, laminin or extracellular matrix derived from bovine aortic endothelial cells (Tennekoon *et al.*, 1987). In fact, the results of one study suggested that the addition of laminin to dissociated Schwann cell cultures stimulated cell proliferation in a dose dependent manner (McGarvey *et al.*, 1984). However, as Schwann cell proliferation was observed to proceed in young cultures in the presence of basement membrane, it is possible that proliferation may be more directly related to cell density or cell shape (Carey *et al.*, 1986a).

1.0 LITERATURE REVIEW

1.4 MYELINATION

1.4.1 Introduction

There are two types of nerve fibre in the mature animal, based on the type of axonal ensheathment; the fibre may be myelinated or unmyelinated. It is the same type of Schwann cell which forms the two different types of fibre and it is the axon that dictates which type will be formed. One type of axon can induce the Schwann cells to myelinate and the other cannot (Aguayo *et al.*, 1976a; Brockes *et al.*, 1981; Thomas and Ochoa, 1984; Weinberg and Spencer 1976).

1.4.1.1 Location

The myelin sheath is the compacted plasma membrane of the Schwann cell that has spiralled around the axon to form multiple concentric lamellae. The myelin sheath in the CNS is formed by the oligodendrocyte and in the PNS by the Schwann cell. There are a number of significant morphological, biochemical and biologic differences between these two forms of myelin (Peters, 1976). The junction of the nerve roots with the spinal cord or with the brain stem forms the border between the oligodendrocytes of the CNS and the Schwann cells of the PNS (Jenkins 1978). It has been estimated that the rat sciatic nerve contains about 2 to 3 non-myelin-forming Schwann cell for every myelin-forming Schwann cell (cited in Jessen *et al.*, 1987c).

1.4.2 Morphology of the myelin sheath

The tube formed by the myelin sheath encloses the axon and its continuity is interrupted at regular intervals by the nodes of Ranvier. By light microscopy, these nodes appear as constrictions in the myelin sheath along the length of the axon. The axon continues across the nodes without interruption. The nerve fibre immediately adjacent to a node is the paranode and the internode is the myelin sheath formed by one Schwann cell between successive nodes (Peters, 1976).

The sheaths of myelinated fibres in the PNS are thicker than those in the CNS for an equivalent axonal diameter (Peters, 1976). The thickness of the myelin sheath also varies between different nerve fibres and, in general, is proportional to the diameter of the axon (reviewed in Peters, 1976). More specifically, the internodal myelin volume is directly proportional to the surface area of the axon beneath the

myelin sheath. This holds true for normal internodes and remyelinated internodes which often seem to have an inappropriately thin myelin sheath. The relationship also accurately predicted that unmyelinated fibres would be unmyelinated as the surface area beneath the ensheathing Schwann cell is very small (Smith *et al.*, 1982). An oddity has been noted in the axon close to the neuronal somata of the spinal ganglia in neonatal and adult animals. This peculiarity consists of large diameter axons with thin myelin sheaths, adjacent internodes which are unequal in the number of myelin lamellae, and short internodes. These internodes were thought to represent a transition from the unmyelinated initial segment of the axon to the myelinated portion of the axon. These phenomena imply that axonal diameter is not the only determining factor for myelin sheath formation and thickness (Spencer *et al.*, 1973). These nodes may still subscribe to the relationship described by Smith and colleagues (1982) as Spencer described these transitionary nodes as being short as well as being poorly myelinated.

The internodal length is normally proportional to the thickness of the fibre with the average length being 100 to 200 times the average diameter of the fibre (reviewed by Peters, 1976; Jenkins 1978). However, internodal length varies throughout the development of the animal and also between fibres in different locations. By the time a developing axon has made connection with its target organ, it has acquired its full complement of Schwann cells and internodes. Subsequent axonal growth would necessitate the elongation of existing internodes. Axons destined to be of large diameter acquire their full complement of Schwann cells first and will, thus, have longer internodes than axons of smaller diameter. The internodal length may vary along a fibre, either, physiologically with shorter internodes near the axonal termination, or after pathologic change to the myelin sheath. In the latter case, shorter internodes may be formed (reviewed by Peters, 1976).

It has been estimated that the myelinating glial cell makes greater than three times its own body weight in myelin each day (cited Mezei, 1987).

1.4.2.1 Microscopic appearance

In routine preparations of peripheral nerve fibres examined by light microscopy (LM) the lipids of myelin are leached out leaving an artifactual appearance. All that remains is the neurokeratin which is the non-lipid component of the myelin sheath. This appears like spokes of a wheel with finger like projections surrounding the axon. The nucleus of the Schwann cell which forms the myelin is peripheral to the myelin sheath. These nuclei are large, vesicular and have clumped chromatin. A basal lamina covers the outside of the myelin sheath and separates it

from the surrounding connective tissue (Banks, 1981; Peters, 1976; cited by Thomas and Ochoa, 1984). Myelin is eosinophilic on routine haematoxylin-eosin sections. Special stains, including Weigert, Weil, osmic acid (osmic tetroxide) and luxol fast blue, have an affinity for the lipid portion of myelin. A fixative such as potassium dichromate is useful for preserving myelin lipids (Jenkins, 1978).

The internode

Myelin is composed of spiral layers of compacted Schwann cell membrane sandwiched between an inner and outer layer of Schwann cell cytoplasm. The compaction is achieved by fusion of the plasma membranes with extrusion of the intervening cytoplasm. The outer and inner terminations of this spiralled membrane are the outer and inner mesaxons respectively. At the mesaxons two outer plasma membranes closely appose to form the intraperiod line, while the inner plasma membranes fuse to form the more obvious major dense line. Thus the compacted myelin sheath is composed of radially arranged repeating units which, in fresh myelin, are spaced 17 to 18.5 nm apart. Artifactual shrinkage induced by osmic acid fixatives renders this space to measure only 11.5 to 13.0 nm on electron microscopy. The unit measures 13 to 14 nm after preparation with aldehyde fixatives. On aldehyde fixed preparations, the major dense line is 3.5 nm in width, whilst the less prominent intraperiod line has a width of 5.5nm (reviewed by Peters, 1976).

With aging, morphological changes occur in the myelin sheath. These primarily affect the ventral root, but abnormalities have also been noted in the dorsal roots and peripheral nerves in rats. Changes include ballooning, infoldings, and duplications of the myelin sheath. Remyelination and intralysosomal myelin debris within macrophages was also noted. The cause of these changes within the myelin was unknown (Knox *et al.*, 1989).

Remodelling of the compact myelin sheath occurs during development which eliminates many of the initial irregularities and short internodes (10 to 150 μm) (cited in Peters, 1976).

1.4.3 Composition of myelin

Fresh myelin has a water content of 40%. One third of the dry weight is made up of proteins and the rest is lipids. The proteins include P_0, myelin basic protein (MBP) and myelin associated glycoprotein (MAG). Glycolipids and sterols, especially cholesterol, each account for 1/6 of the dry weight. The lipids include

galactocerebroside (GalC), its ester sulphatide, and cholesterol. Myelin composition varies both between species and between central and peripheral myelin within the species (reviewed by Norton, 1981).

The principal biochemical features of myelin that distinguish it from other membranes include the following: a high lipid to protein ratio; the lipids are predominantly long-chain saturated fatty acids; only 2 to 3 proteins make up 80% of the total protein and it has a slow metabolic turnover. The lipid composition of PNS myelin is quite different from CNS myelin of the same animal with the concentration of glycoprotein being high in the PNS and low in the CNS. The CNS myelins of rats and mice have two proteins not present in most other species and the PNS myelins of rats and mice have an extra basic protein. The fluid-mosaic model is presumed to apply to myelin such that the membrane consists of a lipid bilayer with some proteins fully or partially embedded in it and others attached to one or other surface by weaker linkages (reviewed by Norton, 1981).

The rate of turnover depends upon the age of the individual and the myelin component. Increasing myelin turnover is observed with increasing age, such that myelin formed early in life is more stable than myelin of more mature animals. Therefore newly formed myelin is catabolized faster than "old" myelin. The assembly of the sheath is asynchronous with the entry of different compounds into the myelin sheath occurring at different rates. Likewise it is not catabolized as a unit (reviewed by Norton, 1981).

1.4.3.1 Myelin proteins

P_0 protein

This is a 28 kDa glycoprotein and is the major structural protein of the peripheral myelin, accounting for over 50% of the protein in the sheath. It is expressed by myelinating glial cells of the PNS. It is not detected in cultured Schwann cells unless the Schwann cells are freshly dissociated, nor is it observed in unmyelinated nerve, nor embryonic nerve prior to myelination (Brockes *et al.*, 1981; cited in Lemke, 1988a; reviewed by Norton, 1981). The mRNA encoding for this protein, but not the protein itself, is expressed transiently by non-myelin forming Schwann cells surrounding the neurons in the trigeminal ganglia of the neonatal rat (Lamperth *et al.*, 1989). Its expression, during development, seems to depend on the establishment of a 1:1 relationship between the neurons and Schwann cells (Ranscht *et al.*, 1987). P_0 and two of the myelin basic proteins (see later) P_1 and P_2, first appear *in vivo* at the time of, or just after, the formation of compact myelin; this is at birth or

or postnatal day 1. Significantly more Schwann cells express P_0 than P_1 or P_2 at this time (Winter *et al.*, 1982). High levels of P_0 mRNA (Lemke and Axel, 1985) and lower levels of protein (Trapp *et al.*, 1981) have been detected in the sciatic nerve at birth before significant myelination has occurred, so it has been suggested that the protein may contribute to interaction between adjacent Schwann cell membranes prior to compaction (Lemke and Axel, 1985; Owens and Bunge, 1990a). Significant levels of the protein were detected only after two (Lamperth *et al.*, 1990) or three (Trapp *et al.*, 1981) compact lamellae were formed. P_0 protein was also detected in the Schwann cell cytoplasm during active myelination perinatally. The protein was located over the areas of the Golgi apparatus, but not the rER which was presumed to be its primary site of synthesis. This was considered possibly due to rapid transport from the rER to the Golgi apparatus and thence to the sheath (Trapp *et al.*, 1981). Radiolabelled fucose was used to study production and incorporation of P_0 protein. Within 1 to 4 hours of injection into adult mouse peripheral nerve, fucose was localized over the juxtanuclear area of the Schwann cell cytoplasm which was thought to be over the Golgi apparatus. Within several weeks the label was distributed over the myelin sheath, expecially the outer aspects. In neonatal rats the label was also found over the Schwann cell membrane and outer regions of the myelin sheath within 2 hours of injection (Gould, 1977).

The major role of P_0 appears to be in membrane compaction (Owens *et al.*, 1990a, Lemke and Axel, 1985). This protein has extracellular, intramembranous and cytoplasmic domains. The transmembrane orientation and function of the different domains play essential roles in both the elaboration and subsequent compaction of the myelin sheath. P_0 is a member of the immunoglobulin gene superfamily and as such it contains a single variable immunoglobulin-like region in its extracellular domain (see section 1.5.1.2. on CAMs). Compaction at the intraperiod line is hypothesized to result from homophilic interactions between these extracellular domains (Filbin *et al.*, 1990; Lemke and Axel, 1985; Lemke *et al.*, 1988c; reviewed by Salzer and Colman, 1989). In this respect, P_0 protein may act as a cell adhesion molecule (D'urso *et al.*, 1990; Filbin *et al.*, 1990). It has been proposed that the intracytoplasmic domain interacts electrostatically with acidic lipids on the apposing membrane face to serve as a membrane adhesion molecule (Lemke and Axel, 1985; Lemke *et al.*, 1988c). Compaction at the major dense line is probably achieved through the combined action of MBP (see later) and the cytoplasmic domain of P_0 (Lemke *et al.*, 1988c).

P_0 protein exhibits microheterogeneity due to post-translational glycosylation, acylation, sulphation and phosphorylation (cited in Mezei, 1987). In sciatic nerve samples obtained from 9 day old rat pups, the estimated time required for P_0 to travel

from the site of translation to the myelin sheath was 33 minutes. The protein becomes fucosylated in that time (Rapaport and Benjamins, 1981). The L3 molecule is a carbohydrate epitope found associated with P_0 in compact myelin. Along with P_0, it may have a role in the maintenance of compact myelin (Martini *et al.*, 1988b).

Adult Schwann cells do not require axons for specifying P_0 gene expression, although it is likely that axons play a role in amplifying the expression (Poduslo and Windebank, 1985b). Recent studies have identified that this may also be true for neonatal Schwann cells. Endoneurial explant cultures from 4, 8 and 12 day old rat pups were found to synthesize P_0 protein in the absence of axonal contact, but this newly formed protein was rerouted to the Schwann cell lysosomes for catabolism within 3 hours of manufacture. Therefore failure of detection of this protein in Schwann cell isolated from axonal contact is probably due to efficient catabolism of it as well as a low level of expression (Brunden *et al.*, 1990b).

Myelin basic protein (MBP)

The myelin basic proteins are a family of at least six closely related extrinsic membrane proteins which together account for 30 to 40% of CNS myelin protein and 5 to 15% of PNS myelin proteins. The proteins are relatively small 14.0 to 21.5 kDa and very basic. They have been localized immunocytochemically to the major dense lines of myelin and are therefore confined to the cytoplasmic region of the membrane. At least four forms of differing molecular weight MBP have been identified in the rat, and a fifth form has been described in the mouse. The different forms of MBP are thought to arise from alternative splicing of the same gene which is composed of 7 exons (reviewed by Norton, 1981). Recently, two other minor variants of MBP (19.7 and 21 kDa) were described (Aruga *et al.*, 1991). In the PNS, the 18.5 kDa protein is termed P_1 which is identical to the large basic protein of CNS myelin. P_2, with a molecular weight of 14.8 kDa, is unrelated to either P_1 or the small basic protein of rodent CNS. It is an antigen of experimental allergic neuritis. The ratio of P_1:P_2 is extremely species variable. The small rodent basic protein, P_r, is 14 to 15 kDa and was thought for a long time to be the same as P_2. However, it contains only a trace of P_2 and is immunologically related to P_1 but not to P_2 (reviewed by Norton, 1981). As for P_0, the appearance of P_1 and P_2 seems to depend on the establishment of a 1:1 relationship between neurons and Schwann cells (Ranscht *et al.*, 1987). P_0 and P_1 were first oserved in rat sciatic Schwann cells at birth and seemed to require continued axonal contact for expression. During the first five postnatal days the number of Schwann cells expressing both P_1 and P_0 rapidly increased (Winter *et al.*, 1982).

By immunoperoxidase techniques, all peripheral myelin sheaths in the rat were found to contain P_0 and P_1, whereas P_2 had an uneven distribution and was found to be associated predominantly with the sheaths of the larger nerve fibres and was absent from sheaths of smaller axons (Winter *et al.*, 1982).

Proteolipid protein (PLP)

Proteolipid protein is a 30 kDa integral membrane lipoprotein that accounts for approximately 50% of the protein present in CNS myelin. Schwann cells express PLP but at a much lower level. In Schwann cells, the protein is apparently not incorporated into compact myelin, but is instead restricted to Schwann cell cytoplasm which may suggest a role other than a structural one in the myelin sheath (Puckett *et al.*, 1987). PLP exhibits a lower molecular weight variable, DM-20, which results from alternative mRNA splicing. The functional significance of this variable is not known (Lemke, 1988a).

Myelin associated glycoprotein (MAG)

Myelin associated glycoprotein is found only in the myelin-forming Schwann cells of the PNS and accounts for 0.02 to 0.10% of myelin proteins. It is an important cell adhesion molecule of 98 to 100 kDa and is located on uncompacted regions of the sheath such as Schmidt-Lantermann incisures, outer mesaxon, paranodes and normal periaxonal space (Owens and Bunge, 1989; Ranscht *et al.*, 1987; Trapp *et al.*, 1989). Like P_0 it is a member of the immunoglobulin superfamily (reviewed by Salzer and Colman, 1989). It exists in two forms; large (626 amino acids) and small (582 amino acids) which differ on the C terminal of the cytoplasmic domain and may differ in carbohydrate compostion. Both forms are present in the CNS through development, but, in young animals, MAG-L predominates, whilst in the adult MAG-S is more abundant. The functional significance of these two forms is not known (Brady and Quarles, 1973; Lai *et al.*, 1987).

The expression of MAG relies on axonal contact and is a prerequisite for Schwann cells before myelination can occur (Owens and Bunge 1989). In SV-40 transformed Schwann cell lines however, MAG is expressed at low levels in the absence of axonal contact (Chen *et al.*, 1987). With development, its expression is down-regulated on ensheathing, non-myelin-forming Schwann cells, but persists on the surface of myelin-forming Schwann cells, although it is reduced over the surface of mature myelin. It is concentrated in the paranodal and periaxonal areas and the Schmidt-Lanterman incisures in mature myelin segments (Owens and Bunge, 1989; Trapp and Quarles, 1984a). MAG is thought to function as a cell adhesion molecule

in myelination and to promote the initial investment of the axons destined to be myelinated (Brady and Quarles, 1973; Owens *et al.*, 1990b; Ranscht *et al.*, 1987). In the quaking mouse mutant (see section 1.4.4.2.) derangements of the periaxonal space are common. In areas where the periaxonal space was normal, (12 to 14 nm) MAG immunostaining was positive, however, if the distance was greater than this MAG was not detected. MAG seems to be essential to maintain the periaxonal space and the periaxonal Schwann cell cytoplasmic collar. In the absence of MAG, the periaxonal Schwann cell membrane collapses to fuse with the inner lamellae of compact myelin forming a major dense line (Trapp *et al.*, 1984b).

In vivo, MAG was detected at sites where the Schwann cell was only just starting to contact the axon and had only partially surrounded it (Trapp *et al.*, 1984b). In cocultures grown in defined medium basal lamina was absent and the Schwann cells were just associated with the neurites. Under these conditions MAG was still expressed prior formation of a 1:1 relationship of the Schwann cell with the axon or ensheathment of the axon (Owens and Bunge, 1989). These data suggest that MAG is critical to the early investment of the axon by the Schwann cell. Maintenance of the Schwann cell periaxonal cytoplasmic collar and the periaxonal space in the mature animal is possibly achieved by the cytoplasmic and extracellular domains of the molecule, respectively (Trapp, 1988a). MAG is thought to have a large, heavily glycosylated extracellular domain, a trans-membranous domain and an intracellular polypeptide domain. The postulated polarity and bulk of this molecule may prevent the close contact of the Schwann cell plasmalemma and the axolemma (Trapp *et al.*, 1984b). MAG contains the tripeptide with the sequence of Arg-Gly-Asp (RGD). This tripeptide mediates the binding of many extracellular matrix adhesion molecules to their receptors (Lai *et al.*, 1987; Lemke 1988a). MAG is found to colocalize with the microfilament components actin and spectrin. It is possible that these microfilaments form stable contacts with the cytoplasmic domain of MAG. These MAG-microfilament linkages might serve to maintain Schwann cell cytoplasmic continuity and the uncompacted state of the membranes. MAG may also interact with a motility system based on microfilaments, which potentially provides the mechanism by which the mesaxon rotates to form the myelin sheath (Trapp *et al.*, 1989).

1.4.3.2 Myelin lipids

The myelin of the PNS contains less cerebroside, less sulphatide and more phospholipids that that of the CNS (reviewed by Norton, 1981).

Galactocerebroside (GalC)

GalC forms 17% of the total PNS myelin lipid (Yao, 1984) and is the major myelin lipid of the PNS and CNS. Freshly dissociated cultured Schwann cells expressed GalC on both myelin-forming (GFAP negative) and non-myelin-forming (GFAP positive) cells. It was also expressed by teased fibres prepared from sciatic and cervico-sympathetic trunk (Jessen *et al.*, 1985). GalC is first seen on non-myelin-forming Schwann cells in the sciatic nerve at embryonic day 18 and in the sympathetic trunk at embryonic day 19. Peak expression was observed in the cervico-sympathetic trunk on postnatal day 10, but in the sciatic nerve peak expression was not seen until postnatal day 35 (Jessen and Mirsky, 1987a). Both myelin-forming and non-myelin-forming Schwann cells require ongoing axonal contact for GalC expression, but when freshly dissected, will actively synthesise significant quantities of GalC. The role of GalC in non-myelin-forming Schwann cells is not clear, but it appears as these cells are approaching maturity, whereas in myelinating Schwann cells it appears when the 1:1 ratio of Schwann cells:axons is reached. Its role does not appear restricted to myelination, but it is possibly signficant in maintaining stable adhesion between axons and Schwann cells (Jessen *et al.*, 1985; Jessen and Mirsky, 1987a; Mirsky *et al.*, 1980; Mirsky and Jessen, 1987). As both types of Schwann cell express GalC it is unlikely that the signal initiating GalC expression is the same as the myelinating signal (Jessen *et al.*, 1985). Transformed Schwann cells will also incorporate [^{3}H] galactose into GalC in the absence of axonal contact (Chen *et al.*, 1987).

GalC may have a role in ensheathment and initial spiralling of the mesaxon. Treatment of Schwann cell/neuron cocultures with low levels of the antibody interfered with the elongation of the mesaxon and prevented the production of the myelin spiral. At higher concentrations ensheathment itself was prevented (Ranscht *et al.*, 1987). In another study GalC antibodies were found to prevent myelination, but did not affect the expression of MAG nor the achievement of the 1:1 Schwann cell:axon relationship. In many cells the inner mesaxon began spiralling around the axon, but did not complete more 1.5 turns (Owens and Bunge, 1990a). The effect of this antibody was reversible (Owens and Bunge, 1990a; Ranscht *et al.*, 1987). However, the antigen-antibody complex may have indirectly caused improper ensheathment and mesaxon spiralling by interfering with other membrane components involved in ensheathment. Alternatively, the effect may have been due to the antibody interfering with the formation of sulphatide which is a metabolite of GalC (Ranscht *et al.*, 1987).

Sulphatide

Sulphatide is the sulphate ester of GalC and appears on differentiating Schwann cells shortly after the appearance of GalC at embryonic day 19 (Ranscht *et al.*, 1987). Both primary and SV-40 transformed cultured Schwann cells actively synthesise sulphatide as detected by incorporation of [^{35}S] sulphate (Chen *et al.*, 1987).

Cholesterol

In comparison with other membranes, myelin lipids are enriched cholesterol (reviewed by Norton, 1981). Labeled cholesterol administered intraperitoneally appeared in the outer and inner zones of the myelin in the sciatic nerve within 20 minutes. By 90 minutes after injection it was evenly distributed throughout the myelin sheath indicating rapid diffusion or migration of cholesterol within the sheath. Therefore cholesterol, used in the formation and maintenance of nerve fibres, enters from the Schwann cell cytoplasm, possibly from the axon or periaxonal space or crosses from outer Schwann cell cytoplasm through Schmidt-Lanterman incisures to the adaxonal layer. After degeneration, cholesterol is utilized from an exchangeable pool of myelin debris for the formation of regenerating fibres (Rawlins, 1973).

1.4.3.3 Myelin associated enzymes

Myelin has a large number of enzymatic activities. This implies that it is active in metabolism and transport and possibly functions that are quite separate from its accepted properties as an insulator. There is a class of enzymes specific to myelin, and a class also known to be present in other subcellular fractions (reviewed by Norton, 1981).

2'3'-cyclic nucleotide phosphohydrolase

2',3'-cyclic nucleotide 3'-phosphohydrolase is a myelin specific enzyme which hydrolyzes 2',3'-cyclic nucleotides specifically to form 2'-phosphates (cited in Mezei, 1987). Its level increases during myelination and it is deficient in the myelin-deficient mouse mutants, quaking and jimpy. The enzyme is found in low levels in the PNS and the specific activity of PNS myelin is even lower than whole nerve. It is a dimer with a molecular weight of 94 to 98 kDa having subunits of 44 to 48 kDa (reviewed by Norton, 1981). Although it comprises several percent of the total myelin protein, its physiological function is unknown (reviewed by Norton, 1981; Trapp *et al.*,

1988c; Yoshino *et al.*, 1985). Schwann cells which had been cultured for up to 2 months were labelled with a CNPase antibody, but fibroblasts were not. Both murine and human cultured Schwann cells possess CNPase activity. PNS CNPase activity was found in the periaxonal and abaxonal Schwann cell cytoplasms, the mesaxons and Schmidt-Lanterman incisures. It was not found in compact myelin (Trapp *et al.*, 1988c). The activity appeared to be associated with the plasma membranes and may be specifically localized on the cytoplasmic face, since the cells were not stained by anti-CNPase unless they were initially fixed. As expression of CNPase persists in culture, continued axonal contact is not required and the enzyme must be intrinsic to the Schwann cells in the absence of myelin production. The persistence of the enzyme suggests it has a role in the general metabolism of the Schwann cell (Yoshino *et al.*, 1985). CNPase was primarily located within the cytoplasm of SV-40 transformed Schwann cells (Chen *et al.*, 1987).

Cholesterol esterase

The majority (70 to 80%) of the activity of the cholesterol ester hydrolase (pH 7.2) is located in isolated myelin. Its function is also unknown (Norton, 1981). However, in chicks the activity of this enzyme increases in three stages from 14 to 17 days in ovo; from 19 days in ovo to 2 days post-hatching; and from 7 days post-hatching to adult. There is good correlation between the increase in cholesterol esterase activity, commencement of myelination and deposition of free cholesterol in the avian sciatic nerve (Mezei, 1987).

Other

At least 13 other enzymes, not specific for myelin, are known to exist. Carbonic anhydrase is one of these; it has the hypothetical function of facilitating carbonic acid removal from the axon. It is located in CNS glia, especially in the oligodendrocytes, and myelin of both the CNS and PNS. The enzyme 5'-nucleotidase has been detected in CNS glia and myelin and it is presumed to function in hydrolyzing extracellular 5'-AMP and facilitating transport of adenosine into neurons to act as a neurotransmitter. Other non-specific enzymes, such as Na^+/K^+ ATPase, have been detected in CNS myelin (reviewed by Norton, 1981).

1.4.4 Function and dysfunction of myelin

1.4.4.1 Function of myelin

The speed of nerve impulse conduction in the mammalian unmyelinated fibres is of the order of 1 m/sec. Without significantly increasing axonal diameter, higher conduction speeds cannot be achieved. The structural specialization of myelination allows for much greater conduction velocities without significantly increasing the axonal diameter. It is possible that increased transmission frequency can also occur (Thomas and Ochoa, 1984). The rate of nerve impulse conduction tends to be directly proportional to the thickness of the myelin sheath. The fibre diameter of the fast conducting (70 to 120 m/sec) monosynaptic fibres ranges from 12 to 20 μm. These fibres are lower motor alpha neurones and Ia proprioceptive - primary (annulospiral) muscle spindle fibres. In comparison, the preganglionic autonomic visceral afferents have a diameter range of 1 to 3 μm with a conduction velocity of 3 to 15 m/sec (Jenkins, 1978).

Demyelinating diseases of the PNS result in markedly delayed nerve conduction velocities. Intraneural injection of lysolecithin causes demyelination with resulting conduction blockade despite a maintained axon (Rubenstein and Shrager, 1990). In the dystrophic and trembler mouse mutants, myelin abnormalities cause slowed nerve conduction velocities. The dystrophic roots were abnormally excitable and spontaneous electrical activity occurred (Aguayo *et al.*, 1979).

1.4.4.2 Dysfunction of peripheral myelin - myelin mutants

Dystrophic mice

In dystrophic mice there is a very obvious loss of myelinated fibres in the dorsal and ventral spinal nerve roots and clusters of naked axons without Schwann cell contact (Aguayo *et al.*, 1979; Bradley and Jenkison, 1973). The diameter of these unmyelinated axons ranged up to 5 to 6 μm. The neuronal perikarya and most of the axons appeared normal, although some axonal degeneration and ballooning was observed. Rare attempts at myelination were made by the glial cells manifesting as several turns of mesaxon and some bizarre sheath formation. Intracytoplasmic myelin debris was also noted (Bradley and Jenkison, 1973). The lumbosacral and cervical spinal roots are most affected, however, defects have been observed in cranial nerves, proximal sciatic and peroneal nerves (Aguayo *et al.*, 1979; Jaros and Bradley, 1979).

Studies on the common peroneal nerve of dystrophic mice have identified abnormal Schwann cell behaviour with respect to Schwann cell-axon interaction, abnormal myelination and abnormal myelin sheath formation. Basement membrane deficits and axonal abnormalities were also observed (Jaros and Bradley, 1979). A defect in the formation of the basal lamina by the Schwann cell is thought to underlie this abnormality (see section 1.3.3.1). Nerve grafting experiments resulted in myelination of both the dystrophic segment grafted into normal mice, and the normal mouse segment grafted into dystrophic mice. These results suggested that Schwann cells from both mice could myelinate normally given the right circumstances. An axolemmal abnormality may play a role in these mice (Aguayo *et al.*, 1979).

Quaking mice

This is a recessively inherited mutation affecting primarily the CNS but also affecting the PNS (cited by Costantino-Ceccarini *et al.*, 1980). Initial attempts at myelination by the Schwann cells may be adequate, but, with time, hypomyelination and demyelination occur. Abnormalities of the Schwann cell are noted in the nodal and Schmidt-Lanterman areas and include long nodal gaps and some areas of unensheathed axon, which is just surrounded by basal lamina. Focal dilation of the periaxonal space, vacuolation of the myelin or axon, and myelin debris within the Schwann cell are also observed. Persistently elevated Schwann cell proliferation rate may be subsequent to myelinolysis (Aguayo *et al.*, 1979; Trapp *et al.*, 1984b). Insertion of P_0 protein into the expanding mesaxon appears to be delayed in the quaking mouse mutant. Even after the mesaxon had encircled the axon up to 5 times, P_0 protein was not detectable by immunocytochemical methods and thus the major dense line was not formed (Trapp 1988a).

Shiverer mice

MBP deficiency in shiverer mice affects compaction of the myelin in the central nervous system, resulting in hypo- or amyelination. However lack of this protein only subtly affects the peripheral nervous system probably because the cytoplasmic domain of P_0 protein substitutes functionally for it (reviewed by Lemke, 1988a). Therefore myelin formation, compaction and stabilization in the PNS does not require the presence of the basic protein (Ganser and Kirschner, 1980).

Trembler mice

In this dominantly inherited mutant, Schwann cells produce little or no myelin in the spinal roots and peripheral nerves. The CNS does not appear to be affected.

The myelin which was produced was thin and poorly compacted. Some Schwann cell processes were concentrically arranged around the axons giving an appearance reminiscient of an onion bulb, and some Schwann cells were, themselves, surrounded by multiple layers of basal lamina. The presence of debris in Schwann cells of animals less than one month old suggested that active demyelination was occurring. The poor myelination was functionally reflected in the clinical signs of ataxia and trembling, and delayed nerve conduction velocities. The fast axonal transport was not affected by the demyelination. Approximately 2/3 of axons less than 1.0 μm in diameter lacked myelin in these animals whereas in normal animals, axons of this size were myelinated. The unmyelinated (Remak) fibres exhibited normal morphology. Persistence of Schwann cell proliferation was reflected in the elevated [^{3}H] thymidine labelling. This was only observed in myelinated nerves. The axonal diameter was also reduced in these mice. Nerve grafting studies indicated that this is a Schwann cell defect resulting in inability to produce myelin (hypomyelination) and inability to sustain any myelin that was produced (demyelination) (Aguayo *et al.*, 1979).

1.0 LITERATURE REVIEW

1.5 SCHWANN CELL - AXON INTERACTION

Continuous Schwann cell-axon interaction appears to be required for maintenance of normal nerve structure and function. The recognition and interaction is thought to be mediated via specific chemical interaction between the axolemma and the plasmalemma of the Schwann cell (Hall, 1978a). The mediators of this interaction depend upon the developmental stage. In the embryo outgrowth of naked axonal sprouts from the CNS is rapidly followed by migrating Schwann cells from the neural crest. Local axolemmal mitogens stimulate Schwann cell proliferation and axon/Schwann cell attachment is mediated by adhesion molecules expressed on both components. The axon dictates the next sequence of events which results in either ensheathment or myelination. If myelination is to ensue, then a variety of DNA sequences must be transcribed into RNA which is then translated into the various components and enzymes of the myelin sheath. The rate of myelination and the thickness of the sheath is also directed by interaction of the Schwann cell with the axon (reviewed by Hall, 1978a; Smith *et al.*, 1982). However, the Schwann cell also influences the differentiation of the axolemma (section 1.2.3) (Rosenbluth, 1979; Tao-Cheng and Rosenbluth, 1983) and contributes to the function (nerve conduction velocity) of the axon (Hoffman *et al.*, 1984). Many of these stages of Schwann cell-axon interaction have been studied, but the full story has not been elucidated. In this section, various aspects of this interaction will be reviewed including that of Schwann cell-axon interaction pertaining to development.

1.5.1 - Development of the peripheral nerve

1.5.1.1 - Embryology of the peripheral nerve

During development or regeneration of the PNS many events involving Schwann cell-axon interaction occur. These maybe physical or chemical and result in the formation of myelinated or unmyelinated fibres.

The formation of the peripheral nerve is initiated by the outgrowth of a pioneer axon from a neuron. This is soon followed by outgrowth of other axons. Schwann cells of neural crest origin migrate along the nerve fibres (reviewed by Peters, 1976). Studies undertaken on distal nerve ends in tadpoles (Billings-Gagliardi *et al.*, 1974) have identified that the migrating Schwann cells move sporadically at a

speed of up to 114 μm/day. At this point the Schwann cells are ovoid, lack a basal lamina and have blunt ended cytoplasmic processes. They move sporadically by process extension, displacement of cytoplasm and retraction of trailing processes. They may migrate rapidly for up to 20 minutes at a time and then remain static for hours. The net direction of movement is in the proximo-distal direction, but bidirectional migration has been noted. Often the tips of these processes are the only portion of the Schwann cell contacting the axon and it is thought that surface interaction between the cell and the axon occurs through these tips during the migratory phase of the Schwann cell (Billings-Gagliardi *et al.*, 1974). In primary Schwann cell cultures the cells were noted to be migrating discontinuously often by sliding along the length of each other and by translocating the nucleus along the cells' processes followed by an extension of the leading processes and retraction of the trailing processes. The path of migration was often oriented along a constant axis for each cell whereas the migration of fibroblasts was random. The average speed of migration was 120 μm/hour. When mitotic agents were added to expand these cultures, the migratory activity was greatly reduced (Dubois-Dalcq *et al.*, 1981).

Initially columns of Schwann cells align along the outside of large nerve bundles. During this stage of development the main procedure is sorting of axons and development of axon-Schwann cell relationships (Peters and Muir, 1959). This requires a substantial increase in Schwann cell numbers, which is achieved by proliferation in response to the axolemmal mitogen (Wood and Bunge, 1975). During prophase the Schwann cell retracts its sheet like cytoplasmic processes and adopts a simple spindle shape. The axis of mitosis is parallel to the long axis of the axon so that after division, both daughter cells are aligned along the axon. The daughter cells adopt a spindle shape and then extend out the sheet like cytoplasmic processes to participate in ensheathment again. Eventually, the Schwann cells do completely envelop the fibre bundle (reviewed by Peters, 1976; Webster *et al.*, 1973).

In rat peripheral nerve, Schwann cell migration must occur prior to embryonic day 16.5 as, at that time point, the nerve consists of naked, tightly packed axons with the Schwann cells located at its periphery. Isolation of individual axons and ensheathment occurs over the next few days (Peters and Muir, 1959). Once the Schwann cells become associated with axons, they become spindle shaped and acquire a basal lamina. The degree of ensheathment is variable and changing in this early developmental stage. Groups of Schwann cells, called families, are delimited by a common basal lamina and these families become associated with groups of 5 to 20

axons (Peters, 1976; Webster *et al.*, 1973).

The Schwann cells send out processes to subdivide the bundles. A 1:1 relationship of axons to Schwann cells maybe established usually with axons of a larger diameter (Peters, 1976). Eventually, the axon to be myelinated and the Schwann cell become an individual unit separated from the developmental bundle by a basal lamina and extracellular collagen fibrils. The myelinating Schwann cell ceases division once it has established a 1:1 relationship with an axon segment. Other Schwann cells may associate with several axons destined to become unmyelinated fibres and the glial cell provides furrows in its surface in which the axon resides (Peters, 1976; Peters and Muir, 1959; Ranscht *et al.*, 1987; Webster *et al.*, 1973).

Myelinated nerve fibres

The larger axons segregate first at the periphery of the small bundle and eventually locate into a separate furrow in the Schwann cell surface. To become myelinated, the axon needs to achieve a 1:1 relationship with all the Schwann cells which are associated with it along its length (Peters, 1976; Peters and Muir, 1959; Ranscht *et al.*, 1987). A study performed on the developing sixth cranial nerve in the rat has identified that myelination begins first in the large fibres and these fibres have a well developed sheath before myelination of the small fibres begins. The minimum diameter of the axon for myelination was 0.6 μm, but axons were also larger at the onset of myelination (Hahn *et al.*, 1987). Elongation of the Schwann cell is observed during this time just prior to the onset of myelination and this may establish the future internode length. Schwann cells compete for space on the axon and some may be displaced as a result of this competition (Bunge *et al.*, 1989). The furrow deepens in the Schwann cell until the lips of the cytoplasm completely wrap around the axon and the mesaxon is formed. At the outer surface of the mesaxon the outer plasma membranes appose and a tight junction is formed. The mesaxon begins to elongate and spiral around the axon (Peters, 1976; Ranscht *et al.*, 1987; Webster, 1971). The mechanism(s) underlying the spiralling of the mesaxon to form the concentric layers of myelin is uncertain. In general, it is considered that the Schwann cell, or part of it, rotates around the axon (cited by Webster, 1971) and this continues even after compact myelin has been formed. The Schwann cell nucleus also revolves slowly around the developing axon during the formation of the myelin sheath. In one study, nuclear movement was mainly in one direction, although some reversal was occasionally observed. The direction of nuclear movement was always the same as that of the inner mesaxon and it was thought that the movement of the nucleus is due to it being "towed" by the advancing inner spiral. Eventually the outer tip of the

Schwann cell cytoplasm may be anchored to the basal lamina (Bunge *et al.*, 1989). Initially, the spiralling of the mesaxon is not uniform, either with respect to number of turns or even the direction of spiralling (clockwise versus anticlockwise) within, and between, internodes (Bunge *et al.*, 1989; Peters, 1976; Ranscht *et al.*, 1987; Webster, 1971). The mesaxon continues to spiral and when it has turned 3 to 4 complete turns around the axon, then the cytoplasm between the inner membranes is lost so that compact myelin begins to be formed. The only cytoplasm that persists within the developing compact myelin is in the Schmidt-Lanterman clefts. As the cytoplasm is lost from between the inner plasma membranes, these membranes fuse to form the major dense line. In the mature compacted myelin sheath, the inner and outer mesaxons retain cytoplasm (reviewed by Peters, 1976; Ranscht *et al.*, 1987). Compact myelin is first observed between day 0 and day 1 postnatal in rat sciatic nerve (Winter *et al.*, 1982). In the first week postnatal there was little correlation between the axonal diameter and the thickness of the myelin sheath. However, irregularities in the appearance of the sheath were lost within the first few weeks (Hahn *et al.*, 1987). The myelin sheath must also be able to expand to accomodate increases in axonal diameter. This is possibly achieved by the myelin lamellae slipping over each other. A number of inhibitors are known to retard myelination including cholesterol inhibitors, undernutrition and experimental thryroidectomy (reviewed by Peters, 1976).

Unmyelinated nerve fibres

Axons less than 1 μm in diameter, destined to remain unmyelinated becomed embedded in troughs on the Schwann cell surface with 1 to 100 axons being associated with one Schwann cell (Berthold, 1978; cited in Peters, 1976). The Schwann cells associated with unmyelinated fibres become separated from each other by collagen (see also section 1.3.1.2) (Webster *et al.*, 1973).

1.5.1.2 Molecular interactions between the Schwann cell and the axon:

Cell adhesion molecules

Cellular interactions, such as cell-cell adhesion and cell-cell recognition are vitally important in organ system development. Cell adhesion molecules (CAMs) are involved in induction and formation of organ rudiments in early embryonic life; detailed histogenesis of the organs; and in the adult, CAMs may be concerned with surface regulation of cellular metabolic states (Edelman, 1983). Within the nervous system cell surface molecules are responsible for the histotypic organization of

germinal zones, cellular migration, and for the formation of the numerous specific connections between various types and subtypes of neurons (Terkelsen *et al.*, 1989). Surface mediated interactions between Schwann cells and axons are necessary for cellular recognition prior to and during the initial stages of myelination (Nieke and Schachner, 1985).

Specific intercellular adhesion is the result of the interaction of complementary structures. It requires that at least one of the interacting molecules is a protein for these are the only molecules that have the required specificity. It is generally assumed that homotypic adhesion is preferred, and that cells from a defined anatomical region prefer to adhere to each other rather than to cells from a different anatomical region. In a number of instances, dissociated cells from the same organ or tissue, but from different species will coaggregate. If cell surface molecules are antigenic, then antibodies may be raised to them and these should inhibit cell aggregation if the antigenic sites and the adhesive site are in close enough proximity (Frazier and Glaser, 1977)

Neural cells may express just one class of CAMs on their surface (cited by Rutishauser and Jessell, 1988) but most cells express more than one. Combinations of, or competition between, different adhesion mechanisms probably result in cell-cell adhesion and different fasciculation patterns of fibre systems. Variations in the relative contribution of multiple adhesion mechanisms may also result in different fasciculation patterns (Rutishauser and Jessell, 1988). For example, L1 and NCAM are coincidentally expressed on many types of neurons and axon surfaces. Antibodies against each molecule, decreased axonal fasciculation in a different manner (reviewed by Edelman, 1983; reviewed by Rutishauser and Jessell, 1988; reviewed by Yoshihara *et al.*, 1991). Likewise antibodies to N-cadherin and L1/Ng-CAM individually inhibited neurite outgrowth on Schwann cell substratum and, when used simultaneously, strongly inhibited outgrowth from chick ciliary ganglia (Bixby *et al.*, 1988). CAMs may also have a role in the formation of neuro-muscular junctions (Edelman 1983). Inhibitory mechanisms may exist that influence the path and destination of migrating cells and developing axons (Rutishauser and Jessell, 1988).

Cell adhesion molecules may be classified as general (N-CAM, N-cadherin) or restricted (axon-associated CAMs and saccharide-mediated CAMs) based on their spatial and temporal distribution, or chemical characteristics (Rutishauser and Jessell, 1988). There appear to be few general CAMs, whilst there are likely to be a large number of restricted CAMs. General CAMs are expressed frequently and commonly during development of the nervous system. They are likely to mediate more global

interactions affecting diverse and fundamental aspects of development. As a result, their genes appear to be highly conserved during evolution. Restricted CAMs are found in distinct tissue subregions and are therefore likely to mediate more specialized and localized adhesive events. There is likely to be some overlap between moleules assigned to these two classes (Rutishauser and Jessell, 1988).

A common extracellular polypeptide domain is expressed in many cell surface proteins including CAMs (N-CAM, L1) and MAG. This structure is about 100 amino acids long, contains many cysteine residues and is referred to as the immunoglobulin homology unit. The constant (C) portion of the immunoglobulin molecule contains amino acid sequences similar to the polypeptide chains of the cell surface molecules of the nervous system. It is the C region that is mainly utilized in binding of the immunoglobulin to cell surfaces. Myelin associated glycoprotein, L1 and N-CAM have very similar amino acid sequences in their extracellular domains. These sequences are composed of multiple immunoglobulin homology units and are mediators of cell surface recognition events. Homophilic interaction can occur between extracellular domains. Both P_0 (see section 1.4.3.1) and N-CAM interact with like molecules to mediate the apposition of membranes. The members of the immunoglobulin superfamily also have transmembrane spanning and cytoplasmic domains. However, the cytoplasmic domains of these molecules do not exhibit similarity to each other. These portions may be involved in interacting with cytoskeletal elements or molecules involved in transduction of signals (reviewed by Salzer and Colman, 1989; reviewed by Yoshihara *et al.*, 1991).

Neural CAMs have been divided into three basic groups based on amino acid sequence homologies. This suggests that, ancestrally, a limited number of polypeptides gave rise to the neural CAMs. The three groups are members of the immunoglobulin super family, or the cadherin or integrin families. The majority of the neural CAMs belong to the immunoglobulin super family (reviewed by Yoshihara *et al.*, 1991).

General CAMs

1) N-CAM:

N-CAM is a large sialoglycoprotein found mainly on the cell surfaces of neurons and striated muscle precursors (myoblasts and myotubes) and on glial cells of the central and peripheral nervous system. In the PNS of the adult rat, mouse and chicken, it is found only on non-myelin-forming Schwann cells (Daniloff *et al.*, 1986; Jessen *et al.*, 1987b, Nieke and Schachner, 1985; Rieger *et al.*, 1986). N-CAM purified from chick embryo brains is a relatively abundant molecule accounting for

1.0% of the total membrane protein (Hoffman *et al.*, 1982). After separation on SDS-PAGE, three distinct bands were noted; 180, 140 and 120 kDa (Edelman, 1983; Rutishauser and Jessell, 1988). Alternative splicing of the N-CAM gene results in the different isoforms of the same molecule (Salzer and Colman, 1989). The 180 and 140 kDA variants are integral transmembrane proteins which vary in the length of their cytoplasmic chains. N-CAM-120 is easily removed from membranes, does not contain antigenic sites associated with cytoplasmic domains and therefore does not appear to be an integral membrane protein (Edelman, 1983; Rutishauser and Jessell, 1988). N-CAM exists in embryonic form with high carbohydrate content ($\approx$30% sialic acid, E or H form), and in the adult form with a low carbohydrate content ($\approx$10% sialic acid, A or L form). The amino acid and sugar content of the two forms is very similar (Edelman, 1983). Conversion of N-CAM from the embryonic to adult form occurred between embryonic days 14 and 18 in the developing mouse sciatic nerve (Reiger *et al.*, 1986). The large amount of sialic acid may be required for the activity of N-CAM as a ligand, or to maintain the integrity of the molecule on the cell surface (Hoffman *et al.*, 1982). During brain development, the embryonic form is converted to the lower carbohydrate adult form. This chemical modulation may either stabilize contacts between neurones (cited in Daniloff *et al.*, 1986) or arrest the developmental processes (Edelman, 1983). It has been suggested that primary control of N-CAM expression occurs at the transcriptional level. Post-translational modification may be involved in, for example, conversion of embryonic to adult forms of CAM (cited in Daniloff *et al.*, 1986).

N-CAM is one of the earliest markers for differentiation following gastrulation and may play a role in early inductive interactions as well as in shaping of early organ rudiments (reviewed by Edelman, 1983, reviewed by Rutishauser and Jessell, 1988). During development, NCAM is broadly distributed, appearing in many embryonic tissues both neuronal and non-neuronal. It acts as a versatile "glue" keeping the membranes of appropriate cells in close proximity to each other and allows other cell recognition molecules to operate (Edelman, 1983; Rustihauser and Jessell, 1988; Terkelson *et al.*, 1989). N-CAM appears to be the major neuron-neuron and neuron-muscle adhesion molecule in many vertebrate species (Edelman, 1983). It is persistently present on neurones and variably expressed on some glial and muscle cells (reviewed by Rutishauser and Jessell, 1988). N-CAM appears to contribute to muscle innervation and to the formation of synapses between muscle and nerves (Rustihauser and Jessell, 1988; Terkelson *et al.*, 1989). The suppression of N-CAM expression on mature myelinating Schwann cells is dependent upon axonal contact (Jessen *et al.*, 1987).

2) N-CADHERIN:

This is a single polypeptide of 130 kDa. Its appearance or disappearance often occurs at sites of active cell rearrangement, segregation, or association. It may be involved with the outgrowth of neuronal processes and is much more concentrated on sensory compared with motor axons. This differential concentration could contribute to the formation of separate fascicles of sensory and motor axons. Antibody directed against N-cadherin causes an almost complete disruption of cell-cell contacts (reviewed by Rutishauser and Jessell, 1988).

N-CAM is calcium independent, and N-cadherin is calcium dependent implying a fundamental functional difference between the two molecules (Rutishauser and Jessell, 1988).

Restricted CAMs

1) AXON ASSOCIATED ADHESION MOLECULES:

These are axonal surface glycoproteins and include L1, NILE (nerve growth factor-inducible large external glycoprotein), ASCS4, 69A1 and TAG-1 (transiently expressed axonal glycoprotein) in the rat and mouse; and G4, NgCAM, F11, 8D9 and neurofascin in the chick. These CAMs may also be present in soluble as well as the membrane-bound forms. They are thought to contribute to fasciculation and axonal guidance as antibodies directed against these molecules promote defasciculation and decrease the rate of axon elongation (reviewed by Rutishauser and Jessell, 1988). L1 is immunocytochemically identical to NILE, which is identical to Ng-CAM (cited in Martini *et al.*, 1988a). Different axon-associated CAMs may coexist on the same axons during development suggesting that they may function in concert to mediate axon-axon interactions (Rutishauser and Jessell, 1988).

In the rat and mouse the L1 antigen exists in two molecular masses: 140 and 180 kDa. It is involved in calcium independent adhesion, neurite fasciculation and cerebellar granule cell migration (reviewed by Nieke and Schachner, 1985). In the rat and mouse it is expressed on non-myelin-forming Schwann cells and unmyelinated axons (Martini *et al.*, 1988a; Nieke and Schachner, 1985; Seilheimer and Schachner, 1987). Chicken Ng-CAM is a structurally and functionally different molecule to N-CAM, but does share at least one epitope with N-CAM (Grumet *et al.*, 1984).

2) SACCHARIDE-MEDIATED CELL ADHESION MOLECULES:

Cell surface oligosaccharides probably have a role in cell adhesion and recognition in vertebrate development. The saccharide-mediated CAMs have been

defined on the basis of recognition events that involve complex saccharide structures. Three major classes of oligosaccharides have been recognized defined by their polysaccharide backbone sequences, and have been termed lactosyl, globosyl and gangliosyl (Rutishauser and Jessell, 1988).

A number of glycoproteins (L1, N-CAM, MAG) implicated in cell adhesion express a common oligosaccharide structure recognized by monoclonal antibodies such as HNK-1 and L2 (Martini and Schachner, 1986; cited in Nieke and Schachner, 1985). HNK-1 is a frequent marker of immunoglobulin related molecules (Salzer and Colman, 1989; reviewed by Yoshihara, 1991). However, not all N-CAM molecules express the L2/HNK-1 epitope (Martini and Schachner, 1986; cited in Nieke and Schachner, 1985). The functional significance of the L2/HNK-1 family is not clear (Rutishauser and Jessell, 1988). J1 antigen is a another glycoprotein recognized by the monoclonal antibody L2 (Kruse *et al.*, 1985).

Cytoactin is another protein thought to be involved in neural glial cell interaction. It is an extracellular matrix protein synthesised by the glia of both CNS and PNS and is expressed most strongly at the node of Ranvier. It may be synthesised by the Schwann cell and transported to the nodal area for stabilization of the node (Rieger *et al.*, 1984).

Expression of CAMs during the development of the nervous system

L1 and N-CAM are the first molecules to appear during peripheral nerve development and have an almost identical cellular and subcellular localization (Martini and Schachner 1986; Nieke and Schachner, 1985). Large axons are positive only in early development before the mesaxon has turned more than 1.5 loops. After this time, L1 expression is lost and N-CAM expression is reduced as myelination proceeds. It is still detectable at the peri-axonal region and weakly detectable in compact myelin (Martini and Schachner, 1986; Seilheimer and Schachner, 1987). In one study, both antigens were detected on embryonic day 17 mouse Schwann cells, but the expression of the antigens declined so that by day 10 postnatal, the molecules were expressed only on non-myelinating Schwann cells (Nieke and Schachner, 1985). Rieger and coworkers (1984) detected N-CAM and Ng-CAM at embryonic day 14 on small calibre fibres. Schwann cells dissociated from sciatic nerve of 17 to 18 day old rat embryos or neonatal rats were positive for N-CAM expression. Galactocerebroside was coincidentally expressed with N-CAM in the neonates, but P_0 protein was not. This suggests that N-CAM expression is suppressed when Schwann cells start myelinating, presumably by a signal of axonal origin (Jessen *et al.*, 1987b). At the

node of Ranvier the axolemma was always L1 and N-CAM negative in one study (Martini and Schachner, 1986), however, both N-CAM and Ng-CAM were detected at the node of Ranvier by Rieger *et al.*, (1984). The colocalization of Ng-CAM and N-CAM with the sodium channels of the node of Ranvier raised the possibility that the distribution of these CAMs is related to the development of that functional region along the axon (Rieger *et al.*, 1984). Myelin associated glycoprotein was first detected when L1 expression was lost after the mesaxon had encircled the axon and was observed not only at the axon-Schwann cell interface, but also on the opposing cell surface membranes of the turning glial loops. It was lost from compacted myelin and the finger like processes at the node of Ranvier, but remained expressed in non-compacted myelin and at the periaxonal region. The loss of MAG coincides with the appearance of MBP which was detectable only in compact myelin (Martini and Schachner, 1986).

The localization of the L2/HNK-1 epitope does not always coincide with L1 and N-CAM distribution (Martini and Schachner, 1986; Nieke and Schachner, 1985) nor was it observed coinciding with MAG expression during sciatic nerve myelination (Martini and Schachner, 1986). Hence, the L2/HNK-1 epitope is not expressed by all L1 or N-CAM molecules. It is probable that this epitope is a member of functionally important adhesion molecules, but its actual function is yet to be elucidated (Nieke and Schachner, 1985).

Both L1 and N-CAM are expressed on mature non-myelin-forming Schwann cells and unmyelinated axons (Martini *et al.*, 1988a; Nieke and Schachner, 1985; Seilheimer and Schachner, 1987). Based on the chronology of expression, it seems that L1 and N-CAM are involved in axon fasciculation and mediate initial contacts between axons and Schwann cells. Then, for fibres destined to be myelinated, MAG appears to take over these Schwann cell-axon and Schwann cell-Schwann cell interactions. It is possible that P_0 and MBP are involved in the apposition of the cytoplasmic sites of the Schwann cell membranes, while P_0 protein maintains the interlamellar associations at the intraperiod line (see section 1.4.3.1). L1 and NCAM expression appear prior to myelination, while MAG, P_0 and MBP expression occur after the Schwann cell is committed to myelin formation (Martini and Schachner, 1986). This pattern of events is recapitulated after nerve transection during the subsequent re-innervation (Seilheimer and Schachner, 1987).

Expression of CAMs during the regeneration of the nervous system

The expression of CAMs has also been studied during degeneration and regeneration of peripheral nerve after nerve transection or crush experiments in the

adult mouse sciatic nerve. Re-expression of these molecules may be a prerequisite for nerve regeneration. Non-myelinating Schwann cells and Schwann cells associated with degenerating myelin expressed N-CAM and L1 in the distal and proximal nerve stumps. The antigens reappeared in the Schwann cells of the distal stump within 3 days of transection and peak expression occurred at 14 days post-transection. Both L1 and N-CAM were strongly expressed on the bands of Bungner, but not on cellular debris (Nieke and Schachner, 1985). N-CAM expression is regulated by functional contact with the nerve. The time course of CAM expression was closely correlated with the extent of innervation. The amount of N-CAM returned to near normal adult levels in nerves at approximately the same time as the initiation of myelination. A sialic-rich form of N-CAM (similar to the embryonic form) was expressed during regeneration after nerve crush (Daniloff *et al.*, 1986). However, in permanently transected nerve, 90% of Schwann cells in the distal stump were still expressing N-CAM 2 months after injury (Jessen *et al.*, 1987b). As in development, L1 expression became negative after the Schwann cells had enveloped the axon, whilst the N-CAM expression was still weakly positive and MAG was first detected after the mesaxon had enveloped the axon approximately twice. Contact of the regenerating axon with the Schwann cell was L1 and N-CAM positive, but was negative, or only weakly positive, for these CAMs if the axon just contacted basement membranes (Martini *et al.*, 1988a). Thus the expression of these molecules during regeneration recapitulates development (Nieke and Schachner, 1985).

It appears from all of these studies, that the expression of CAMs is closely correlated with tissue morphology and cell differentiation during development and regeneration. Neurotrophic factors may also have a role in expression of CAMs (Daniloff *et al.*, 1986).

Cell adhesion molecules *in vitro*

Cell adhesion molecules are re-expressed on adult Schwann cells that had been forming myelin if they lose axonal contact. The number of N-CAM positive cells from dissociated 10 day old sciatic nerve increased from 10 to 56% within 9 hours of culturing and was not dependent upon cell division for re-expression. However, it was probably dependent upon new proteins being synthesised (Jessen *et al.*, 1987b). Alternatively, these molecules were down-regulated on both cell types when pure populations of Schwann cells and neurons were cocultured. Within 3 days, L1 expression was reduced on Schwann cells by 91% and by 36% on neurones. The N-CAM expression was reduced by 43% on Schwann cells and unchanged on neurones. When the neurones were removed from the cocultures, the expression of L1 and N-

CAM on Schwann cells was reversed and increased significantly. Cell to cell contact was required for this interaction as just the supernatant of spinal ganglia cultures had no effect. Fibroblasts also had no effect in this interaction (Seilheimer *et al.*, 1989). Neonatal rat sciatic Schwann cells expressed L1, N-CAM, and the L2/HNK-1 epitope when cultured on laminin. The expression of L1, but not N-CAM, was increased by the addition of nerve growth factor (NGF) to the cultures. This may suggest a role for NGF regulation of Schwann cell CAM expression (Seilheimer and Schachner, 1987). After nerve transection, increased expression of CAMs by Schwann cells may promote interactions with the regenerating axon (Daniloff *et al.*, 1986; Seilheimer and Schachner, 1987). It is known that Schwann cells, compared with other cell types, provide the best substratum for neuronal growth from chick ciliary ganglia. Integrins are neuronal cell surface proteins that mediate interactions between the neurones and extracellular matrix components. Antibodies to integrins, N-CAM or N-cadherin restricted neurite outgrowth. Therefore axonal outgrowth requires interaction between cell adhesion molecules expressed on the Schwann cells and also extracellular molecules (Bixby *et al.*, 1988)

1.0 LITERATURE REVIEW

1.5 SCWANN CELL-AXON INTERACTION

1.5.2 Wallerian degeneration

> "*We know that when the connexion of a spinal nerve with the spinal marrow is interrupted, its elementary parts become disorganised*" (Waller, 1852).

Wallerian degeneration was first described in frogs by Waller in 1850. Gross morphologic changes were observed in the transected nerve at 3 to 4 days and consisted of "*a slightly turbid or coagulated appearance of the medulla*". This was more obvious at 6 days with "*curdling*" of the medulla, and by the twentieth day, the nerve had a granular appearance "*like the beads of a necklace*" (Waller, 1850). Wallerian degeneration may occur secondary to physical trauma, ischaemia, compression and also active segmental demyelination (Dyck *et al.*, 1984).

In rats, permanent nerve transection results in an orderly sequence of events characterized by axonal degeneration, break down of myelin (myelinolysis), which is seen by 4 days, cell proliferation (especially of Schwann cells), peaking at 2 to 3 weeks, and reorganization of the Schwann cells into longitudinal columns known as bands of Bungner (Poduslo *et al.*, 1985a). The debris is ultimately removed by phagocytosis (Hall, 1989). Changes in the neuronal somata are also oberved (see section 1.5.2.4).

Distal stumps have been reported to remain excitable for 4 to 5 days after transection (Landau, 1953; cited in Pellegrino *et al.*, 1986), although compound action potentials could only be obtained from C57Bl/6J mice for one day after transection (Perry *et al.*, 1990a). Functional changes were also studied using saxitoxin which binds to nodal voltage-sensitive sodium channels of myelinated nerves. The sharp decline in saxitoxin binding at 4 days post-nerve transection closely corresponded to the loss of nerve excitability in the distal stump at this time. This was thought to occur prior to axolemmal dissolution. (Pellegrino *et al.*, 1986).

After transection, all nerve fibres in the distal stump undergo degeneration both of the axon and the myelin sheath. Both components are eventually removed from the nerve fascicle. At 3 weeks post-transection in the rat sciatic nerve, transverse section identifed that each fascicle was filled with myelin debris, lipid droplets and an increased number of cell nuclei. Schwann cell columns were well separated from each

other by an enlarged endoneurial space. Lipid laden cells were often seen near blood vessels, but not within the perineurium. Schwann cells of degenerating fibres had irregular processes, containing increased endoplasmic reticulum, filaments and, occasionally, Golgi apparatus and rough endoplasmic reticulum. Lipid vacuoles and, more rarely, myelin debris or lamellar inclusions were observed in the Schwann cell cytoplasm. A closely applied basal lamina bordered each tube and, outside that, fibroblastic processes encircled individual or groups of Schwann cell columns. Small columns with irregular contours were also noted and these probably represented degenerating, unmyelinated fibres. Lipid droplets were noted in both fibroblasts and macrophages. By 5 weeks post-transection most of the myelin debris was either gone or located intracellularly. The Schwann cell columns were largely free of debris and some flattened Schwann cells were observed partially encircling larger columns. Residual debris and lipid laden cells disappeared during 4 to 5 months and perineurial cells were noted encircling the columns giving a reticulated appearance to the nerve. This network of cells was very prominent after one year and the density of Schwann cell nuclei was decreased with the cells being replaced by collagen and elastin. Therefore, after nerve transection, Schwann cells divide, align to form columns and eventually atrophy and disappear due either to cell death or migration. Hence the maintenance of these cells within the distal stump is probably dependent on axonal contact. This study also suggests that macrophages are extraperineurial and that endoneurial fibroblasts are actively involved in clearing the myelin debris. These cells also encircle the columns of cells and give rise to the perineurial type cells (Weinberg and Spencer, 1978).

Whether nerve degeneration proceeds in a proximo-distal direction or disto-proximal direction has been debated for a number of years. In one study, Wallerian degeneration was found to occur in the peripheral nerve stump in a proximo-distal direction at velocities dependent on fibre diameter. The degeneration occured in jumps from one internode to the next, but within one internode there was no directionality to the degeneration (Lubinska, 1982). However, this study referred mainly to the myelin sheath and not the axon.

Axonal degeneration was found to occur in a disto-proximal direction, probably due to trophic substances originating from the proximal nerve reaching a critical low level first at the nerve terminals (Lunn *et al.*, 1990). Progressive loss of metabolic reserves may trigger lysozymal activity and activation of axonal degeneration (reviewed by Joseph, 1973). Also, the invasion of myelomonocytic cells into the distal terminal region is greater than for other regions of the nerve (see later

1.5.2.2). Subsequent to initial axonal terminal degeneration, macrophage and polymorphonuclear cells are recruited which exacerbate the damage. This may then be compounded by calcium influx through damaged axolemma (Lunn *et al.*, 1990).

It has also been proposed that a trophic factor, supplied to the Schwann cells by an intact axon, maintains the Schwann cells in a "quiescent" state and if this factor is lost, as in axonal degeneration, then the Schwann cells' degenerative activities are initiated (Lubinska, 1982).

Wallerian degeneration also occurs *in vitro*. Within 24 hours of excision of spinal ganglia from mature myelinating cocultures, irregularities appeared in the myelin, the nodes of Ranvier widened and the myelin fragmented to form ovoids over the next few days. By 5 days much of the myelin had been removed despite the absence of macrophages. This was thought to be phagocytosed by the Schwann cells. Schwann cells that had been forming myelin prior to the excision of the ganglia were stimulated to divide by axotomy, but those that were not myelin related did not proliferate in response to axotomy (q.v. section 1.3.1.4) (Salzer *et al.*, 1980a).

1.5.2.1 Myelinolysis

Loss of myelin is a significant characteristic of Wallerian degeneration. As myelin is composed largely of lipids, the decrease in the lipid fraction of the nerve is marked. Within 14 days of tibial nerve transection in the rabbit, all lipid fractions had decreased by greater than 50%. The decrease continued until at least 100 days post-transection at which time the lipid fractions were only 3 to 17% of the control level (McCaman and Robins, 1959a).

By EM, degenerative changes in the myelin sheath were observed within 1 hour of sectioning and included widening of the nodal gap and dilation of the peri-incisural myelin in some sheaths (Peters, 1976). It was initially thought that myelin ovoid formation was initiated over Schmidt-Lantermann incisures which dilated in response to axotomy. Electron microscopy studies have indicated that it is the peri-incisural myelin that undergoes dilation by a separation of the myelin at the intraperiod line and the incisures themselves remain intact. The number of incisures does not increase during the early phase of Wallerian degeneration as was previously thought (Ghabriel and Allt, 1979). The formation of ovoids has been observed within 24 hours of nerve crush and was quite obvious within the first 3 days of Wallerian degeneration (Holtzman and Novikoff, 1965). By 4 days post-axotomy, intrafascicular oedema and endoneurial mononuclear cells were noted. Loss of axoplasm, overt

myelin abnormalities and vacuolation of Schwann cell cytoplasm were noted and myelin debris was found within Schwann cells (Nathaniel and Pease, 1963; Pellegrino *et al.*, 1986). Fragmentation of the full thickness of the myelin sheath was well advanced within 7 days (Nathaniel and Pease, 1963). By nearly 2 weeks post-transection, the myelin was severely degraded and almost all intratubal cells contained myelin debris (McCaman and Robins, 1959b; Pellegrino *et al.*, 1986). At 16 days, the only myelin debris was within phagocytes; Schwann cells did not contain much debris. By 5 weeks relatively little myelin was still present (Mc Caman and Robins, 1959b) and by 7 weeks, there was little evidence of myelin. The Schwann cells had formed basal lamina covered tubes (Pellegrino *et al.*, 1986).

Radiolabelling of forming myelin has been used to establish which myelin is lost first in Wallerian degeneration. The most recently formed myelin is usually lost first (Patsalos *et al.*, 1980; Simon, 1969) although extensive myelin loss also occurs in layers representative of synthesis at all ages (Patsalos *et al.*, 1980).

Myelinolysis is achieved through cellular and enzymatic mechanisms. The relative contribution of these mechanisms to the clearance of myelin debris have been extensively studied and debated, especially with respect to myelin phagocytosis by Schwann cells versus macrophages.

Within 96 hours of the onset of Wallerian degeneration, the cytoplasm of the Schwann cells was hypertrophied and more active with development of the endoplasmic reticulum and ribosomes and an increase in cytoplasmic protein. The hypertrophy and increased protein content indicated the active state of the cells and remained as long as degenerating myelin was present (Nathaniel and Pease, 1963). Acid phosphatases were thought to be provided by Schwann cell Golgi vesicles for digestion of engulfed ovoid fragments and axonal remnants. Lysosomes were noted within Schwann cells 2 to 3 mm distal to the crush site within 2 hours of lesioning and phagocytic vacuoles arose in Schwann cell filopodia engulfing degenerating myelin (Holtzman and Novikoff, 1965). The peak level of a number of enzymes occured at 2 weeks, probably associated with the cellular infiltration and proliferation. The increase in enzyme activity was thought to reflect an increase in the number of cells containing enzyme or an increase in the metabolic state of the cells, rather than a release of bound enzyme from the degenerating tissue (McCaman and Robins 1959b).

Pleomorphic lipid inclusions were observed in Schwann cells and macrophages beginning within 2 weeks of nerve transection. Between 10 and 14 days there were many inclusions which were mainly comprised of multiple lamellae with a periodicity

of 400 to 500 nm. These droplets were presumed to contain cholesterol esters and triglycides. Between 3 and 6 weeks inclusions were mostly small and membrane bound, containing osmiophilic material, but some layered inclusions and inclusions containing just a few membranes separated by clear clefts, were also noted. The level of lysosomal activity associated with the inclusions was variable. Both arylsulfatase and acid phosphatase activity was noted (Lassman *et al.*, 1978). Degenerating myelin within the Schwann cell cytoplasm was variably bound by membrane (Nathaniel and Pease, 1963).

The presence of lipid debris in both macrophages and Schwann cells argues for both of them being involved in phagocytosis of degenerating nerve fibre, but, as previously suggested, the relative contribution of each of these cells is unclear. *In vivo*, treatment of the distal stumps with the antimitotic Mitomycin C, at the time of nerve transection greatly retarded thymidine incorporation and clearance of myelin debris. But it did not alter the time course of axonal degeneration, decline in the synthesis of the P_0, or the onset of ovoid formation. This suggested that replication of Schwann cell DNA has a role in clearance of myelin debris during Wallerian degeneration (Pellegrino *et al.*, 1986). The effect of a Schwann cell toxin on sciatic explants also betokens a role for the Schwann cell in Wallerian degeneration. After addition of 2-deoxyglucose-sodium cyanide to cultures no myelin break down was observed within 5 days and degeneration was delayed for many weeks (Crang and Blakemore, 1986).

The resident population of cells with the appearance and functional characteristics of macrophages within the peripheral nerves is less than 5% (Oldfors, 1980). However, by 2 weeks after transection, a high level of macrophage infiltration, phagocytosis and Schwann cell proliferation was noted. At 45 days there were fewer macrophages and a similar number of Schwann cells, but by 100 days there were few macrophages, the Schwann cells had formed cords and fibroblasts were prominent (McCaman and Robins 1959b). Therefore, after nerve crush or transection some of the increase in endoneurial count is due to rapid recruitment of myelomonocytic cells into the damaged and degenerating area (Hall, 1989). If circulating myelomonocytic cells were excluded from the degenerating nerve then myelin phagocytosis did not occur by the Schwann cells. Rather the Schwann cells appear to reject their sheaths and myelin debris removal was extremely slow (reviewed by Hall, 1989).

With *in vitro* culture of sciatic nerve explants there would be no recruitment of myelomonocytic cells in response to nerve degeneration. However, an increase in the number of phagocytes was observed in cultured explants which implies some local origin and proliferation (Bonnekoh *et al.*, 1989). Despite this, the rate of myelinolysis under these conditions was much slower than *in vivo*. This was thought to emphasize the role of recruitment of macrophages in myelinolysis. Under these *in vitro* conditions, although the Schwann cells were observed to contain neutral fat as well as small myelin figures, they were not thought to phagocytose myelin debris to any extent. Instead, degenerating myelin was just transposed to the endoneurial space from within the myelin sheath (Crang and Blakemore, 1986).

Other studies have been performed suggesting that haematogenous monocyte/macrophage cells are the main agents in myelin removal during Wallerian degeneration. Antimitotics such as colchicine and cytosine arabinoside were added to eliminate the proliferation of the resident macrophage population in cultured sciatic explants. Exposure of nerves to colchicine on the first day of culture reduced myelin phagocytosis and also the number of phagocytic cells. The nerve sheaths appeared to be better preserved in cultures to which these agents were added. Addition of peritoneal monocytes caused a dramatic increase in myelin phagocytosis (Bonnekoh *et al.*, 1989). The addition of these agents would also inhibit Schwann cell proliferation.

During Wallerian degeneration *in vivo*, both non-membrane-bound myelin debris and membrane-bound debris has been observed within Schwann cells. The former suggests autophagy of the sheath, whilst the latter probably arose from phagocytosis of extracellular debris. This work combined with Crang and Blakemore's (1986) results suggested that macrophages are primarily responsible for myelin debris clearance during Wallerian degeneration, but that Schwann cells also have a role (Bigbee *et al.*, 1987). Rejection of the sheath by the Schwann cell is an early feature of Wallerian degeneration and does not require macrophages (Beuche and Friede, 1984; Crang and Blakemore, 1986). This may involve withdrawal of the cytoplasm from the incisures and the paranodes. Myelinolysis may then be initiated by proteases with the Schwann cells having a role in the processing and presentation of myelin debris to the macrophages for initiation of subsequent myelin breakdown. The macrophage-processed myelin may then be presented to the Schwann cell as a mitogen or to be reused for remyelination (Hall, 1989).

Despite the destruction of the myelin sheath during Wallerian degeneration, quiescent Schwann cells continue to produce at least some myelin components

(Poduslo *et al.*, 1985). At 35 days post-transection, the distal nerve stump continued to produce P_0 protein although myelin assembly by the Schwann cells did not occur (Poduslo, 1984a; Poduslo *et al.*, 1984b). Studies involving permanent nerve transection in rats have identified that by 4 days after transection, two bands of P_0 can be observed with molecular weights of 28,500 and 27,700 on SDS-PAGE gels. Both bands are also found at 7 days, but between 7 and 14 days, the 28,500 form disappears and the major species is the 27,700 P_0 glycoprotein. Therefore down-regulation of P_0 biosynthesis by Schwann cells is accompanied by a molecular weight transition from 28,500 to 27,700 P_0 species with a progressive increase in mannose incorporation into the glycoprotein. It is likely that the 27,700 high-mannose containing P_0 is an intermediate in the production of the mature form and that post-translational modifications account for the molecular weight shift from the 28,500 species. With increasing time after nerve transection, the intermediate continues to accumulate, suggesting a decreased rate of processing through the Golgi apparatus (Poduslo *et al.*, 1985a). Similar results were seen when endoneurial slices were cultured from adult rats (Poduslo, 1984; Poduslo *et al.*, 1985a). The decline in synthesis of this protein after axotomy is less extreme than immunofluorescence studies suggest. The protein is still being produced, albeit from a lower level of the mRNA. However, within 1 to 2 hours of synthesis, it is degraded by Schwann cell lysosomes. The post-translational control is altered by axotomy, but the translation of the mRNA is not (Brunden *et al.*, 1987).

1.5.2.2 Axonal fragmentation

Axonal changes have been observed within the first few hours of nerve crush, but most changes are observed within the first few days (Holtzman and Novikoff, 1965). Within 48 hours axonal degeneration had resulted in a residue of amorphous or granular debris (Nathaniel and Pease, 1963); it was almost complete by 2 weeks and all debris had gone by 45 days (McCaman and Robins, 1959b).

Factors such as electrical stimulation of nerves and an increased ambient temperature have been associated with an increased metabolic activity of the nerve. Under these conditions an increased rate of Wallerian degeneration was observed. The temperature sensitive nature of axonal fragmentation raised the idea of an enzymatic basis for Wallerian degeneration. These findings suggested that axonal degradation was inaugurated by release of hydrolytic enzymes secondary to the depletion of metabolic reserves. Other mechanisms such as the influx of calcium ions, resulting from increased permeability of axonal membrane, could also partially account for the

disaggregation of neurofibrils (reviewed by Joseph, 1973). Increased levels of hydrolytic enzymes have been noted in the distal segments of transected sciatic nerve of rabbits in the early stages of Wallerian degeneration. These enzymes were thought to be axonal in origin (Bubis and Wolman, 1965).

In crushed sciatic nerve, axonal degeneration was noted to begin in the most distal part of the nerve (furthest from the site of injury) and to proceed proximally. Similarly, recruitment of myeloperoxidase positive cells was into the distal portion and in areas where this was extensive, neurofilament disintegration had occurred. The cause of the distal disintegration was either due to the entry of a substance at the lesion site traveling distally to the endplate and initiating degeneration or, more likely, the loss of trophic support from the cell body reaching a critical level first in this region (Lunn *et al.*, 1990). It was hypothesized that macrophages may be recruited in response to degeneration of the nerve terminal due to lack of trophic support. This enlistment signal(s) may be released from the axons, or Schwann cells, or resident macrophages. The lack of blood-nerve barrier in this area may aid this cellular influx. The cell membrane damage initiated by phagocytic cells may allow calcium influx and activation of proteases leading to further degeneration.

Axonal degeneration occurred more slowly after nerve crush than after a cut. This was seen in the slowed disintegration of neurofilaments and prolonged ability to conduct action potentials. Ligation and freezing of the nerve had the same effect on speed of degeneration as cutting the nerve, unless the ligature was only temporarily applied in which case degeneration was slowed. The difference between the rates of degeneration after cut and crush lesions was thought due to whether or not the distal stump was in functional contact with living cells in the proximal stump. The benefit of contact with the proximal stump was unclear (Lunn *et al.*, 1990).

Three days post-transection of cat tibial nerve, only occasional floccular changes were noted in the axoplasm of some myelinated fibres, but, by 4 days, there was loss of axoplasm, although internodal axolemma of myelinated fibres was mostly present. By 5 days, 50% of internodal axolemma was degenerating; at 7 days only 1/3 of fibres had internodal axolemma and by 11 days, few intact fibres were present. At 5 days, most unmyelinated fibres and their axolemma, were intact with occasional floccular changes of the axoplasm noted. By 11 days, unmyelinated axons and internodal axolemma were mostly degenerated (Pellegrino *et al.*, 1986). In another study on rat dorsal root nerves, many axons were gone by 2 days, but sometimes traces of axons and mesaxons remained for up to 4 days. Schwann cells were observed to occasionally disgorge the degenerating axons if they were close enough to the cell surface (Nathaniel and Pease, 1963).

Axonal degeneration of unmyelinated fibres of the cervico-sympathetic trunk occured earlier than the unmyelinated fibres of the mixed sciatic nerve described above. Occasional axoplasmic changes were observed in these fibres by 9 to 11 hours after nerve crush in the rat and degenerative changes were well established by 34 hours. The changes included a watery appearance to the axon, loss or clumping of axonal contents and sometimes electron dense material. The axolemma was discontinuous or lost by this time. Very distended axons (4 to 5 μm diameter) were occasionally noted. An increase in the number of nuclei was noted by 2 to 3 days after crush and occasional intact fibres were also seen. These were thought to be undamaged axons originating from neurons that were located within the cervico-sympathetic trunk distal to the crush site (Dyck and Hopkins, 1972).

1.5.2.3 Traumatic degeneration

In 1928 Ramon y Cajal noted that "*external violence*" causes traumatic degeneration of the distal portion of the proximal stump adjacent to the wound (Ramon y Cajal, 1928). This may occcur with either nerve crush or transection. It occurs rapidly in both the proximal and distal stump tips and may be secondary to derangements of ions, such as calcium, potassium and sodium, within the area (Hodgkin and Katz, 1949; Schaepfler, 1971; Seckel, 1990). The extent of derangement in the proximal axon after axotomy is variable. Minimially it extends back to the next node of Ranvier or, maximally, may result in neuronal death (Seckel, 1990). Within 24 hours of transection, the axon immediately above and below the cut swells and contains mitochondria and dense lamellar bodies. By 5 days the mitochondria were degenerating, the microtubules had disappeared and more irregular shaped vesicles and lamellar bodies were noted. The lamellar bodies may have arisen from degenerating lipid. The enlarged axonal tips persisted for up to 2 weeks although the surrounding myelin sheath disintegrated within 1 to 3 days (Blumcke *et al.*, 1966).

Mechanical injury to the sciatic nerve caused an increase in the RNA and DNA content at both ends of the severed nerve within 8 to 24 hours of trauma. This increase was considered to be mainly due Schwann cell synthesis as it occurred prior to invasion by other cells and Schwann cell proliferation, but the possibility that axonal RNA contributed to this increase was also considered. The injury stimulated nucleic acid synthesis not only in the Schwann cells directly damaged, but also in those from neighbouring internodes. The authors speculated that the increase was an example of the general response of cells to direct injury (Oderfeld-Nowak and

Niemierko 1968). Reorganization of the tip of the proximal axon occurs prior to regeneration and this may be aided by lysosomes (Holtzman and Novikoff, 1965).

1.5.2.4 Axonal reaction

After axotomy, changes are observed within the neuronal somata. These are variable and may include eccentric displacement of the nucleus, enlargement of the nucleolus, swelling of the perikarya and dispersion of the Nissl substance to the periphery of the cell (central chromatolysis). Chromatolysis consists of fragmentation and dispersion of the rough endoplasmic reticulum that comprises the Nissl substance. Ultimately the endoplasmic reticulum may disappear, but the ribosomes are extant (Singer *et al.*, 1982; Torvik, 1976). Central chromatolysis represents a change in the metabolic activity of the neuronal soma from maintenance activity and neurotransmitter synthesis to axonal repair and synthesis (Seckel, 1990). Folds may develop in the nuclear membrane and a nuclear cap may form from ribosomal aggregates. This axonal reaction may occur in neurons of both the PNS and CNS. The neurons may recover, regardless of whether axonal regeneration occurs, or the reaction may result in neuronal death (Torvik, 1976). Other changes may include a variable amount of neurofilamentous hyperplasia, and increases in autophagic vacuoles and lysosomal structures have been described. Organelles such as the Golgi apparatus, mitochondria and cytoskeletal elements may be affected. The degree of the reaction is increased after nerve transection compared with nerve crush. Younger animals usually have a more pronounced reaction than older animals although in neonates the neuron may just rapidly atrophy. The severity is proportional to the amount of axoplasm removed so the onset of the reaction is earlier with more proximal lesions. There is also a species variation to the changes, with mice exhibiting dispersion of the Nissl substance and subsequent increased cytoplasmic basophilia rather than chromatolysis. This is probably due to the accumulation of short segments of rough endoplasmic reticulum and free ribosomal clusters. There may also be a variation in the reaction depending upon the region of the body in which the nerves arise (Torvik, 1976).

Regardless of whether the neurons are destined to die or not the initial reaction appears to be the same. The signal for the axon reaction is not known, although a possible hypothesis involves retrograde axoplasmic transport either through a quantitative change in the normal transport, or by transport of some abnormal molecules. Colchicine blocks retrograde axonal transport and injection of it into the proximal stump of a transected nerve inhibited the axonal reaction. This defined a role

for retrograde axoplasmic transport of substances from the axotomy site in the axonal reaction (Singer *et al.*, 1982). The reaction is blocked by administration of actinomycin D which inhibits DNA-dependent RNA synthesis. This indicates that the reaction may be started through the formation of new RNA. It is known that regeneration of peripheral axons is accompanied by a net increase in RNA and protein synthesis by the nerve cell bodies (reviewed by Torvik, 1976). Nerve growth factor (NGF) is a protein that stimulates growth of peripheral neural crest derived sensory and sympathetic neurons (cited in Bandtlow *et al.*, 1987). Application of NGF to the proximal nerve stump after nerve transection limited some of the axonal reaction implying that loss of retrogradely transported factor may have a role in the axonal reaction (Fitzgerald *et al.*, 1985). Local application of nerve growth factor to the cut end of the proximal stump of rat sciatic nerve prevented the down-regulation of substance P, a neurotransmitter. However, axotomy-induced changes in cytoskeletal components was not affected. Reduction in retrogradely transported nerve growth factor is, therefore, not the only signal that is needed to induce the axonal reaction (Wong and Oblinger, 1991). The mRNA for nerve growth factor receptor is induced in the cell bodies of adult rat spinal cord motor neurons following crush lesion of the sciatic. This mRNA was down-regulated 6 weeks after the lesion, coinciding with improved motor function of the leg. This data may also suggest that the regeneration or survival of these neurons is affected by nerve growth factor (Ernfors *et al.*, 1989).

The effect of axotomy on the neuronal nucleus was examined in a marine slug because of its large neuronal size. Changes in [^{35}S] methionine incorporation were observed within 5 hours of transection. These early changes in nuclear proteins may subsequently initiate later changes in the production of proteins associated with axonal regeneration (Buriani *et al.*, 1990). The first observed change in motor neuron cell bodies after axotomy was an increase in ornithine decarboxylase activity. This enzyme is involved in polyamine synthesis and hence nucleic acid synthesis. Also observed at a biochemical level was an early and sustained increase in glucose uptake by the neuronal perikarya which may aid lipid synthesis whilst the enzymes involved in transmitter metabolism are decreased after axotomy (reviewed by Tetzlaff *et al.*, 1986).

Under normal conditions phosphorylated forms of neurofilament are not found in the perikarya (reviewed by Hollenbeck, 1989; Rosenfeld *et al.*, 1987). Phosphorylation of neurofilaments occurs close to the somata, but additional phosphorylation occurs during subsequent axonal transport of the neurofilaments. The level of phosphorylation is variable and may be associated with the speed of the neurofilament within the axon (reviewed by Hollenbeck, 1989). Within 1 to 5 days of

nerve crush of the sciatic nerve, phosphorylated epitopes were apparent in the perikarya. The maximum staining percentage was observed 7 days after axotomy and that pattern persisted for at least 21 days with phosphorylated neurofilaments being observed in 30 to 40% of neurons. The staining occurred in large and small neurons and was irrespective of whether chromatolysis was occurring. By 12 weeks post-axotomy, phosphorylated neurofilaments were no longer observed in the perikarya. After sciatic transection/ligation, phosphorylated neurofilaments were observed in the perikarya 1 to 10 days later. However, by 3 weeks post-transection the distribution of phosphorylated neurofilaments was the same as the intact, control nerve. Therefore, changes in processing of neurofilaments in the neuronal somata may be induced by axotomy, but the cause of the appearance of phosphorylated epitopes in the neurons is not known. It is possible that aberrant phosphorylation of neurofilaments abnormally retained in the soma occurs, or that phosphorylated neurofilaments are not being transported from the perikarya. Likewise the basis for the difference of phosphorylated neurofilament distribution at 3 weeks post-transection compared with post-crush, was not known. Axotomy had no effect on the distribution of non-phosphorylated neurofilaments (Rosenfeld *et al.*, 1987).

After nerve crush in mice the degree and nature of axonal reaction was different in the neurons of the spinal ganglia versus those of the ventral horn cells. A more prominent dispersal of the Nissl substance and nuclear eccentricity was observed in the spinal ganglia, but the increase in numbers of neurons staining positive for neurofilament was greater in the ventral horn cells than in the spinal ganglia. The increase in perikaryal neurofilament is thought to be a regenerative phenomenon (Moss and Lewkowicz, 1983).

1.5.2.5 Schwann cell proliferation

> "*It looks as though the destruction of the conducting filament were the signal for the emancipation of the cells of Schwann from their nutritive function; their nuclei enlarge and divide.....*
>
> *The nucleus does not remain idle.....on the fourth days mitoses begin.*"
>
> (Ramon y Cajal, 1928 referring to the Schwann cell nucleus during Wallerian degeneration.)

Nuclear increase within the endoneruium is mainly due to Schwann cell and macrophage proliferation. After macrophage processing, the products of myelin degradation may be presented to the Schwann cell as a mitogen (see section 1.3.1.4) or the components may be reutilized for remyelination (Hall 1989).

Division of Schwann cell nuclei was not observed until 36 hours after nerve severance (Oderfeld-Nowak and Niemierko, 1968) although other workers did not observe this until 72 hours (Pellegrino *et al.*, 1986). However, using the freeze-fracture technique, changes in the plasmalemma and sub-plasmalemmal cytoplasm of the Schwann cell have been noticed 24 hours after nerve crush. These changes were thought to represent dedifferentiation of this cell in association with cell division (Abrahams *et al.*, 1980).

Loss or acquisition of axonal contact induces Schwann mitosis. Intraneural [^{3}H] thymidine incorporation, indicating DNA replication, and cell mitosis was found to peak at 4 days post-transection at 30 to 50 fold normal levels. The incorporation was mainly by the Schwann cells (Pellegrino *et al.*, 1986). The DNA content is an index of the number of cell nuclei/mm^3 and in the degenerating nerve this increased to 271% of the control nerve DNA content within the first 14 days of transection. After this time it decreased, so that by 45 to 100 days there was no significant difference to the control level. The increase in DNA was considered to be due to cell invasion and proliferation during Wallerian degeneration (McCaman and Robins, 1959a). The increased DNA content and labelling with [^{3}H] thymidine corresponded to morphologic studies on rat sciatic nerve after permanent transection. A noticable increase in the number of nuclei, especially of Schwann cells, was noted by 7 days. The nuclear increase had peaked by 14 to 21 days and after 3 to 4 months, nuclear numbers were fewer (Poduslo *et al.*, 1985a).

There are two, often-cited stimuli for Schwann cell proliferation. The first is in response to degenerating myelin and the second due to stimulation by the axolemmal mitogen (see section 1.3.1.4). The supernatant from cultured macrophages that have phagocytosed a myelin-enriched fraction is mitogenic for Schwann cells. Supernatant from axolemmal-enriched fraction is not mitogenic. These results suggest a critical role of the macrophage in the Schwann cell proliferation during Wallerian degeneration (Baichwal *et al.*, 1988). Myelin induced division would occur in both nerve transection and after crush, but the proliferation in response to axonal contact would obviously require axonal regeneration, which is most likely to occur after nerve crush. Delaying coaptation of severed nerve enabled Schwann cell mitosis due to myelinolysis, and that due to regenerating axonal contact, to be studied individually. In nerve rejoined after 7 weeks Schwann cell proliferation was stimulated by contact

with regenerating axons. If this wave of mitosis was prevented by intraneural injection of Mitomycin C, then myelination was delayed. Therefore, axonally induced Schwann cell proliferation may be required for remyelination (Pellegrino and Spencer, 1985).

Another suggestion for the cause of Schwann cell proliferation revolves around their epithelial nature and requirement to establish and maintain cell-to-cell contact. This continuity may initially be lost after sheath rejection during degeneration. This would suggest a direct relationship between fibre size and the extent of Schwann cell proliferation as degeneration in larger fibres would leave a larger void to fill within the basal lamina tube (Crang and Blakemore, 1986).

1.5.2.6 Basal lamina

In the normal nerve fibre, basal lamina surrounds the columns of Schwann cells investing each axon. *In vivo* after Wallerian degeneration, this basal lamina tube may be detected in the distal stump of a non-reinnervating nerve for months (Nathaniel and Pease, 1963; Weinberg and Spencer, 1978). It has an important role in nerve fibre regeneration providing a scaffold for migration, orientation and adhesion of regenerating cells. The Schwann cell basal lamina tube probably aids the linear orientation of Schwann cells, providing a guiding pathway along which regenerating axons are encouraged (Giannini and Dyck, 1990; Peters, 1976).

In vitro explants of cat sciatic nerve were established to study Wallerian degeneration and changes in the basal lamina in the absence of bulk macrophage activity. The debris from the rejected myelin sheath was released from a tortuous lamina tube either through small breaks in it or due to total focal dissolution of the basal lamina. Disintegration of the tube released free myelin and Schwann cells into the endoneurial space. Following this translocation the basal lamina tube collapsed. Residual intratubal Schwann cells formed cellular aggregates bounded by a single basal lamina. These intratubal aggregates then rearranged to form structures resembling bands of Bungner (Crang and Blakemore, 1986).

However, the basement membrane in the distal stump of the transected nerve alters with chronicity of transection. Changes were observed in its integrity within a few days of nerve transection and included collapse of the tube and development of occasional breaks within it. With increasing time after transection, it fragmented and became dispersed throughout the endoneurium and was partially lost. This altered the scaffold along which neurites could grow back during reinnervation. Thus, progressive changes of the basement membrane may contribute to the poor regeneration when nerve reconnection is delayed (Giannini and Dyck, 1990).

1.5.2.7 Regeneration

During the first 2 days of injury, there is axonal sprouting from the proximal stump. If possible, these neurites will grow across the gap and enter the distal stump and the bands of Bungner formed by the Schwann cells. Ideally, they will ultimately grow towards the periphery and reinnervate their target organs (Hall, 1989). The DNA and RNA content of the distal stump of crushed sciatic nerve increased by 600% and 400% respectively 2 weeks after crush. This was thought to be partially due to Schwann cell proliferation that accompanies regeneration (Alberghina *et al.*, 1983). Myelination of some of these sprouts occurs in clusters and morphologically clusters of remyelinated sprouts implies regeneration. The regeneration occurs concurrently with degeneration (reviewed by Dyck, 1984). Myelination of the new axons results in the new sheaths being thinner, less compact and having shorter internodes (reviewed by Peters, 1976). The myelination had started by 30 days after nerve crush and peaked between 3 and 4 months (Alberghina *et al.*, 1983; Alberghina *et al.*, 1985).

The distal stump is thought to play a significant role in the attraction of regenerating fibres. Several days after transection axon-glia interaction molecules such as L1 and N-CAM are re-expressed on the Schwann cell and probably have an important role in the support of neurite growth. NGF receptor expression by the Schwann cells is also upregulated. This may provide a NGF-rich surface to which NGF dependent axons can grow. The S100 protein content of the Schwann cell in the distal stump falls within the first few weeks of injury and will subsequently increase when regenerating neurites invade these Schwann cells. Axon-glia contact may regulate the expression of this protein and it has been suggested that S100 may have a role in axonal outgrowth. Basement membrane components are also thought to have a role in axonal regrowth and Schwann cell migration along axons and axonal ensheathment (reviewed by Hall, 1989).

In the peripheral nerve both NGF receptor and NGF increase after axotomy (Heumann *et al.*, 1987b; Raivich and Kreutzberg, 1987; Taniuchi *et al.*, 1988). The rapid accumulation of the protein in the distal stump within the first 24 hours was probably due to ongoing retrograde axonal transport. This then declined, presumably due to protease mediated axonal degradation. An increase in NGF protein was observed subsequent to this, due to local synthesis by the Schwann cells and fibroblasts. This coincided with the increase in NGF mRNA in these cells, implying

axonally induced suppression of local non-neuronal synthesis of this protein (Heumann *et al.*, 1987a; Heumann *et al.*, 1987b). The increase in the NGF protein mRNA was biphasic with a rapid and transient increase at 6 hours and a second peak at 2 days (Heumann *et al.*, 1987b).

A similar early increase in NGF receptor was observed after nerve cut or crush in sensory nerves. It was noted at the site of injury (proximal and distal) with accumulation beginning 2 hours after injury, peaking at 24 hours and rapidly decreasing after 2 days. When an isolated middle segment of nerve was created by proximal and distal cuts then NGF receptor was only expressed at the ends of the segment. This early, local accumulation of NGF receptor was thought to be due to bidirectional axonal transport and not local synthesis of the receptor after nerve injury. Four days after the injury a second, more massive, increase in the protein was observed in the distal stump of both sensory and motor nerves. This increase was due to local synthesis by the Schwann cells (Raivich and Kreutzberg, 1987). The mRNA for the receptor also increased after axotomy, but the increase was delayed for 24 hours and more prolonged. The increase in the mRNA for the receptor after transection was considered evidence of some degree of sciatic nerve dedifferentiation (Heumann *et al.*, 1987b). Re-expression of NGF receptor protein was observed on Schwann cells of NGF-unresponsive neurons such as lower motor neurons, parasympathetic neurons and placodally derived sensory neurons in response to axotomy. This suggests that regardless of axonal type, all Schwann cells respond to axotomy by upregulating expression of this protein (reviewed by Johnson *et al.*, 1988; Taniuchi *et al.*, 1988). However, the re-expression of NGF receptor by motor neurons occurred only after nerve crush and not after transection (Wood *et al.*, 1990).

The levels of both receptor and protein mRNA declined with reinnervation. The down-regulation of NGF receptor mRNA occurred in a proximo-distal gradient and closely correlated with the ingrowing regenerating axons (Heumann *et al.*, 1988b; Raivich and Kreutzberg, 1987; Tanuichi *et al.*, 1988). The loss of receptors occured approximately 0.5 to 1.0 mm in front of the axonal front or approximately 12 hours in front of the growing axonal tip (Tanuichi *et al.*, 1988). The proposed function of the NGF receptors expressed on the Schwann cells of the distal stump is to become laden with NGF which provides haptotactic guidance for the regenerating axon. (reviewed by Johnson *et al.*, 1988; Raivich and Kreutzberg, 1987; Rush, 1984; reviewed by Seckel 1990; Taniuchi *et al.*, 1988). The binding of NGF protein to a surface receptor is essential for its action (Yan and Johnson, 1988). These receptors are low affinity, and fast dissociating. The axolemma expresses slow dissociating

NGF receptors and can bind and retain NGF molecules as they are released from the Schwann cell membrane. The NGF molecule is internalized within the axon and retrogradely transported to the neuronal soma to provide trophic support for the regenerating neuron (Tanuichi *et al.*, 1988). In the absence of axonal regeneration, Schwann cells distal to the axotomy continued to exhibit strong expression of the receptor (Raivich and Kreutzberg, 1987; Taniuchi *et al.*, 1988).

Proximal to the transection, an increase in the NGF mRNA occurs in the non-neuronal cells but only adjacent to the cut. The distal end of the proximal stump may thus be a "substitute target" for regrowing axons. Local production of NGF is probably essential for axonal regrowth (Heumann *et al.*, 1987a).

Increased levels of the mRNAs for both receptor and protein were observed *in vitro* in transected nerve. It may be that macrophages have a role in maintaining the increased levels of the protein mRNA as their presence was required *in vitro* for the increase to occur (Heumann *et al.*, 1987b). Dissociated neonatal rat sciatic nerve Schwann cells were also positive for NGF receptor by 3 days in culture. Treatment of cultures with dibutryl cyclic-AMP, 8-bromo cyclic-AMP, or forskolin, resulted in a down-regulation of NGF receptor and its mRNA over an 11 day period. The effect was reversible with withdrawal of treatment. Glial growth factor had no effect on NGF receptor expression. Thus cyclic-AMP may mediate the effect of the axon on Schwann cell NGF receptor gene expression (Mokuno *et al.*, 1988).

Other factors associated with the distal stump have effects on neural regeneration. Improved regeneration occurred when the distal stump was preserved by injection with the protease inhibitor, leupeptin. This was either due to retention of the neurotrophic influence of the axon or the Schwann cells in the distal stump. Improved preservation of retrograde axoplasmic flow for one week post-transection was also observed (Hurst *et al.*, 1984).

The matrix that forms between severed nerve ends is also an important substrate encouraging the migration of endoneurial cells, such as Schwann cells, into the gap. It is composed primarily of fibrin polymers and provides a scaffold for cellular migration (reviewed Seckel 1990; Williams *et al.*, 1983). Soluble factors are released by cells participating in regeneration of peripheral nerve. These factors were obtained from a silicone tube encasing the proximal and distal stump of a transected nerve. The soluble factors promoted Schwann cell adhesion, motility, and proliferation (Le Beau *et al.*, 1988). The Schwann cell and its basal lamina components then provide a supportive environment for axonal regeneration. The tip of the outgrowing neurite is referred to as the growth cone and is thought to play an

essential role in guiding the axon into the periphery. It releases a protease to aid passage through the matrix, which then guides it to the periphery (reviewed by Seckel, 1990). The diameter of the regenerating nerve is determined by several factors. It has the potential to be large if it makes a connection with an end organ, or if the size of the central nerve that grows into the distal stump is large, or if the size of the Schwann cell tubes in the peripheral stump is large (Simpson and Young, 1945).

After nerve crush, Schwann cell proliferation results in excess numbers of cells required to ensheath regenerating axons. Excess axonal sprouts are also formed and as regeneration proceeds, redundant sprouts degenerate. By 2 months after crush, excess Schwann cells had become attenuated forming only 1 or 2 processes and had arranged themselves circumferentially, in a crescentic shape, around a central myelinated axon. It is possible that eventually these extra Schwann cells, like the axonal sprouts, disappear. Schwann cells associated with remyelination had increased nuclear chromatin and cytoplasmic organelles (Ohara and Ikuta, 1988).

Myelination of regenerated axons recapitulates many events of developmental myelination. Following regeneration of axons into chronically transected feline sciatic nerve, MAG was again re-expressed at high levels during the initial stages of axon-glia contact and before the reappearance of P_0 and P_1 (Willison *et al.*, 1988). The C4 protein increased during reinnervation (Neuberger and Cornbrooks, 1989). The re-expression of myelin protein mRNAs was closely regulated, both spatially and temporally, by regenerating axons. Upregulation of the mRNAs occured 3.2 mm behind the advancing axonal tip. During this spatial lag axon-glia contact would be made, with its associated adhesion and molecular interaction for signalling. The axonal signals encoding the initial stages of myelination were expressed on the axon just behind the advancing tip (Mitchell *et al.*, 1990). Galactocerebroside upregulation occurred before upregulation of the mRNAs encoding myelin-specific molecules. Myelination ensued after this (Mitchell *et al.*, 1990; Politis *et al.*, 1982). The myelin basic proteins (P_1 and P_2) were expressed before P_0 protein due to either later synthesis or later expression of that protein (Politis *et al.*, 1982).

Schwann cells are quite versatile and given the right opportunities will remyelinate axons even after being unusually manipulated. They will still myelinate regenerating axons even if the Schwann cells have been cultured artificially as a nerve explant *in vitro* for 10 days prior to being reintroduced to an *in vivo* situation (Bonnekoh *et al.*, 1989).

Regenerating unmyelinated axonal sprouts were observed between 5 and 15 days after crush of the cervico-sympathetic trunk in the rat. These were sometimes difficult to distinguish from Schwann cell processes. Between 20 and 30 days after crush many regenerating axons were interspersed amongst Schwann cell processes, but by 57 and 60 days post-crush fewer axons were noted. Some of the axons had formed a normal mature relationship with the Schwann cells being ensheathed by the glial cell cytoplasm. By nearly 4 months post-crush injury, most axons were in a normal relationship with the Schwann cell either completely or partially ensheathed by them. Unensheathed portions were covered by a basal lamina. These regenerated fibres were smaller than normal uninjured fibres with a modal diameter of 0.25 μm compared with 0.4 μm (Dyck and Hopkins, 1972).

1.5.2.8 Delayed degeneration

In his early studies on the nervous system, Waller used nerve transection to investigate the anatomic origin of peripheral nerves. Bilateral transection of the glossopharyngeal nerve resulted in death of the frog, but what Waller also noted was that the timing of death was seasonally dependent. Frogs survived up to 20 days in winter but only 5 days in summer (Waller, 1850; Waller, 1852). This implied maintained function of the nerve and therefore slowed Wallerian degeneration in cooler temperatures. Studies performed over 130 years later, elaborated on this observation. In frogs kept at 11^{o}C, compound action potentials and the resting membrane potential could be elicited from transected sciatic nerve for up to 8 weeks. If frogs were kept at room temperature, then morphologic and functional degeneration began at 10 days whilst keeping them cool at 4^{o}C maintained function in the transected stump for up to 12 weeks. No morphologic degeneration was observed in these latter nerves. The membrane bound Na-K-ATPase pumps must be maintained until Wallerian degeneration starts for the resting membrane potential to be maintained. Isolated nerve segments kept in frog Ringer's solution at 4^{o}C maintained structural and functional integrity for 19 days and then degenerated. This study linked the speed of Wallerian degeneration to the body temperature in poikilothermic animals. In homeothermic animals with 37^{o}C body temperature, functional and morphologic loss of nerve integrity was noted at 3 days post-transection (Wang, 1985).

The prolonged preservation of frog sciatic nerves post-transection was used to study the ability of the transected nerve to engage in remyelination. Intraneural

injection of lysolecithin causes demyelination of the nerve. The progression of events of both demyelination and remyelination in cut axons was indistinguishable from that in uncut fibres. Macrophages appeared and phagocytosed myelin debris simultaneously for cut and uncut demyelinated fibres. The demyelination was largely complete in many axons at 6 days. Proliferating Schwann cells appeared in the second week post-injection in both cut and uncut fibres and began remyelination. Nerve conduction was lost during the demyelinated phase, but began to reappear during remyelination of both cut and uncut nerves (Rubinstein and Shrager, 1990).

Both these experiments imply that if Wallerian degeneration is delayed then normal axonal activites can proceed. This carries the connotation that membrane proteins involved in nerve conduction (Wang, 1985) and in encoding myelination do not require contact with, nor control by the neuronal somata. It may also imply that myelin encoding molecules are stable with a low turn over rate.

1.0 LITERATURE REVIEW

1.5 SCHWANN CELL-AXON INTERACTION

1.5.3 Schwann cell-axon interaction for myelination

> "...*Schwann cell expression of myelin genes shows a remarkable plasticity that is almost entirely controlled by neurons.*" (Lemke and Chao 1988b).

The basis of myelin-specific molecule induction in Schwann cells by axons may be based upon an axonal stimulus which interacts with a Schwann cell receptor, and the signal is transduced, possibly by a second messenger such as cyclic-AMP, to the Schwann cell nucleus which initiates gene transcription (Lemke, 1988a). The exact nature of this signal is not known, but the role of the axon in triggering myelination has been studied extensively. The general conclusion is that the axon dictates whether it is associated with Schwann cells which will ensheath or myelinate it. This implies that there is one type of Schwann cell and potentially two types of axons. However, this may not be the full story.

The axonal signal for myelination appears to be the same for both the peripheral and central nervous systems in that Schwann cells can myelinate central fibres. This was observed normally in the vagal rootlets at the junctional area of the brain stem and the peripheral nervous system. Schwann cells were found myelinating portions of the axons deep within the brain stem. These Schwann cells were intercalated as semi-islands amongst the oligodendroglia, but maintained a connection with peripheral Schwann cells. The internodes formed by these cells were, on average, 60% shorter than peripheral internodes which may relate to the astrocytic barrier surrounding these cells and curtailing their elongation (Fraher and Rossiter, 1991). Demyelinated axons of the CNS have also been successfully remyelinated by Schwann cells that have invaded or been transplanted into the area (Blakemore, 1975; Blakemore, 1982b; Blakemore, 1984; Blakemore and Crang, 1985, reviewed by Blakemore and Franklin, 1991).

It has been suggested that cultured Schwann cells will ensheath and myelinate any cylindrical object (Ernyei and Young, 1966). However, EM studies were not performed to accurately assess the morphologic events in detail. In later EM studies on cultured CNS glia by Field *et al.*, (1968) no myelination of polyester fibres was identified. In other studies using cultured oligodendroglia and carbon fibres, a form of

myelin was produced (Althaus *et al.*, 1987). However, oligodendrocytes do not seem to require an ongoing axonal signal to make detectable amounts of myelin-specific molecules (Dubois-Dalcq *et al.*, 1986, Mirsky *et al.*, 1980). True myelination of artificial cylindrical elements by cultured Schwann cells has not been achieved rendering it unlikely that myelin induction is just a physical process independent of axon-glial chemical interaction.

1.5.3.1 The role of axonal calibre

Axonal calibre has been suggested as one of the factors in determining whether or not the Schwann cell would produce myelin. Mathews (1968) noted that enclosure of an axon by a Schwann cell was a necessary prerequisite to myelination, and that axons with a diameter of 1 to 3 μm that were singly-enclosed were already surrounded by several myelin lamellae. Anatomically similar nerve fibres were compared between three mammalian species of different sizes to assess the effect of axonal calibre on the nature of Schwann cell ensheathment. In the rat the thoracic ventral root fibres were very small and approximately half were unmyelinated. In the cow, the diameter of these fibres was much greater and very few were unmyelinated. The same fibres in the cat were half way between these two extremes with respect to axonal diameter and nature of glial ensheathment. The author concluded that a critical diameter of axon for myelination did exist which was between 1 to 2 μm and seemed to be irrespective of the age, size or species of the animal, or function of the fibre (Mathews 1968). The findings of Duncan (1934) were similar in that the critical myelination diameter was 1 to 2 μm. Axonal diameter greater or lesser than this range was associated with myelinated or unmyelinated fibres respectively (Duncan, 1934).

A more recent study re-addressed this theory by altering axonal diameter and studying the effect of this manipulation on myelination. Axonal diameter can be increased by increasing the ratio of target size to number of innervating axons. The effect of this on the predominantly unmyelinated postganglionic sympathetic fibres innervating the rat submandibular gland was dramatic, resulting in a 12 fold increase in the number of myelinated fibres within the nerve. In the normal nerve the average fibre diameter was 1.15 μm. The critical axonal diameter for myelination, in this case, was 1.6 μm. Above this diameter 90% of fibres were myelinated and below this diameter 95% of fibres were unmyelinated. The effect of decreasing the axonal diameter of unmyelinated fibres was to increase the number of axons ensheathed by one Schwann cell. Between these two ends of the spectrum was the effect of increasing unmyelinated axonal diameter and this was accompanied by a decreasing

number of axons per Schwann cell. The point at which the axon to Schwann cell ratio approached one was around the axonal diameter associated with myelination. The conclusion drawn from this was that as axonal diameter increased it reached a point where the Schwann cell could no longer accomodate further increases in axonal size by decreasing the number of ensheathed axons. Significantly, this seems to imply that there is no qualitative difference between axons that are myelinated or unmyelinated, but rather it is the axonal calibre that dictates the state of Schwann cell ensheathment (Voydovic, 1989).

However, there is significant overlap in axonal diameter of non-myelinated and myelinated fibres so axonal calibre cannot be the only determinant (Aguayo *et al.*, 1976b). Also, in the developing sixth cranial nerve of the rat, myelination began on axons with diameters of 0.6 to 1.0 μm (Hahn *et al.*, 1987). Even as far back as the 1930's the question of overlapping sizes was raised (Duncan, 1934). Then and now, available evidence cannot definitively answer this question.

1.5.3.2 *In vivo* studies

The question of axonal induction of the type of Schwann cell envelopment has been studied for many years by cross anatomosing myelinated and unmyelinated fibres. In 1898 Langley joined the fifth cervical nerve to the distal stump of the cervical sympathetic. He thought that:

> "*the histologic condition of the sympathetic peripherally of the point where it was joined to the 5th cervical nerve was noteworthy. In both experiments it consisted almost entirely of medullated nerve fibres, and in both cases a considerable number of the fibres were larger than those normally found in the cervical sympathetic.*" (Langley and Anderson, 1903).

Subsequent cross anastomosis and nerve grafting experiments between a myelinated (phrenic, sternohyoid or sural nerves) and an unmyelinated nerve (cervico-sympathetic trunk) have identified that the type of nerve regenerating from the proximal stump determines whether or not the distal stump will be myelinated (Aguayo *et al.*, 1976a; Simpson and Young, 1945; Weinberg and Spencer, 1975; Weinberg and Spencer, 1976). The Schwann cells in the distal stump of the nerve resemble those in the proximal stump as they adopt the glial cell characteristics of the nerve which reinnervates them (Aguayo *et al.*, 1976a Aguayo *et al.*, 1976b). If a proximal myelinated nerve is anastomosed to a distal unmyelinated stump, then as the proximal nerve grows into the distal stump, the Schwann cells of the distal stump will

be stimulated to myelinate the ingrowing fibres (Aguayo *et al.*, 1976a Aguayo *et al.*, 1976b; Simpson and Young, 1945; Weinberg and Spencer, 1976). If a graft from the cervico-sympathetic trunk is placed as a midsection in the sural nerve, then regeneration of the proximal sural, myelinated fibres into the unmyelinated cervico-sympathetic trunk segment will occur and this segment becomes partially myelinated (Aguayo *et al.*, 1976a). However, non-myelinated fibres that grew into a distal myelinated stump did not become myelinated (Simpson and Young, 1945). This fact adds further proof that the proximal stump controls the status of the distal stump and not vice versa.

The origin of the Schwann cells was investigated to ascertain whether they were of local origin and thus changing the nature of their envelopment of the nerve, or whether they had migrated in with the regenerating axons. The myelinating Schwann cells were found to be locally derived and not migrating in with the ingrowing nerve (Aguayo *et al.*, 1976b; Weinberg and Spencer, 1976). Some labelled Schwann cells from the proximal stump were observed in the region of the anastomosis, but, distal to that, it appeared that regenerating axons became associated with local Schwann cells of the distal stump (Weinberg and Spencer, 1976).

Schwann cell proliferation occurs in the distal stump prior to invasion by the regenerating axons from the proximal stump (Aguayo *et al.*, 1976b). Schwann cells may re-express their developmental multipotentiality after dividing in response to injury (Aguayo, 1976a) and thus undertake a new form or association with the axon. Hence, it would seem that the myelinated type of axon is able to trigger myelin production by Schwann cells, whereas this trigger is absent in unmyelinated axons. The myelinating signal may involve molecule(s) present on the surface of the axon or it may be a diffusible signal. The latter idea is probably less valid as both myelinated and unmyelinated axons are intimately associated with each other in a mixed peripheral nerve (Brockes *et al.*, 1981). Schwann cells do not express myelin-specific molecules or their mRNAs in advance of regenerating axons. This also suggests that the axons do not release a diffusable factor acting over centimetre distances and stimulating myelin component expression by Schwann cells. It instead points to an axonally controlled stimulation of Schwann cells that requires cell-to-cell contact (Mitchell *et al.*, 1990; Politis *et al.*, 1982).

The interaction between axon and sheath cell has also been widely studied on a molecular level. Induction of myelin-specific molecule expression is very specific and is not induced by certain types of axons, (q.v.) nor if the Schwann cells surround tissue other than axons. Schwann cells that were satellite cells to trigeminal neurons

expressed P_0 mRNA at decreasing levels up to 15 days postnatal (Lamperth *et al.*, 1989) but were negative for both P_0 and MBP mRNAs in early adulthood (Lamperth *et al.*, 1989; Trapp *et al.*, 1987).

Axotomy and withdrawal of axonal contact has important ramifications on the expression of various molecules in the distal stump. The effect of the axon on P_0, MBP and NGF receptor is expressed at the level of the mRNA and has either a positive or negative effect on these molecules (Lemke and Chao, 1988b). The concentration of P_0 protein expressed in the distal stump of peripheral nerve after crush injury decreased and then increased as a function of time after crush. This corresponded to the loss of myelin induced by axonal degeneration and subsequent myelination after axonal regeneration (Poduslo, 1984a). The expression of the mRNAs encoding for both P_0 and MBP is reduced dramatically in the distal stump of sciatic nerve within the first week after transection (Gupta *et al.*, 1988; Trapp *et al.*, 1988b). In fact, the down-regulation of these two mRNAs occured within 24 hours of crush injury indicating the sensitive nature of the interdependence of the Schwann cell and axon (Gupta *et al.*, 1989). This down-regulation of P_0 and MBP message was confirmed at 35 days post-transection by northern and dot blots (Le Blanc *et al.*, 1987). *In vitro* translation experiments indicated that the decrease in the mRNAs was not due to poor quality of the transcripts (Gupta *et al.*, 1988). The perinuclear distribution of P_0 message in the Schwann cell was not changed by transection, but the intensity of it, and the MBP message, declined so dramatically that by 5 days, these mRNAs were difficult to detect. Therefore, axons appear to regulate Schwann cells' expression of P_0 and MBP protein at the transcriptional level (Trapp *et al.*, 1988b).

After reinnervation subsequent to crush injury, the P_0 and MBP mRNAs increased with peak elevation at 10 to 14 days and a return to adult control levels by 3 weeks. The level of P_0 protein followed a similar trend to the mRNA but with a temporal lag of about one week. The decreases in these two transcripts occurred during the degenerative phase and the subsequent increases during the remyelinating phase of regeneration (Gupta *et al*, 1988). At 35 days post-nerve crush, axonal regeneration into the distal stump was well advanced and this was reflected in P_0 mRNA levels which were similar to normal nerve (Le Blanc *et al.*, 1987). Within 2 days of nerve crush, the level of MAG mRNA had decreased to 22% of the control nerve. It returned to near normal levels in the distal portion of crushed nerve by 3 weeks, but in permanently transected nerve, no increase was observed. The MAG protein level followed a similar trend but was temporally delayed by 7 days (Gupta *et al*, 1989).

It has been suggested that Schwann cells in the distal stump of a transected peripheral nerve become functionally naive and do not synthesise myelin-specific proteins in the absence of axonal contact (Politis *et al.*, 1982). However, it appears that synthesis of P_0 protein at least, occurs in this situation, but just at very low levels. This suggests that Schwann cells *in vivo* are still able to express myelin components in the absence of axonal contact (Poduslo *et al.*, 1985a).

Axonal contact also effects expression of molecules other than myelin-specific ones. Galactocerebroside expression is lost in axotomized sciatic nerve, although the rate of loss is slower from myelin-forming compared with non-myelin forming Schwann cells. It was present up to 9 weeks after denervation in the former, but not found after 3 weeks in the latter. Similarly, loss of axonal contact after crushing or transection of rat cervico-sympathetic trunk leads to loss of GalC expression in these non-myelin-forming Schwann cells, whilst axonal regeneration into the distal stump was accompanied by re-expression of GalC (Jessen *et al.*, 1987c). But as GalC appears on both myelin-forming and non-myelin-forming cells, the axonal signal required for its expression is not the same as the myelinating signal (Jessen *et al*, 1985). N-CAM and L1 expression tapers off on the myelin-forming Schwann cells in the developing sciatic nerve as myelination proceeds, but is quickly re-expressed after denervation and loss of axonal contact (Jessen *et al.*, 1987b; Nieke and Schachner, 1985). Likewise, in contrast to the myelin-specific molecules, NGF receptor and NGF expression is upregulated by loss of axonal contact (Heumann *et al.*, 1987b; Lemke and Chao, 1988b; Raivich and Kreutzberg, 1987; Taniuchi *et al.*, 1988). With myelination after re-innervation the expression of these molecules declined (Heumann *et al.*, 1987b; Nieke and Schachner, 1985; Raivich and Kreutzberg, 1987; Taniuchi *et al.*, 1988). The synthesis of these molecules in myelin-forming Schwann cells appears to be suppressed by ongoing axonal signals.

Similarly, the O series of antigens seems to rely on axonal contact for their expression. After nerve transection O4 expression was markedly down-regulated in the distal stump, but was upregulated with reinnervation after nerve crush (Mirsky *et al.*, 1990).

1.5.3.3 *In vitro* studies

(See also section 1.3.2.3)

Components of the myelin sheath

In vitro, loss of myelin component expression by Schwann cells may be observed in cells enzymatically dissociated from neonatal rat sciatic nerve. This dissociation causes loss of Schwann cell-axon contact which results in cessation of myelin membrane synthesis and down-regulation of expression of myelin components such as P_0, MBP and GalC, within 5 days of culture (Brockes *et al.*, 1981; Jessen *et al.*, 1985; Lemke and Chao, 1988b; Mirsky *et al.*, 1980; Morrison *et al.*, 1991; Owens and Bunge, 1989; Winter *et al.*, 1982). Loss of GalC with time *in vitro* is also observed in cells dissociated from the cervico-sympathetic trunk (Jessen *et al.*, 1985). The expression of MAG also relies on axonal contact *in vitro* (Owens and Bunge, 1989).

The loss of myelin protein expression is a reflection of the down-regulation of the mRNAs encoding for them. By 5 days *in vitro*, dissociated Schwann cells express the P_0 and MBP mRNAs at a level approximately 40 fold lower than that of cells in the 2 day postnatal sciatic nerve. This basal level of expression was maintained for greater than 4 months.

The effect of the axons on these two proteins could be partially mimicked by the addition of the drug, forskolin, which raises the intracellular level of cyclic-AMP. P_0 mRNA was induced at a much lower level than MBP, but neither mRNA was expressed at its full level in response to forskolin. Cholera toxin, which also raises intracellular cyclic-AMP, had a similar effect. However, a tumour promotor which activates protein kinase C, had no effect (Lemke and Chao, 1988b). Similarly, treatment of neonatal dissociated Schwann cells with 8-bromo cyclic-AMP or dibutryl cyclic-AMP induced synthesis and expression of GalC on them. Without treatment, these cells were negative for surface GalC by 4 days *in vitro*. Only a maximum of 48% of these cells were positively induced and this required supraphysiologic doses of the drugs (Sobue and Pleasure, 1984). These data suggest that cyclic-AMP may have a role as a second messenger in the transduction of the myelination signal of the axon, within the Schwann cell (Lemke and Chao, 1988; Sobue and Pleasure, 1984). Both AOO7 and O4 expression on Schwann cells is lost almost entirely within 8 days when

cells are dissociated from the axons for culture. Again, cyclic-AMP may have a role in the transduction of this interaction as cholera toxin and forskolin can induce upregulation of both molecules in dissociated cultures of embryonic and neonatal Schwann cells (Mirsky *et al.*, 1990).

The effect of losing axonal contact on sulphatide synthesis was not found to be quite so well defined. Using thin layer chromatography and labelled sulphur, it was found that sulphatide continued to be synthesised by neonatal dissociated Schwann cells which had been cultured in the absence of axonal contact for greater than 2 weeks (Fryxell, 1980). The techniques used in Fryxell's study were apparently more sensitive than immunofluorescence as dissociated Schwann cells were negative for sulphatide by immunofluorescence by 4 days *in vitro* (Mirsky *et al.*, 1980).

New data suggests that, in fact, the expression of many of these apparently axonally dependent molecules is not completely lost after deprivation of axonal contact. Studies performed by Rutkowski and colleagues (1990) on cholera toxin expanded cultures have identified the persistence of expression of P_0 and MBP and the lipids GalC and sulphatide. However, a marked reduction in staining intensity was noted in comparison with primary cultures and detection of the lipids required more sensitive techniques. The effect of the cholera toxin was not thought responsible for the continued presence of these molecules. It was concluded that these cultured Schwann cells did not dedifferentiate in culture, but just down-regulated these components and continued to produce and insert them in their plasma membrane (Rutkowski *et al.*, 1990).

It is unlikely that the low level of expression of these components is due to just the small surface area of the Schwann cell membrane as work by Morrison *et al.*, (1991) has identified high levels of P_0 mRNA expression in Schwann cells cocultured with neurons under non-myelinating conditions. Under those conditions, Schwann cells barely make contact with the axons and have not undergone massive membrane expansion characteristic of the myelin sheath (Morrison *et al.*, 1991).

Cultured sciatic nerve explants have been shown to incorporate radiolabelled sulphate and methionine into P_0 protein in the absence of axonal contact (Halligan, 1985). Recently, using endoneurial explants it was also shown that Schwann cells isolated from axonal contact in young rat pups do synthesize P_0 protein, but this protein is rapidly catabolized and its appearance may be missed (Brunden *et al.*, 1990b). The situation in mice may be slightly different to that in rats as dissociated,

cultured neonatal mouse Schwann cells do continue to express basal levels of P_0, P_1 and P_2 for up to 21 days in culture. This was detected by immunostaining and radiolabel incorporation. The conclusion of this study was that the interdependence of the Schwann cell on the axon was "relaxed" in mice (Burroni *et al.*, 1988).

If axon-Schwann cell contact is maintained or re-established (by coculturing the Schwann cells with spinal ganglia neurites) then Schwann cells express myelin componenents (Brockes *et al.*, 1981; Brunden *et al.*, 1990a, Morrison *et al.*, 1991). Only limited contact appears to be required for induction of P_0 mRNA expression (Morrison *et al.*, 1991).

Axonal contact of Schwann cells has other effects also. Ratner and coworkers (1987) noted that Schwann cells react with changes both in their morphology and behaviour when contacted by growing neurites. A colony of pure spinal ganglia cells were established and transplanted into a dish containing Schwann cells which were located at a distance. Neurites grew out from the spinal ganglia and approached the Schwann cells which were dividing at a minimal rate ($<0.2\%$). Within 5 hours of contact being established, the Schwann cells began to realign on the ingrowing neurites; significant alteration in Schwann cell orientation was observed between 5 and 13 hours, and Schwann cell division began at 14 hours after contact (Ratner *et al.*, 1987). The effect of axons on Schwann cell division was discussed in section 1.3.1.4.

In contrast to the proteins which are upregulated by contact with myelin inducing axons, there are a set of Schwann cell marker molecules which are down-regulated in their expression by contact with such an axon. These surface proteins included N-CAM, Ran-1/217c/NGF receptor, GFAP and A5E3. They were quickly manifested after loss of axonal contact without requiring cell division. These molecules are not suppressed in non-myelin-forming Schwann cells as they mature. Their suppression by myelin inducing axons is important in the induction of the myelinating phenotype which is P_0, MBP and MAG positive (Jessen *et al.*, 1990).

The role of the axon in dictating molecule expression by the Schwann cell is thus well documented. However, the assembly of the myelin sheath appears to be a different matter. It is interesting to note that cocultures of Schwann cells and spinal ganglia in defined medium will express both the P_0 mRNA and protein (Brunden *et al.*, 1990a; Brunden and Brown, 1990c; Morrison *et al.*, 1991). So whilst the

expression of the P_0 gene is dependent upon contact with the axon, the assembly of the myelin sheath requires the presence of basement membrane. The basement membrane may also have a role in amplification of production of myelin specific molecules (Brunden *et al.*, 1990a; Brunden and Brown, 1990c). Therefore, the control of myelin sheath formation does not rest entirely upon the axon, but is also influenced by other factors such as assembly of the basement membrane.

1.0 LITERATURE REVIEW

1.6 SCHWANN CELL GENE EXPRESSION

1.6.1 Myelin associated genes

The conditions under which genes encoding myelin-specific molecules are expressed have been discussed (see sections 1.3.2.3 and 1.5.3.). The intensity of expression of the mRNAs encoding P_0, PLP and MBP was greatest during the most active period of myelination, either developmental or during nerve regeneration (Gupta *et al.*, 1988; Gupta *et al.*, 1989; Trapp *et al.*, 1987). Myelination begins perinatally in the PNS of rodents, peaks at approximately 14 to 16 days and then drops to low levels (Webster, 1971).

1.6.1.1 P_0

It has been proposed that the control of P_0 gene expression is at the transcriptional/translational level in neonates, compared with at the post-translational level in adults (Poduslo and Windebank, 1985b). This is supported by the work of Morrison and colleagues (1991) which demonstrates that P_0 mRNA may be present in significant amounts in the absence of the protein.

Analysis of the gene encoding P_0 has identified that the gene is split into six exons, the segregation of which is consistent with its three domains. The 5' untranslated mRNA seqence and the majority of the amino-terminal signal sequence of the protein is encoded by exon I. Exons II and III encode the extracellular domain; exon IV the transmembrane section of the protein and exons V and VI the cytoplasmic domain and the 3' untranslated region (Lemke and Axel, 1985; Lemke *et al.*, 1988c). The gene is transcribed as a single 1.9 kb mRNA species (Gupta *et al,* 1988; Lemke and Axel, 1985). A 1.85 kb cDNA has been cloned from this RNA (Lemke and Axel, 1985).

By light and electron microscopy, P_0 mRNA was observed on cisternae of many rough endoplasmic reticulum distributed throughout the internodal and paranodal cytoplasm in the earliest stages of myelination before compact myelin lamellae had formed. The location of the mRNA over a diffuse area of the abaxonal mesaxon suggested that there are many sites of synthesis and entry of the protein into the developing myelin sheath. The paucity of P_0 mRNA along the inner mesaxon and the Schmidt-Lanterman suggested that P_0 protein synthesis was much lower in these areas. It is possible that at this stage some P_0 protein is incorporated directly into the

sheath without post-translational processing. At later stages after peak myelination the rER is restricted to the perinuclear region and it is unlikely that the protein is directly incorporated into the sheath without further processing through the Golgi apparatus (Lamperth *et al.*, 1990). Peak P_0 mRNA expression was found to occur at 3 weeks postnatally, which is relatively late in the temporal course of PNS myelination. This may reflect less efficient translation of this gene in the later aspects of myelination (Stahl *et al.*, 1990). The number of cells expressing P_0 mRNA, and intensity of cell expression, declines with development and maturation of the myelin sheath (Lamperth *et al.*, 1990; Stahl *et al.*, 1990). The mRNA also became more perinuclear in distribution (Griffiths *et al.*, 1989; Lamperth *et al.*, 1990; Trapp *et al.*, 1987; Trapp *et al.*, 1988b). The density of the message appears to be directly proportional to nerve fibre size (Griffiths *et al.*, 1989; Griffiths *et al.*, 1991). This proportionality may be based on the axolemmal surface area and abundance of the putative axolemmal associated myelinating signal with which the Schwann cell may interact. Hence larger diameter fibres would be associated with increased expression of P_0 mRNA. The intensity of hybridized labelled P_0 mRNA probe is not uniform in the perinuclear cytoplasm but is more dense on one side than the other (Griffiths *et al.*, 1991).

P_0 cDNA probes have identified the presence of a minor 4 kb band in some Schwann cell-neuron cocultures. This may represent a species of P_0 mRNA expressed during the early stages of myelination whilst the 1.9 kb species is predominant in the adult animal (Morrison *et al.*, 1991)

1.6.1.2 MBP

The size of the MBP transcript is 2.1 kb (Gupta *et al.*, 1988). The mRNA for MBP was found to be more concentrated at the perinuclear and paranodal areas in teased fibre preparations with a relatively low intensity signal along the internode (Griffiths *et al.*, 1989). This supported previous studies which also identified a diffuse distribution of the mRNA during active myelination of the CNS. It is thought that the mRNA is transported to sites near the myelin sheath for translation (Trapp *et al.*, 1987).

1.6.1.3 MAG

The MAG mRNA transcript from rat sciatic nerve is 2.5 kb, however from 21 to 23 day old rat brains the transcript is 3.0 kb (Gupta *et al.*, 1989). Alternative splicing of the MAG gene yields the two protein isoforms (reviewed by Salzer and Colman, 1989). The peak level of MAG mRNA expression occurred one week before that of the mRNAs encoding MBP and P_0 which would be consistent with its earlier

role in myelination (Stahl *et al.*, 1990). The mRNA for MAG was found to be located in the perinuclear area of the Schwann cell in teased fibre preparations, but appeared diffuse on paraffin sections. It was not observed in the paranodal region on teased fibres (Griffiths *et al.*, 1989).

1.6.1.4 PLP

By *in situ* hybridization methodology, PLP mRNA was observed in the perinuclear area of the Schwann cell (Griffiths *et al.*, 1989; Trapp *et al.*, 1987).

1.6.2 Control of myelin gene expression

The prime determinant of myelin gene expression is contact with an appropriate axon that calls for myelination. This axonal dependence is reflected in the initial induction of these genes, the steady state levels them, and pathologically after axotomy and during regeneration (see section 1.5.3.2) (Gupta *et al.*, 1988; Gupta *et al.*, 1989; Le Blanc *et al.*, 1987; Lemke, 1988a; Lemke and Chao, 1988b; Trapp *et al.*, 1988b). The axonal dependence of the mRNAs was also noted in Schwann cells dissociated from axonal contact and has been discussed in the a previous section (section 1.5.3.3). The level at which these genes is manifested is an indication of the functional activity of the Schwann cell with respect to myelination (Gupta *et al.*, 1988). The control of P_0, at least, is likely to be at the transcriptional level (Gupta *et al.*, 1988; Le Blanc *et al.*, 1987; Poduslo and Windebank, 1985b). Some post-translational regulation of any transcribed mRNA may also occur (Le Blanc *et al.*, 1987; Poduslo and Windebank, 1985b). As the level of MBP message follows that of the P_0 message, co-regulation of these two genes is likely (Le Blanc *et al.*, 1987; Mitchell *et al.*, 1990).

MAG mRNA regulation is likewise thought to be at the transcriptional level, but some post-transcriptional processing may also occur. However, after nerve crush, this mRNA is detected before P_0 and MBP suggesting that it is not under the same control as the latter two genes (Gupta *et al.*, 1989). Whilst SV-40 transformed Schwann cells do not express P_0 in culture despite expressing a significant amount of P_0 mRNA they do synthesise MAG and CNPase. These data also supports the view of different regulatory mechanisms (Chen *et al.*, 1987).

The role of a second messenger system in the regulation of these genes is possible as both MBP and P_0 genes were induced in cultured Schwann cells by agents that raise intracellular cyclic-AMP concentrations (Lemke, 1988a, Lemke and Chao,

1988b). However, the action of a second messenger on the P_0 promoter may not be direct, but may be transduced via yet another molecule. SCIP (suppressed cyclic-AMP inducible POU) mRNA is one such possible mediator. It is primarily expressed in developing brain and peripheral nerve, but is only found at very low levels in non-myelin forming Schwann cells. After stimulation of Schwann cells by cyclic-AMP, peak SCIP expression occurs before peak myelin gene expression. Thus, the possible cascade of events that occur during axonal stimulation of Schwann cell myelin-specific molecule expression may be that the axonal myelinating signal interacts with the Schwann cell plasmalemma and induces an intermediary molecule such as SCIP. This causes induction of cyclic-AMP which stimulates the P_0 promotor and causes up-regulation of P_0 gene transcription (Monuki *et al.*, 1989).

CHAPTER 2: MATERIALS AND METHODS

The exact processing schedules and the compositions of various buffers and solutions are cross-referenced and given in the appendices. Cross-references appear as (A_._). Distilled water was also deionized.

2.1 TISSUE CULTURE

All tissue culture media and reagents, other than those specified, were obtained from either Flow laboratories or Sigma.

2.1.1 Dissociated neonatal Schwann cells

The method used for producing dissociated Schwann cells from neonatal sciatic nerve of either rat or mouse was modified from that devised by Brockes *et al.*, (1979).

Neonatal animals ranging in age from 2 to 9 days were killed by an overdose of halothane anaesthetic and decapitated. The heads were immediately frozen as a tissue source from which axolemmal-enriched fractions were produced (see chapter 7). The animals were placed in sternal recumbency, washed with 70% ethanol in distilled water and the sciatic nerves exposed. These were removed bilaterally from the level of the greater sciatic notch to just distal to the stifle and placed in L15 medium at 37^{o}C. In a laminar flow hood, the nerves were minced using two scalpel blades in scissor like fashion and placed in 1.5 ml of L15 to which was added 2 x 20 minute, 60 μl pulses of 0.1% collagenase. This was incubated at 37^{o}C. After the 40 minute collagenase incubation, 2 x 10 minute, pulses of 0.25% trypsin were added. The first pulse was 1000 μl and the second was 500 μl. Both trypsin and collagenase were diluted in L15 medium. Incubation was at 37^{o}C after which 1 ml of SD solution (A1.1) was added to stop the enzymatic activity and prevent tissue clumping. The suspension was triturated 6 times through a 21G needle, 4 times through a 23G needles and transferred to a centrifuge vial. It was adjusted to 15 mls with Schwann cell growth medium (Dulbecco's modification of Eagle's medium, 7.5% foetal calf serum and 1 % glutamine) and centrifuged at 1200 rpm on Wifug bench centrifuge for 10 minutes. The pellet was resuspended in sufficient SCGM to obtain a cell concentration of 10-20,000/50 μl and 50 μl of the suspension was plated out on 13

mm coverslips. These sterile coverslips had been immersed in 100 μg/ml poly-L lysine/SDW to encourage cell adhesion (Mc Keehan and Ham, 1976), washed three times in water and allowed to air dry in the wells of the culture plate. The cells were plated out and and fed 30 minutes later with 500 μl of SCGM. Cells were fed twice weekly with SCGM. In some instances, 0.01 mM cytosine arabinoside was added 24 hours after plating and removed on the 4th day after plating to control fibroblastic proliferation.

2.1.2 Preparation of rat tail collagen

Type I collagen obtained from tails of Sprague-Dawley rats was used as a substrate for Schwann cell culture (Masurovsky and Peterson, 1983). All procedures were performed under aseptic conditions in a laminar flow hood. Tails were soaked in 70% ethanol for 30 minutes, broken with rongeurs and the long collagenous strands pulled out of them. The fibres were broken down by teasing using two pairs of forceps for 10 minutes, and placed in 300 ml of 0.1% glacial acetic acid at 4^{o}C for 2 days during which time the fibres dissolved. Centrifugation at 16000 rpm on a Beckman J2-21 M/E centrifuge for 2 hours at 4^{o}C resulted in a clear, viscous supernatant which was aliquoted and stored at 4^{o}C until required. The collagen was tested for sterility at this point by placing a 1 ml aliquot in 5 mls of tissue culture medium and incubating at 37^{o}C for 7 days.

Prior to use, the collagen was dialysed against large volumes of SDW for 48-72 hours at 4^{o}C to neutralize the pH. Sterility was again checked by a 7 day incubation at 37^{o}C. The gelling agent was 0.012% riboflavin. The prepared collagen was stored in light tight bijoux at 4^{o}C and, when used, gelled within several minutes of exposure to light.

2.2 ANIMAL STUDIES

2.2.1 Perfusion fixation

Both rats and mice were killed by an overdose of halothane anaesthetic and immediately after death were pinned out in dorsal recumbency. The ventral wall of the thorax was removed and perfusion was initiated through the left side of the heart after a small cut had been made in the right atrium. The initial perfusate used was 0.85% NaCl until the most of the blood was flushed from the body, when it was

changed to 4% paraformaldehyde with either 0.1% (A2.3) or 2.5% glutaraldehyde (A2.2) in 0.1 M phosphate buffer (A2.1.1). These two fixatives were used for tissues destined for light and electron microscopy respectively. After one hour at 4°C, the required tissue was removed and stored in the appropriate fixative.

2.3 MORPHOLOGIC STUDIES

2.3.1 Light microscopy

2.3.1.1 Immunofluorescence

Immunofluorescence was performed both on cultured cells and teased nerve fibres. To stain for cell surface markers unfixed tissue was used and tissue was fixed at -20°C for 15 minutes with 5% glacial acetic acid in cold ethanol after immunostaining. For cytoplasmic markers, the cells were permeabilized by fixing with 4% paraformaldehyde in phosphate buffered saline (A2.1.1) prior to immunostaining.

The basic methodology for immunostaining of either surface or cytoplasmic markers was the same. The cells or teased fibres were washed in a balanced salt solution such as 0.1 M phosphate buffered saline (A2.1.1) or Hank's balanced salt solution and the primary antibody added. In initial studies prior to addition of the primary antibody, non-specific staining in the samples was blocked by a 2 minute incubation of the samples in undiluted serum, or in 10% serum/balanced salt solution. However, this seemed to be associated with increased non-specific staining, and so this step was subsequently deleted. Pre-fixed samples were incubated overnight with the primary antibody at 4°C whereas unfixed samples were only incubated for an hour with the primary antibody at room temperature. For both tissue types, the samples were then washed three times briefly with the balanced salt solution and the secondary antibody added for one hour at room temperature. Unfixed tissue was fixed at this stage (q.v.). The samples were washed twice briefly with the salt solution and once with distilled deionised water and mounted in PPD mountant (A3.1.3). Examination was performed under UV light. The concentrations at which antibodies were used is given in table 2.1. A balanced salt solution was used for dilutions.

Nuclei were counter-stained with propidium iodide (Jones and Kniss, 1987) which fluoresces under UV light. A stock solution of 10 mg propidium iodide was diluted in 2 mls of PBS and this was diluted 1 in 250 in PBS for use. Fixed tissue was

PRIMARY ANTIBODY	DILUTION	SOURCE	SECONDARY ANTIBODY	DILUTION	SOURCE
S100	1:900	Dakopatts	GAR-FITC	1:130	Sigma
O4	1:10	Dr. I. Sommer (Southern General Hospital, Glasgow)	GAM IgM-FITC or TRITC	1:200	Dr. I. Sommer
H8 (GalC)	1:5	Dr. I. Sommer	GAM IgG-FITC	1:200	Dr. I. Sommer
MBP	1:500	Dr. J-M. Matthieu (Lausanne Switzerland)	GAR-FITC GAR-TRITC	1:130 1:200	Sigma Sigma
Laminin	1:800 for cells, 1:300 for teased fibres	Sigma	GAR-FITC GAR-TRITC	1:130 1:200	Sigma Sigma
N-CAM	1:100	Sigma	GAM IgG_1-FITC	1:100	Southern Biotechnology
GFAP	1:750	Dakopatts	GAR-FITC	1:130	Sigma
NGF receptor	1:40	Boehringer	GAM-IgG_1-FITC	1:100	Southern Biotechnology

Table 2.1: **Antibodies, their dilutions, and sources used in immunofluorescence studies.** The Sigma antibodies, raised in goats, were the IgG fraction. G = goat, M = mouse, R = rabbit, F = fluorescein, TR = rhodamine

stained for 20 minutes at room temperature in a light tight box and washed briefly in tap water. The samples were briefly dehydrated through 2 changes of alcohol and xylene and mounted in DPX mountant, or wet mounted in 90% glycerol/PBS.

2.3.1.2 Peroxidase-antiperoxidase (PAP)

Peroxidase-antiperoxidase staining was performed on paraffin-embedded sections (A3.1.1). This methodology is based on the binding of a primary antibody to the test antigen in the sample. A second antibody, raised against the species in which the primary antibody was produced, was added in excess and bound to the primary antibody by one of its Fab arms. The other free arm bound to a peroxidase-antiperoxidase complex raised in the same species as the primary antibody. Thus the secondary, or "link" antibody, bound to both the primary and the enzyme-antibody complex. 3'-diaminobenzidine tetrahydrochloride (DAB) bound to the enzyme and in the presence of hydrogen peroxide was oxidized to a brown insoluble stain visible by light microscopy (Boenisch, 1989). Endogenous peroxidase activity was first quenched by the incubation of the slide with H_2O_2 prior to the addition of the primary antibody. Non-specific binding was blocked by the presence of normal goat serum in low concentrations throughout the procedure. The exact protocol is given in A3.2.

The negative controls used non-immune mouse and rabbit sera in lieu of primary antibodies. Table 2.2 identifies the antibodies that were used and their dilutions.

2.3.2.2 Electron microscopy

Blocks of tissue were subject to a routine processing schedule (A3.3) for embedding in araldite resin. Sections for light microscopy were cut at 1 μm on a Reichardt-Jung FC4E Ultracut E cryotome and stained with methylene blue/azure II (A3.1.2). Thin sections for electron microscopy were cut at 70 nm and placed on 200 mesh copper grids. In some instances grids were precoated with parlodion for support (A3.3). Grids were examined in an AEI (Associated Electrical Industry) EM6B electron microscope.

PRIMARY ANTIBODY	DILUTION	SOURCE	PAP COMPLEX	SOURCE	LINK
Actin	1:750	Amersham	mouse	ICN	goat-anti-mouse
RT97	1:10,000	Wood and Anderton, (1981)	mouse	ICN	goat-anti-mouse
P_0	1:750	Mr. D. Kirkham (University of Glasgow)	rabbit	ICN	goat-anti-rabbit
S100	1:500	Dakopatts	rabbit	ICN	goat-anti-rabbit
beta-tubulin	1:1,500	Amersham or Sigma	mouse	ICN	goat-anti-mouse
NGF receptor	1:40	Boehringer	mouse	ICN	goat-anti mouse

Table 2.2: Antibodies, their dilutions and sources, used for immunostaining by the PAP technique. ICN = ICN immunobiologicals

2.4 MOLECULAR BIOLOGY TECHNIQUES

2.4.1 Isolation of nucleic acid from tissue samples

2.4.1.1 Genomic DNA isolation

Genomic DNA was prepared from liver tissue by a method modified from Blin and Stafford (1976). The tissue was frozen in liquid nitrogen immediately and ground in a mortar and pestle. Homogenization was performed in a glass hand-held homogenizer in a solution of 0.01 M EDTA, 0.01 M Tris-HCl (pH 8.0), 0.01 M NaCl, 4% N-lauryl sarcosine (w/v), proteinase K (100 μg/ml) was added after homogenization and incubated overnight at 37^{o}C.

After incubation, an equal volume of phenol/chloroform in 0.01 M EDTA, 0.5 M Tris-HCl (pH 8.0), 0.01 M NaCl and 0.5% SDS was added and mixed gently for 30 minutes at 50^{o}C. The solution was centrifuged for 10 minutes on a Beckman J2-21 M/E centrifuge, 1000 rpm, at room temperature, and the aqueous phase containing the DNA was precipitated using 0.3 M sodium acetate and 2.5 x volume of ethanol for 30 minutes. The precipitated DNA was spooled out of solution using a sterile glass rod. It was washed twice by immersion for 1 minute in 75% ethanol, and 95% ethanol and then once in chloroform. The DNA was air-dried and resuspended overnight in 10 ml of 0.1 x SSC (A2.1.3). Contaminating RNA was removed by RNase treatment with boiled RNase A (50 μg/ml) and RNase t (1 μg/ml) in 0.01 M EDTA, 4% sarcosine, 50 μg/ml proteinase K for 3 hours at 37^{o}C. The DNA was extracted with phenol/chloroform, precipitated and washed as previously described. The DNA was resuspended in Tris-HCl (pH 8.0) and dialysed against large volumes of T/E buffer (A4.2.4) over 2 days. Samples were diluted 1:100 and the DNA concentration calculated from the OD values at 260 and 280 nm. 10.0 μg samples were digested with Bam HI, Eco RI, Pst I and Hin dIII restriction enzymes overnight at 37^{o}C. The digested DNA was electrophoresed through a 1% agarose gel (A4.2.3) at 140V for 4.5 hours. It was blotted onto nitrocellulose paper (A4.2.6) and probed with a [^{32}P] cDNA fragment (2.4.5.1).

2.4.1.2 RNA extraction

The isolation of RNA from both tissue and cellular samples was performed using RNAzol B$^{(R)}$ (Biotecx Laboratories, Inc.) and following the manufacturer's instructions. This technique is a modification of the single step procedure of Chomczynski and Sacchi (1987). This method promotes formation of RNA complexes with guanidium and water molecules and renders the DNA and protein hydrophobic. Thus the RNA remains in the aqueous phase and the rest of the cellular debris remains in the organic solvents.

Briefly, 800 μl of RNAzol was added to the cells from 8 x 13 mm coverslips or 1.0 ml was used for 50 mg of nerve tissue. Cultured cells formed monolayers covering between 1/3 to 3/4 of the area of the coverslip. A 1/10 x volume of chloroform was added to the solubilized material, which was vortexed for 15 seconds, incubated on ice for 5 minutes and microfuged at 13000 rpm, 4^{o}C for 15 minutes. The supernatant was harvested and an equal volume of isopropanol added. The RNA was precipitated by incubating the sample on ice for 15 minutes and pelleted by microfuging at 13000 rpm, 4^{o}C for 15 minutes. The pellet was washed in 75% ethanol/DEPC treated SDW and dried under vacuum. Samples were resuspended in 800 μl DEPC treated SDW and their quality and concentration were determined spectrophotometrically at OD 260 and 280 nm. The ratio of 260/280 was ideally greater than 1.8 indicating minimal contamination of the RNA with phenol or aromatic amino acids. Samples with a ratio of < 1.7 were reprecipitated to increase the purity of the RNA. All samples were reprecipitated with 1/10 x volume of 3.0 M sodium acetate and 2 x volume of cold ethanol overnight at -20^{o}C. The pellet was washed in 75% ethanol/DEPC treated SDW, vacuum-dried and resuspended in DEPC treated SDW at the required concentration.

2.4.2 Preparation of DNA

2.4.2.1 S100 DNA

Microbiological techniques

Microbiological techniques were performed as described in (Sambrook *et al.*, 1989).

L-broth culture medium

1.0 l of L-broth contained 10.0 g bactotryptone, 10.0 g NaCl, 5.0 g yeast

extract and was buffered to pH 7.5 with 1.0 M NaOH before being autoclaved. Prior to use for culturing, ampicillin was added to a final concentration of 100 μg/ml.

L-Agar culture plates

To prepare 1.2% agar plates, 6.0 g of bacteriological agar powder (Oxoid Ltd) was added to 500 ml of L-broth and autoclaved. After cooling to 55^{o}C, ampicillin was added (100 μg/ml) and the plates were poured.

Propagation and maintenance of bacterial cultures

For short term storage and ready usage, bacteria were streaked out on L-agar culture plates containing 100 μg/ml ampicillin, cultured at 37^{o}C overnight and stored at 4^{o}C in an inverted position. For long term storage 200 μl of an overnight culture in L-broth, containing ampicillin (100 μg/ml), was added to 800 μl of sterile glycerol and stored at -70^{o}C.

Bacterial cell transformation

The method of bacterial cell transformation employed was modified from Hanahan (1983). S100 cDNA which encodes the Schwann cell marker, S100, was kindly supplied by Professor Marks (University of Toronto) as a 264 base pair insert in the plasmid pBP2 (see fig 2.1). To amplify the DNA the plasmid was introduced into the competent E.coli strain JM103 kindly supplied by Dr. Paul Montague (University of Glasgow).

100 μl of competent cells in 100 mM $CaCl_2$/15% glycerol (OD_{600} 0.3-0.4) were used for each transformation. The DNA was diluted with SDW to produce concentrations of 0.01, 0.1, 1.0, 10 ng/ml and one negative control was also included which contained no DNA. 100 μl of bacterial cell suspension was added to each concentration of DNA and suspension was incubated on ice for 30 minutes with gentle tapping of the tube to mix the contents every 10 minutes. The cells were then heat shocked (42^{o}C for 45 seconds) and placed on ice. The bacteria were transferred to a bijou containing 900 μl of L-broth and incubated on a roller mixer for an hour (cell doubling time was approximately 20 minutes). 200 μl aliquots were plated onto bacteriological L-agar plates containing ampicillin (100 μg/ml) and cultured overnight, in an inverted position, at 37^{o}C. The transformation efficiency was approximately 2 x 10^6 colonies/μg DNA. Single colonies were transferred using a sterile toothpick to a bijoux containing 2 ml of L-broth contining ampicillin (100 μg/ml) and grown overnight.

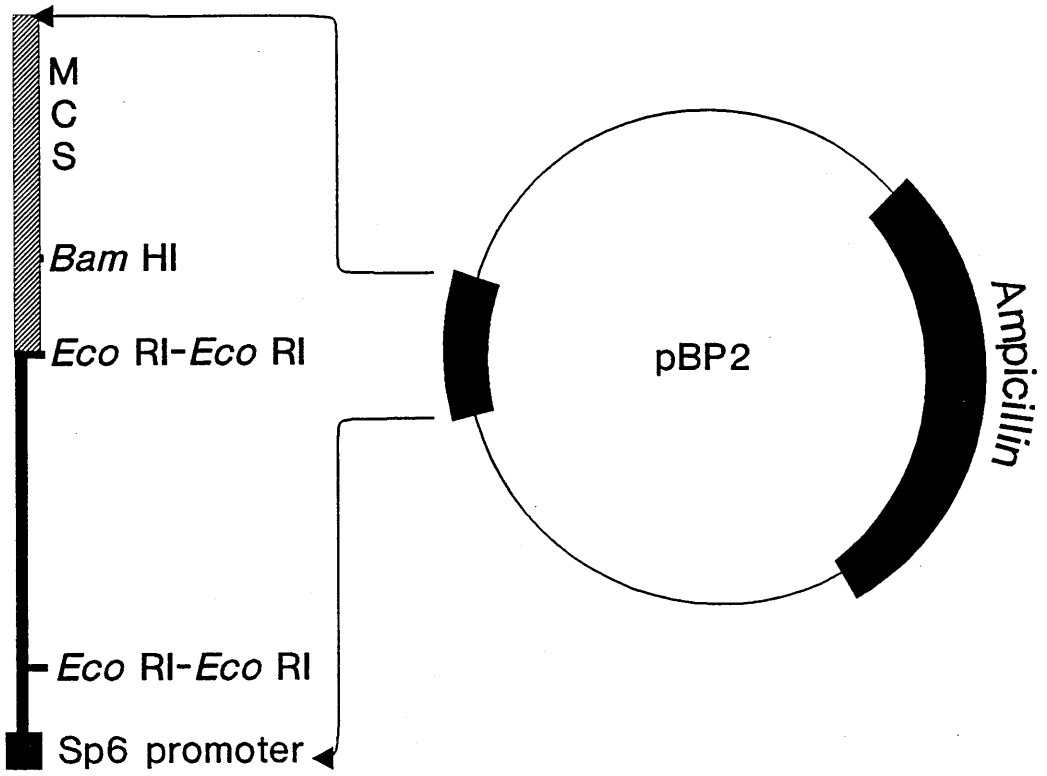


Figure 2.1: pBP2 plasmid. The 264 bp S100 cDNA was subcloned into the *Eco* RI site of the pSP65 RNA synthesis vector (Melton *et al.*, (1984)) and donated by Professor Marks (University of Toronto). MCS = multiple cloning site. (Graphics by Mr. A. Miller, University of Glasgow).

Small scale preparation of plasmid DNA

The alkaline lysis method modified from Birnboim and Doly (1979) was employed to prepare plasmid DNA. All microfuge centrifugations for this procedure were performed on an MSE microcentrifuge. 1.0 ml of culture was pelleted by centrifugation (30 seconds, 13000 rpm, room temperature) and the supernatant carefully removed. Bacterial cell wall lysis was induced using 100 μl of solution 1 (0.05 M glucose, 0.025 M Tris-HCl pH 8.0, 0.01 M EDTA) followed by vigorous vortexing and incubation on ice for 30 minutes. Denaturation of both the plasmid and chromosomal DNA was achieved by the addition of 200 μl of solution 2 (0.2 M NaOH, 1.0% SDS) and incubated on ice for 5 minutes. Selective renaturation of the DNA was achieved by the addition of 150 μl of solution 3 (3.0 M sodium acetate (pH 5.2), followed by vigorous vortexing and incubation on ice for 60 minutes. The suspension was centrifuged at 13000 rpm, for 4 minutes at room temperature and 400 μl of the clear supernatant was removed followed by the addition of 1 ml of cold (-20^{o}C) ethanol. This was left at -20^{o}C for 30 minutes and the DNA was subsequently pelleted by centrifugation for 5 minutes. The pellet was resuspended in 100 μl of Tris-acetate buffer (A4.2.4) to which was added 200 μl of cold ethanol, and stored at -20^{o}C for 30 minutes. The precipitated DNA was collected by centrifugation, washed in 800 μl of 70% ethanol and centrifuged. The pellet was vacuum-dried and a phenolic extraction performed for purification purposes. The pellet was resuspended in 100 μl of SDW and 100 μl of phenol was added. The solution was vortexed, and centrifuged for 2 minutes. The aqueous DNA was precipitated by addition of 1/10 volume of 3.0 M sodium acetate (pH 5.2) and 3 volumes of ethanol and left at -20^{o}C for 10 minutes. This was then centrifuged for 5 minutes and washed with 70% ethanol, centrifuged and vacuum-dried.

Restriction enzyme digestion analysis

Restriction enzymes were purchased from BRL or NBL and used at the manufacturer's instructions. The pellet was resuspended in 20 μl SDW and split into two samples; one for digestion and another as a non-digested control. 2.0 μl of EcoR I enzyme (10 units/μl) was added to the former sample and both were made up to 20 μl with 2 μl of the appropriate enzyme buffer and SDW. Both samples were incubated at 37^{o}C for 3 hours; excess conditions were employed to ensure complete digestion. After this, 1 μl of RNase (10 mg/ml in 10 mM sodium acetate) was added to ease the identification of the low molecular weight insert. The samples, and DNA markers (lamda Hin dIII and Phi X174 Hae III) were electrophoresed at 70 V for 60-75

minutes, through a 1% agarose analytical mini-gel (A4.2.3). Visualization was affected by the addition of ethidium bromide (0.05μg/ml) to the buffer (1 x TAE) (A4.2.4) used in gel preparation and electrophoresis. The isolated S100 insert was identified co-migrating with the 271/281 bp doublet of the Phi X174 Hae III marker (fig 2.2).

Large scale preparation of DNA

150 μl of the original 2.0 ml culture was innoculated into 10 mls of L-broth containing ampicillin (100 μg/ml) and incubated on a roller mix overnight at 37^{o}C. 2 x 2.0 ml aliquots of the overnight culture was added to 2 x 200 mls of L-broth with ampicillin and cultured overnight at 37^{o}C. The OD_{600} value of 1.515 was indicative that the culture had reached a stationary phase. The bacteria were pelleted by centrifugation on a Beckman J2-21 M/E model at 5000 rpm for 5 minutes at 4^{o}C and all pellets were resuspended in 50 mM Tris HCl (pH 8.0).

Plasmid DNA was prepared from 400 ml of bacterial culture by passage over a Qiagen column$^{(R)}$ using the manufacturer's instructions (A.4.3). Briefly, the cell wall was broken using buffer P1 containing RNase (100 μg/ml). The SDS in buffer P2 caused cell membrane lysis and the alkali denatured the DNA. The DNA was selectively renatured by addition of buffer P3. The DNA was separated from the cell lysate on a Qiagen affinity column and then precipitated with isopropanol, washed in 70% ethanol and vacuum-dried. The DNA was resuspended in T/E buffer (A4.2.4) and concentration ascertained at OD_{260}. Centrifugation was performed on a Beckman J2-21 M/E centrifuge at 16,500 rpm.

S100 DNA preparation

After a small scale digestion of the plasmid with Eco RI, a large scale digestion of 200 μg of plasmid DNA was undertaken.

The DE81 filter affinity chromatography method for isolation of DNA from agarose gels was employed to isolate the S100 insert DNA (Dretzen *et al*, 1981). The digested plasmid was electrophoresed on a 1.0% agarose gel (A4.2.3) for 50 minutes at 50 V. Using the UV light source to visualize the insert fragment, slits the same width as the fragment were made in the gel 1-2 mm below the fragment. Trimmed pieces of DE81 filter paper (Whatman) were positioned into the cuts and electrophoresis of the gel continued until the insert DNA had migrated onto the paper (approximately 5 minutes). The transfer was monitored by UV light. The filters were washed in 1 x T/E buffer (A2.2.4) and placed in sterile 1.5 ml eppendorf centrifuge

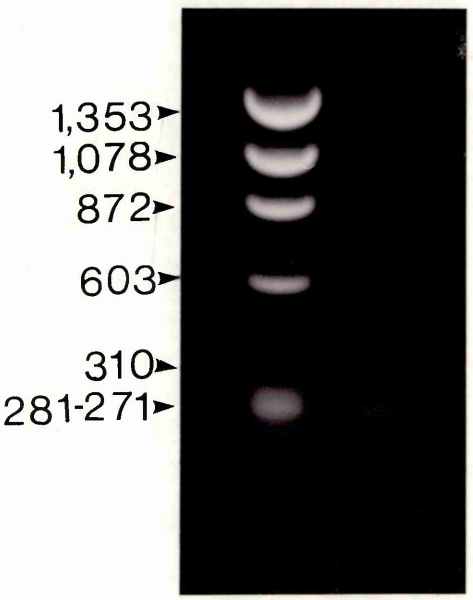


Figure 2.2: Isolated S100 cDNA insert. The Schwann cell marker S100 cDNA, inserted into the pBP2 plasmid, was kindly supplied by Professor Marks (University of Toronto). The 264 bp insert was excised from the plasmid using *Eco* RI restriction enzyme, stained with ethidium bromide (0.05 ug/ml) and identified on a 1.0% agarose mini-gel comigrating with the 271/281 bp doublet of the Phi X174 *Hae* III marker. The left lane is the Phi X174 *Hae* III marker and the isolated S100 cDNA is visible in the right lane (x 2).

vials. The DNA was eluted from the paper with 250 μl of elution buffer (A4.2.4) and incubated at 55^{o}C. for 30 minutes. The supernatant was transferred to another vial and the elution repeated with another 150 μl of buffer. The pooled samples were microfuged for 15 minutes, 13000 rpm to pellet any filter paper fibres. The supernatant was transferred to a fresh eppendorf and the DNA precipitated by the addition of 2 x volume of cold ethanol at -20^{o}C. The DNA was pelleted by microfugation (13000 rpm for 15 minutes) and the pellet washed twice in 70% ethanol. The amount of the isolated insert was estimated by comparison on a gel with known concentrations of the Phi X174 Hae III marker.

2.4.2.2 P_0 DNA preparation

Dr. Lemke kindly donated the cDNA (pSN63c) encoding P_0 which was subcloned by Dr. Mitchell (University of Glasgow) into the Eco RI multiple cloning site of the pGEM4 plasmid giving a 4.7 kb recombinant containing a 1.85 kb insert referred to as P_0-1. It was engineered so that the Sp6 promoter drove transcription of the sense strand and the T7 promoter directed transcription of the antisense strand (fig 2.3). The plasmid was digested with Eco RI at 37^{o}C for 2 hours under conditions of 3 fold enzyme excess. 200 ng samples of the digested and the non-digested DNA, and the markers (lamda Hin dIII and Phi X174 Hae III) were electrophoresed on an analytical, 1% agarose mini-gel (A4.2.3) to monitor digestion. The digested DNA was loaded onto a 1.0%, low-melting point preparative gel (A4.2.3) and electrophoresed at 70 V for 2.5 hours. The portion of the gel representing the P_0 insert was cut out and 200-250 mg amounts placed in 1.5 ml sterile eppendorfs to which was added 400 μl T/E buffer (A4.2.4). The DNA was purified from the agarose by the following extraction procedures (Sambrook *et al.*, 1989). The agarose was melted at 65^{o}C over a 15 minute period and 0.5 ml warm Tris-saturated phenol was added to each tube and vortexed. The suspension was incubated at room temperature for 5 minutes and microfuged at 13000 rpm for 5 minutes. The supernatant was aspirated, the phenolic extraction repeated and followed by extraction with 400 μl of phenol/chloroform and centrifuged for 3 minutes. A final extraction was performed with 400 μl chloroform. A 1/10 x volume of 3.0 M sodium acetate (pH 5.2) was added to each eppendorf, followed by 1.0 ml cold ethanol and the solution the DNA allowed to precipitate at -20^{o}C for 15 minutes. The DNA was pelleted by microfugation for 10 minutes and subsequently washed with 75% ethanol. After removal of the supernatant, the eppendorfs were incubated at 65^{o}C, with their tops open, to evaporate the ethanol. The DNA was resuspended in T/E buffer

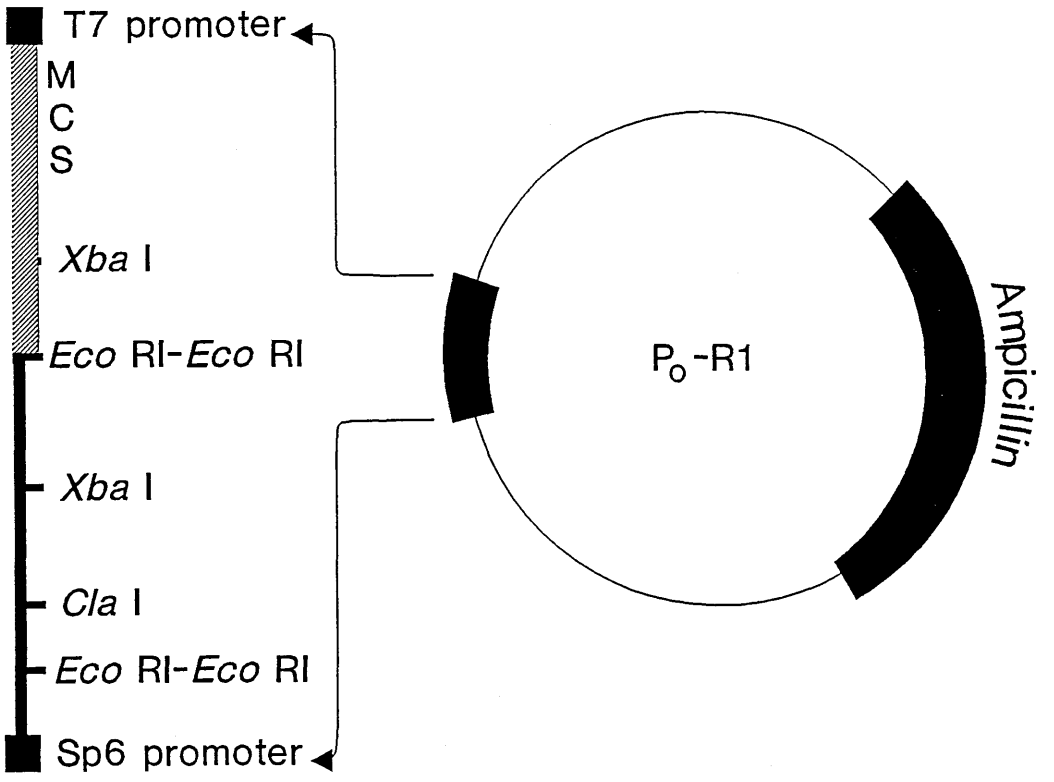


Figure 2.3: **P_0-R1 transcription vector.** (A) The original P_0 cDNA was inserted as a tandem into the pSN63c plasmid and donated by Dr. Lemke (The Salk Institute, California). Dr. Mitchell (University of Glasgow) subcloned the P_0 cDNA into the *Eco* RI site of the multiple cloning site (MCS) of the pGEM4 plasmid, forming P_0-R1, so that the T7 promoter directed the transcription of the antisense strand and the Sp6 promoter directed transcription of the sense strand. (Graphics by Mr. A. Miller, University of Glasgow).

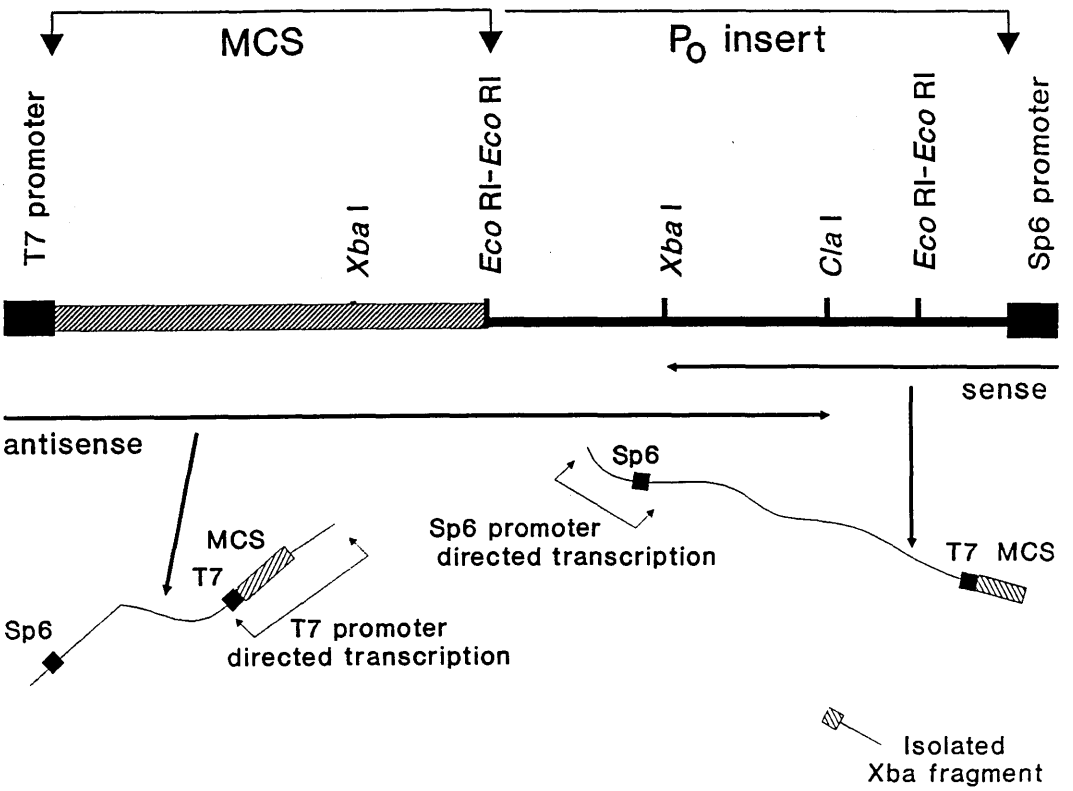


Figure 2.3 (cont.): Transcription of sense and antisense RNA from P_0-R1. (B) For the antisense strand, the plasmid is linearized with *Cla* I restriction enzyme and transcription occurs in the presence of T7 polymerase, directed by the T7 promoter. For the sense strand, the plasmid is linearized with *Xba* I restriction enzyme, Sp6 polymerase is used and the Sp6 promoter directs the transcription from the the plasmid. Because of a second Xba I site within the multiple cloning site (MCS) of the plasmid, a short, non-utilized fragment of the plasmid is also created by digestion of the plasmid with this restriction enzyme.

(A4.2.4) and samples pooled. The concentration of the isolated insert was estimated by comparison with known concentrations of the starting DNA digested with Eco RI on a gel. This DNA was used as a template for random primed labelling to create [^{32}P] probes.

2.4.2.3 NGF receptor DNA preparation

The NGF receptor fragment (Radeke *et al.*, 1987), subcloned into the Eco RI/Bam HI sites of pBluescript II KS, was kindly supplied by Dr. A. Toews (University of North Carolina) (fig 2.4) as plasmid linearized with Eco RI. A fragment of approximately 700 bp was excised from the plasmid following digestion with Bam HI and was electrophoresed through a 1.0% agarose gel (A4.2.3). The DNA was isolated using a Spin-X centrifuge filter unit (0.22 μm cellulose acetate tubes, Costar, UK) according to manufacturer's instructions and based on the freeze-squeeze method initially reported by Polman and Larkin, 1989. After fragment separation by electrophoresis, the agarose containing the isolated fragments was excised and loaded into Spin-X centrifuge units. These were incubated at -20^{o}C. for 15 minutes and microfuged at 13000 rpm for 5 minutes at room temperature. The filtrate was collected from the bottom chamber and the DNA concentrated by precipitation with 1/10 volume of 3.0 M sodium acetate and 2 x volume of cold ethanol and washed with 70% ethanol. The concentration of the isolated insert was estimated by comparison with known concentrations of the Phi X174 Hae III marker.

2.4.3 Preparation of radiolabelled probes

2.4.3.1 cDNA probes

The technique developed by Feinberg and Vogelstein (1983) was used in preparing [^{32}P] probes for northern and dot blots by the random primed DNA labelling method$^{(R)}$ kit from (BCL Mannheim). Isolated P_0 insert DNA (200 ng) was denatured by incubating at 90^{o}C for 10 minutes and then placed on ice. In a reaction volume of 20 μl, the following were added: the required volume of P_0 DNA, 1.0 μl each of dATP, dGTP and dTTP (each at 0.5 mM/l in Tris buffer), plus 2.0 μl of the reaction mix (containing all possible primer hexanucleotides in a 10 x concentrated reaction buffer), plus 5.0 μl (50 μCi) of the isotope ([α^{32}P] dCTP, specific activity of 370 MBq/ml; 10 mCi/ml, Amersham), 1.0 μl of Klenow enzyme and SDW. This mixture was incubated at 37^{o}C for 30 minutes and then at 65^{o}C for 10 minutes to

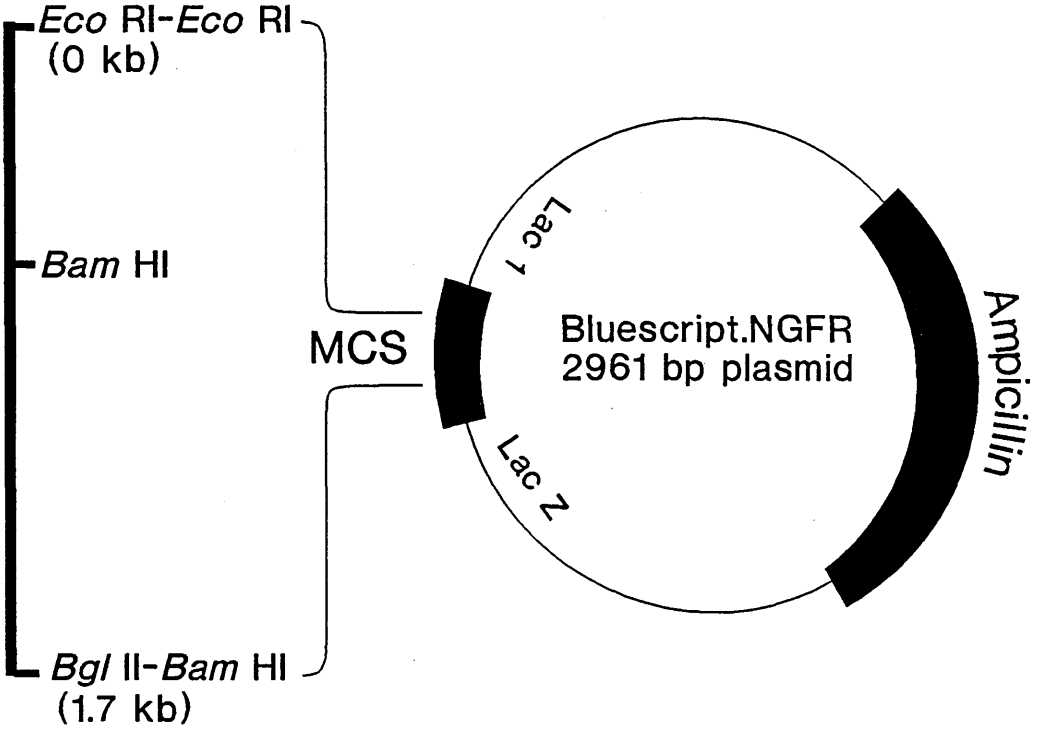


Figure 2.4: **Bluescript.NGF receptor plasmid.** The original 3.2 kb plasmid is from Dr. Shooter (California) as described in Radeke *et al.*, 1987. Dr. Toews (North Carolina) kindly donated the plasmid which he had subcloned as a 1.7 kb *Eco* RI/*Bgl* II fragment into the *Eco* RI/*Bam* HI sites of the pBluescript II KS (+/-) vector (Stratagene). After linearization of this plasmid with *Bam* HI, it was possible to transcribe mRNA from a 0.7 kb fragment. MCS = multiple cloning site. (Graphics by Mr. A. Miller, University of Glasgow).

stop the reaction. An agarose (Bio-gel A-5m, 100-200 mesh) column in a sterile pasteur pipette, plugged with siliconized glass wool was equilibrated with 0.1 x SSC (A2.1.1). The efficacy of the column was checked by elution of dye (A4.2.5). 5.0 μl of dye was added to the probe prior to application to the column. The probe eluted with the blue dye and was collected in an 1.5 ml eppendorf tube.

Hybridisation

Hybridisation was performed as described in Sambrook *et al.*, (1989). Prior to hybridisation, the filter was soaked in 6 x SSC (A2.1.1)and then treated with a prehybridisation solution (A4.2.1) in a sealed plastic bag for 2 hours at 42^{o}C and the prehybridisation buffer was discarded. The probe was denatured by heating to 90^{o}C for 10 minutes and added to the hybridisation mix (A4.2.1). Hybridisation was performed in a sealed bag overnight at 42^{o}C with gentle shaking.

After this period, the filter was removed from the bag and gently washed in increasingly stringent salt solutions until an acceptable level of background was achieved (< 7 to 10 cps). The following procedure was used with all washes being performed at 65^{o}C except the first which was at room temperature: 2 x SSC (A2.1.1) for 15 minutes with occasional agitation; 2 x 30 minute washes in 2 x SSC/0.1 % SDS; 2 x 30 minute washes in 0.1% x SSC/0.1% SDS; and if required a further wash in this solution for 60 minutes. The filter was then given a final brief wash in 0.1% x SSC at room temperature to remove the SDS, air-dried for 30 minutes and placed in a plastic bag against radiographic film for autoradiography at -70^{o}C.

2.4.3.2 Riboprobes for ISH

P_0-1 DNA was linearized by digestion with Xba I for a sense probe, or Cla I for an antisense probe. The linearization was performed under conditions of enzyme excess by digesting with the enzyme for 2-3 hours at 37^{o}C. After this period, samples of the digested and the undigested DNA were electrophoresed through a 1.0% agarose mini-gel (A4.2.3) to monitor digestion (fig 2.5).

Riboprobes were produced using the Sp6/T7 Transcription kit$^{(R)}$ from (BCL Mannheim), in the presence of [^{35}S] CTPα, 10 μCi/μl. This method is derived from Melton *et al.*, (1984) (figure 2.3). A 20 μl labelling reaction contained the following: 1-2 μg of linearized DNA, 1.0 μl each of the nucleotides (ATP, GTP, UTP (each at 10 mM/l in Tris buffer)) plus 5.0 μl (50 μCi) of [^{35}S] CTPα (specific activity of 37TBq/mM; >1000 Ci/mM; Amersham), 2.0 μl of enzyme buffer, 1.0 μl of RNAse inhibitor, sufficient DEPC treated water to make up the volume and 2 x 1.0 μl of

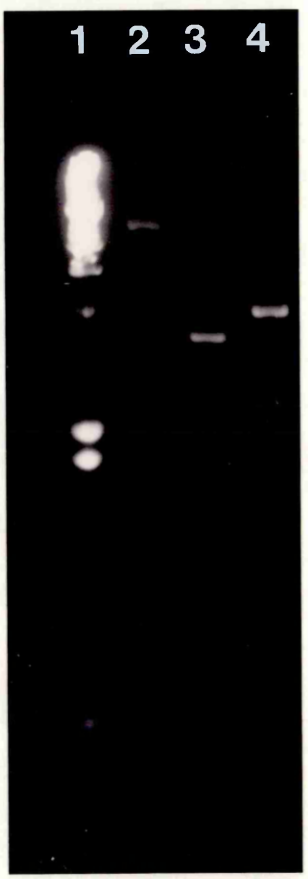


Figure 2.5: Linearized and non-linearized P_0 cDNA. The P_0 cDNA was kindly supplied by Dr. Lemke (The Salk Institute, California) and was subcloned by Dr. Mitchell (University of Glasgow) into the *Eco* RI multiple cloning site of the pGEM4 plasmid to form P_0-1. The plasmid was linearized using *Xba* I restriction enzyme for transcription of the sense strand, and cut with *Cla* 1 restriction enzyme for transcription of the antisense strand. Linearized plasmid, non-linearized plasmid and lambda *Hin* dIII DNA marker were electrophoresed on a 1.0% agarose mini-gel, in the presence of ethidium bromide (0.05 ug/ml), and visualized under UV light to assess the efficacy of digestion prior to transcription. The lamda *Hin* dIII marker is in the left lane; non-digested plasmid in lane 2; plasmid linearized with *Xba* I in lane 3 and *Cla* I linearized plasmid in lane 4 (x 2). The two bands in lane 2 represent the open circle (top) and super-coiled (bottom) species of the plasmid. In lane 3, the faint upper band represents non-linearized plasmid; the middle band is the main fragment produced by digestion with *Xba* I, and the faint bottom fragment is the short excised portion of DNA (see figure 2.3B). In lane 4 a small amount of non-linearized plasmid is present (upper faint band) and the linearized DNA is visible just below that.

enzyme was added at the beginning of the procedure and 30 minutes later. The T7 enzyme was used to make the antisense probe and the Sp6 enzyme used to make the sense probe. The mixture was incubated at 37°C for one hour, then 2.0 μl of DNase was added, to digest the template, leaving only the radiolabelled RNA. This was incubated for 15 minutes at 37°C. An equal volume (22 μl) of phenol/chloroform T/E buffer (A4.2.1) was added and the mixture vortexed for 10 seconds then centrifuged for 3 minutes at 13000 rpm at room temperature. The aqueous phase was removed and a back-extraction performed by adding 22 μl of DEPC treated SDW to the precipitated layer, vortexing and microfuging. The aqueous phases from the two extractions were combined. The RNA was precipitated at -20°C for 2 to 3 hours by the addition of 1/10 x volume of 7.5 M ammonium acetate and 2.5 x volume of cold ethanol and storage. 5.0 μl of the sample was removed and added to 5.0 ml of Ecoscint for scintillation counting. The radiolabelled RNA was pelleted by microfugation at 13000 rpm for 20 minutes at 4°C and the supernatant harvested and retained. The pellet was resuspended in 100 μl of DEPC treated SDW and 5.0 μl of this and 5.0 μl of the supernatant were collected and added to different 5.0 ml volumes of Ecoscint. The riboprobe was reprecipitated overnight at -20°C. by the addition of 0.3 M sodium acetate and 2.5 x volume of cold ethanol. Counting the vials of Ecoscint was performed on Beckman LS 1801 scintillation counter. The percentage of isotope incorporated into transcribed RNA was calculated as a ratio of the counts of the pelleted solution over the total counts. The amount of isotope incorporated into RNA was calculated by a standard formula and the riboprobe resuspended in 0.01 M dithiothreitol to achieve a concentration of 1.0 ng/μl/kb. The probe was stored at -20°C until required with a maximum shelf life of 8 weeks if made from freshly manufactured isotope.

2.4.4 *In situ* hybridisation (ISH)

The aim of *in situ* hybridisation is to identify RNA of interest within the target tissue. The basic procedure was as described by Cox *et al.*, (1984) and modified by Wilkinson *et al.*, (1987). The schedule for ISH (A4.1) has three phases. The first (A4.1.1) is a pre-treatment that renders the target (mRNA) accessible to the probe with morphologicalal preservation of the tissue. Proteinase K treatment of the sections removes proteins bound to the target mRNA. There is also an acetylation stage (Hayashi *et al*, 1987) aimed at decreasing non-specific binding of the probe due to electrostatic interactions between the probe and the basic proteins. The second phase (A4.1.2) is the hybridisation of the riboprobe (0.1 ng/μl/kb) to the cellular mRNA

which is performed under low stringency conditions that favour nucleic acid hybrid formation. The third phase (A4.1.3) consists of washing to remove unbound probe and produce an acceptable background. An RNAse step is also included to remove non-base-paired RNA from the tissue sections (Tecott *et al.*, 1987). The resultant samples were stored until processed for autoradiography (section 2.4.6)

2.4.5 Blotting techniques

Quantitation of RNA, extracted from pooled samples of tissue or cells, was performed using dot or northern blotting (Thomas, 1983). The latter was also used to assess the size and integrity of RNA. The RNA was probed with a [^{32}P] labelled cDNA probe (section 2.4.3.1) and detected by autoradiography against Cronex (Dupont) medical screen radiograph film exposed at -70^{o}C.

2.4.5.1 Northern blots

The required amount of RNA (0.5-2 μg) was freeze-dried and resuspended in 9 μl of denaturation buffer (A4.2.4), heat denatured at 65^{o}C for 15 minutes and placed on ice. 1.0 μl each of ethidium bromide (1 mg/ml) and northern gel dye (A4.2.5) were added to each sample which was loaded into a well of a denaturing 1.5% agarose gel (A4.2.3) and electrophoresed, at convenience, in a Gibco BRL 20-25 horizontal gel electrophoresis apparatus using a Pharmacia electrophoresis power supply EPS 500/400. The RNA was transferred overnight to nitrocellulose paper by capillary action (A4.2.6). The filter was air-dried and baked under vacuum for 2 hours at 80^{o}C. The filter was ready for pre-hybridisation.

2.4.5.2 Dot blots

Two approaches were employed to assess the integrity of the RNA used for dot blotting. The RNA was electrophoresed on a 1.5% analytical denaturing mini-gel (A4.2.3). Non-degraded RNA had clean, non-smeared bands representing the two major RNA species - 28 and 18S and a less intense band representing the 5S species. 1.0 μl of ethidium bromide (1 mg/ml) was added to some RNA samples and the resulting bands detected under UV light on a Fotodyne 240 V ultraviolet light source. Alternatively, after electrophoresis in the absence of ethidium bromide, the RNA was transferred overnight to nitrocellulose paper (A4.2.6) and baked at 80^{o}C. for 2 hours. The filter was immersed in 5% acetic acid and agitated for 15 minutes, the acid was

removed and replaced with 0.04% methylene blue/0.5 M sodium acetate dye and shaken gently for 15 minutes. The filter was then rinsed over a 15 minute period in distilled deionized water. The dye bonded specifically to RNA.

Dot blots were performed for the axolemma experiments because of the low level of P_0 mRNA in the samples. The dot blotting method was selected because it is more sensitive for detecting low levels of mRNA. The RNA was heat denatured at 65°C for 15 minutes to break the secondary structure, then transferred to ice. The nitrocellulose paper (Sartorius 0.1 μm) was sequentially pretreated by immersion in DEPC-treated water, equilibrated in 20 x SSC (A2.1.1) for 30 minutes and air-dried. After sample application, the filter was air-dried and baked at 80°C for 2 hours under vacuum on a BioRad slab dryer to bond the RNA.

2.4.5.3 Stripping of filters

Filters were stripped of bound probe prior to re-labelling with a second probe, by 2 x 30 minute incubations at 55°C. in 70% formamide, 0.1 mM EDTA and 10 mM Tris (pH 7.4). The efficacy of stripping was monitored with a radiation monitor.

2.4.6 Autoradiography

2.4.6.1 [^{35}S] (for ISH samples)

Prior to coating the slides in emulsion, they were placed against Cronex (Dupont) medical screen radiograph film in a radiograph cassette at room temperature overnight. The intensity of image on the developed film the next day determined the length of exposure of the slides after dipping in emulsion.

Clean, dry slides were dipped in a solution of Ilford K5 emulsion mixed in a 1:1 ratio with distilled water containing 1.0% glycerol. The emulsion was maintained at 42°C. After dipping the slides were air-dried for 4 to 6 hours and then stored in light-tight slide boxes, containing a sachet of silica gel, at 4°C for the required exposure time as determined by the test exposure against the radiographic film.

For quantitative studies using the Quantimet image analysis system, slides were coated in AR10 stripping film which coated the samples with a uniformly thick emulsion. By red safe light, emulsion strips were floated onto the surface of the sample slide in a water bath at 25°C. The emulsion was exposed at 4°C. and

developed in D19 developer for 5 minutes, washed for 1 minute, fixed in 30% sodium thiosulphate for 10 minutes and washed in gently running water for 10 minutes.

2.4.6.2 [^{32}P] (dot and northern blots)

Filters which had been probed with [^{32}P] were enveloped in thin plastic and sandwiched between two sheets of radiograph film in a cassette with intensifying screens and exposed at -70^{o}C. The first film was removed after the estimated required period of time and processed. The second film was left for a longer exposure if required. The optical density was measured on a Quantimet 970.

2.4.6.3 [^{3}H]

Tritiated thymidine was used in mitotic index studies. After fixation in 4% paraformaldehyde (A2.3) the cells were dipped in Ilford K2 emulsion concocted in the same manner as the K5 emulsion (1:1 with water and 1.0% glycerol). The storage and processing of these samples was the same as for the K5 dipped slides.

2.4.6.4 Development of slides

For developing, the slides were allowed to come to room temperature and processed in Kodak D19 developer for 4 minutes. Development was stopped in 1% glycerol/1% acetic acid for 1 minute and fixation in 30% sodium thiosulphate was for 2.5 minutes. The slides were washed 3 times in water and allowed to air dry completely prior to staining. Teased fibres were stained with sudan black or H & E, whilst cells were stained with haematoxylin (A3.1.2).

2.5 PROTEIN ANALYSIS TECHNIQUES

2.5.1 Protein concentration assay

Protein concentrations were determined by Pierce Protein assay (Pierce & Warriner (UK) Ltd.) using the manufacturer's instructions for the standard protocol at 37^{o}C for 30 minutes (A5.1). Protein standards were prepared using bovine serum albumin diluted in the same medium as that in which the samples were suspended.

Samples and standards were read at a wavelength of 562 nm. A standard curve was drawn and the samples read against the standards.

2.5.2 SDS-polyacrylamide gel electrophoresis

Protein electrophoresis was performed on vertical polyacrylamide gels produced by polymerization of the acrylamide polymer crosslinked by N,N'-methylene bisacrylamide (Hames, 1981). The accelerator for the reaction was N,N,N',N',-tetramethylethylenediamine (TEMED) and the catalyst was ammonium persulphate (Hames, 1981; Johnston and Thorpe, 1982).

Initial sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gels were composed of a 2.5% stacking gel and a 12.5% resolving gel. However poor separation of the higher molecular weight proteins (> 100 kDa) occured with this protocol. The use of a gradient gel was much more successful in separating these proteins. A 5% solution (A5.2.1) of acrylamide-bisacrylamide was placed in one well of a Biorad mode 385 gradient former and a 20% (A5.2.2) solution in the other well which also contained a magnetic stirrer. The outlet from the gradient former passed through a peristaltic pump (LKB Bromma 2120 Varioperpex II pump) and into the glass plate sandwich. The gradient former delivered the most concentrated acrylamide into the sandwich first and as the level of this rose, then the concentration of the acrylamide decreased. The concentration of TEMED was decreased to 15 μl so that polymerization was slowed to allow for delivery of the mixture into the gel rig. The rate of delivery was 3.0 ml/min (Hames, 1981). This produced gels which were 17.5% at the bottom and 5% at the top. The top of the resolving gel which interfaced with the stacking gel was made even by layering 3.0 M Tris-HCl (pH 8.8) and 0.2% SDS over the top of the resolving gel before it sets. This was discarded before layering the 2.5% bis-acrylamide stacking gel (A5.2.3) over the resolving gel. This gel had minimal sieving effect on the proteins because of its large pore size. It thereby concentrated them into a relatively narrow band at the stacking/resolving gel interface (Hames, 1981; Johnstone and Thorpe, 1982). Thirty minutes at room temperature was allowed for the polymerization of each gel.

50 μg protein samples were prepared for electrophoresis by adding 100 μl of SDS-PAGE sample buffer (A5.3.2) and boiling for 5 minutes. Heating of the protein in the presence of excess SDS and mercaptoethanol resulted in denaturation of polypeptides. The SDS bound to them in a constant weight ratio so that they had essentially a similar charge and just migrated through the gel according to size (Hames, 1981). The samples were cooled to room temperature and microfuged at

13000 rpm for 30 seconds to pellet any insoluble protein. Samples were electrophoresed through a vertical LKB electrophoresis apparatus at 35 mA until the blue dye band had reached the foot of the gel (2-4 hours). Passage of an electrical current through the gel produces heat which affects ion mobility and results in protein band distortion (Hames, 1981). To nullify this, electrophoresis was performed at 8^{o}C using an LKB Multicool.

A discontinuous buffer system was used such that the gel buffer and the tank buffer were different (A5.3.1). The difference between the tank buffer and the gel buffer helped to concentrate the proteins prior to their entry into the resolving gel (Hames, 1981).

The gel was stained with coomassie brilliant blue dye (A5.4) for 30 minutes at room temperature and non-specific staining removed by overnight incubation in destaining solution (A5.4).

2.5.3 Western blotting

For western blotting, the size-fractionated proteins were transferred to nitrocellulose paper using a Bio-Rad Transblot cell$^{(R)}$. The paper and the gel were initially equilibrated in western blot transfer buffer (A5.5) and then assembled sandwiched in the Transblot cell and transferred overnight at 100 mA.

The filters were air-dried, and blocked with blocking buffer (A5.5) for 3 hours at room temperature or overnight at 4^{o}C. After air-drying again, they were incubated at 4^{o}C with the primary antibody diluted in blocking buffer. This was followed by washing three times in 15 minutes with blocking buffer and then incubating with the secondary antibody (anti-rabbit/anti-mouse IgG conjugated to peroxidase) diluted in blocking buffer for 1 hour at room temperature. After 2 x 5 minute washes with the blocking buffer, the papers were washed in 0.05 M phosphate buffer (pH 7.4) for 5 minutes. The colour was developed with 50% 3,4,3',4'-tetra-aminobiphenyl hydrochloride in phosphate buffer containing 0.01% H_2O_2 until the colour developed (up to 10 minutes). Development was discontinued by extensive washing in water.

CHAPTER 3: SCHWANN CELL CULTURE

3.1 DISSOCIATED NEONATAL SCHWANN CELL CULTURE

3.1.1 Introduction

Dissociated neonatal Schwann cell culture methodology has been in widespread use since its innovation by Brockes *et al.*, in 1979. The purpose of this section was to establish the method of dissociated Schwann cell culture for this thesis and study the morphologic characteristics of the cells produced. Because of questions raised by studies involving exposure of Schwann cells to axolemmal fragments (see chapter 7) the behaviour of neonatal dissociated Schwann cells on various components of basement membrane was also investigated.

3.1.2 Materials and method

The technique used is given in section 2.1.1 and was a modification of that described by Brockes *et al.*, (1979). Both neonatal rats and mice were used. Due to occasional limitations on animal availability, the brachial nerves were also utilized as a source of Schwann cells. To obtain this nerve bundle, the animal was prepared aseptically in dorsal recumbency and a skin incision was made extending across the chest from elbow to elbow. The insertion of the deep pectoral muscle on the proximal humerus was incised to expose the proximal, brachial nerves. The brachial nerves were then removed, trying to avoid the brachial artery and vein, from the level of the shoulder joint to the level of the elbow and dissociated in the same way as the sciatic nerves.

Morphological studies of the Schwann cells were mostly made on living cultures using a Wilovert inverted microscope.

A variety of different substrates for cell culture were used with the most common one being poly-L lysine. However, rat tail collagen and Matrigel$^{(R)}$ were used solely or in a 1:1 combination. The volume of substrate added to a 13mm coverslip was 12 μl. To assess the effect of these compounds on investing the cells with basement membrane, immunfluorescence studies for surface laminin (section 2.3.1.1) expression were performed. The primary antibody was anti-laminin raised in rabbits (Sigma) used at a dilution of 1:800 with L15 medium and the secondary antibody was goat-anti-rabbit conjugated with fluorescein or rhodamine (Sigma) used

at 1:200 diluted with L15. For negative controls, the primary antibody was replaced with L15 medium.

3.1.3 Results

The Schwann cells were obtained from neonatal rat or mouse sciatic nerve. Brachial nerves were also utilized from some rat pups and the cell yield was similar to, or slightly less than, that from the sciatic. The usual cell yield from 4 to 5 day old pups was 80 to 100,000 cells per pair of sciatic nerves. Schwann cells could be obtained from rat pups aged 1 to 9 days with the optimum age range of 3 to 6 days. After a week of age, the dissociation was more difficult, and cell loss higher, presumably due to both the formation of myelin sheaths by the majority of Schwann cells, and the increase in fibrous component of the nerve. It is likely that dissociation of Schwann cells that have engaged in significant myelination strips the sheath off the Schwann cell resulting in cell death.

Morphologically, the cells changed somewhat with time in culture. For the first 12 to 18 hours after plating out, they were rounded without cell processes. In the latter stages of this time, they started to put out processes, generally two in number, to form the typical bipolar, spindle shaped cell. However tripolar cells were also noted. Over the next few days, the Schwann cells would flatten out and enlarge somewhat whilst retaining the bipolar or tripolar appearance. The nucleus was oval with little lateral cytoplasm. The cytoplasm was concentrated at either end of the nucleus and flowed out into the poles of the cell.

The growth pattern of the Schwann cells, and relationship they adopted with each other, was dependent on the substrate upon which they were cultured. When cultured on poly-L lysine, the cells tended to maintain only a loose, haphazard, association with each other for the first 4 to 5 days. After this time, they occasionally formed clusters with the cells radiating out from a central nidus. If cell density was sufficient, then the cells would be loosely associated with each other and a lacey, reticular pattern formed. When cultured on rat tail collagen, the cells behaved in a similar manner to those on poly-L lysine, but the cell processes were somewhat longer and finer. Schwann cells cultured on substrate containing Matrigel (pure Matrigel or mixed with collagen in a 1:1 ratio) had processes that were even longer and finer and on this substrate, the cells aligned themselves in an orderly swirling pattern (fig. 3.1, and fig. 3.11). The cell numbers were affected by the substrate on which they were grown with cell numbers being greatest on Matrigel. The difference between collagen and poly-L lysine was not so noticeable.

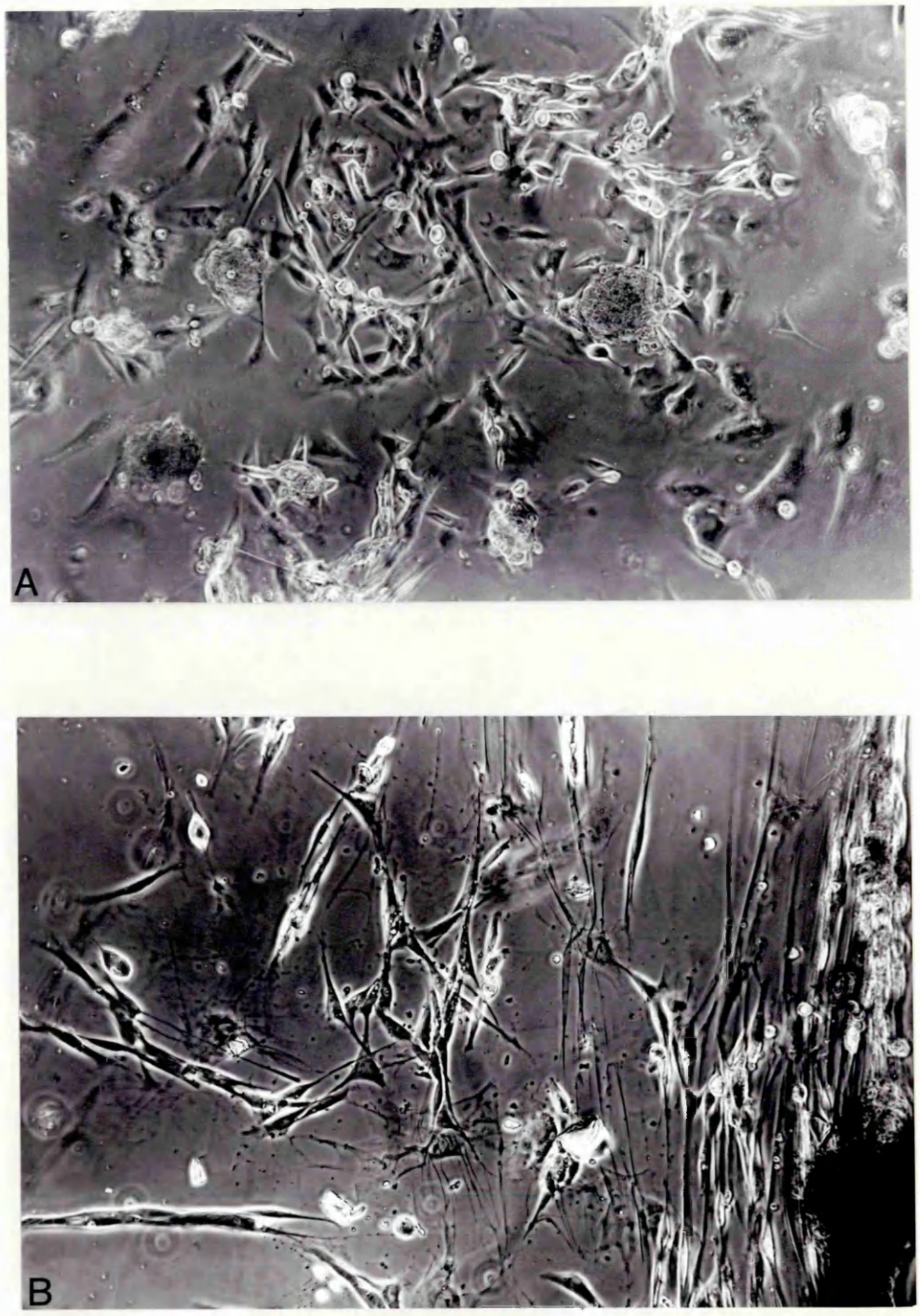


Figure 3.1: The effect of substrate on Schwann cell growth characteristics. (A) Dissociated neonatal Schwann cells cultured poly-L lysine for 4 days, (B) the same cells cultured on rat tail collagen for 4 days. Living cultures (x 75).

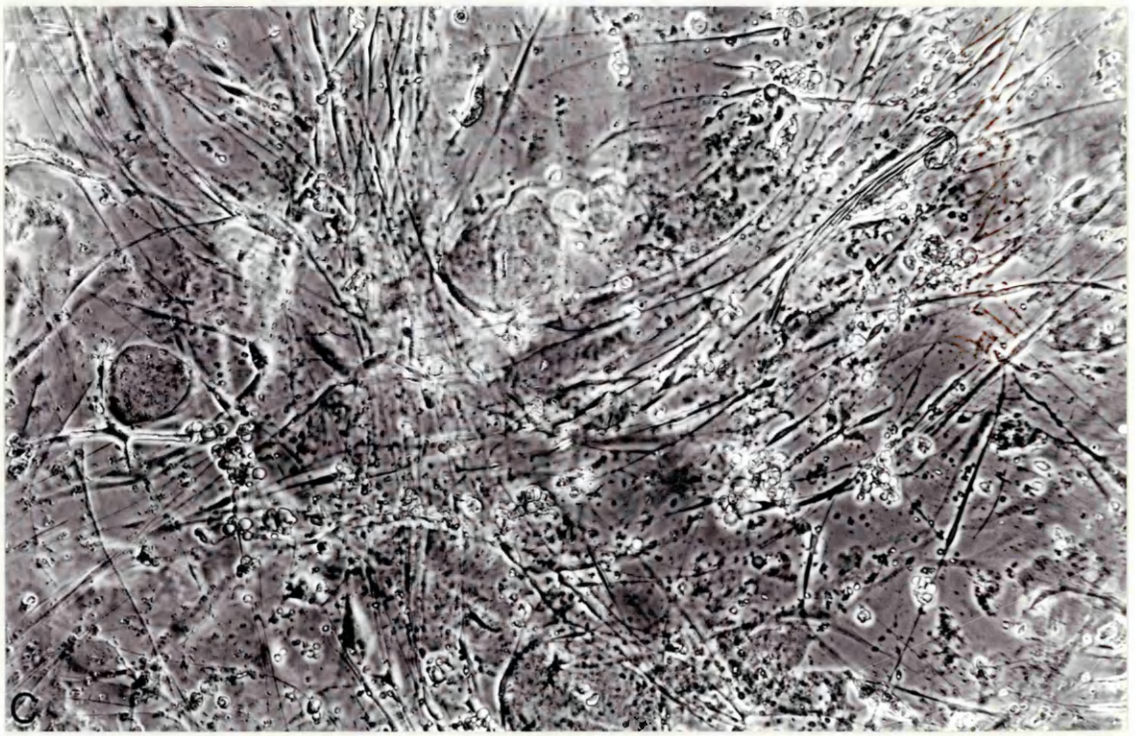


Figure 3.1 (cont.): The effect of substrate on Schwann cell growth characteristics. (C) Dissociated neonatal Schwann cells cultured on collagen plus Matrigel for 4 days. The cells have much longer, finer processes when cultured on this substrate. Living cultures (x 75).

Fibroblasts were mostly morphologically distinguishable from the Schwann cells. They were visible within 24 hours of plating out and, if not controlled by antimitotics, would overgrow the glial cells within 3 to 4 days and form a confluent monolayer under them. The fibroblasts were larger cells with bigger nuclei and broader, sheet-like cytoplasm (fig. 3.8). Generally, these cells would extend several short fat processes, but occasionally, they would form a tripolar or more spindle shaped cell with longer, finer processes. Thus, there was some overlap in the cell morphology of fibroblasts and Schwann cells. So, although these cells could mostly be distinguished by morphologic features, absolute discrimination required the use of cell specific markers such as S100 (see section 3.4).

Coverslips coated with a collagen/Matrigel mixture stained brightly with anti-laminin. Dissociated Schwann cells within the first 24 hours *in vitro* had a thick coating of laminin if plated onto collagen/Matrigel substrate, but only a patchy, discontinuous one if plated onto poly-L lysine. By 5 days *in vitro*, surface laminin was virtually undetectable on dissociated Schwann cells plated onto poly-L lysine. What was present was very fine and granular. Dissociated cells plated onto collagen/Matrigel substrate were enveloped in a thicker, confluent layer of laminin (see also section 3.4).

3.1.4 Discussion

Schwann cells were readily obtainable from neonatal rats or mice from the sciatic nerve. They were also successfully harvested from the brachial nerves. Utilizing both sets of nerves the Schwann cell yield from one animal could easily be increased or even doubled. Growth characteristics in culture were similar to those previously reported (Brockes *et al.*, 1977; Brockes *et al.*, 1979; Murray and Stout, 1942; Wrathall *et al.*, 1981a). However, the addition of basement membrane compounds to the cultures changed the morphology and orientation of the cells with respect to each other. Longer cell processes and a more orderly, palisading arrangement of the cells were observed when they were cultured on the complex basement membrane, Matrigel. The effect of basement membrane components on Schwann cell morphology *in vitro* has been described for SV-40 transfected cells (Yoshino *et al.*, 1990). The transfected cells grown on basement membrane grew in small, multilayered colonies with some cells aligning to form fascicles. However, the characteristics of these transfected cells, on any of the substrates, were not the same as those observed in these studies for primary, dissociated neonatal Schwann cells. This difference may have been due to the effect of transfection which does change

other cell characteristics such as cell marker expression (Chen *et al.*, 1987 (see "MAG" in section 1.4.3.1); Tenekoon *et al.*, 1987 (see section 1.3.2.3)).

Schwann cells *in vivo* are invested by basement membrane (Peters, 1976; Thomas and Ochoa, 1984). *In vitro*, the presence of this structure depends upon culture conditions. Significantly, the ability of the Schwann cell to fully differentiate and myelinate axons, *in vitro*, depends upon the presence of a complete basement membrane. Addition of exogenous basement membrane components to cocultures of Schwann cells and neurons adequately replaces endogenously formed basement membrane and permits full Schwann cell differentation (see section 1.3.3). In the experiments performed here, the different morphology and behaviour of dissociated Schwann cells grown on Matrigel, compared with those grown on type I collagen or poly-L lysine, possibly represents a more complete development and expression of mature Schwann cell behaviour. As laminin is a major component of basement membranes (Carey *et al.*, 1986a), anti-laminin was used to detect the presence of basement membrane around cells. A significant basement membrane coating of dissociated cells was observed only if the cells had been cultured on a substrate containing Matrigel.

Poly-L lysine has been widely used as a substratum for many types of cell culture and for attaching cells to glass for subsequent manipulations. It is a polycationic agent that bonds strongly to various solid surfaces and the exposed cationic sites combine with the anionic sites on cell surfaces (Mazia *et al.*, 1975). So whilst it is a useful agent in promoting cell adhesion *in vitro* it does little for promoting Schwann cell differentiation.

3.2 CULTURE OF SCIATIC EXPLANTS

3.2.1 Introduction

Other studies in this work (see chapter 7) made it necessary to ascertain whether Schwann cells could be cultured from adult and neonatal animals without subjecting the cells to the rigors of mechanical and enzymatic dissociation. Enzymatic treatment is known to affect the expression of Schwann cell surface molecules such as Ran-1 (Mirsky *et al.*, 1980), the ability of the Schwann cell to respond to the neuritic mitogen (Ratner *et al.*, 1987) and the expression of the mitogen on the neurite (Ratner *et al.*, 1987; Salzer *et al.*, 1980c). Mechanical dissociation would have an injurious effect on any cells that had elaborated a myelin sheath as it is unlikely that the cell

would survive the loss of that sheath resulting from the shearing forces applied during dissociation. Ideally, Schwann cells unassociated with a myelin sheath, and not subjected to enzymatic treatment were required.

After chronic axotomy, in the latter stages of Wallerian degeneration, the myelin sheath has been removed primarily by phagocytosis. The Schwann cells are left in a quiescent, non-proliferative, non-myelin-forming state, aligned within the tube of basal lamina as bands of Bungner (see section 1.5.2) (Poduslo *et al.*, 1985a; Weinberg and Spencer, 1978). Peak myelination during development in rats occurs at 2 weeks (Lemke and Axel, 1985). In neonatal animals, less than 7 days old, myelination is occurring but many of the Schwann cells have not formed elaborate myelin sheaths. Therefore, it seemed plausible that Schwann cells without mature myelin sheaths could be harvested from neonatal tissue or chronically denervated adult tissue. To avoid the harsh mechanical and enzymatic conditions of dissociation, these Schwann cells were cultured from the tissues using the explant method. Schwann cell cultures have been established from peripheral nerve tissue explants and these explants have been transplanted at various times during culture to attempt enrichment of the Schwann cell content of the outgrowth (see section 1.3.2.1). Explants were set up from both adult and neonatal sciatic nerve to investigate the Schwann cells' morphology and antigen expression.

3.2.2 Materials and method

To obtain Schwann cells unassociated with the myelin sheath from adult rats, neurotomies were performed. Anaesthesia was induced and maintained with halothane, in adult Sprague-Dawley rats of either sex. The hair over the left lateral thigh was clipped and aseptically prepared using chlorhexidene solution. A 1.0 cm skin incision was made extending distally from the greater trochanter of the femur. The underlying fascia and biceps femoris muscle was bluntly dissected apart and the sciatic nerve exposed in its position caudal to the femur. The nerve was transected just distal to the greater trochanter and the proximal end of the distal stump was reflected distally to decrease the chances of the nerve rejoining. The muscle and skin were closed with 3/0 vicryl or silk. Post-operatively the animals' water was supplemented with terramycin antibiotic. Generally, the distal stump of the nerve was left to undergo Wallerian degeneration for 6 weeks before use, however, other time frames were also used. At the appropriate time after transection, the animals were killed with an overdose of anaesthetic, or cervical dislocation, or both. The incision site was reopened and the distal stump of the nerve removed and placed in L15 medium. If the

nerve had regained continuity with the proximal stump, it was not used. For a different reason, (see chapter 7) in two separate experiments, explants were established from nerves that had been cut only 4 days previously or not cut at all. Neurotomies were not performed in neonatal animals and in separate experiments explants were created from 2 to 10 day old animals.

Under a dissecting microscope the non-nervous extraneural connective tissue and the perineurium was removed from adult nerves. Stripping the perineurium in neonatal animals was not routinely performed because it was too fragile and the procedure usually resulted in nerve fragmentation. For both neonatal and adult animals the subsequent procedures were the same and performed under aseptic conditions.

13 mm glass coverslips were prepared by washing in ethanol, distilled water and then leaving them to clean overnight in 1.0 M nitric acid. They were rinsed in distilled water, ethanol and then sterilized. After sterilization, they were immersed in 100 μg/ml poly-L lysine for 1 hour, rinsed in several changes of SDW and then placed in the wells of a 24 or 4 well tissue culture plates and left to air dry in a laminar flow hood. They were coated in 12 μl of either rat tail collagen or Matrigel, or a mixture of 6 μl each of collagen and Matrigel. The substrate was spread across the coverslip with the rubber plunger end of a 1 ml syringe. In a small amount of L15 medium, the nerves were chopped into 1 to 2 mm fragments and several pieces of minced nerve were placed on the substrate. Three methods were tried to encourage adhesion of the tissue to the substrate. Firstly, the explants were left to settle onto the substrate for 1 to 2 hours. The moisture associated with the explant evaporated during that time and the substrate and the explant became "tacky" and would stick together. However, the adhesion was not strong and the explant often floated off during subsequent feeding. The second method involved letting the explants settle onto the substrate for 10 to 15 minutes. A second coverslip was prewetted with SCGM and gently lowered on top of the explant. Subsequently the explants were fed with 0.5 ml/well of SCGM. 24 hours later, the second coverslip was removed using forceps and a hook fashioned from a 20G needle. In the third method, 2 to 4 μl of Matrigel was allowed to gel on top of the explants prior to feeding. The cultures were maintained at 37^{o}C in a humid environment and fed twice weekly with fresh medium. Attempts were made to enrich the cultures by the explant/transplant method. This consisted of allowing the Schwann cells and fibroblasts to grow out for 7 days, then excising the explant and transplanting it to a fresh, substrate coated coverslip to produce a secondary outgrowth (see section 1.3.2.1 - culture of non-dissociated Schwann cells).

Schwann cell antigens such as O4, GalC and S100 were identified by immunofluorescence techniques (see section 3.4). The distribution of the basement membrane, both on the coverslip and the cells surface, was similarly assessed using anti-laminin for which the methodology is given in section 2.3.1.1. For positive and negative controls, a collagen/Matrigel combination was spread across some coverslips, they were air dried and fixed in 4% paraformaldehyde before being washed in a balanced salt solution and processed for immunofluorescence. The primary antibody was used at 1:800 for the positive controls, and the tissue culture medium was used in place of the primary antibody for the negative control.

3.2.3 Results

The animals were not overtly compromised by the neurotomy. The only complication was mutilation of the foot on the side of the surgery apparently due to the aberrant innervation. However, this occurred in less than 3% of cases and the animal was euthanased if trauma was noticed.

Using the "tacky" method, most explants detached upon addition of SCGM at the time of first feeding. Using the second coverslip method to press the explant onto the substrate, approximately 60 to 75% of the explants attached although with time in culture, some would subsequently lift off because of detachment of the substrate. If detachment did occur, then the explants could usually be encouraged to reattach by repeating the adhesion procedure. Using the "gluing" down method by gelling Matrigel on top of the explants, attachment rates approached 100%.

Anti-laminin staining of both neonatal and adult Schwann cells identified a moderate, albeit somewhat patchy, coating of laminin, and presumably basement membrane, on the cell surface even after 5 to 10 days *in vitro* (fig. 3.2).

3.2.3.1 Adult explants

By immunofluorescence, the collagen/Matrigel substrate, without cells, appeared as a thick, 3-dimensional gel arranged in a fine lacey pattern. Neither collagen alone, nor poly-L lysine stained with anti-laminin. After several days in culture, the collagen/Matrigel substrate became patchy, forming thick strands and islands interspersed by negatively staining areas. Schwann cell density was much greater on the basement membrane covered portions of the coverslip.

After 4 days, a small amount of outgrowth was observed from the explant from neurotomized nerves and virtually none was observed from non-neurotomized

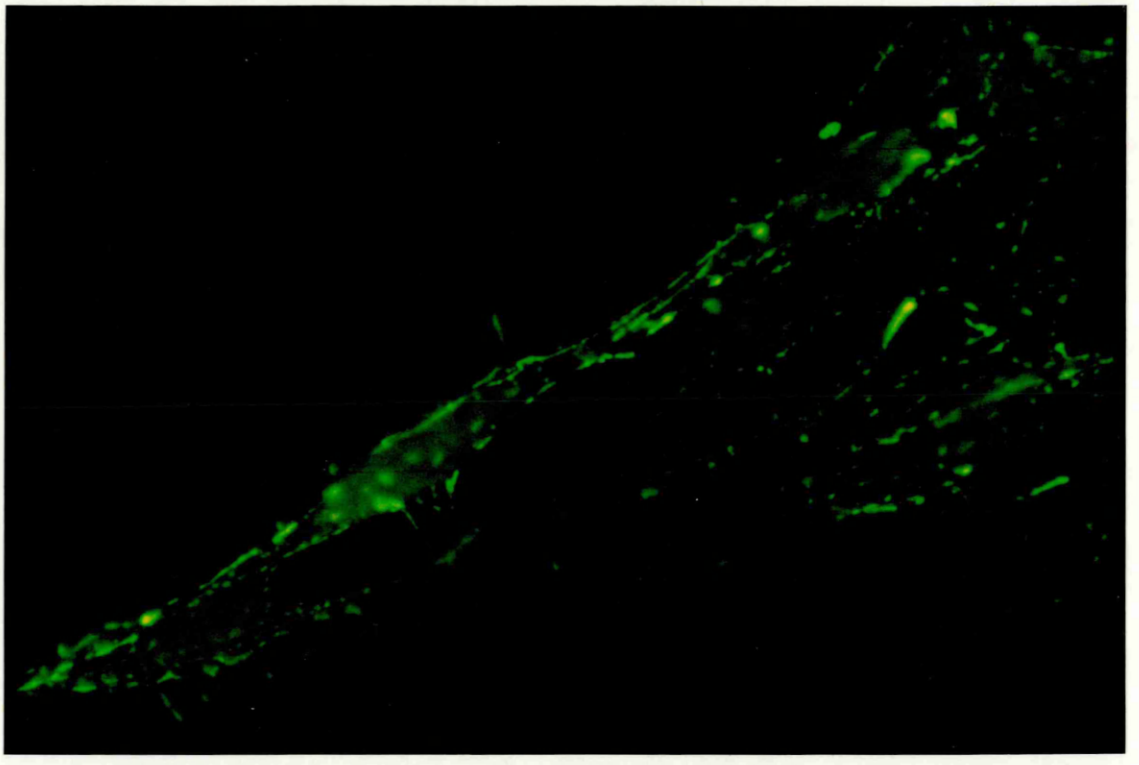


Figure 3.2 Anti-laminin stained Schwann cells derived from adult sciatic nerve explant. Explants of sciatic nerve which had been transected 6 weeks previously and allowed to degenerate *in vivo*, then established on collagen plus Matrigel substrate, cultured for 9 days and stained for surface laminin as described in the text. Original in colour (x 420).

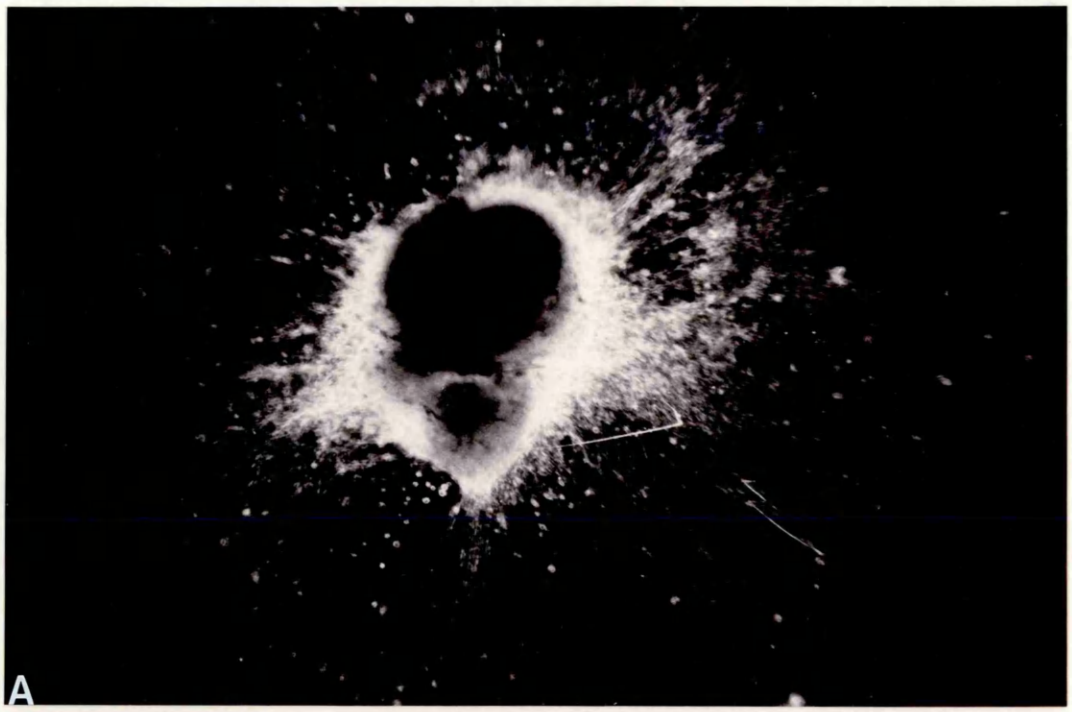
explants. Even after 1 week in culture, little outgrowth was observed from the latter explants. *In vitro* degeneration of the non-neurotomized explant was presumed to have occurred, as frayed out individual nerve fibres at the edge of the explant became vesiculated and "bubbly" in appearance after several days. The outgrowth from the neurotomized explants consisted of both Schwann cells and fibroblasts. By 8 days, significant outgrowth had occurred with Schwann cells migrating up to several millimetres away from the explant (fig. 3.3). No antimitotics were used, so by this time point the fibroblasts were well spread out migrating large distances away from the explant. They often formed a confluent monolayer under the explant and glia and could cover the whole coverslip. However, if all perineurial tissue was meticulously dissected from the nerve prior to mincing, the proportion of fibroblasts in culture was markedly decreased. The secondary outgrowth achieved from the explants after transplanting them at 7 days *in vitro* was less vigorous and had lower cell numbers than the primary outgrowth, but there were relatively less fibroblasts in these cultures..

The Schwann cells had the typical bipolar or tripolar shape with long processes sometimes extending several millimetres when growing on fibroblasts or on substrate. This was as observed by other workers (Askanas *et al.*, 1980; Wrathall *et al.*, 1981a). The outgrowth tended to be better on substrates containing Matrigel rather than just collagen. If basement membrane was present, the Schwann cells tended to align to form cords or chains of cells oriented in the same direction. These cords were several cells deep and corresponded well to the distribution of the basement membrane. A similar "twisted string" arrangement of Schwann cells grown on gelatin-plasma was noted in the outgrowth from human peripheral nerve (Askanas *et al.*, 1980). In areas devoid of substrate the Schwann cells were plumper with significantly shorter processes, and had a more haphazard arrangement, tending to form clusters or aggregate in a lacey, disoriented fashion. The distribution and characteristics of fibroblasts did not appear to be affected by basement membrane.

3.2.3.2 Neonatal explants

Outgrowth from the neonatal explants was brisker than outgrowth from adult explants and began within 24 hours of culture establishment (fig. 3.4). Like the adult explant cultures, the growth of the neonatal Schwann cells was determined by the substrate. Schwann cells cultured on just collagen had very poor outgrowth compared with those cultured on collagen/Matrigel. At 6 days *in vitro*, outgrowth was only 1/3 to 1/2 that observed on collagen plus Matrigel and many of the cells had lost their

Figure 3.3: Schwann cells cultured from adult sciatic nerve explant. The explant of predegenerated sciatic nerve tissue forms the nidus from which Schwann cells can emmigrate. The explant (bottom left) was harvested from transected sciatic nerve which had been allowed to degenerate for 6 weeks *in vivo*, prior to culturing for 8 days on collagen plus Matrigel substrate. The Schwann cells (fine white particles) can be seen radiating out from the fragment of nerve. Living culture (x 20).



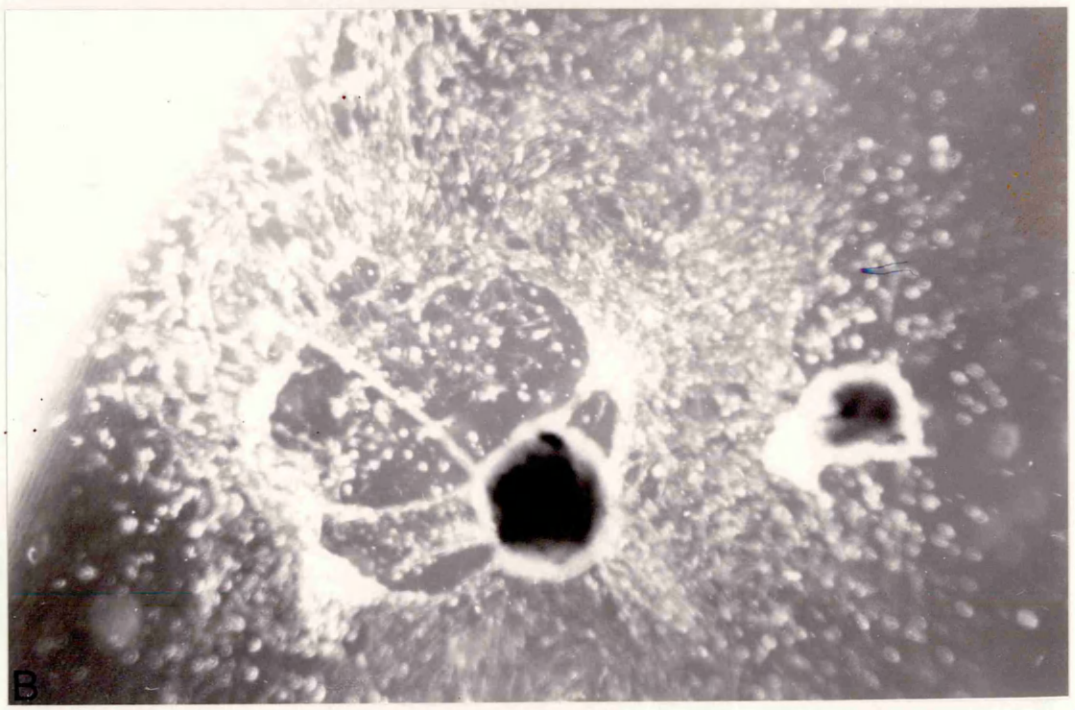
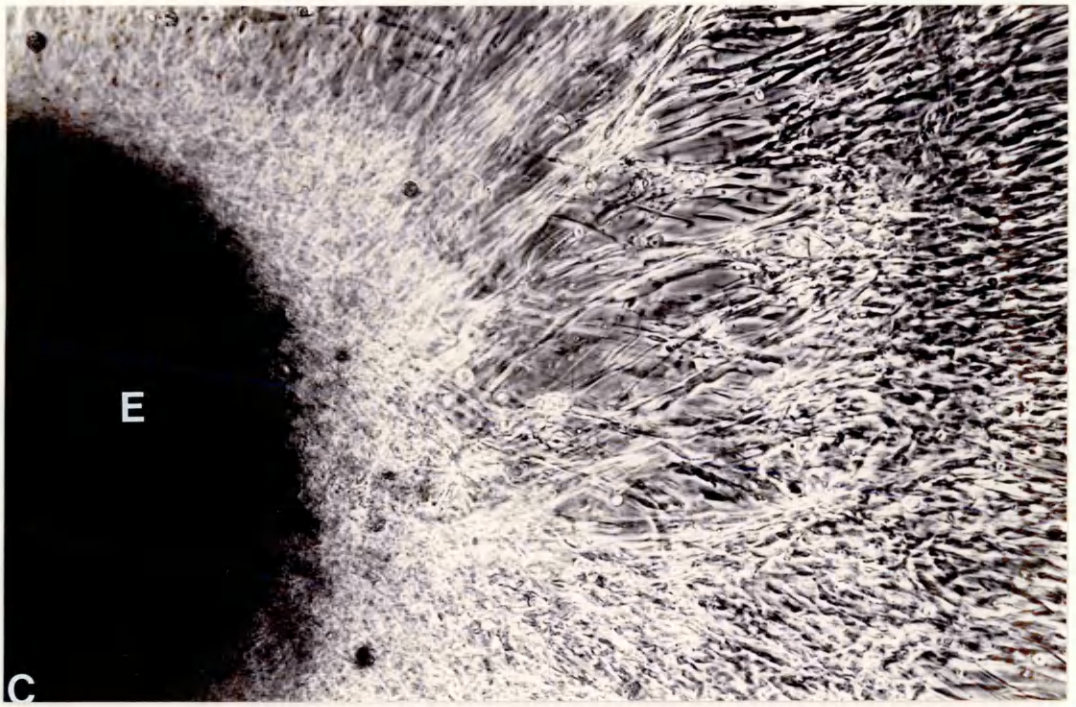


Figure 3.4: Neonatal sciatic nerve explants. Fragments of tissue (centre of photograph) were cultured on either rat tail collagen substrate or a mixture of collagen and Matrigel for 4 days. (A) Explant on collagen plus Matrigel; (B) Explant on collagen only. Living cultures (x 20).



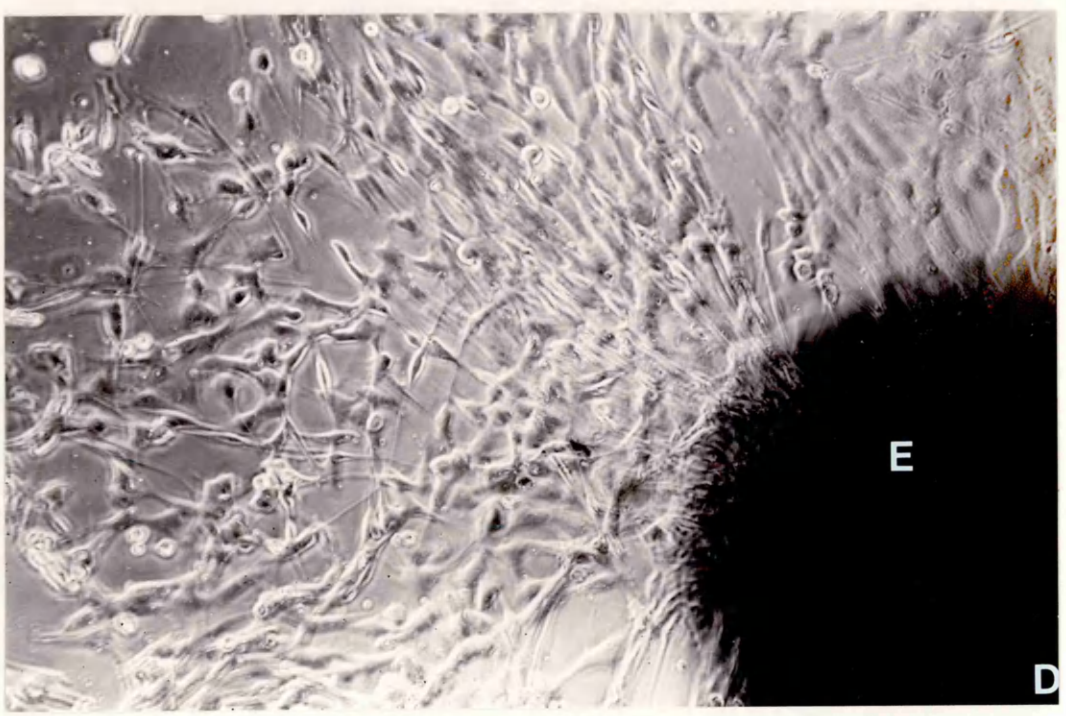


Figure 3.4 (cont.): Neonatal sciatic nerve explants. Fragments of tissue were cultured on either rat tail collagen substrate or a mixture of collagen and Matrigel for 4 days. (C) Explant (E) on collagen plus Matrigel; (D) Explant (E) on collagen only. Living cultures (x 75).

processes and were detaching (dying) (fig. 3.4). Fibroblastic contamination was not marked in these cultures. Within 7 days, the outgrowth on collagen/Matrigel was generally very good with 1/2 to 2/3 of the coverslip being covered by Schwann cells grown out from 1 or 2 explants. However, as for adult explants, after transplantation, the secondary outgrowth was reduced compared to the primary outgrowth. Excision of the explant for transplantation left behind a good culture of Schwann cells which continued to grow and expand despite removal of the explant.

3.2.4 Discussion

My studies confirmed previous work (Abercrombie and Johnson, 1942; Askanas *et al.*, 1980; Murray and Stout, 1942; Poduslo and Windebank, 1985b; Wrathall *et al.*, 1981a; see section 1.3.2.1) that Schwann cells can be satisfactorily obtained from explants of peripheral nerve tissue without mechanical or chemical dissociation. However, I found that if explants were obtained from adult nerves, then outgrowth was poor if the nerves had not been transected prior to culture. Similarly, in previous studies, poor outgrowth occurred in the nerves that had not been transected and allowed to degenerate, whereas Schwann cells did migrate out from predegenerated nerves (Abercrombie and Johnson, 1942). Comment was not passed on the exuberance with which Schwann cells outwandered from unpredegenerated explants established from human sural nerve biopsies. It is likely that outgrowth was not marked as after 7 to 10 days, a mixed cell population had only emerged 3.0 mm from each explant (Askanas *et al.*, 1980).

The lack of cellular outgrowth (Schwann cells and fibroblasts) from non-neurotomized explants may be related to the presence of the intact myelin sheath in this tissue. The Schwann cells would still be attached to these sheaths and not be "free" to migrate. Even in explants derived from nerves cut in the preceding week, the outgrowth was better. Wallerian degeneration, myelinolysis and break down of the sheath is well under way within 1 week of axotomy (Nathaniel and Pease, 1963; Pellegrino *et al.*, 1986; Poduslo *et al.*, 1985a; see section 1.5.2.1). It has been demonstrated that Schwann cell migration begins approximately 3 days after nerve transection and Schwann cells migrated at 0.1 to 0.3 mm/day (Billings-Gagliardi *et al.*, 1974; cited in Le Beau *et al.*, 1988). Cultured neonatal Schwann cells have been shown to migrate also (Dubois-Dalcq *et al.*, 1981).

Little information has been given in the literature on encouraging attachment of peripheral nerve explants. My initial studies consisted of placing the explant on the substrate with the minimum quantity of medium required to keep the cells moist. The

explants were thus maintained for 2 to 4 hours at 37^{o}C, in an attempt to create a bond with the substrate by virtue of the "tackiness" of the two components. However, even gentle, slow addition of the SCGM often resulted in immediate detachment of the explants. Using the second coverslip method, a significant proportion of the explants were be pulled off with the top coverslip when it was removed. This was especially so if the second coverslip was not prewetted prior to application on the explant. Wetting seemed to decrease the sticking of the explant to the top coverslip. Presumably, there is sufficient diffusion of medium under the second coverslip to the explant to maintain it during these first 24 hours as the Schwann cells were still subsequently viable. The substrate was placed on the coverslip just prior to the addition of the explant, aiming for it still to be not quite gelled so that the explant may stick to it better. I found that gelling basement membrane on to the explant was the most successful method for encouraging attachment. However, the volume of the complex had to be kept to a minimum as more than a few microlitres seemed to smother the explant and result in poorer outgrowth.

In the adult explants from neurotomized nerve, within 1 week significant outgrowth of Schwann cells from the explant occurred, but fibroblasts could overgrow the coverslip and form a monolayer underneath the Schwann cells. The fibroblast contamination can interfere with subsequent manipulations, such as RNA extraction, by contaminating the Schwann cell RNA with their own. Also upon reaching confluence, I noted that they could cause the substrate to detach and the whole culture was then lost. Fibroblastic contamination has been a problem in the past when explants have been used for culturing, so enrichment of the cultures by transplanting the explants to a fresh culture surface has been tried (Askanas *et al.*, 1980; Wrathall *et al.*, 1981). However, it was found here that the secondary outgrowth from adult or neonatal explants was less vigorous than the primary outgrowth and low cell numbers were obtained. Instead, if utmost care was taken to remove the perineurial elements, then fibroblastic contamination was minimized.

In the studies performed here, the primary outgrowth from the neonatal explants was very vigorous with the Schwann cells forming loosely swirling masses of cells. This contrasted with the slower outgrowth of adult cells which radiated away from the explant. In the sixth cranial nerve of the developing rat, myelination of the small fibres does not begin until the third postnatal week (Hahn *et al.*, 1987). It is possible that some Schwann cell migration and axonal sorting is still occurring within the nerve prior to this time. Potentially, the brisker outgrowth from the neonatal sciatic nerve compared with the adult nerve may be related to this. Poduslo and coworkers (1985a) observed rapid outgrowth from both neonatal and denervated adult

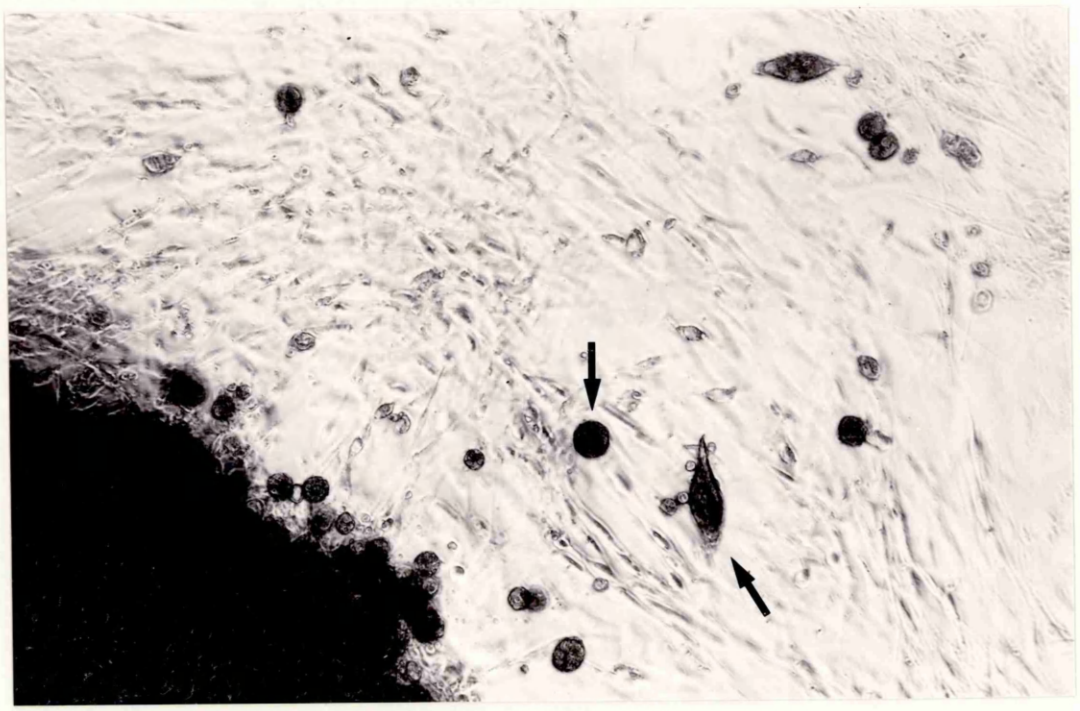


Figure 3.5: Adult sciatic nerve explants - possible phagocytic cell. In cultures established from adult rat sciatic nerve which had been transected only 4 days prior to harvesting, plump, debris filled cells (arrows) were occasionally noted in the outgrowth. These were thought to be phagocytic cells, although special stains were not performed to verify this. Living culture (x 75).

membrane on Schwann cell differentiation and maturation has been discussed previously (see section 1.3.3). As laminin is a major component of this compound (q.v.) the use of anti-laminin to identify its distribution is vindicated.

Schwann cells grown in isolation from neurons do not produce a basement membrane (Bunge *et al.*, 1980; Bunge *et al.*, 1982; Dubois-Dalcq *et al.*, 1981). By electron microscopy, Poduslo and Windebank (1985b) observed that basement membrane and collagen fibrils were present in the body of the adult explant but were absent at the culture periphery. In my studies, culturing Schwann cells on collagen/Matrigel resulted in a bright, thick laminin deposition over the cell surface. This did not occur if cells were grown on collagen only or poly-L lysine. This implies that addition of Matrigel to the substrate provides the cells with at least a coating of laminin. As Matrigel is a complex substrate, it is also likely that the cells are coated with complex basement membrane. The successful effect of exogenous basement membrane addition on Schwann cell differentiation has been discussed (see section 1.3.3.4). In my experiments, both adult and neonatal Schwann cells grew better on substrate containing Matrigel producing longer, finer processes than when grown on just collagen alone. In neonatal dissociated cultures grown on Matrigel-containing substrate they also adopted a different relationship to each other, tending to form a swirling, palisading mass. They are also in greater numbers on this basement membrane, either because Schwann cell mitosis is encouraged or because there is less cell attrition.

However, one problem I encountered with using any of the basement membrane components is the tendency for the substrate to detach from the coverslip. A number of experiments were tried before the volume of 12 μl/13 mm coverslip was decided upon. At volumes greater than 15 μl, the substrate tended to slough off the coverslip quite quickly in culture, but volumes less than 10 μl/coverslip were also unsatisfactory. Even with volumes of 12 μl/coverslip, after a few days in culture, anti-laminin staining identified that the distribution of the substrate basement membrane had become patchy suggesting dissolution of the components into the medium. Smaller starting volumes would contribute to this evanescence. In an attempt to increase the strength of bonding of the basement membrane, some coverslips were immersed in 40% anhydrous hydrofluoric acid to etch them and roughen their surfaces. Coverslips were exposed to the acid for 30, 60, 90 and 120 seconds and then washed in water. Definite etching of the coverslip was observed under the microscope after 90 seconds exposure to acid. Excessive etching was avoided as the optical

quality of the coverslip needed to be maintained. Normal and etched coverslips were then sterilized, coated in a 1:1 mixture of collagen and matrigel, and covered with SCGM. They were maintained at 37^{o}C for 6 days, fixed and stained with anti-laminin for immunofluorescence. The degree of coating of the coverslip was assessed and no obvious difference was found between etched and non-etched coverslips. In earlier studies, coverslips were not coated with poly-L lysine and the substrate frequently detached. Coating them with poly-L lysine, prior to coating with substrate, did appear to help the bonding of the substrate.

At the time of writing, a paper by Morrisey *et al.*, (1991) was published describing the production of Schwann cells from adult rat and human peripheral nerve explants. The explants were plated onto tissue culture plastic or occasionally, poly-L lysine. Difficulty in producing adhesion of the explants was alluded to, however, the success rate of attachment was not given. Adult nerve was not allowed to degenerate *in vivo*, as in my experiments, but rather "*a form of in vitro degeneration was encouraged*". These workers also employed the idea of Askanas *et al.*, (1980) using the explantation/transplantation technique to enrich the percentage of Schwann cells in the cellular outgrowth. Morrisey *et al.*, (1991) found that cell yields produced by the explantation method were 20 fold higher than those produced by attempting enzymatic dissociation of adult nerve. The first cells (fibroblasts) outwandering from the unpredegenerated explant were seen by 2 to 4 days *in vitro*, but it was not until the fifth or sixth transplantation that the Schwann cells were the predominant cell type. Schwann cell division was noted to be poor on plastic necessitating the use of Schwann cell mitogens (glial growth factor and forskolin) (Morrisey *et al*, 1991).

These results parallel mine in that outgrowth from unpredegenerated nerves was poor and primarily fibroblastic. My experiments identified better outgrowth from predegenerated adult nerves, and even better outgrowth from neonatal nerves. There are, of course, practical constraints to doing this with human nerve. The use of substrates such as collagen plus Matrigel, would have enhanced the culture conditions of my experiments compared with Morrisey *et al.* However, all of my experiments were performed under the same culture conditions and, for my purposes, I found that neonatal nerves were a vastly superior tissue source compared with adult nerve, predegenerated or not. The Schwann cells produced by Morrisey *et al*, (1991) were still functional with respect to interacting with neurites and myelination.

3.3 CULTURE OF CERVICO-SYMPATHETIC TRUNK

3.3.1 Introduction

Because of the requirement to find Schwann cells which were not expressing P_0 mRNA, a method was devised to culture Schwann cells from the cervico-sympathetic trunk (CST) which is virtually entirely unmyelinated. Studies on the rat CST have identified an average of just over 4000 fibres per nerve, of which an average of 1.01 % of fibres are myelinated. The unmyelinated fibres occur in cluster of 1 to 20 fibres and each fibre is embedded within Schwann cell cytoplasm. The fibres range in diameter from 0.1 to 1.1 μm with a modal diameter of 0.4 μm. Occasional neurons have been observed along the course of the CST outwith the ganglia (Dyck and Hopkins, 1972).

3.3.2 Materials and Method

Adult Sprague-Dawley rats were killed by overdose of halothane anaesthetic. With the animal in dorsal recumbency, a ventral incision was made from the manubrium to the larynx and blunt dissection technique was used between the ventral muscles of the neck and continuing deep to either side of the trachea. The cranial cervical ganglion was located deep to the carotid artery just caudal to the mandible. Caudally from this ganglion, the CST could be traced running in the carotid sheath to the thoracic inlet. It was excised just caudal to the ganglion and just cranial to the thorax and placed in L15 medium. Under a dissecting microscope, the nerve was carefully desheathed, and minced using two scalpel blades in a scissor like fashion in a sterile plastic petri dish and then placed on substrate-coated coverslips as for sciatic explants. Approximately 8 to 12 small explants could be obtained from one CST.

3.3.3 Results

Significant Schwann cell outgrowth occurred from these explants within about 1 week. They were not quite as vigorous in their outgrowth as those from the adult, neurotomized sciatic nerve, but were more vital than those from unpredegenerated adult sciatic nerve. Fibroblastic contamination was almost non-existent in these cultures. The Schwann cells exhibited the same morphology as those derived from myelinated fibres and tended to adopt the loose swirling pattern or a reticular pattern (fig. 3.6). Occasional cells had both long fine processes and short, broader processes.

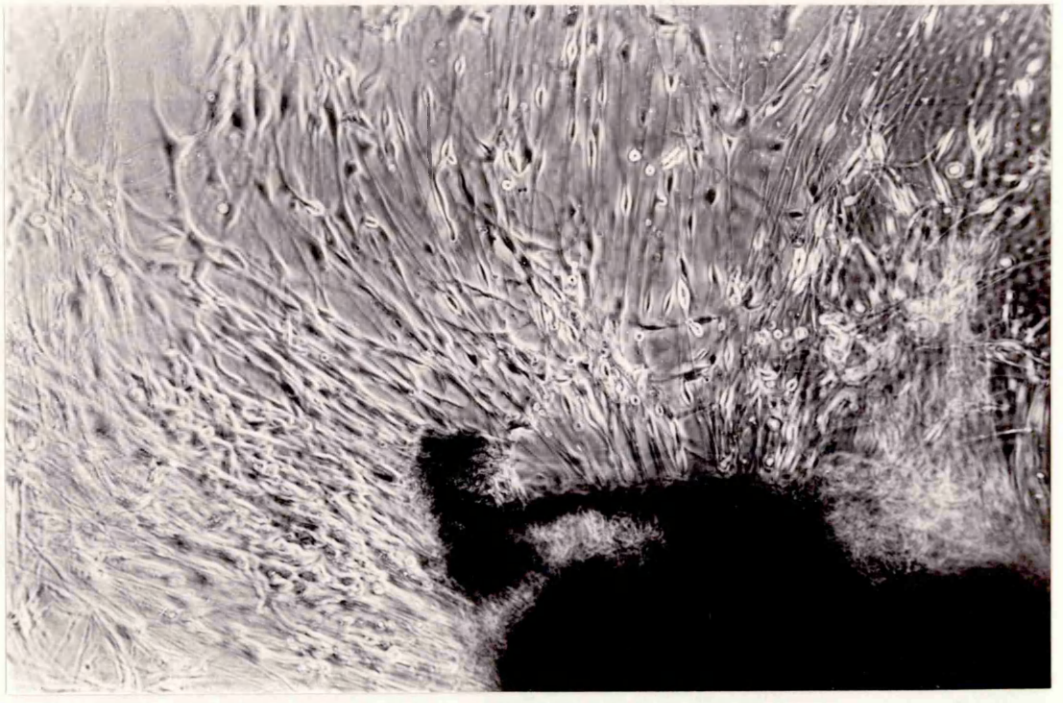


Figure 3.6: Schwann cells derived from cervico-sympathetic trunk explants. Explants (bottom right) of the predominantly unmyelinated cervico-sympathetic trunk were cultured on collagen plus Matrigel for 11 days. The Schwann cells are characteristically spindle shaped, and very few fibroblasts are noted. Living cultures (x 75).

Multiple layers and not just monolayers were sometimes noted.

3.3.4 Discussion

The CST provides a source of tissue from which non-myelin-forming Schwann cells can be derived. Although the Schwann cells took longer to grow out from the explant than from predegenerated sciatic nerve explants, they still exhibited similar morphologic features to those of sciatic origin. Their outgrowth was more exuberant than that from uncut sciatic nerve. This may be due to their axonal relationship which is one of ensheathment rather than myelination. Thus, sheath degeneration would not have to occur prior to migration. This tissue provides a useful source of Schwann cells which are naive with respect to formation of myelin-specific molecules.

3.4 SCHWANN CELL MARKERS

3.4.1 Introduction

Positive identification of cells in culture really requires use of cell-specific markers as morphologic characteristics overlap between Schwann cells and fibroblasts (Brockes *et al.*, 1979; Brockes *et al.*, 1981; Ernyei and Young, 1966). The markers used in these studies included S100, GalC, O4 and laminin (see section 1.3.2.2)

3.4.2 Materials and Method

Schwann cells were dissociated from neonatal rat pup sciatic nerves, or explants were created. The dissociated cells were established on poly-L lysine substrate or collagen/Matrigel mixture, whilst the explants were cultured on collagen or the mixture. Adult Schwann cells were cultured from neurotomized sciatic nerves or CST explants.

The general methodology for immunofluorescence and PAP techniques is given in section 2.3.1. Briefly for surface markers such as O4, GalC and laminin, the cells were immunostained prior to fixation in glacial acetic acid, whilst for S100, which is a cytoplasmic marker, the cells were fixed in 4.0% paraformaldehyde prior to staining. This prior fixation was necessary to permeabilize the cell and allow the antibodies to enter. For double labelling, the cells were labelled with one primary antibody; washed and then labelled with the second primary antibody; washed and

incubated with the secondary antibodies (linked to different dyes) combined in the same diluent. Positive controls for GalC and O4 were dissociated neonatal Schwann cells which had been *in vitro* for less than 24 hours. The positive control for laminin was Matrigel spread over a coverslip, without cells, and fixed in 4.0% paraformaldehyde. Negative controls were freshly (< 24 hours) dissociated cells on which the primary antibody was replaced with tissue culture medium. Intracytoplasmic distribution of cell surface markers (GalC and O4) was also investigated after prior fixation of the cells.

3.4.3 Results

Results are given in table 3.1. Negative controls were all negative. Dissociated neonatal Schwann cells cultured for less than 24 hours (day 0 cells), on poly-L lysine or collagen/Matrigel, exhibited bright surface fluorescence for O4 and GalC. At 5 days *in vitro*, both dissociated and explant cells were negative for surface GalC. However, only the dissociated cells on poly-L lysine were negative for O4, whilst some explant (neonatal, adult sciatic, or, rarely, CST) cells had bright, fine granular surface staining and many of day 5 dissociated cells on collagen/Matrigel had moderate to bright staining for O4 (fig. 3.7). There was also a moderate amount of intracytoplasmic binding of the antibody to O4 on denervated sciatic Schwann cells.

Day 0 dissociated cells on poly-L lysine had fine, patchy laminin staining, whereas those on collagen/Matrigel had a thicker, brighter laminin coating which persisted during culture (fig 3.8A and B). Explant cells grown on collagen/Matrigel also retained a coating of laminin during culture (fig. 3.2., section 3.2.3), but no such staining was seen on dissociated cells grown on poly-L lysine or collagen (fig. 3.8C and D).

Adult sciatic and CST Schwann cells were negative for surface GalC. However, on fixed sciatic cells, anti-GalC identified the presence of bright intracytoplasmic globules at the perinuclear poles in the sciatic Schwann cells (fig. 3.9). The S100 cytoplasmic stain was specific for Schwann cells of any origin and brightly stained them, but not the fibroblasts (fig. 3.10).

3.4.4 Discussion

Schwann cell expression of GalC and O4 is dependent upon axonal contact, and when Schwann cells are removed from contact, they down-regulate these molecules (Eccleston *et al.*, 1982; Mirsky *et al.*, 1980; Mirsky *et al.*, 1990a). In the

CELL TYPE ADULT	LAMININ Surface	Cytoplasmic	O4 Surface	Cytoplasmic	GALC Surface	Cytoplasmic	S100 Cytoplasmic
Sciatic - 9 days IV	+ on C/M - on collagen	weak + ND	small patches of cells +	+	-	+ (globules)	+
CST - 8 days IV	ND	ND	rare +	ND	-	ND	+

NEONATAL	LAMININ Surface *Poly-L lysine*	*C/M*	O4 Surface *Poly-L lysine*	*C/M*	GALC Surface *Poly-L lysine*	*C/M*	S100 Cytoplasmic *Poly-L lysine*	*C/M*
Dissoc. - <24h IV	faintly +	+	+	+	+	+	+	ND
Dissoc. - 5d IV	-	+	-	variably +	-	-	ND	ND
Explant - 5d IV	ND	+	ND	variably +	ND	-	ND	+
Explant - 11d IV	ND	ND	ND	variably +	ND	-	ND	ND

Table 3.1: Immunofluorescence results on Schwann cells cultured from chronically neurotomized adult sciatic explants, adult cervico-sympathetic trunk (CST) explants, or neonatal sciatic nerve. Cells from the neonatal animals were either dissociated or cultured as explants. Surface staining (Surface) was performed prior to fixation and cytoplasmic staining (Cytoplasmic) was performed after fixation with 4% paraformaldehyde. Dissociated neonatal cells were grown on either poly-L lysine or collagen plus Matrigel (C/M). Adult Schwann cells were grown on collagen plus Matrigel, or rarely collagen substrate only. IV = *in vitro* and refers to the duration of culture; ND = not done; d = days; h = hours.

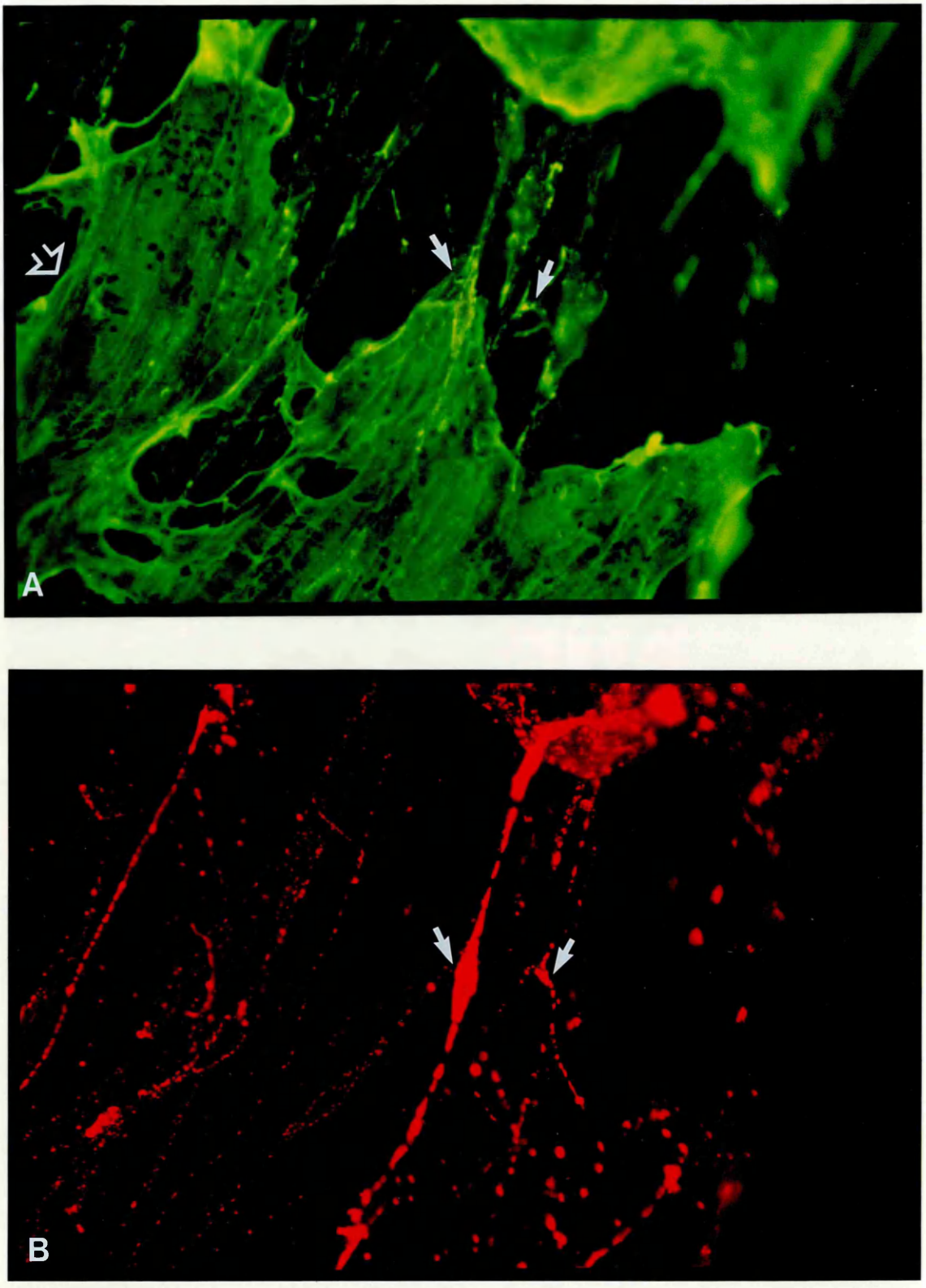


Figure 3.7: **Dissociated neonatal Schwann cells on collagen plus Matrigel double-stained for laminin and O4 surface marker.** (A) Dissociated cells cultured for 5 days and stained for surface laminin using fluorescein labelled antibody. Both the substrate (open arrow) and the Schwann cells (arrows) stain positively for laminin. Cells located within thick deposits of substrate can just be discerned (arrows). (B) The same culture stained for surface O4 using rhodamine labelled antibody. The same cells in both pictures have been arrowed. Original in colour (x 420).

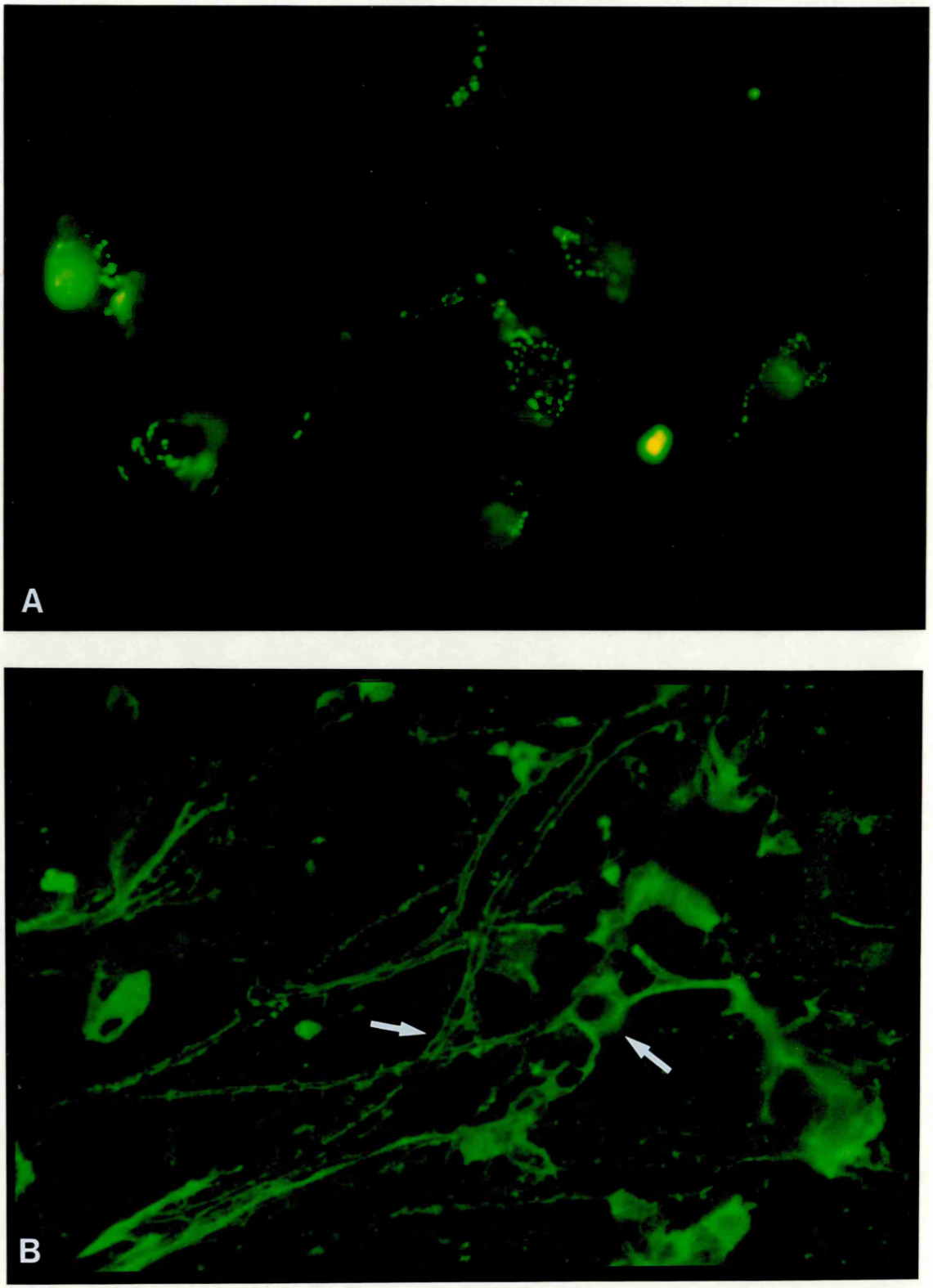


Figure 3.8: The effect of culture substrate on laminin coating of cultured Schwann cells. Cells are stained for surface laminin and photographed under fluorescein optics. (A) Dissociated neonatal Schwann cells cultured for less than 24 hours on poly-L lysine; the laminin coating is patchy and incomplete. (B) Cells from the same dissociation cultured for less than 24 hours on collagen plus Matrigel; the laminin coating on the cells is thick (arrows) and virtually continuous. Original in colour (x 420).

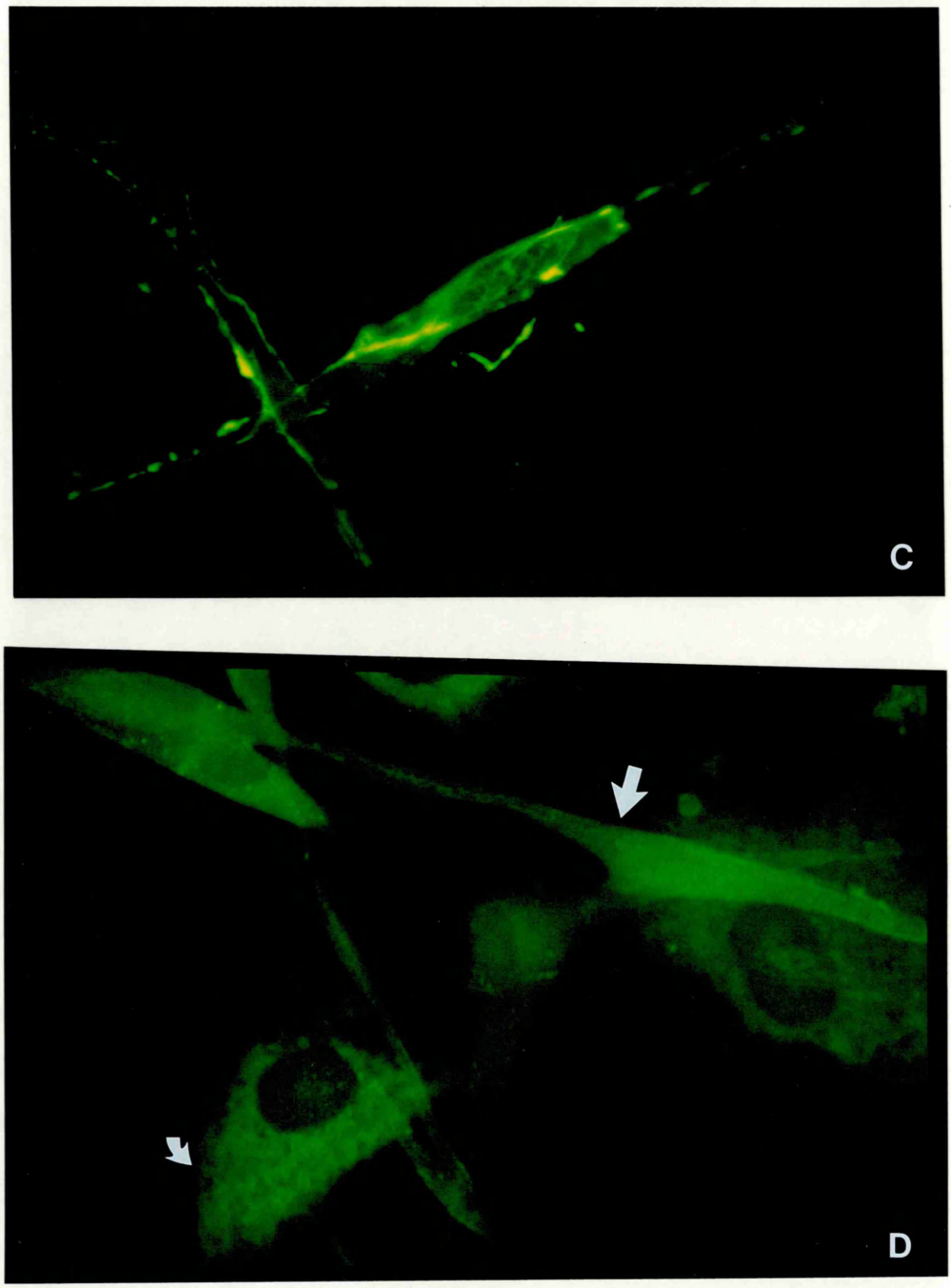


Figure 3.8 (cont.): The effect of culture substrate on laminin coating of cultured Schwann cells. Cells are stained for surface laminin and photographed under fluorescein optics. (C) Dissociated neonatal Schwann cells cultured for 10 days on collagen plus Matrigel substrate. The laminin coating is still very obvious, although somewhat patchy. (D) Cells from the same dissociation cultured for 10 days on collagen only. The Schwann cells are not expressing surface laminin (arrow); nor are the fibroblasts (curved arrow). Original in colour (x 420).

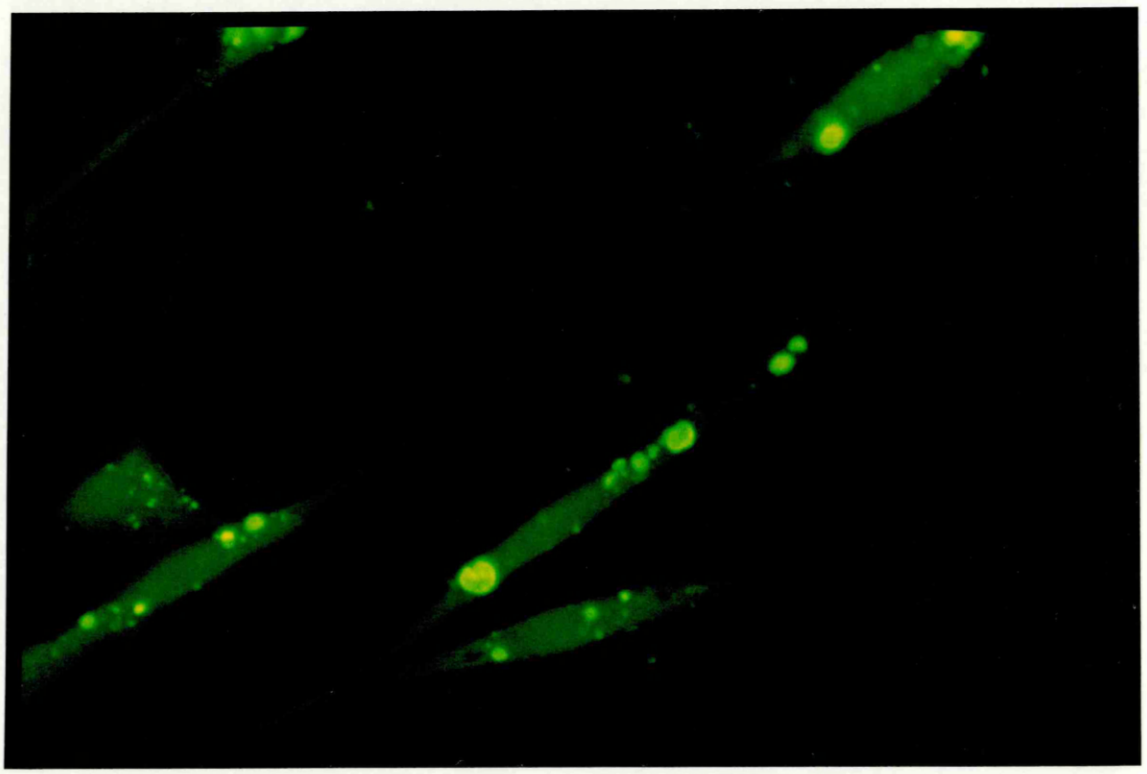


Figure 3.9: Intracytoplasmic staining for GalC in cultured adult sciatic Schwann cells. Schwann cells derived from adult sciatic nerve transected 3 weeks prior to establishing cultures. The explants were cultured on collagen plus Matrigel substrate, for 9 days prior to fixing with 4% paraformaldehyde and staining for intracytoplasmic GalC. Bright GalC positive globules are visible in the poles of the Schwann cells, presumably representing myelin debris. Original in colour (x 420).

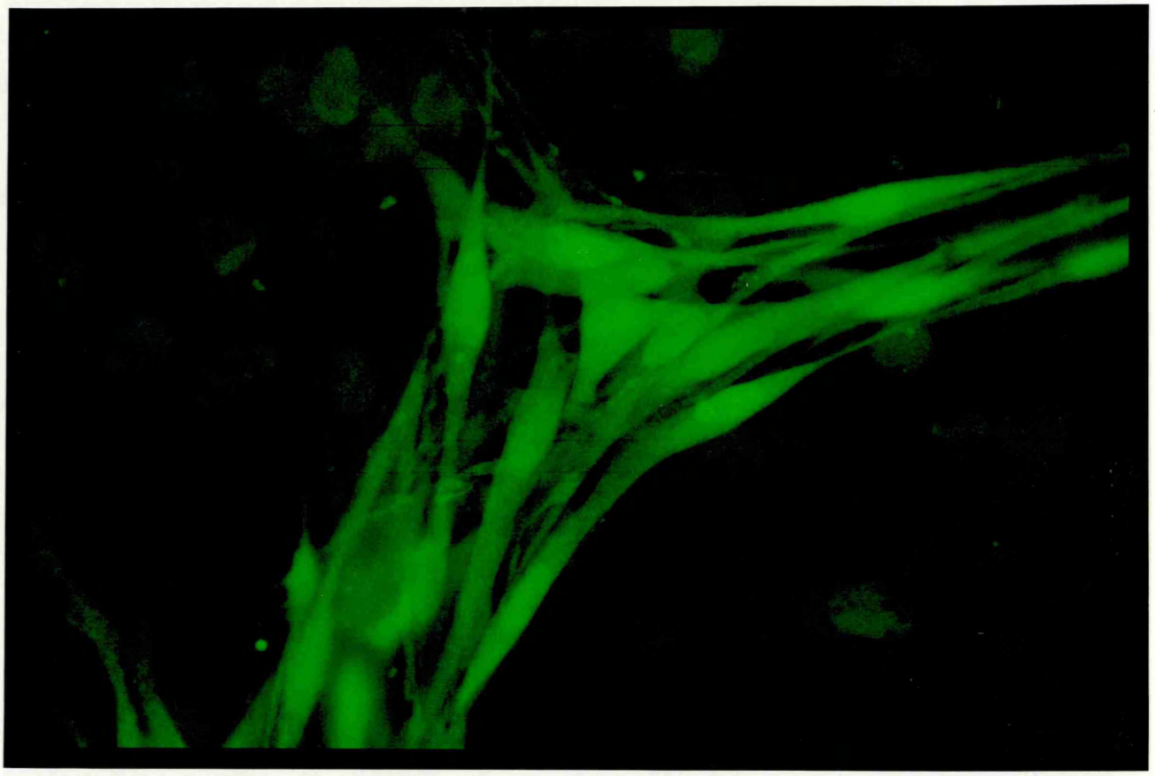


Figure 3.10: Schwann cells stained for S100. Cultures of Schwann cells were established from adult sciatic nerve which had been transected 6 weeks prior to harvesting the nerve. The explants were cultured on collagen plus Matrigel for 11 days prior to fixing with 4% paraformaldehyde and staining with S100. The cells were photographed under fluorescein optics. The spindle shaped Schwann cells are positively stained, but the broader, more sheet like fibroblasts are essentially negative. Original in colour (x 420).

present studies, these phenomena were observed on both adult and neonatal Schwann cells. There was one interesting modification to this rule; the slowed down-regulation of O4 expression on neonatal Schwann cells cultured on basement membrane. Double labelling studies using O4 and laminin identified the presence of surface laminin on O4-positive cells. The amount of O4 staining was not always proportional to the intensity of laminin staining. However, dissociated neonatal Schwann cells cultured on poly-L lysine under identical conditions did not express the O4 marker, nor any significant amounts of laminin, at day 5 *in vitro*. Only those cells that were cultured on basement membrane expressed this marker. It has been suggested that the development of O4 and AOO7 expression on Schwann cells may be related to the appearance of basal lamina. Both these molecules and basal lamina probably appear at a similar time in the developing rat sciatic nerve (Mirsky *et al.*, 1990a). The results of the studies in this work support that theory. Alternatively, the expression of O4 may represent a more differentiated state of the Schwann cell as it is not expressed much at embryonic day 15, but 99% of Schwann cells express it at birth (Mirsky *et al.*, 1990a) and the presence of basement membrane may contribute to this differentiation.

The loss of surface GalC from the cells was expected as explant cells are not in contact with an intact axon. However, the presence of intracytoplasmic globules in adult Schwann cells obtained from axotomized nerves was interesting but, again, not unexpected. Small membrane bound inclusions of myelin debris have been observed within Schwann cells between 1 and 2 months after axotomy (Lassman *et al.*, 1978) and Schwann cells were thought to have a role in the clearing of myelin debris during Wallerian degeneration (Pellegrino *et al.*, 1986; see section 1.5.2.1). In cultures of dissociated Schwann cells, myelin debris was still found associated with the Schwann cells after 2 weeks (Dubois-Dalcq *et al.*, 1981).

3.5 SCHWANN CELL PROLIFERATION

3.5.1 Introduction

Addition of antimitotic agents to these cultures would have been beneficial in controlling the proliferation of the fibroblasts. Their use has been discussed in section 1.3.2.1. However, such agents were noted to decrease the outgrowth of Schwann cells from explants (Wrathall *et al.*, 1981a). Concerns about the agents' cytotoxicity interfering with subsequent cellular studies (Askanas *et al.*, 1980) or membrane component biosynthesis (Poduslo and Windebank, 1985b) have also been raised. The

above studies supported their non-usage in the experiments here. Also, fibroblasts may have a supportive role in Schwann cell culture. Basement membrane defects in cultured Schwann cells and axons from dystrophic mice were substantially corrected by culturing the cells on normal fibroblasts (cited in Bunge *et al.*, 1986).

3.5.2 Materials and Method

The mitotic index of the cultures was assessed by labelling the dividing cells with [^{3}H] thymidine. The thymidine was supplied as methyl tritiated thymidine (Amersham) with a specific activity of 75 Ci/mM. It was an aqueous solution, containing 2% ethanol, which was evaporated to dryness under a stream of nitrogen and resuspended in Hank's balanced salt solution at a concentration of 5 μCi/μl. A dose of 1.0 μCi/500 μl of medium was added as a single dose or on consecutive days for a total of 3 days. If consecutive doses were given, the medium was changed prior to the addition of the next dose.

Dissociated Schwann cells were cultured on both poly-L lysine and collagen/Matrigel. Neonatal and adult explants were cultured on collagen/Matrigel. Cytosine arabinoside was added to some dissociated and explanted neonatal Schwann cells on the second day *in vitro* at 0.01 mM for 3 days. Two days after removing the antimitotic, [^{3}H] thymidine was added for 24 hours. The adult explants were produced from sciatic nerve that had been neurotomized 6 weeks previously and thymidine was added on day 11 *in vitro*. The cells were fixed and stained for S100 using the peroxidase-antiperoxidase method (section 2.3.1.2) to distinguish Schwann cells from fibroblasts prior to processing for autoradiography using K2 emulsion (section 2.4.6.3). The cells were exposed for 7 days at 4^{o}C prior to developing. After development the cells were counterstained with haematoxylin (A3.1.2) and 300 to 500 Schwann cells/sample were counted to establish the percent labelled with [^{3}H] thymidine.

3.5.3 Results

The mitotic index of Schwann cells from adult sciatic nerve tissue was 36% for cells labelled for 3 days and 20% for cells labelled for 1 day.

For dissociated Schwann cells cultured on poly-L lysine without cytosine arabinoside, the mitotic index was 25%, whereas the mitotic index for cells cultured with the drug was only 2.1% (table 3.2). For dissociated cells cultured on collagen plus Matrigel, the mitotic index was 35.7% in the absence of cytosine arabinoside,

	MITOTIC INDEX (%)	
SCHWANN CELL SAMPLE	**WITHOUT CYTOSINE ARABINOSIDE**	**WITH CYTOSINE ARABINOSIDE**
Dissociated Schwann cells - on poly-L lysine	25.0	2.1
Dissociated Schwann cells - on collagen/Matrigel	35.7	19.8
Neonatal explants - on collagen/Matrigel	31.7	17.8

Table 3.2: The effect of addition of cytosine arabinoside on the mitotic index of cultured Schwann cells. Twenty-four hours after establishing neonatal explants or dissociated Schwann cell cultures, cytosine arabinoside (0.01 mM) was added to half the cultures for three days. The antimitotic was removed on day 4, and 1.0 uCi/500 ul of medium of [^{3}H] thymidine was added for 24 hours, the cultures fixed, stained by the PAP method for S100, and dipped in K2 emulsion (Ilford) for autoradiography (7 days exposure at 4^{o}C.). After development, nuclei were counterstained with haematoxylin and 300 to 500 Schwann cells/sample were counted to establish the mitotic index.

whereas it was reduced to 19.8% if the cells had been exposed to the drug. Explant-derived neonatal Schwann cells had a mitotic index of 31.7% in the absence of cytosine arabinoside and only 17.8% if cultured in the presence of the drug. The addition of cytosine arabinoside to the explant cultures did decrease the fibroblast component of the outgrowth markedly, but it also decreased the Schwann cell outgrowth and proliferation rate (fig. 3.11).

3.5.4 Discussion

Two pertinent findings are suggested by these studies. Firstly, the addition of the antimitotic agent, cytosine arabinoside, to Schwann cell cultures to control fibroblastic proliferation also inhibits Schwann cell proliferation. Secondly, the mitotic index of Schwann cells cultured on basement membrane is overall higher than if cultured on just poly-L lysine and the addition of cytosine arabinoside, even to these cells, has a deleterious effect on Schwann cell proliferation. The reduction in the mitotic index is not as marked as that observed in Schwann cells cultured on poly-L lysine, but it is still obvious.

It has been suggested that the basal doubling time for pure Schwann cells cultured in growth medium supplemented with 10% foetal calf serum, after treatment with cytosine arabinoside is 7 to 8 days (Raff *et al.*, 1978b). This implies a higher mitotic index (12.5 to 14.2%) for dissociated neonatal Schwann cells cultured on poly-L lysine than observed in the studies described in this chapter. Schwann cells cultured in 10% FCS, after treatment with cytosine arabinoside, had a mitotic index of 6.0% (Eccleston *et al.*, 1987). The mitotic index recorded by Eccleston *et al.* (1987), and Raff *et al.* (1978b), is still lower than that observed here in dissociated neonatal Schwann cells cultured in the absence of cytosine arabinoside. The fact that Schwann cells exhibit strong density-dependent inhibition of cell division (Morgan *et al.*, 1991) may account for some of the variablility in results between different experiments and different laboratories. My own conclusion is that the addition of cytosine arabinoside to Schwann cells cultured under different conditions, does inhibit Schwann cell proliferation as well as fibroblastic proliferation. As many mitotic figures have been observed in Schwann cells outgrowing from neonatal rat sciatic nerve explants (Brunden *et al.*, 1990b) these cells would be vulnerable to the effect of this drug. The presence of myelin debris in dissociated Schwann cell cultures (Dubois-Dalcq *et al.*, 1981) may act as a Schwann cell mitogen (see section 1.3.1.4). So the addition of an antimitotic agent during a period in which Schwann cells have been stimulated to divide is likely to be deleterious to Schwann cell numbers. In my

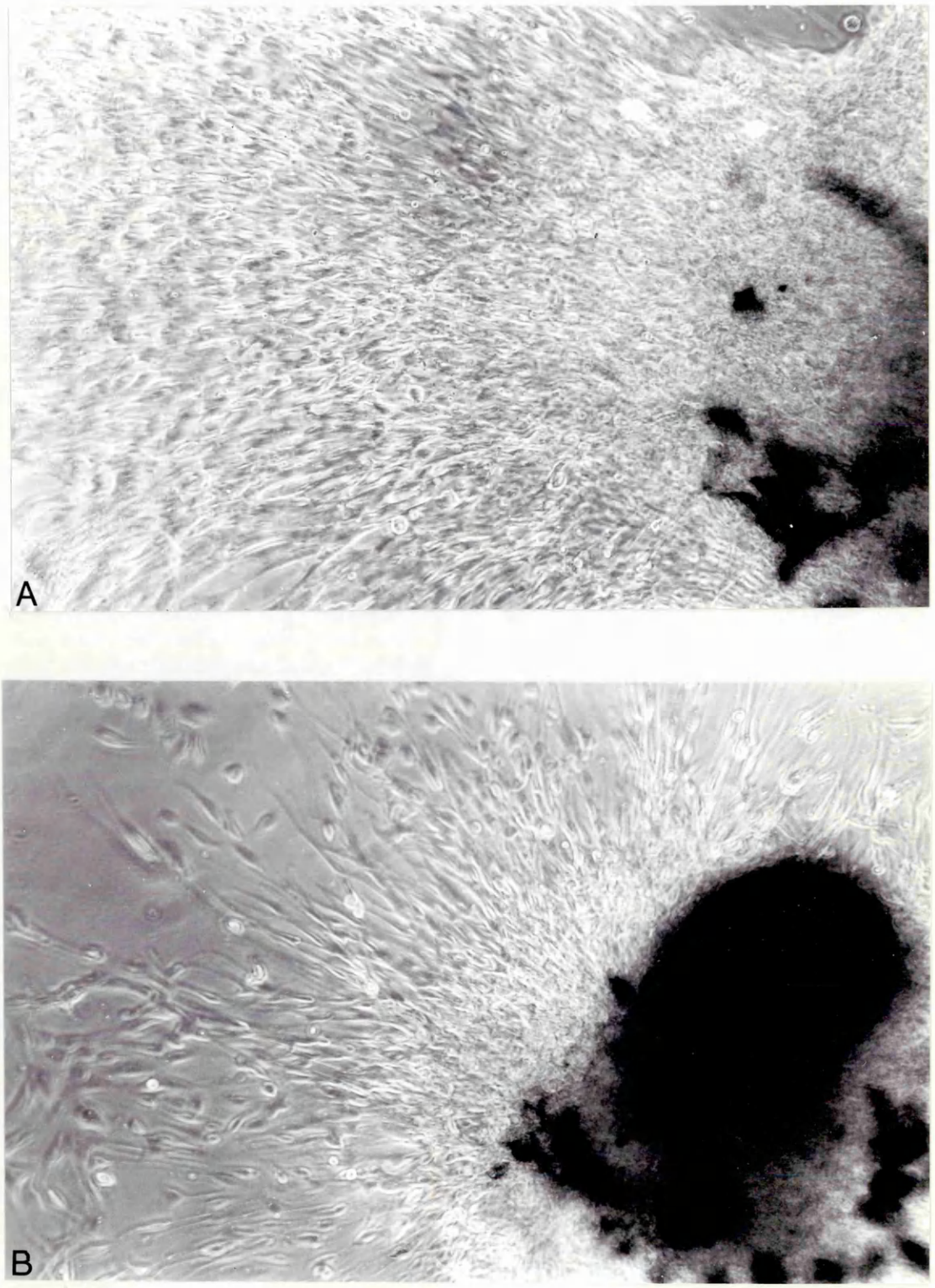


Figure 3.11: The effect of cytosine arabinoside on Schwann cell growth. (A & B) Neonatal explants (bottom right) cultured on collagen plus Matrigel substrate for 5 days. (A) Schwann cells cultured from an explant in the absence of cytosine arabinoside. (B) Schwann cell culture to which cytosine arabinoside (0.01 mM) has been added for 3 days beginning on the second day of culture. The density of the cellular outgrowth is greatly reduced by the addition of the antimitotic agent. Living cultures (x 75).

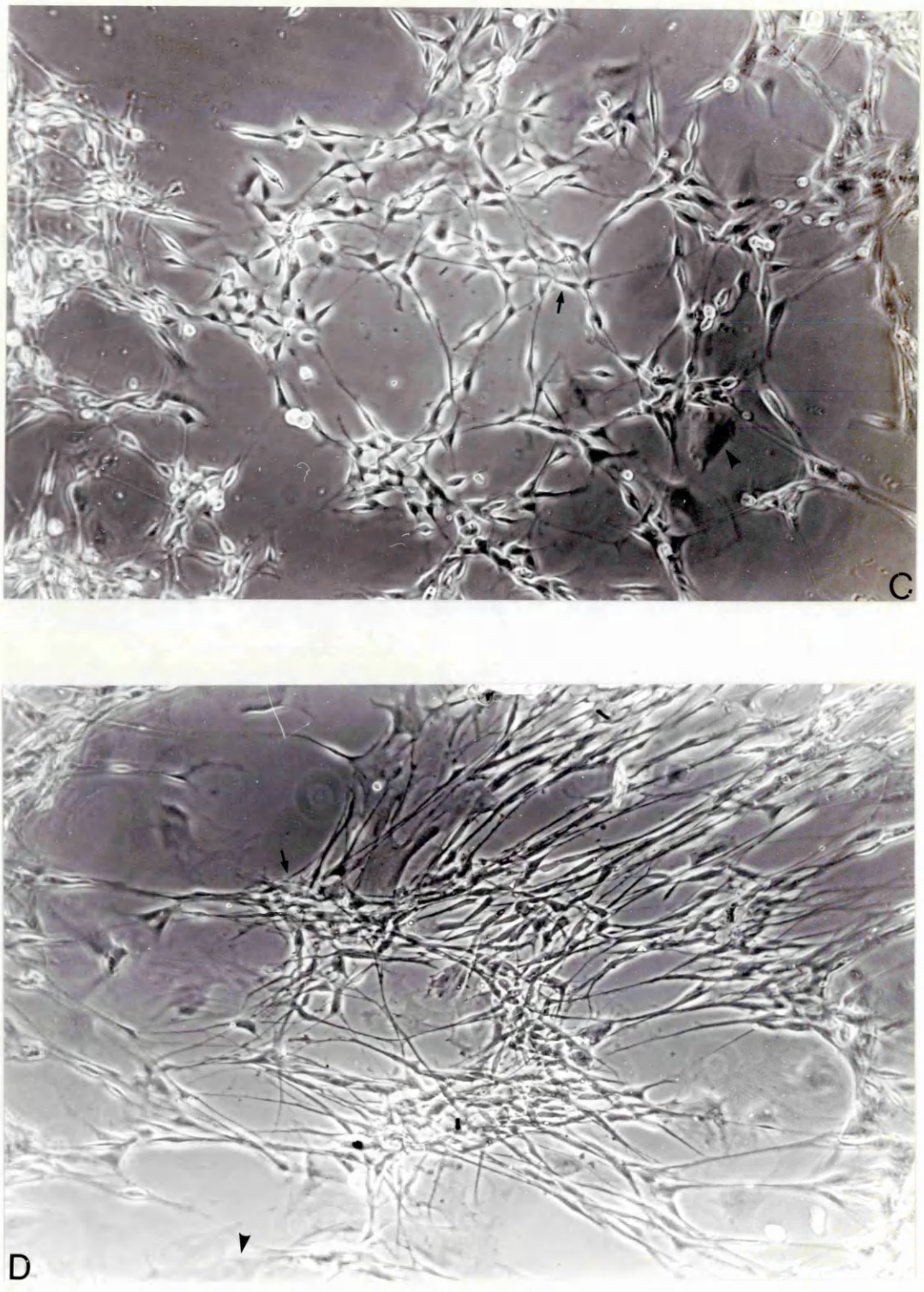


Figure 3.11 (cont.): The effect of cytosine arabinoside on Schwann cell growth. (C) Neonatal dissociated Schwann cells cultured on poly-L lysine for 5 days and (D) on collagen plus Matrigel for 5 days. Cytosine arabinoside (0.01 mM) had been added for 3 days beginning on the second day of culture. The significance of these pictures is the relative purity of the Schwann cell population (arrow) and the lack of fibroblastic contamination (arrow head) due to treatment with cytosine arabinoside. Living cultures (x 75).

studies, the outgrowth of Schwann cells from neonatal explants was reduced to about half when cytosine arabinoside was added which supports the findings of Dubois-Dalcq *et al.*, (1981) and Wrathall *et al.*, (1981a). Other antimitotics have also been shown inhibit Schwann cell proliferation (Hall and Gregson, 1975; Hall and Gregson, 1978b).

Proliferation in dissociated Schwann cell cultures was noted to be markedly increased by the presence of basement membrane components (Eccleston *et al.*, 1987; McGarvey *et al.*, 1984). The overall increased proliferation rate of Schwann cells observed when they are cultured on basement membrane, may relate to some of the lesser components of Matrigel. Growth factor of platelet and fibroblast origin, as well as transforming growth factor, have been shown to stimulate Schwann cell proliferation (Davis and Stroobant, 1990). Matrigel is known to contain both transforming growth factor-ß and fibroblast growth factor (Collaborative Research Incorporated, 1991).

The non-linear relationship of the mitotic index between the 1 day pulse and 3 day pulse of the adult Schwann cells in my studies may, again, relate to the the slowing of proliferation due to contact inhibition (Morgan *et al.*, 1991). It is possible that radioactive thymidine may have an effect on cell proliferation after more prolonged exposure.

3.6 CONCLUDING REMARKS

In recent years, the most popular form of producing Schwann cells for culture has been by mechanical and enzymatic dissociation of neonatal sciatic nerve. My search for another way of culturing Schwann cells was stimulated by questions raised about the ability of neonatal dissociated Schwann cells to respond to various manipulations (see chapter 7). Much time was spent in establishing the "in house" methodology for creating explants of nerve tissue and having them reliably attach. The idea of explant culture is by no means novel.

The level of differentiation of cultured Schwann cells was an important consideration (see chapter 7). Previous work had established that the presence of a basement membrane was an important part of Schwann cell differentiation. Enzymatic treatment of Schwann cells is known to disrupt endogenous basement membrane and, in the absence of neurons, it will not reform around cultured Schwann cells (see section 1.3.3.2). The addition of exogenous basement membrane to cultures of both dissociated and explant Schwann cells was successful in imbuing the cells with a coating of at least laminin, or more likely, a more complete basement membrane. The

justification for using collagen type I as well, is given in chapter 7. The role of basement membrane in encouraging a more advanced state of Schwann cell differentiation is suggested by the prolonged expression of O4, and the better morphologic characteristics of the Schwann cells.

Schwann cells are successfully cultured from explants of both adult and neonatal, myelin-forming and non-myelin-forming tissue. The most vigorous tissue source was the neonatal nerve. Schwann cells could also be readily cultured from adult nerve, but this was best achieved if the nerve had degenerated somewhat *in vivo* prior to establishing cultures. Nerve tissue does degenerate *in vitro* given time, but this is known to be slow in the absence of significant macrophage activity.

The use of the antimitotic cytosine arabinoside did decrease the degree of fibroblastic contamination of all cultures. However, using [^{3}H] thymidine, it was observed that the addition of this agent also markedly decreased the proliferation of Schwann cells.

In conclusion, the production of large numbers of Schwann cells which had not been subjected to the rigors of mechanical and enzymatic dissociation, was possible by producing explants of nerve tissue. The best results were achieved using neonatal tissue and culturing the cells on a substrate containing Matrigel, in the absence of antimitotics.

CHAPTER 4: THE IMPORTANCE OF THE AXON IN EXPRESSION OF MYELIN SPECIFIC MOLECULES BY THE SCHWANN CELL

4.1.INTRODUCTION

Loss of axonal contact by myelin-forming Schwann cells causes down-regulation of genes encoding myelin-specific molecules. This is observed with Wallerian degeneration *in vivo* (Gupta *et al.*, 1988; Lemke and Chao, 1988b; Poduslo 1984; Trapp *et al.*, 1988b; see section 1.5.3.2) and on cultured dissociated Schwann cells (Mirsky *et al.*, 1980; Morrison *et al.*, 1991; Owens and Bunge, 1989; Sobue and Pleasure, 1984; Winter *et al.*, 1982; see sections 1.3.2.3 & 1.5.3.3).

4.2: EFFECT OF PERMANENT OR TEMPORARY AXOTOMY ON SCIATIC NERVE IN RATS AND MICE

4.2.1 Introduction

The effect of either permanent or temporary axotomy on expression of myelin-specific genes has been widely studied (see section 1.5.3.2). The sensitivity of the axon-glia interdependence was reflected in the rapid down-regulation of the mRNAs encoding P_0 and MBP within 24 hours of crush injury (Gupta *et al.*, 1989). This axonal control of the mRNA expression is at the transcriptional level (Trapp *et al.*, 1988b). Re-establishment of axonal contact during reinnervation of the distal stump after nerve crush injury is also reflected in the level of these mRNAs (Le Blanc *et al.*, 1987) and corresponds well with the advancement of the regenerating neurites into the stump (Mitchell *et al.*, 1990). The aim of this section was to establish base line data for the effect of axotomy and reinnervation on P_0 mRNA expression and the accompanying morphological changes.

4.2.2 Materials and method

Sciatic neurotomies were performed as described in section 3.2.2 on adult Sprague-Dawley rats or C57Bl/6 mice of either sex. Nerve crush experiments were performed on similar rats but after exposing the nerve, a pair of Taab (No.5) forceps were clamped over the nerves, using hand pressure, for 10 seconds and a marker

suture of 6/0 silk placed in the perineurium. The crushing resulted in a blanching and dissolution of the perineurial contents and the nerve was only held in continuity by the perineurial covering. The nerves were allowed to undergo Wallerian degeneration *in vivo* for 5 or 7 days at which time the animals were killed and the distal stumps of the nerves harvested. For *in situ* and LM studies, the animals were perfused as described in section 2.2.1 with 4% paraformaldehyde/0.1% glutaraldehyde, and the contralateral normal nerve and the distal stump of the axotomized nerve harvested and immersed in fixative until required. For EM studies strong fixative was used for perfusion (A2.2). Both transected and crushed/reinnervating distal stumps were cut into 2.5 mm lengths working distally from the transection or marker suture. These segments were labelled A to G, beginning proximally, and teased onto poly-L lysine-coated slides for *in situ* hybridization or morphological studies. *In situ* hybridisation, autoradiography and staining with propidium iodide and sudan black were performed as described in chapter 2 and the appendices. Only normal and permanently transected fibres were used for dot blots as blotting during re-innervation had already been performed in our laboratory (Mitchell *et al.*, 1990). Immediately after death, the whole distal stump was cleaned of the perineurial elements and frozen in liquid nitrogen. RNA extraction and blotting was performed as described in 2.4.1.2, 2.4.3.1 & 2.4.5.2. For dot blotting, 2.0 μg of RNA from each sample was loaded onto the nitrocellulose paper. The washing regime after hybridisation consisted of 1 x 15 minute wash in 2 x SSC, 2 x 30 minute washes in 2 x SSC/0.1% SDS, 2 x 30 minute washes in 0.1 x SSC/0.1% SDS and 1 x 60 minute wash in 0.1 x SSC/0.1% SDS. The filter was exposed for 7 days at -70^{o}C. Morphological examination of sections from permanently-transected nerve was only performed in mice and the preparation of these tissue sections is described in appendix A3.1.1.

4.2.3 Results

4.2.3.1 Morphological changes

The histological appearance of normal and transected mouse sciatic nerve in both longitudinal and cross section is described in chapter 5 (5.3.1.1 and 5.3.1.2).

Teased fibres were primarily used to assess the level of P_0 expression in permanently transected and crushed fibres (see 4.2.3.2). This method of fibre preparation can also be used to assess morphological changes affecting the myelin sheath. The sheath of normal myelinated teased fibre stained evenly and intensely with sudan black. It was smooth with parallel sides and the node of Ranvier appeared as an

obvious constriction. A single Schwann cell nucleus was present in the middle of each internode and occasional other nuclei, probably fibroblasts, were associated with some fibres. By 7 days post-transection, the fibre was grossly abnormal. In parts it was very distended and ballooned, whilst in other areas, it was interrupted by multiple narrow constrictions and, as Waller described so many years ago, it appeared "*like the beads of a necklace*" (Waller, 1850). Many fibres were overtly fragmented by this stage. Very little of the myelin sheath was still detected using Sudan black staining. The remaining myelin appeared as dark globules dispersed as ones and twos, or clustered in small groups within the nerve fibres (fig. 4.1B).

Reinnervated fibres were not fragmented, nor were they irregular in diameter like the degenerated fibres. Propidium iodide staining identified a dramatic increase in nuclei along these fibres although the clusters of silver grains obscured some nuclei. The stained myelin appeared similar to that of the transected fibres (fig 4.1D).

4.2.3.2 *In situ* hybridisation

The normal teased fibre had intense accumulations of silver grains in the perinuclear area, which often occluded the nucleus (fig. 4.1A). By 7 days post-transection, the signal had virtually disappeared on most fibres, although occasionally a faint perinuclear distribution of silver grains could be seen. With reinnervation there was strong, focal re-expression of P_0 mRNA at frequent intervals along the nerve fibre (fig. 4.1D). In some instances, the cluster of silver grains associated with the perinuclear region of one Schwann cell, almost contacted the cluster associated with an adjacent cell. By 7 days *in vivo*, after crushing, the re-expression of P_0 mRNA had advanced 10 to 12 mm into the distal stump.

4.2.3.3 Dot blots

Dot blots were performed on both normal rat sciatic nerve and nerve which had been transected 5 days previously and allowed to degenerate *in vivo*. The amount of RNA extracted from the degenerated nerve was approximately 5 fold more than that extracted from approximately the same amount of normal nerve. The down-regulation of P_0 mRNA expression observed on individual teased fibres after transection was substantiated on the dot blots (fig.4.2).

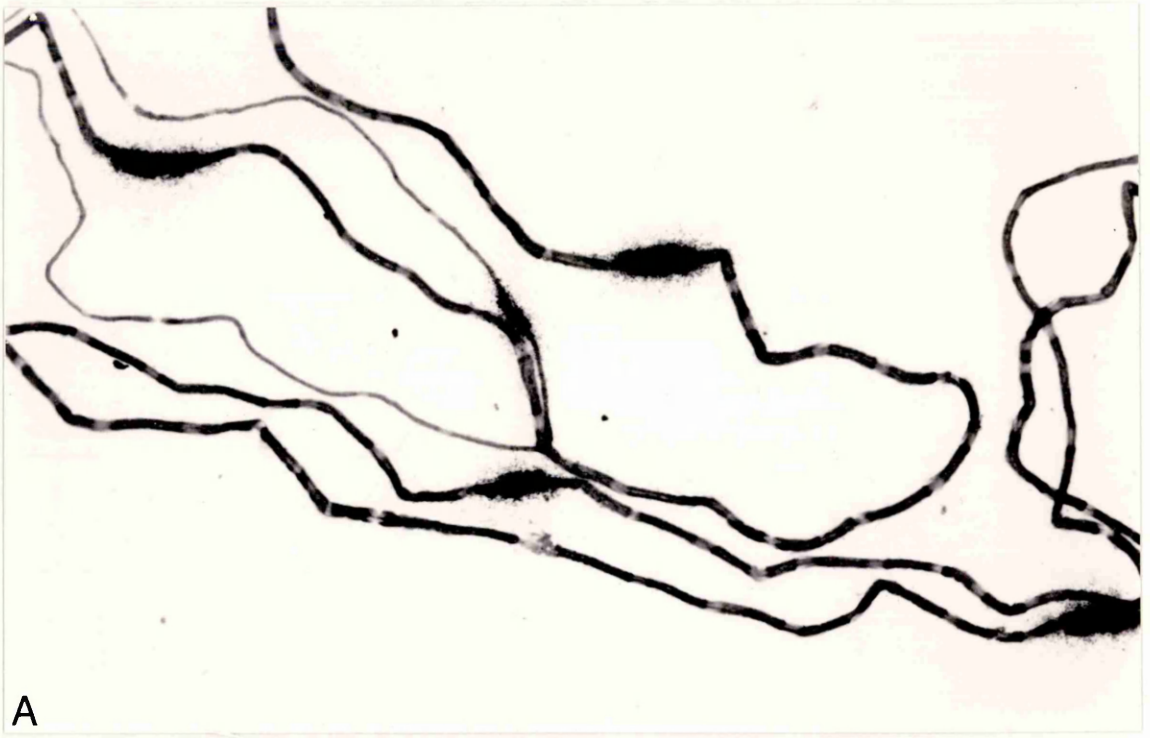


Figure 4.1: The effect of nerve crush on adult sciatic nerve P_0 mRNA expression. The normal right and distal portion of the left sciatic nerves were assessed for P_0 mRNA expression 7 days after crushing the left nerve. Bundles of fibres from the normal nerve, the reinnervated and non-reinnervated portions of the left distal nerve, were teased and processed by *in situ* hybridization. After autoradiography, the fibres were stained with sudan black. (A) Teased fibres from the normal right nerve. There is strong expression of P_0 mRNA as detected by the intense accumulation of silver grains in the perinuclear region. The myelin sheath is uniformly stained with sudan black (x 250).

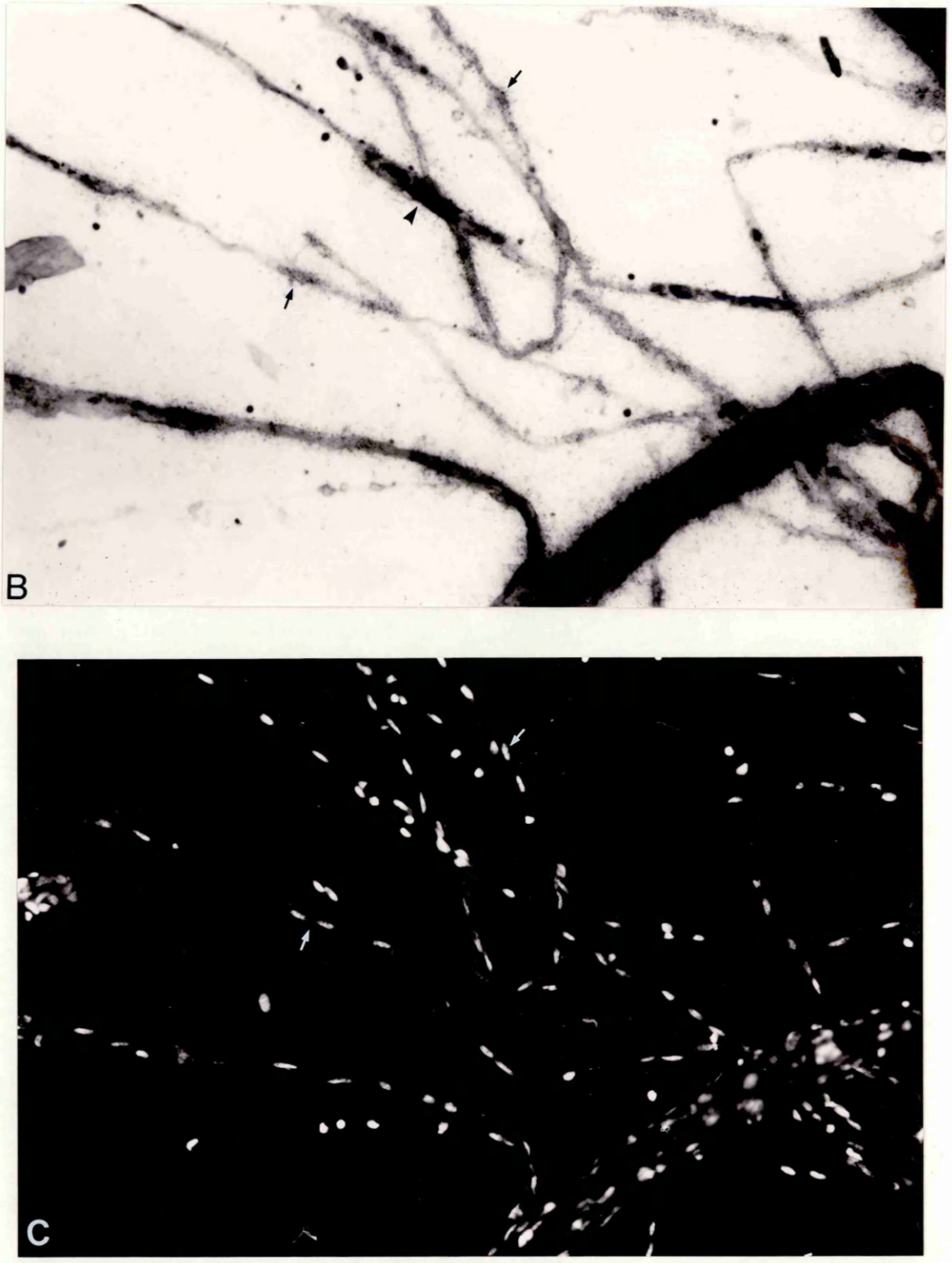


Figure 4.1 (cont.): The effect of nerve crush on adult sciatic nerve P_0 mRNA expression.(B) Teased fibres from the distal non-reinnervated portion of the nerve. Only a low intensity of silver grain expression can be observed (arrows), with no intense focal clusters as in the normal nerve. Globules of degenerated myelin are visible, stained by sudan black (arrow head). (C) The same bundle of teased fibres double stained with propidium iodide and viewed under fluorescence. Nuclei appear as white dots and are dispersed along the fibres. The same nuclei are visible in both pictures (arrows) (x 250).

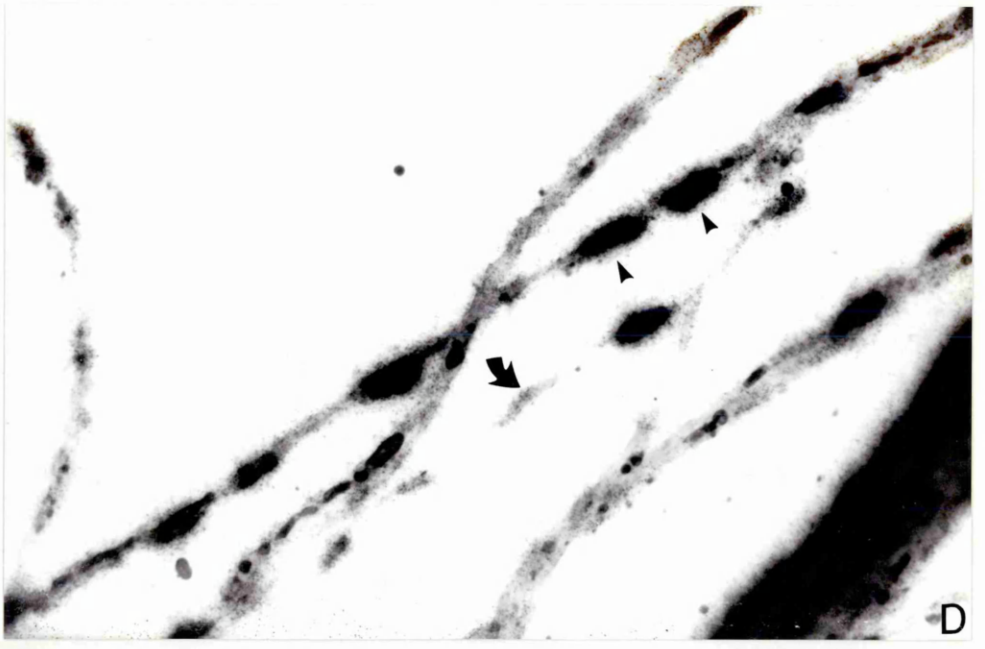


Figure 4.1 (cont.): The effect of nerve crush on adult sciatic nerve P_0 mRNA expression. (D) Teased fibres from the distal reinnervated portion of the nerve. Multiple intense clusters of silver grains occur frequently along the reinnervated fibre (arrow heads). Not all fibres have upregulated P_0 mRNA expression in response to ingrowing axons yet (curved arrow). (E) The same bundle of teased fibres double-stained with propidium iodide and viewed under fluorescence. Nuclei appear as white dots and along the reinnervated fibre, are often occluded by the intense silver grain clusters (arrow) (x 250).

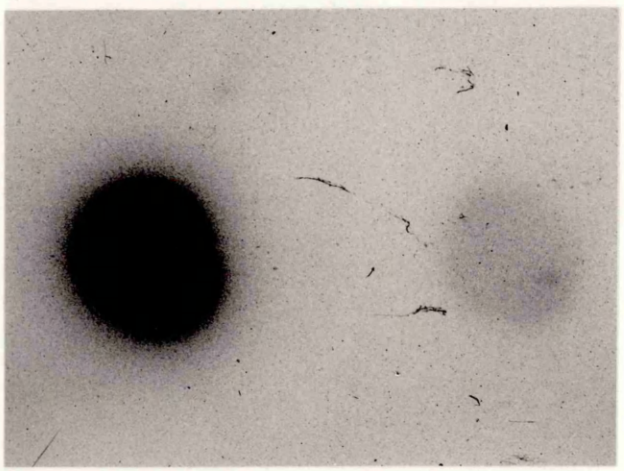


Figure 4.2: The effect of nerve transection on P_0 mRNA expression - dot blots. Sciatic neurotomies were performed on adult rats, and the nerves allowed to degenerate for 5 days *in vivo*. The normal right and transected left nerves were removed, the RNA extracted and dot blotting performed. The filter was probed with a [^{32} P] labelled cDNA P_0 probe. RNA from the normal nerve is on the left of the photograph, that from the transected nerve, is on the right. The down-regulation of P_0 mRNA is dramatic by 5 days (x 7).

4.2.4 Discussion

Cloning of major myelin protein genes has allowed the development of riboprobes to localize and quantitate cellular mRNA. Using such probes the effect of the presence or absence of axons on Schwann cell myelin gene expression can be assessed.

The intimate dependence of P_0 mRNA expression on a healthy axon-glia relationship is demonstrated by these data. Using teased fibres stained for myelin and nuclei, it was observed that within a week of axotomy, the morphological changes of Wallerian degeneration were very advanced. The fragmentation, ballooning and distortion of fibres, and myelinolysis were overt. The morphological changes of Wallerian degeneration have been well characterized (see sections 1.5.2.2 and 1.5.2.3) but mostly on tissue sections. The study of teased fibres offers another way of examining these phenomena. They also allow for simultaneous examination of other aspects of Wallerian degeneration such as down-regulation of genes encoding myelin-specific molecule expression. By 5 to 7 days post-axotomy, if reinnervation had not occurred, P_0 mRNA expression was down-regulated in all fibres. Dot blotting confirmed the dramatic down-regulation of P_0 mRNA expression due to axotomy. Reduction of myelin-specific gene expression to basal levels was observed by 5 days in previous studies on teased fibres (Mitchell *et al.*, 1990).

Previous workers have proven the efficacy of the nerve crush technique in producing complete axotomy. No intact nerve fibres were observed by electron microscopy after forceps crushing of the CST. However, the basement membranes, Schwann cell processes and nuclei were intact. The axoplasm and the myelin was dispersed in both directions away from the crush site (Dyck and Hopkins, 1972). Hence crushing induces complete severance of the axon and Wallerian degeneration ensues in the distal stump.

In this study, during reinnervation after crush injury, fibre continuity was re-established and intense up-regulation of P_0 mRNA expression was obvious. Previous work in this laboratory established that up-regulation of P_0 mRNA expression occurred 3 to 4 mm behind the advancing axonal front, but at the front itself, the level of expression was similar to the still-denervated distal nerve. Myelin sheaths developed 2.5 to 5 mm behind the zone of up-regulation. Dot blotting of RNA from pooled segments of crushed nerves identified the gradation of up-regulation of P_0 mRNA expression in response to the regenerating axon (Mitchell *et al.*, 1990). Based on the data presented by Mitchell and colleagues (1990) the actual extent of axonal regeneration in my studies would probably be 13 to 16 mm distal to the axotomy site

with the Schwann cells in the most distal few millitimetres not having upregulated P_0 mRNA expression. This period between initial axonal contact and upregulation of myelin gene expression is thought to be associated with alignment and adhesion of the cell, and axonal signalling (Mitchell *et al.*, 1990). The gradation in remyelination during reinnervation has also been studied morphologically by Politis *et al.*, (1982). In that study, only the most proximal portion of the distal stump was myelinated, whilst initial axon-glia contacts were being made in the middle portion, and no axonal advancement had occurred into the still-degenerate distal portion (Politis *et al.*, 1982).

Schwann cells also proliferate during Wallerian degeneration (see section 1.5.2.5). Staining with propidium iodide identified a marked increase in nuclear count in degenerating nerve in my studies. The majority, but not all, of these proliferated cells upregulated P_0 mRNA expression in response to reinnervation. This appears to require little axonal contact (Mitchell *et al.*, 1990). The response of the many daughter cells accounts for the dramatic increase in the frequency of silver grain clusters observed on regenerating nerve in the studies performed here.

4.3 THE EFFECT OF AXONAL LOSS ON CULTURED SCHWANN CELLS

4.3.1: Dissociated Schwann cell culture

4.3.1.1 Introduction

By its very nature, production of dissociated Schwann cells separates the Schwann cell from its axon. Previous studies have identified that the disruption of axon-glia contact results in loss of both the myelin-specific proteins and down-regulation of the genes encoding them (see section 1.5.3.3) Experiments were initiated to evaluate the expression of P_0 mRNA at various times in dissociated Schwann cell culture. The effect of culture substrates on P_0 mRNA expression was also assessed.

4.3.1.2 Materials and Method

Schwann cells were dissociated from sciatic nerves of Sprague-Dawley rat pups aged between 2 and 8 days, or from 5 day old C57Bl/6 mice by the method described in chapter 2 (2.1.1). The P_0 mRNA expression of Schwann cells cultured on different substrates (poly-L lysine, collagen, Matrigel) was studied. At various

times after plating out, Schwann cells cultures were fixed in 4% paraformaldehyde and evaluated by ISH and autoradiography to assess the expression of P_0 mRNA. The level of expression, as detected by density of silver grains after autoradiography was rated on an arbitrary scale of 0 to 10+. On this scale, 0 represented no silver grains and 10+ represented a concentration of silver grains such that they were heavily overlapping and formed a dense black cluster obscuring Schwann cell nuclei and background.

4.3.1.3 Results

In cells fixed less than 24 hours after plating out, expression of P_0 mRNA by the Schwann cells was at 5 to 8+ for cells grown on poly-L lysine and tended to be 7 to 8+ for cells grown on a substrate. Approximately 10% of Schwann cells were expressing P_0 on poly-L lysine, but this percentage seemed to be slightly higher (15 to 20%) in cells grown on a substrate. No binding of sense probe was observed on day 0 Schwann cells (fig. 4.3).

When the cells were fixed 5 days after plating out, the level of expression had dropped significantly with cells on poly-L lysine expressing the mRNA at 0 to, rarely, 4+ (fig. 4.4). Cells grown on a substrate, again, had a slightly higher level of expression at 4 to 6+.

The level of expression of P_0 mRNA in cells fixed 10 days after plating out was reduced to 0 to 1+ in all cells regardless of the substrate upon which they were grown.

Schwann cells obtained from mice were grown only on poly-L lysine and were fixed on days 0, 2, 5 and 7. The expression of P_0 mRNA was 10+ on day 0 but had declined to 1+ within 2 days of culturing. This state was maintained through to the seventh day *in vitro,* which was the last time point studied.

4.3.1.4 Discussion

The percentage of cells expressing a strong focal signal for P_0 mRNA at day 0 *in vitro*, was similar to that observed by Morrison and colleagues (1991). The majority of cells were not expressing P_0 presumably because they were in the premyelinating phase or were associated with unmyelinated fibres (Morrison *et al.*, 1991).

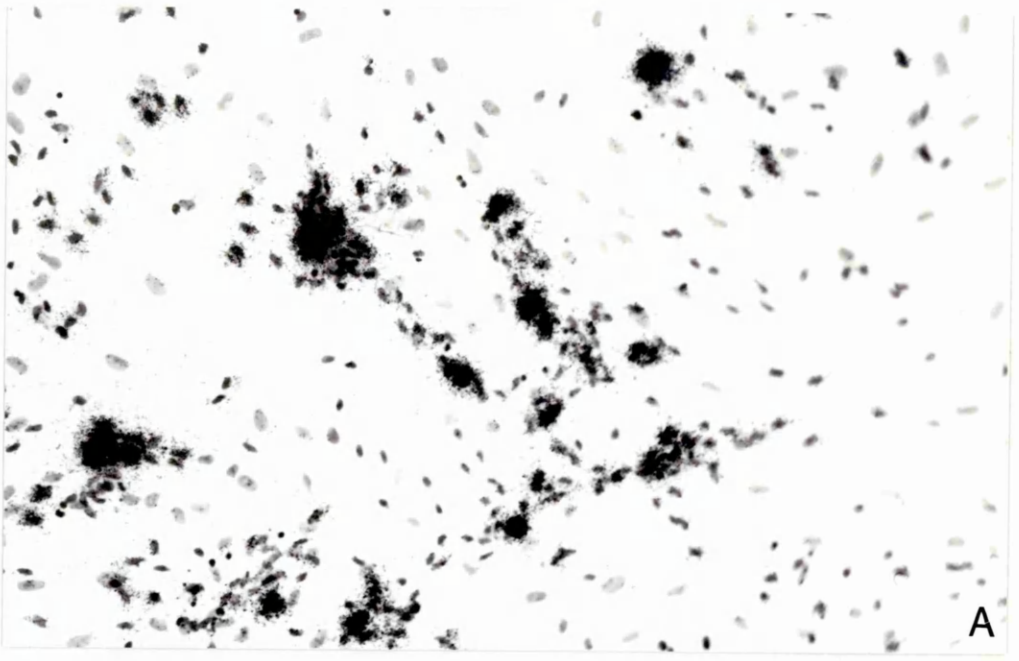


Figure 4.3: The effect of substrate on the expression of P_0 mRNA by dissociated neonatal Schwann cells. Cells were dissociated from neonatal sciatic nerve of rat pups, cultured on different substrates and fixed in 4.0% paraformaldehyde after less than 24 hours *in vitro*. They were probed for P_0 mRNA expression by the *in situ* hybridization methodology. (A) Schwann cells plated onto poly-L lysine and probed with an antisense probe; (B) Schwann cells plated onto poly-L lysine, but probed with a sense probe (x 170).

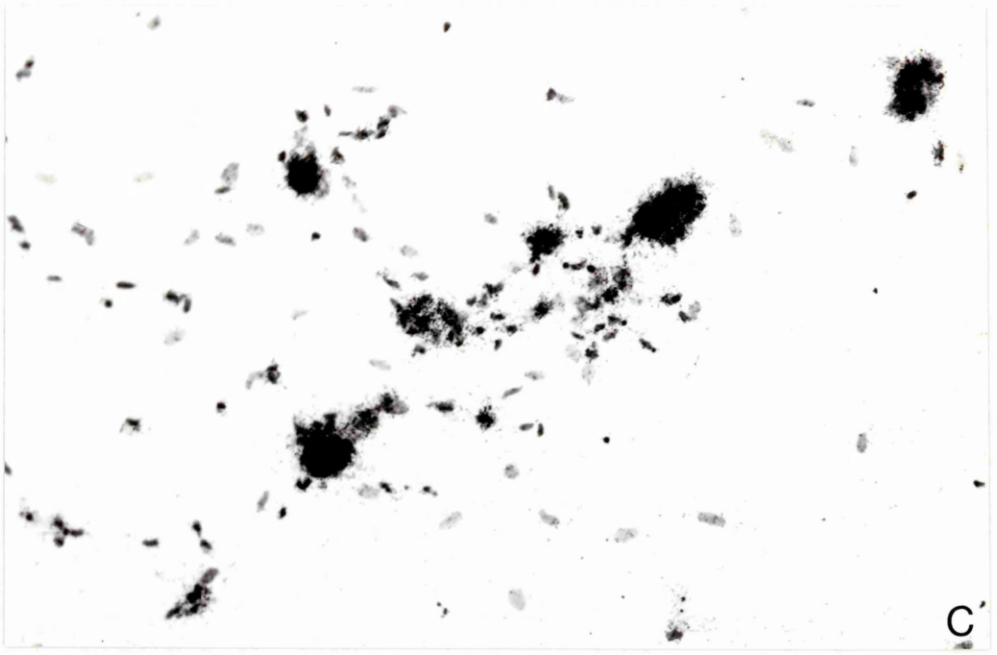


Figure 4.3 (cont.): The effect of substrate on the expression of P_0 mRNA by dissociated neonatal Schwann cells. (C) Schwann cells plated onto rat tail collagen and probed with an antisense probe; (D) Schwann cells plated collagen plus Matrigel substrate mixture and probed with an antisense probe. The intensity of P_0 mRNA expression appears to be greater in the cells plated onto collagen plus Matrigel substrate than on other substrates (x 170).

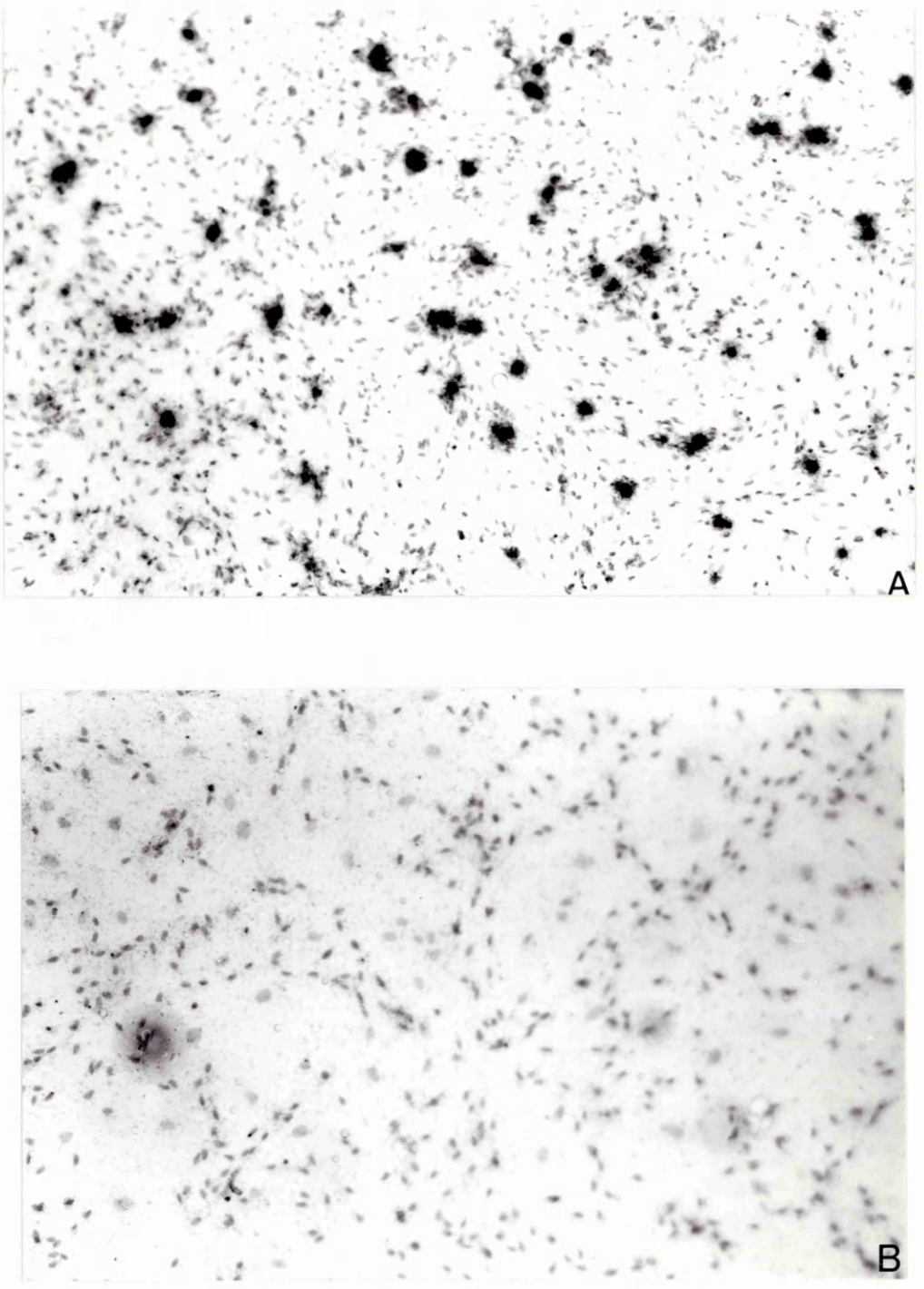


Figure 4.4: The effect of dissociated culture on Schwann cell P_0 mRNA expression. Neonatal sciatic nerve Schwann cells were dissociated and cultured on poly-L lysine. They were fixed at less than 24 hours *in vitro*, and at 4 days *in vitro*, probed for P_0 mRNA expression by *in situ* hybridization, and after autoradiography, the nuclei were counterstained with haematoxylin. (A) Schwann cells express P_0 mRNA strongly when fixed after less than 24 hours in culture; (B) but by 4 days, the expression of this gene has declined to essentially zero (x 90).

When neonatal Schwann cells were dissociated from the axon by enzymatic and mechanical methods, their expression of P_0 mRNA declined within the first week in culture as observed by other workers (Lemke and Chao, 1988b; Morrison *et al.*, 1991). This decline appears to be faster in mice than in rats, however, I have performed fewer experiments in the former animals compared with the latter to verify this fact as constant. Burroni and coworkers (1988), in fact, suggested that P_0 protein was still detectable in mouse Schwann cells cultured in the absence of neurons for 3 weeks. They performed their immunostaining for P_0 protein on prefixed cells, so the protein thus detected may not be newly synthesised. However, it was thought that the Schwann cells were still synthesising the protein in the absence of neuronal contact based on labelling studies with [^{14}C] (Burroni *et al.*, 1988).

In the experiments described in this chapter, the substrate upon which the Schwann cells were cultured seemed to affect the expression of this gene. The initial level of expression was higher and subsequent decline took longer if a basement component was used during culture. However, by the 8 to 10 days in culture, P_0 mRNA expression in neonatal dissociated Schwann cells was reduced to 0 to 1+ regardless of the substrate. This is similar to the finding expressed by Morrison and colleagues (1991) in which all Schwann cells were negative for P_0 mRNA by 10 days *in vitro*, but an increased number and intensity of expression was observed in Schwann cells grown in the presence of laminin (Morrison *et al*, 1991). DeVries *et al.*, (1990) found the opposite result with P_0 mRNA expression being lower in SV40 transfected Schwann cells cultured on Matrigel. This was thought to be due to the increased proliferation rate of the cells due to the Matrigel. These workers were not studying the loss of Schwann cell P_0 mRNA expression after dissociation as I was; rather they were assessing a basal level of expression in the absence of axonal contact. They were also using transfected Schwann cells which exhibit different properties to primary Schwann cells (Chen *et al.*, 1987 (see "myelin associated glycoprotein" in section 1.4.3.1); Tennekoon *et al.*, 1987 (see section 1.3.2.3)). The loss of expression in my study attests to the reliance of neonatal Schwann cells upon axonal contact for expression of the P_0 gene. Alternatively, the method of preparation of the Schwann cell by enzymatic and mechanical dissociation, rather than the dissociation of Schwann cell from axonal contact, may be a factor affecting the expression of P_0 mRNA (see next section).

4.3.2: Culture of sciatic nerve explants

4.3.2.1 Introduction

Schwann cells cultured from explants are derived in a different manner from dissociated Schwann cells. Procuring them relies on the cells' ability to migrate away from the explant onto the culture surface. Due to Wallerian degeneration, explants obtained from rat sciatic nerve that had been axotomized several weeks prior to the initiation of cultures, would contain little or no axonal material. Therefore the Schwann cells from these explants should not be expressing P_0 mRNA. Explants established from non-neurotomized neonatal nerves are still in contact with the axon at the time in which the culture begins. If examined shortly after establishment (within one week) these Schwann cells may still be expressing this gene at low levels as observed in neonatal dissociated Schwann cell cultures. A similar situation would potentially exist in explants taken from non-neurotomized adult nerve, but the Schwann cells only migrate poorly in such cultures and were difficult to assess. However, if the nerve was axotomized only a few days prior to establishment of explant cultures, then adult Schwann cells could be obtained (see section 3.2). This would allow assessment of P_0 expression in adult Schwann cells recently separated from the axon. Theoretically these Schwann cells should also have low, or no, P_0 mRNA expression. The following experiments were performed to assess whether Schwann cells from cultured explants respond in the same way as dissociated Schwann cells to loss of contact with a viable axon.

4.3.2.2 Materials and method

Explant cultures were created as described in section 3.2. Cultures were fixed in 4% paraformaldehyde 8 to 13 days later. Neonatal explants were obtained from animals of different ages ranging between 2 to 10 days old. Adult explants were established from sciatic nerve transected acutely (4 days) or chronically (6 weeks) before establishing explants *in vitro*. After fixation, cultures were assessed for P_0 mRNA expression by ISH and autoradiography as described in chapter 2 (2.4.4 & 2.4.6.1).

4.3.2.3 Results

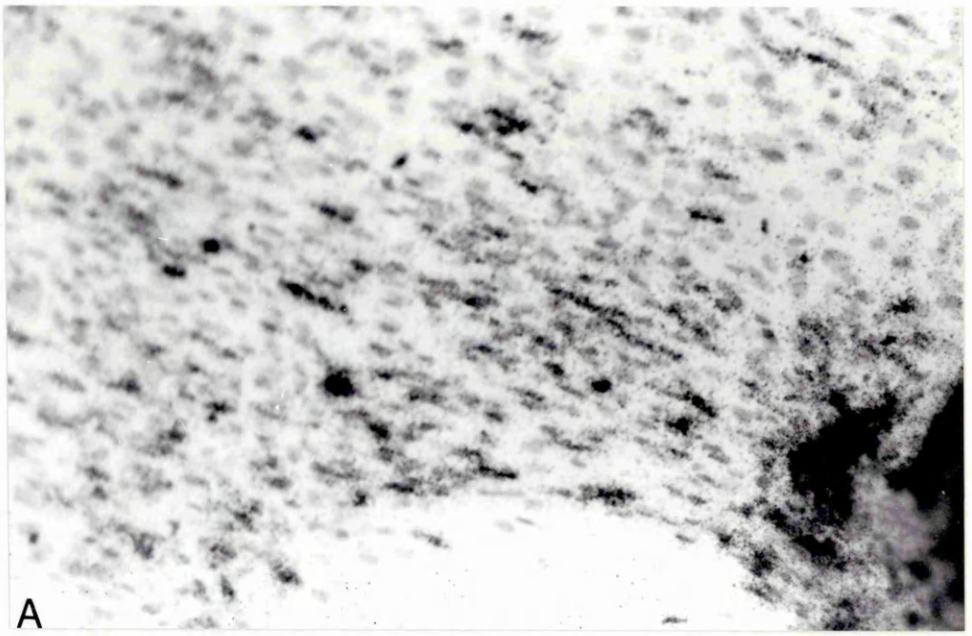
Adult Schwann cells obtained from chronically neurotomized sciatic nerve explants had 0 to 1+ expression of P_0 mRNA. In adult explants derived from acutely (4 day) neurotomized nerve, expression was variable ranging from 2 to 7+ at 9 days *in vitro* (fig. 4.5A). Similarly, in the neonatal explants the expression of P_0 mRNA at 8 to 12 days in culture was variable ranging between 1 to 6+ (fig. 4.5B). Schwann cells from non-neurotomized explants were not assessed.

4.3.2.4 Discussion

Schwann cells derived from chronically denervated adult sciatic nerve in this manner were negative for P_0 mRNA expression by ISH. They had been isolated from axonal contact for 7 to 8 weeks at the point in time when they were fixed. These were Schwann cells which had been in a mature, myelin-maintaining state for a number of months prior to axotomy. Therefore, even mature Schwann cells may require an ongoing signal from the axon to maintain expression of P_0 mRNA at a level detectable by ISH methodology.

Schwann cells derived from explants of a 4 day-neurotomized nerve had variable expression. These cells were in culture for 9 days prior to fixation and thus had been separated from the axon for 13 days in total. In this time, the expression of P_0 mRNA should have declined markedly. However, many cells were observed to have expression of 4 to 6+. Dot blot studies on the level of P_0 mRNA expression in nerve *in vivo* have been performed by Le Blanc *et al.*, (1987). At 35 days post-transection, they found that the distal segment of the transected nerve was expressing this gene at 31% of the level of intact sciatic nerve. Studies performed by Brunden *et al.*, (1990b), Halligan, (1985) and Rutkowski *et al.*, (1990) on P_0 protein have also suggested that it is persistently produced, albeit at a much reduced level, in the absence of axonal contact. This data and the maintained expression of mRNA by Schwann cells cultured from both acutely neurotomized adult, and neonatal explants, suggests that P_0 mRNA expression is not entirely lost but just down-regulated when axonal contact is lost. In this thesis, P_0 mRNA expression in cells cultured from explants derived from chronically transected nerve is at a very low level. These results were obtained by ISH, and not dot blotting. Therefore, it is difficult to compare the level of P_0 mRNA expression by the adult Schwann cells chronically separated from axons in LeBlanc's study with my study.

The neonatal explant Schwann cells also remained variably positive for up to



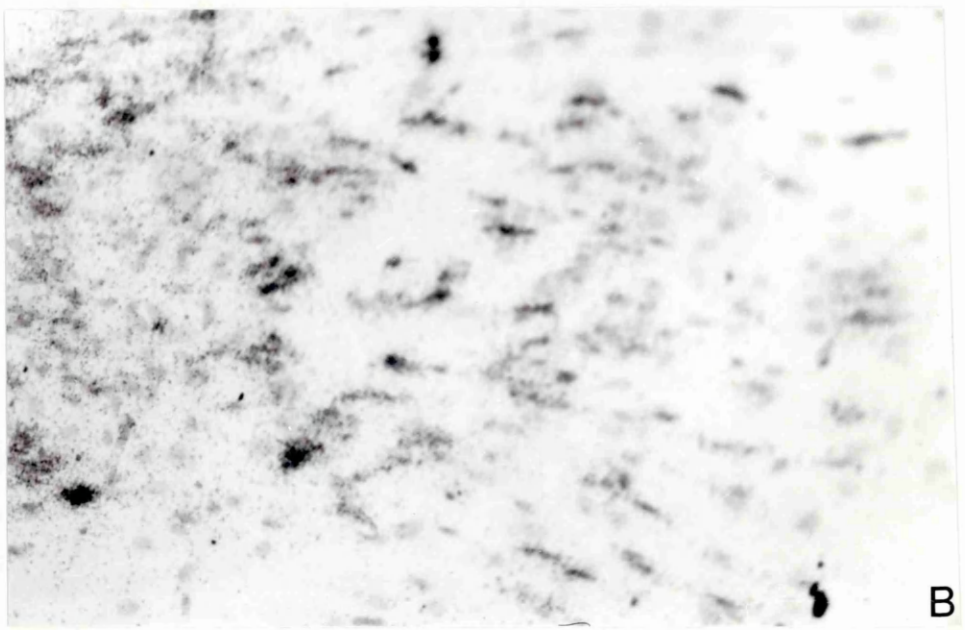


Figure 4.5: Prolonged expression of P_0 mRNA by Schwann cells cultured from explants. Explants were established from neonatal rat sciatic nerve, or neurotomized adult rat sciatic nerve and cultured on collagen plus Matrigel substrate for 9 to 12 days before fixing and probing for P_0 mRNA expression by *in situ* hybridization methodology. After autoradiography, the nuclei were counterstained with haematoxylin. (A) P_0 mRNA expression by Schwann cells derived from explants (lower right) of neonatal sciatic nerve, and cultured for 9 days prior to fixation. Strong, focal expression of P_0 mRNA is still present (x 90). (B) P_0 mRNA expression in Schwann cells derived from explants of sciatic nerve that had been transected and allowed to degenerate for 4 days *in vivo*, before culturing explants for 7 days *in vitro*. These cells had been deprived of axonal contact for 12 days at the time of fixation, yet are still expressing the P_0 gene strongly (x 150).

12 days in culture and this was irrespective of the age, and hence state of myelination, of the donor animals. This is compared with the definite down-regulation of expression in the dissociated Schwann cells described in section 4.3.1.3. It is interesting that the level of expression of P_0 mRNA by neonatal Schwann cells seems to be more intense and better preserved in cells derived from explants than by dissociation. Wallerian degeneration is slowed in the absence of macrophage recruitment both *in vivo* and *in vitro* (see section 1.5.2.1 and 5.1). Could it be that this delayed *in vitro* degeneration of the explant preserved the axonal signalling mechanism(s) such that the down-regulation of P_0 mRNA was delayed? This hypothesis is considered in chapter 6. Alternatively, could the method of production of Schwann cells (dissociation versus explant) affect the expression of P_0 mRNA? Dissociated Schwann cells are subjected to rigorous mechanical and enzymatic treatments. The possibility of enzymes denaturing part of the Schwann cell membrane, and perturbing its metabolism, has been discussed in section 3.2.1. It is possible that slowly degenerating axons or molecules were available to interact with the non-enzymatically treated Schwann cell membrane, thereby maintaining the level of P_0 mRNA expression. In view of the results obtained and discussed in chapter 6, this is very unlikely.

4.3.3: Culture of Cervicosympathetic trunk

4.3.3.1 Introduction

The cervico-sympathetic trunk (CST) is an autonomic unifascicular nerve composed primarily of non myelinated fibres (Aguayo *et al.*, 1976a; Aguayo *et al.*, 1976b; Dyck and Hopkins, 1972). Being non-myelinated, the Schwann cells should not be expressing of P_0 mRNA. Explant cultures and teased fibres from the CST were assessed for P_0 mRNA expression.

4.3.3.2 Materials and Method

Schwann cells of CST explant origin were obtained in the manner described in chapter 3.3. They were fixed in 4% paraformaldehyde after 10 to 12 days in culture and processed for ISH and autoradiography. Intact CSTs were obtained as described in 3.3.2 from perfusion fixed adult Sprague-Dawley rats and bundles of nerve fibres were teased onto poly-L lysine-coated, RNase-free slides.

4.3.3.3 Results

Schwann cells obtained from CST explants were mostly negative (1 to 2+) for expression of P_0 mRNA. Occasional cells were associated with 3+ expression. The teased fibres were mostly negative although an occasional fibre was observed that was expressing the mRNA up to 8+ in intensity.

4.3.3.4 Discussion

As expected, Schwann cells derived from non-myelinating nerve fibres were not significantly expressing the P_0 mRNA at levels detectable by ISH in CST explants. The occasional 8+ expression observed in the teased fibres is consistent with previous reports of rare myelinated fibres with the CST (Dyck and Hopkins, 1972).

4.4 CONCLUDING REMARKS

The close reliance of Schwann cells upon axonal contact to maintain P_0 mRNA expression was demonstrated using *in vivo* and *in vitro* methodology. Axotomy induced a rapid down-regulation of P_0 mRNA expression by Schwann cells *in vivo*. This loss was rapidly reversed if appropriate axons re-established contact with the Schwann cells.

Schwann cells cultured in the absence of axonal contact also exhibited decreased P_0 mRNA expression. This occurred in tissue from both neonatal and adult animals. The presence of basement membrane substrates seemed to delay this loss for a short period in dissociated neonatal Schwann cells.

The level of expression also seemed to be affected by the method of culture with loss being more rapid if the cells were dissociated than if they were cultured from explants. Schwann cells from both acutely neurotomized adult explants and neonatal explants had significantly prolonged expression of P_0 mRNA in culture. The reason for this is unclear. The results of studies discussed in chapter 6, make it unlikely that this delayed down-regulation was due to a preserved axolemmal associated myelinating signal. Could the effect of dissociation have a role in the down-regulation? The possibility that enzymic treatment effects Schwann cell metabolism has been considered by other workers. Could enzymic treatment during dissociation destroy Schwann cell membrane molecules such as a relatively stable

myelinating signal bound to a Schwann cell receptor? This would cause an earlier loss of P_0 mRNA expression in dissociated compared with explant Schwann cells. The addition of basement membrane may help preserve such a membrane associated interaction. Basement membrane is known to have an important role in permitting Schwann cells to respond to the putative myelinating signal. Possibly its presence helped maintain the P_0 mRNA expression in cultured cells.

CHAPTER 5: DELAYED WALLERIAN DEGENERATION IN C57BL/OLA MICE:

5.1 INTRODUCTION

In section 1.5.2.8 the concept of delayed Wallerian degeneration was introduced. As far back as 1850, Waller noted that degeneration in transected frog nerves was delayed during the cooler months of the year (Waller, 1850; Waller, 1852). Subsequent studies by Wang in 1985 elaborated on this concept, identifying maintained nerve conduction in transected myelinated frog nerve. This required an intact and functioning myelin sheath. The duration of maintained function was indirectly proportional to the ambient temperature (Wang, 1985). In 1990, Rubinstein and Shrager demonstrated that in frogs exhibiting delayed degeneration, despite axotomy, the distal stump of the sciatic nerve could remyelinate after lysolecithin-induced demyelination (Rubinstein and Shrager, 1990). These data imply that in the absence of Wallerian degeneration, the axon can still direct metabolic functions of the Schwann cell despite being severed from the neuronal somata. This suggests that the putative myelinating signal of the axolemma is relatively stable, not necessarily requiring continuous contact with the neuron for its viability. Therefore, models of delayed Wallerian degeneration could become useful tools to study the mode of action of the myelinating signal.

In section 1.5.2.1., the various known agents of Wallerian degeneration have been reviewed. The macrophage is presumed to have a significant role in the phagocytosis and clearing of debris. The resident population of macrophages within peripheral nerves is less than 5% (Oldfors, 1980). However, during Wallerian degeneration a high level of myelomonocytic recruitment occurs (Hall, 1989; McCaman and Robins, 1959b). *In vivo*, Wallerian degeneration was delayed if macrophage invasion of the nerve was decreased. This was achieved by placing nerve segments into chambers within the peritoneal cavity of mice. The pore size of the chambers was either small enough to exclude macrophages, in which case degeneration was slowed, or the pore size allowed invasion of phagocytic cells and degeneration proceeded quickly (Beuche and Friede, 1984). Similarly, if macrophage invasion into the transected nerve was decreased by depleting the body's macrophage population, then degeneration was also delayed (Beuche and Friede, 1986). During Wallerian degeneration *in vitro*, recruitment of these phagocytic cells would not

occur, however, the resident population of macrophages is thought to proliferate (Bonnekoh *et al.*, 1989). Despite this, breakdown of the myelin sheath is slower *in vitro* compared with *in vivo*, and this was thought to be a consequence of the low or absent macrophage activity in cultured explants. In these cultures, the myelin debris appeared to just be rejected and transposed outwith the perineurium rather than cleared by phagocytosis (Crang and Blakemore, 1986). When cocultures of peritoneal macrophages with nerve explants were established, then active phagocytosis and clearing of large amounts of myelin debris occurred. However if the opsonization of myelin debris was blocked by cobra venom factor, or the function of the C3 receptor on the macrophage was blocked, then phagocytosis was markedly inhibited and degeneration delayed (Bruck and Friede, 1990b).

In 1989, a series of reports started on a naturally-occurring mouse mutant (C57Bl/Ola) which has poor myelomonocytic cell invasion into peripheral nerves after axotomy. In these mice the distal stump of the sciatic nerve could still conduct action potentials for up to 14 days post-transection whereas conduction failure occurred by 2 days in normal mice. This maintenance of function was accompanied by persisting morphological integrity such that by 7 days post-transection, the majority of axons and myelin sheaths were still intact. The morphological and physiologic integrity of axotomized nerves in normal mice was preserved somewhat by preventing myelomonocytic recruitment using an antibody raised against the complement type 3 receptor on the macrophage (Lunn *et al.*, 1989).

In normal mice and rats, after axotomy, the level of expression of P_0 mRNA by the Schwann cell declines rapidly, presumably as a consequence of loss of axonal integrity and axon/glial cell contact. Likewise, Schwann cells dissociated from axonal contact down-regulate expression of myelin-specific molecule (see section 1.5.3.2, 1.5.3.3 and chapter 4). Rubinstein and Shrager's study identified that the putative myelinating signal persists functionally in frogs if Wallerian degeneration is delayed (Rubinstein and Shrager, 1990). The mouse mutant C57BL/Ola provided an excellent opportunity to study these phenomena in homeothermic animals. The aim of this set of experiments was to assess the effect of delayed Wallerian degeneration *in vivo* on the down-regulation of myelin-specific molecules by Schwann cells in the distal stump of the axotomized nerve. Would the decline in P_0 mRNA expression be delayed if Wallerian degeneration was slow?

5.2 MATERIALS AND METHOD

Nerves of adult C57Bl/Ola and control mice (C57Bl6 or BalbC) were transected as described in chapter 3.2.2. For morphological studies on plastic sections, the mice were perfused through the heart (section 2.2.1) at 5, 10 and 15 days post-transection with strong fixative (A2.2). Samples from the normal and transected side of sciatic nerve, and L4 and L5 spinal ganglia (which give rise to the sensory nerve fibres within the sciatic nerve of C57Bl/Ola mice) were processed as described in section 2.3.2.2. Resin sections, 1 μm thick, were stained with methylene blue/azure II (A3.1.2).

Other mice were perfused with buffered neutral formalin. The L4/L5 spinal cord was removed and paraffin-embedded (appendix A3.1.1). Sections were stained with cresyl violet (appendix A3.1.2) to assess Nissl substance, or with the monoclonal antibody RT97 against the 200 kDa phosphorylated neurofilament protein by the PAP method (McPhilemy *et al.*, 1991). Alternatively, 5 day post-transected sciatic nerve distal stumps were harvested and paraffin embedded (A3.1.1) for immunocytochemistry by the peroxidase-antiperoxidase method (section 2.3.1.2). A portion of the right sciatic nerve was used as the normal control. Negative controls used non-immune mouse and rabbit sera in place of primary antibodies. Nerves were immunostained with the following primary antibodies diluted with phosphate buffered saline: actin, neurofilament 200, P_0 and tubulin.

Dissociated Schwann cells were prepared as described in section 2.1.1, from 5 day old mutant and Balb/C mice and cultured in Dulbecco's modification of Eagle's medium supplemented with 7.5% foetal calf serum and 1% glutamine. They were fixed at less than 24 hours *in vitro* (positive controls) or at 5 days *in vitro*. The distal stump of transected sciatic nerve was harvested at 2, 5 and 7 days and teased onto RNase-free poly-L lysine-coated slides. The normal right nerve was used as a positive control. *In situ* hybridisation (section 2.4.4), using a [^{35}S] P_0 riboprobe made from linearized rat P_0 cDNA (section 2.4.2.2), was performed on both cultured cells and teased fibres. This was followed with autoradiography (section 2.4.6). Some teased fibres were coated with AR-10 stripping film and mounted unstained for densitometry. The Quantimet image analysis system was used for densitometry. It was calibrated using a blank area of the emulsion-coated slide as zero optical density, and a neutral density filter of OD 1.0. A measuring frame was selected which would be completely included within the perinuclear signal of any fibre to be examined. For a given fibre, several OD measurements were made of the perinuclear grain cluster and averaged. The exact number of measurements was determined by the area of the

signal. A selective bias was introduced to the system, such that fibres that were down-regulated so that the signal was unmeasurable were not included. Similarly, small fibres were excluded. The perinuclear signal from at least 120 random fibres in both intact and axotomized nerves of control and mutant mice at 2, 5 and 7 days post-transection was measured directly from the microscope using this system.

Other teased fibres were coated with K5 emulsion and counterstained with haematoxylin and eosin (appendix A3.1.2) for visualization.

Northern blots (section 2.4.5.1) were performed using 10 μg samples of RNA obtained from normal and transected sciatic nerves of 20 mice of each strain.

In other C57Bl/Ola mice a double axotomy was performed. The axotomy sites were at the level of the greater trochanter and 1.0 cm distal to that, creating an isolated middle segment. The distal axotomy was performed by either transection or crushing.

To examine the nature of the Ola mutation, Southern blots were performed on genomic DNA extracted from liver tissue (see section 2.4.1.1) of mutant and normal mice. The DNA was cut with the restriction enzymes Bam HI, Eco RI, Hin dIII and Pst I and probed with [^{32}P] cDNA to screen for gross rearrangements of the P_0 gene in the mutant mouse.

5.3 RESULTS

5.3.1 Morphology of degeneration

5.3.1.1 Light microscopy

Normal mice

Normal non-transected nerve

The myelin sheath surrrounding the axon was compact and of very regular thickness. In longitudinal section, Schmidt-Lanterman incisures and the cloverleaf pattern of the node of Ranvier were observed. Pale blue axoplasm usually filled the space outlined by the sheath, although occasionally it was artifactually pulled away from the sheath. Little extracellular space was observed between the sheaths (fig. 5.1).

5 days post-transection

Most myelin sheaths were collapsed and fragmented, and filled with myelin

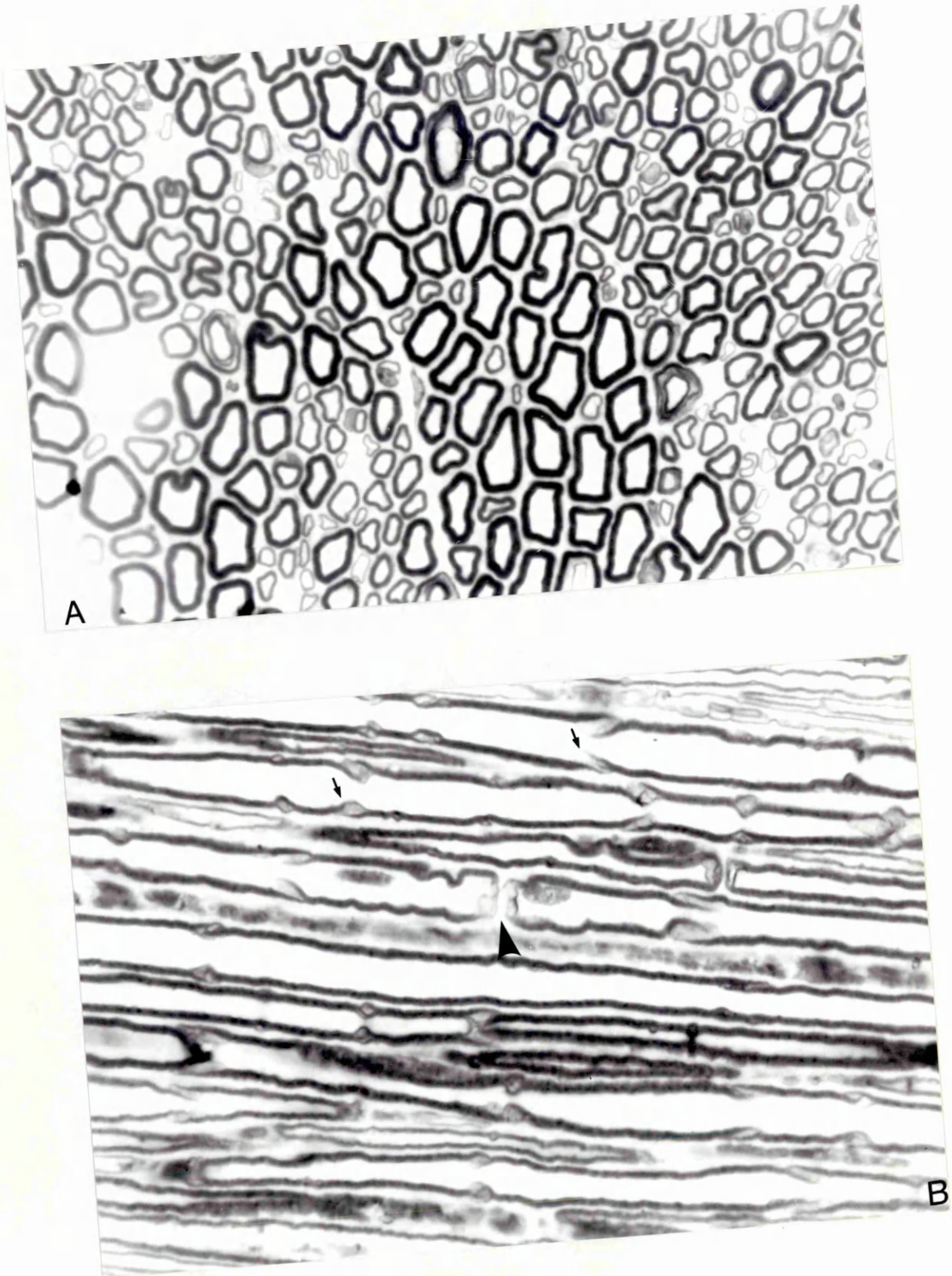


Figure 5.1: Normal C57Bl/Ola mouse sciatic nerve. (A) Transverse section stained with new methylene blue/azure II; (B) longitudinal section, Schmidt-Lanterman incisure (arrows); node of Ranvier (arrow head) (x 420).

debris although some were distended with condensed, fibrillar-appearing axoplasm. Splitting and fragmentation of the myelin sheath was not uncommon and significant numbers of cells containing myelin debris were noted between the sheaths (fig 5.2).

10 days post-transection

Very few residual myelin sheaths were observed still outlining axonal remnants. Most fibres were represented by myelin debris which had condensed into irregular whorls. Engorged phagocytic cells had engulfed much of the debris. Some spindle shaped cells, with elongated nuclei, were present. There were also cells present characterized by a more spherical, euchromatic nucleus with a foamy cytoplasm (possibly macrophages) some of which also contained myelin debris. There was an increased number of cells with small dark homogeneously staining nuclei surrounded by pale blue cytoplasm (possibly fibroblasts) (fig. 5.3).

d) No 15 day post-transection sections were examined.

C57BL/Ola mice

Normal non-transected nerve

No differences between these mice and the normal mice were noted.

5 days post-transection

There was little morphological change from the non-transected nerve. Most fibres appeared normal with preserved Schmidt-Lanterman incisures and nodes of Ranvier. Occasional sheaths had collapsed with fragmentation of the myelin. The axoplasm was visible in most sheaths and cells containing myelin debris were only rarely observed (fig 5.4).

10 days post-transection

The majority of sheaths appeared normal and contained pale blue axoplasm. Some of the sheaths were collapsed and filled with debris. Occasional nodes of Ranvier and Schmidt-Lanterman incisures were observed. Some phagocytic cells containing intracytoplasmic myelin debris were present. Rare cells with spindle shaped nuclei were lodged between fibres (possibly fibroblasts) (fig. 5.5).

15 days post-transection

Approximately 20 to 40% of the sheaths were still intact, but tended to have a corrugated and irregular appearance. Axoplasm was observed within these sheaths.

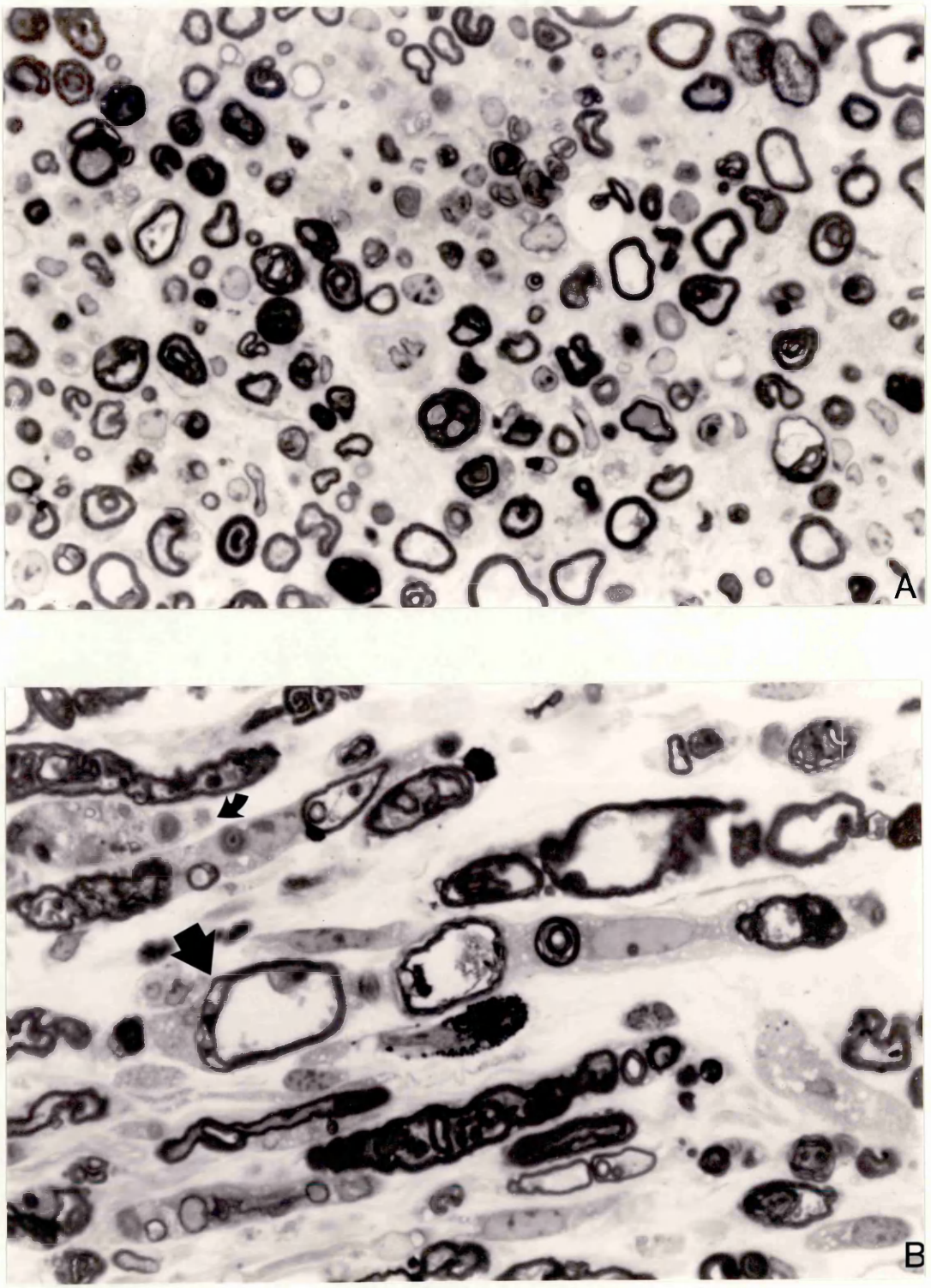


Figure 5.2: Normal mouse sciatic nerve, 5 days post-transection. Fibres are stained with new methylene blue/azure II (A) Transverse section: the majority of myelin sheaths are collapsed and fragmented, whilst some sheaths are distended. (B) Longitudinal section: ballooned sheaths are more obvious in this plane (arrow) and cells filled with myelin debris are apparent (curved arrow) (x 420).

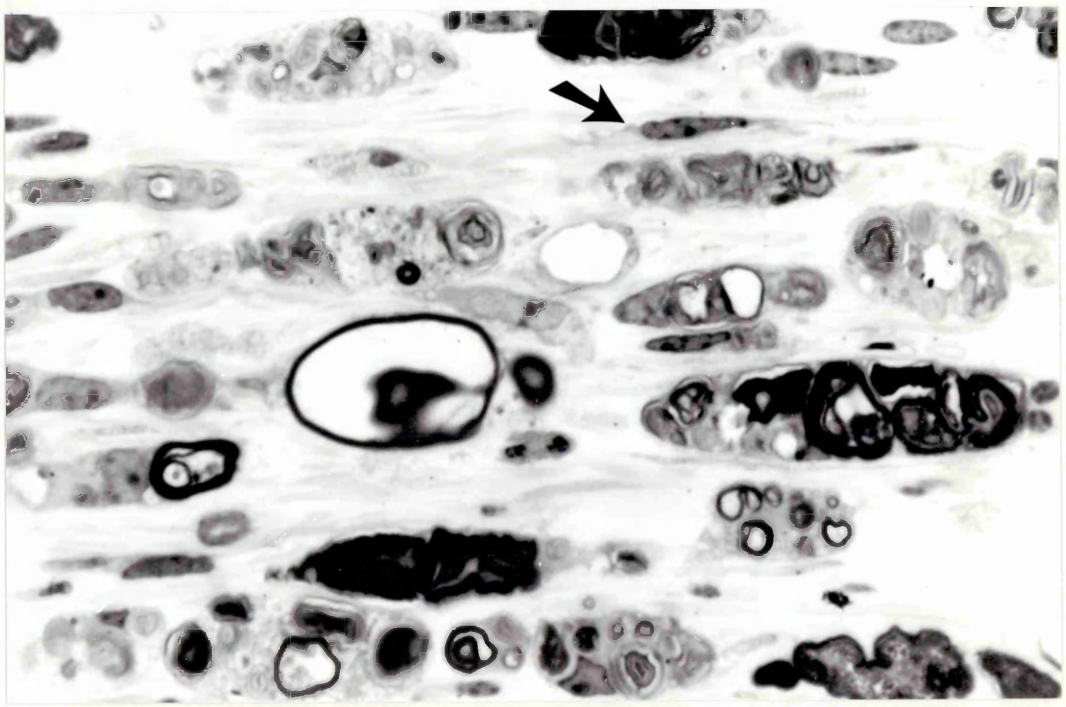


Figure 5.3: Normal mouse sciatic nerve, 10 days post-transection. Fibres are stained with new methylene blue/azure II. Only rare sheaths are still visible at this time, and they are abnormal; the majority of the myelin debris is located intracellularly. An increased number of small cells with elongated nuclei can be seen (arrow); these may be fibroblasts (x 420).

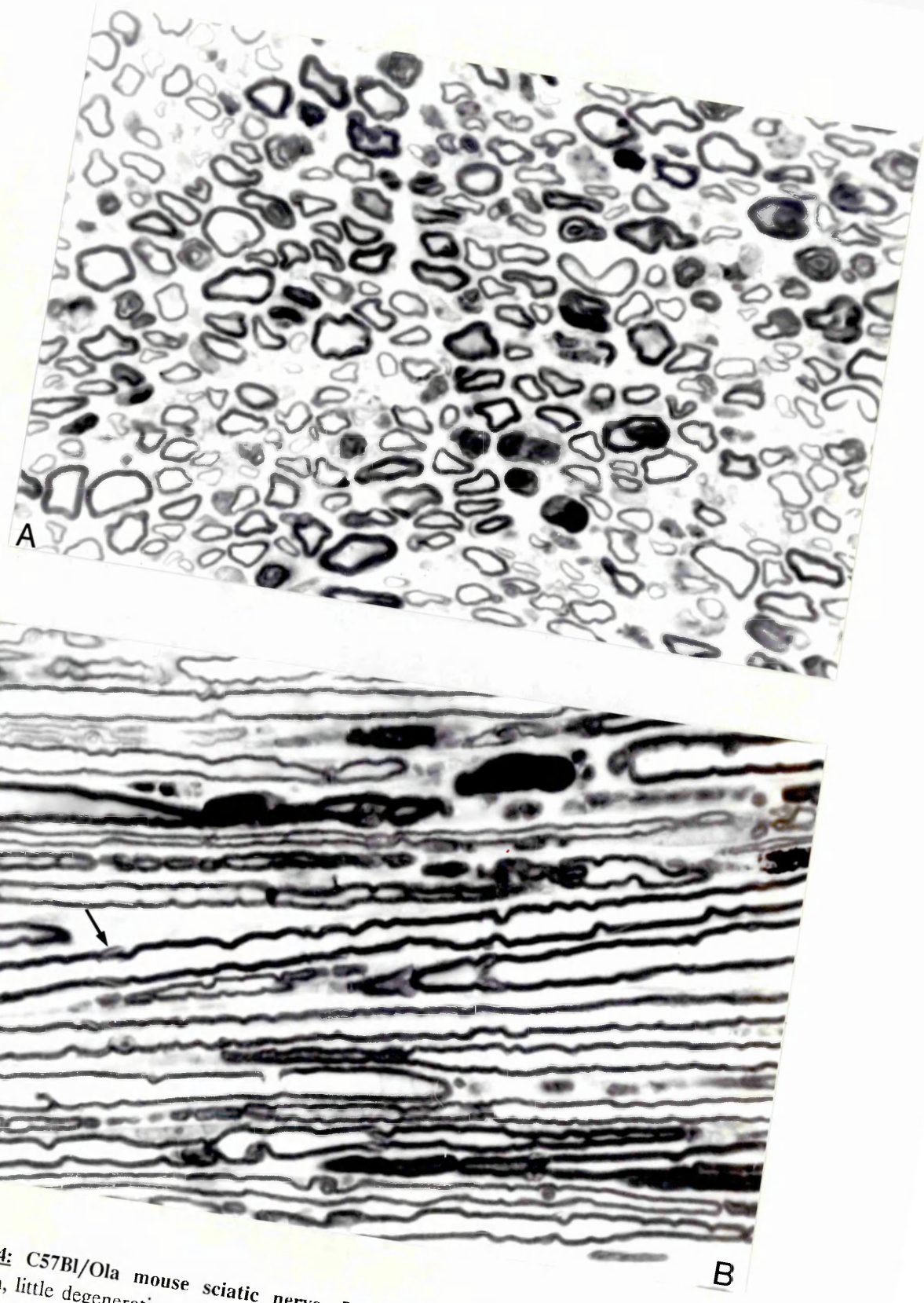


Figure 5.4: C57Bl/Ola mouse sciatic nerve, 5 days post-transection. Five days after transection, little degeneration of the nerve is seen. Fibres are stained with new methylene blue/azure II. (A) Transverse section. (B) Longitudinal section; Schmidt-Lanterman incisure (arrow); (x 420)

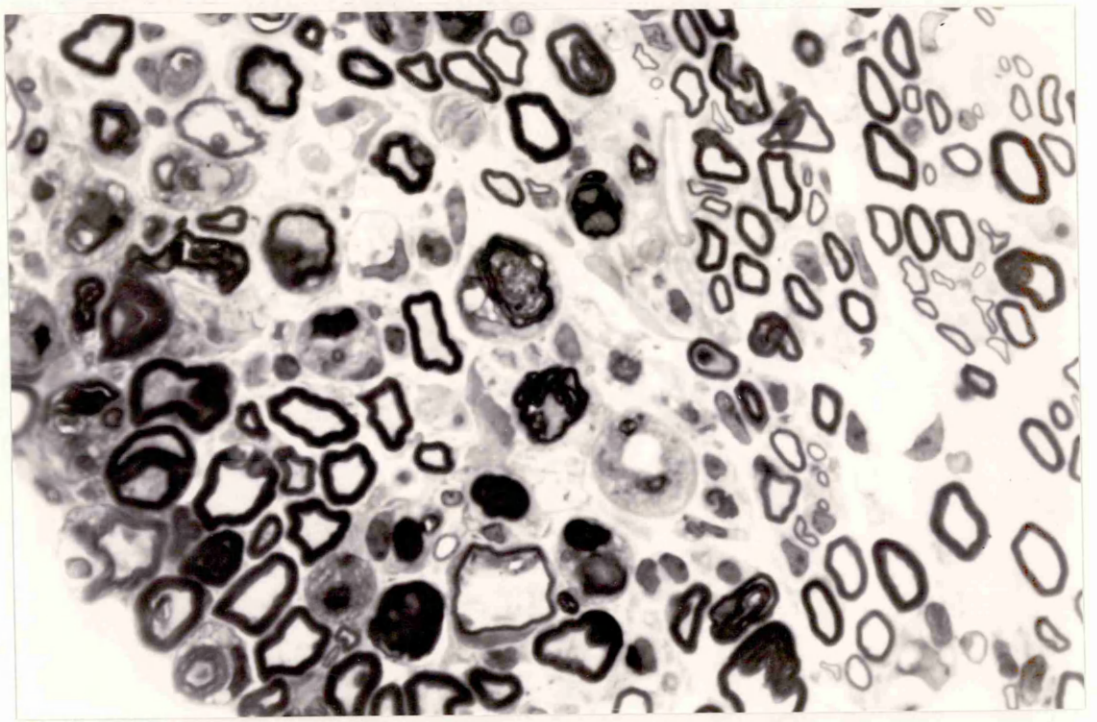


Figure 5.5: C57Bl/Ola mouse sciatic nerve, 10 days post-transection. Even 10 days after transection, degeneration of the nerve was only moderate. Fibres are stained with new methylene blue/azure II. Transverse section: approximately half the myelin sheaths are still intact (x 420).

Large clusters of myelin debris were observed, probably enclosed within engorged phagocytic cells. Phagocytic cells with foamy cytoplasm, and spindle shaped cells were also seen throughout the nerve. Hypercellular areas were seen which may have represented Schwann cell and fibroblastic proliferation.

5.3.1.2 Electron microscopy

Normal mice

5 days post-transection

Electron microscopy confirmed the severity of degeneration of nerve fibres from the transected distal stump of normal mice. At best, only a remnant of axoplasm remained with residual cytoskeletal elements having been reduced to smudged, watery-appearing particles. Often the axon was absent having been replaced by a collapsed myelin sheath. The orderly appearance of the myelin was destroyed in most cases with splitting and disruption of the lamellae, or actual fragmentation and dissolution of the sheath (fig.5.6).

C57BL/Ola mice

5 days post-transection

The majority of nerve fibres of Olac mice in this time zone were intact with maintained sheath and axoplasmic integrity. Mitochondria, neurofilaments and microtubules were visible in most of the axons, as was the axolemma. Some peri-axonal vesiculation and axoplasmic glycogen granules were noted. A few fibres exhibited more pronounced degeneration with loss of compaction of the myelin sheath or even, in the more extreme cases, fragmentation of the sheath to form myelin whorls. The axoplasm of these fibres was also more degenerate with increased vesiculation, degeneration of axoplasmic organelles and sometimes loss of the axon with collapse of the myelin sheath. However, these changes were uncommon. Occasional fibres were somewhere in between these two extremes with incipient dissolution of axoplasmic elements, but relatively well maintained myelin sheath, and preservation of the axolemma (fig 5.7).

10 days post-transection

Even at 10 days post-transection, some relatively normal-appearing fibres were observed with intact axoplasm, axoplasmic contents, and myelin sheath, but with some early periaxonal vesiculation. However some very degenerated fibres were also noted with loss of all aspects of the fibre. Fragments of axolemma were still visible in

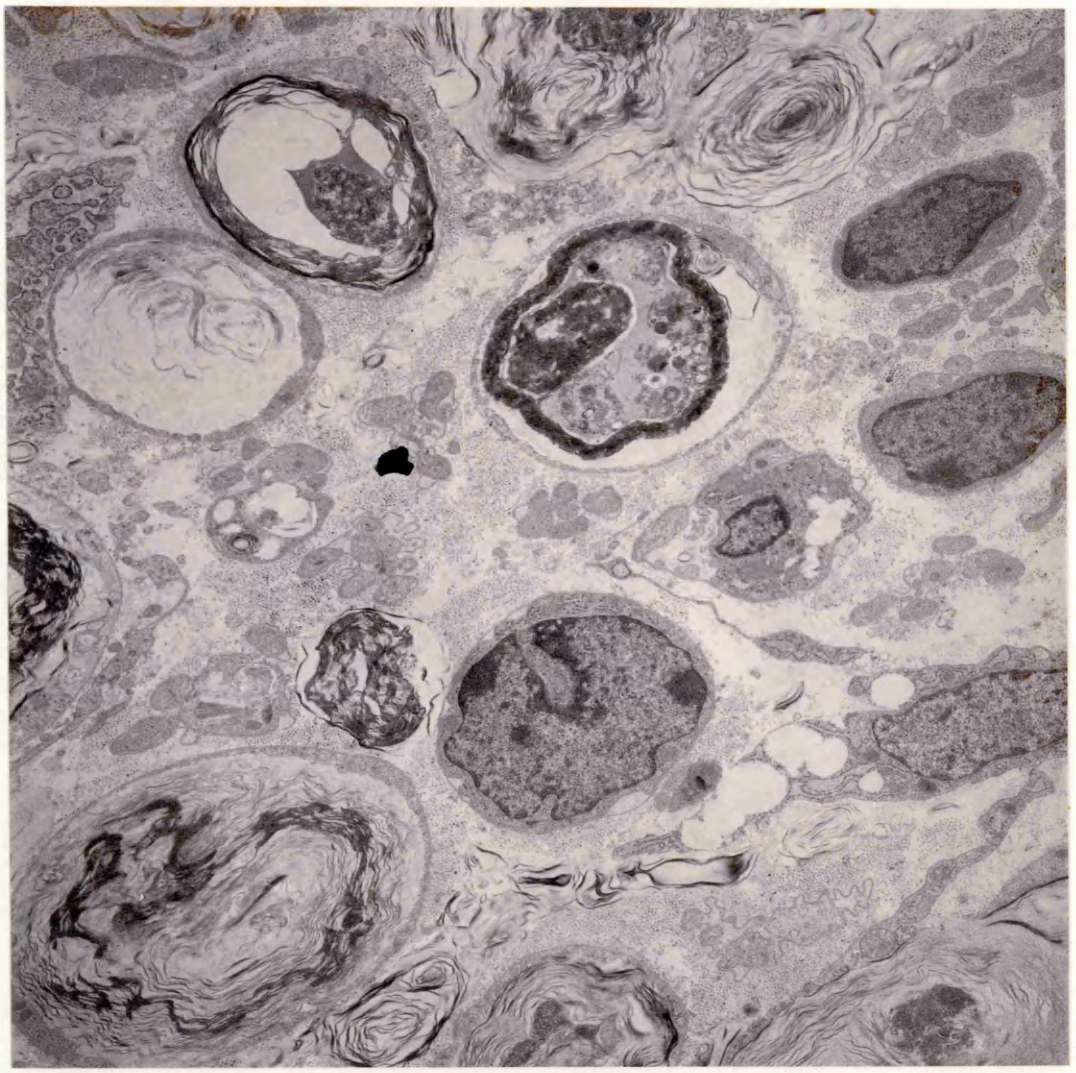


Figure 5.6: Normal mouse sciatic nerve, 5 days post-transection - EM. The majority of myelin sheaths are collapsed and fragmented (x 6000).

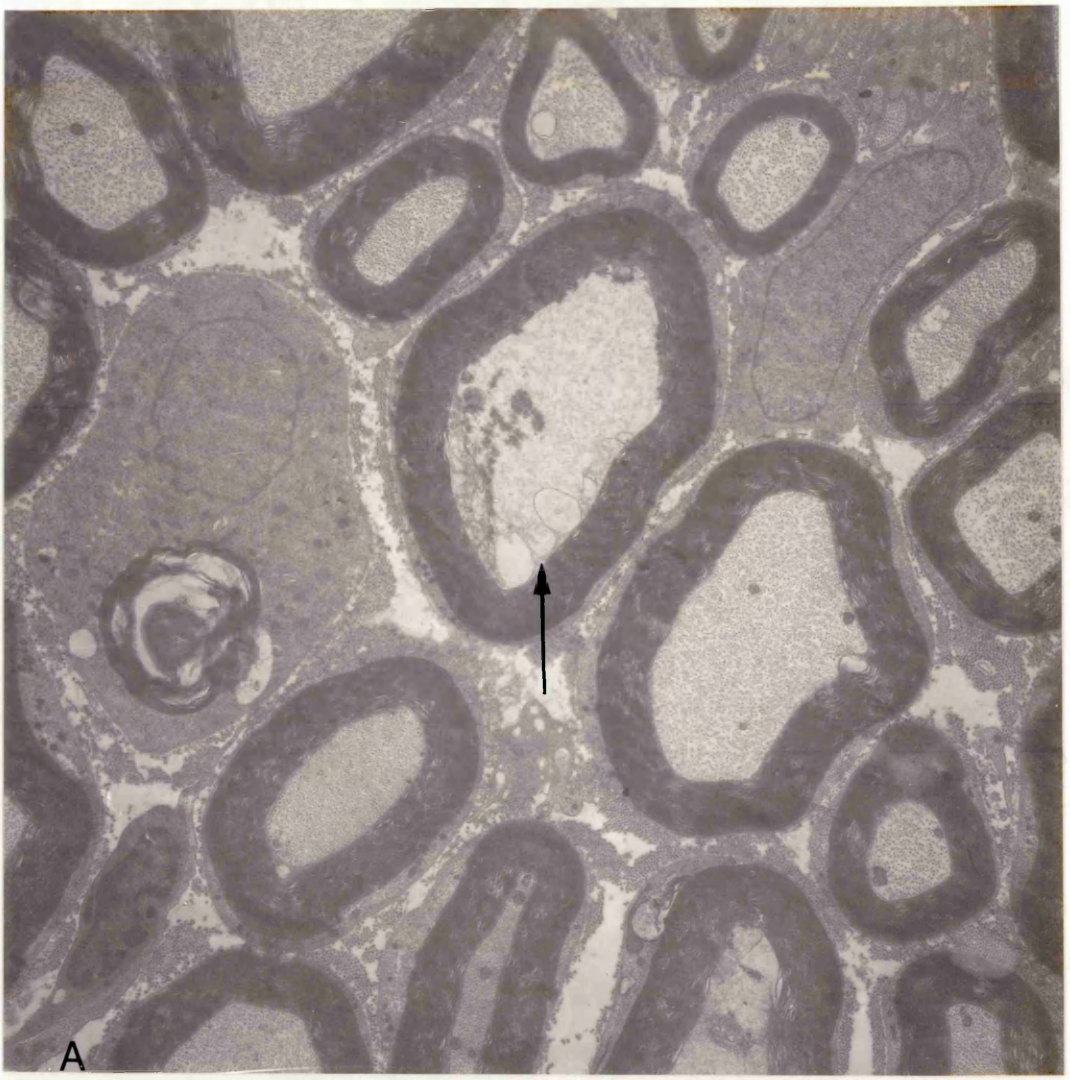


Figure 5.7: C57Bl/Ola mouse sciatic nerve, 5 days post-transection - EM. (A) Transverse section: the majority of myelin sheaths are intact, although early periaxonal vesiculation (arrow) is noted on some fibres (x 6,000).

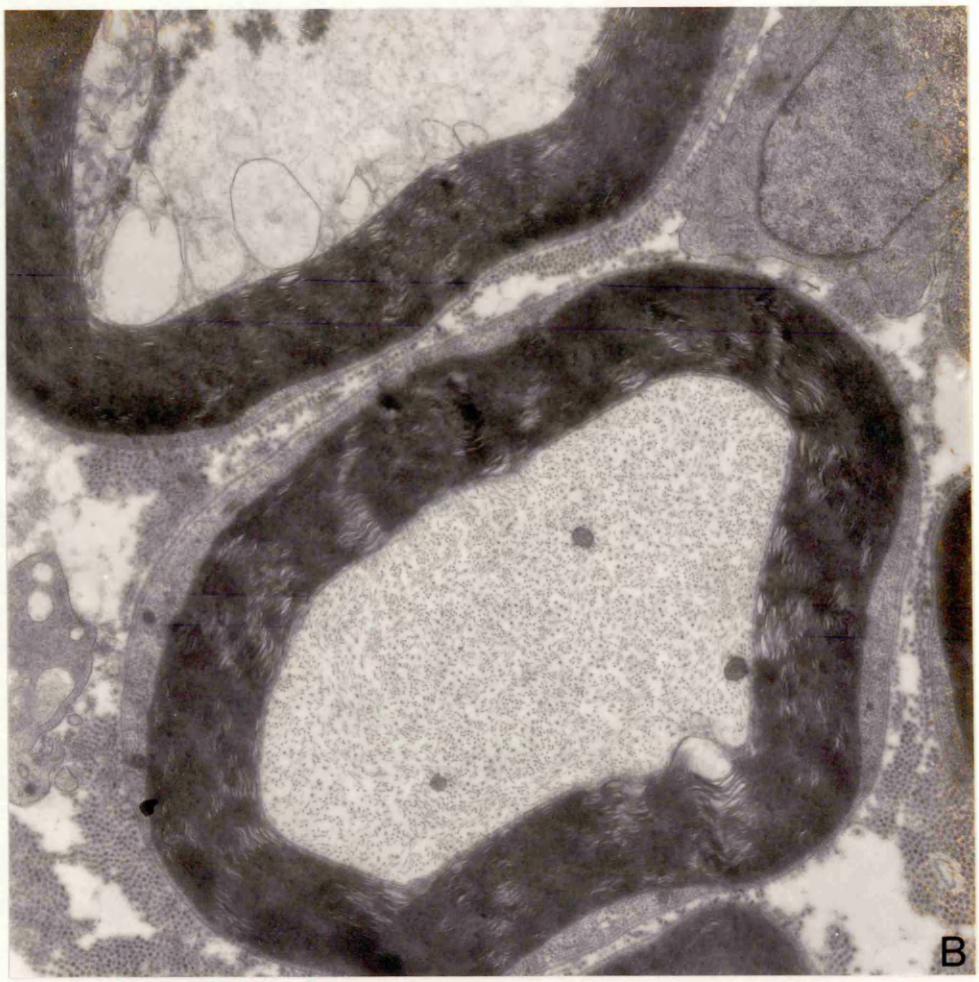


Figure 5.7 (cont.): C57Bl/Ola mouse sciatic nerve, 5 days post-transection - EM. (B) Transverse section: higher magnification of previous section emphasising the preservation of the cytoskeleton (neurofilaments, microtubules and mitochondria) in the intact lower fibre. In the upper fibre, the early periaxonal vesiculation and degeneration of the axoplasm can be appreciated. In both fibres, the myelin sheath appears to be intact (x 15,000).

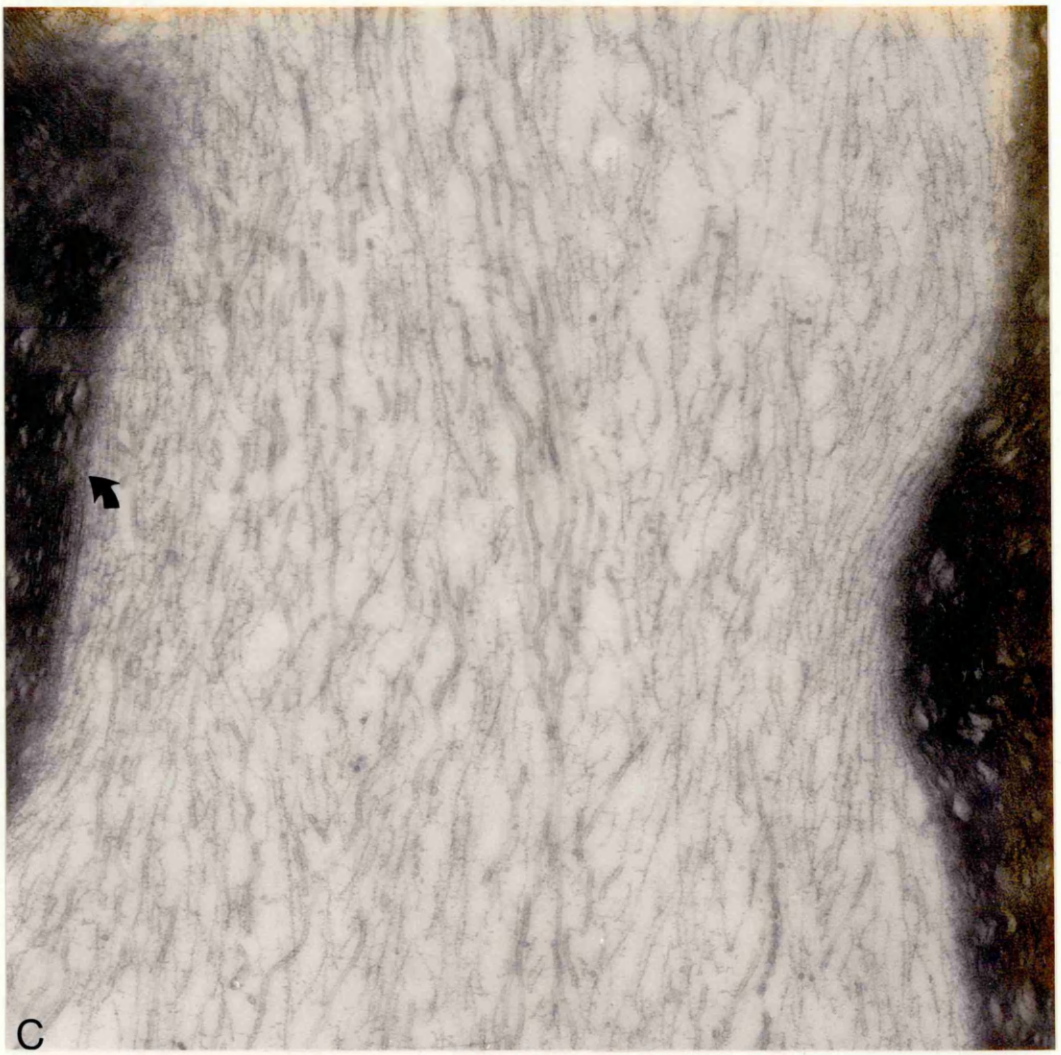


Figure 5.7 (cont.): C57Bl/Ola mouse sciatic nerve, 5 days post-transection - EM. (C) Longitudinal section: the preservation of the cytoskeleton (neurofilaments and microtubules) is dramatic. No peri-axonal vesiculation is present, and the intact axolemma (curved arrow) is barely visible adjacent to the myelin sheath (x 30,000).

fibres exhibiting degeneration of myelin and axons.

5.3.1.3 Immunocytochemistry

The results of immuno-staining for different components of the nerve fibre are given in table 5.1. The negative controls were negative.

5.3.1.4 Axonal reaction

Spinal ganglia

There was no obvious difference in the morphology or degree of the axonal reaction between the normal and the mutant mice.

Normal spinal ganglia

The nuclei of the normal spinal ganglia neurons were mostly centrally located although some were slightly eccentric. The nucleolus was prominent depending upon the plane of the section and the Nissl substance was evenly dispersed. Both light and dark neurons were visible (fig. 5.8A).

5, 10 and 15 days post-transection

Although axonal reaction was present after nerve transection in the mutant mice, the morphological changes in the somata were not dramatic. Nuclei became eccentrically located in 10 to 20% of cells by 5 days and this percentage was slightly increased at 15 days post-transection (fig. 5.8B). Clumping of Nissl substance was observed in the occasional cell and peripheral vacuolation was sometimes noted within the perikarya. In some sections, no changes were noted.

Spinal cord

Normal spinal cord

The ventral horn cells contained large centrally-located nuclei with a prominent nucleolus. Nissl granules were evenly dispersed throughout the cytoplasm.

5, 10 and 15 days post-transection

No difference from normal could be seen in cresyl violet-stained material from either group of mice. However, both groups exhibited many perikarya immunoreactive for phosphorylated 200 kDa neurofilament protein on the side of the transection (fig 5.9).

	AXON		MYELIN SHEATH		OUTER SCHWANN CELL	
ANTIGEN	INTACT	TRANSECTED	INTACT	TRANSECTED	INTACT	TRANSECTED
Actin	+	+(a)	-	-	+	+
NF 200	+	+(a)	-	-	-	-
P_0	-	-	+	+(a)	-	-
Tubulin	+	+(a)	-	-	+	+

Table 5.1: Results of immunostaining by the PAP method on intact and 5 day post-transected sciatic nerve of the C57Bl/Ola mouse.
(a): tissue staining was positive on intact fibres, but not on degenerate fibres. NF 200 = neurofilament 200 protein.

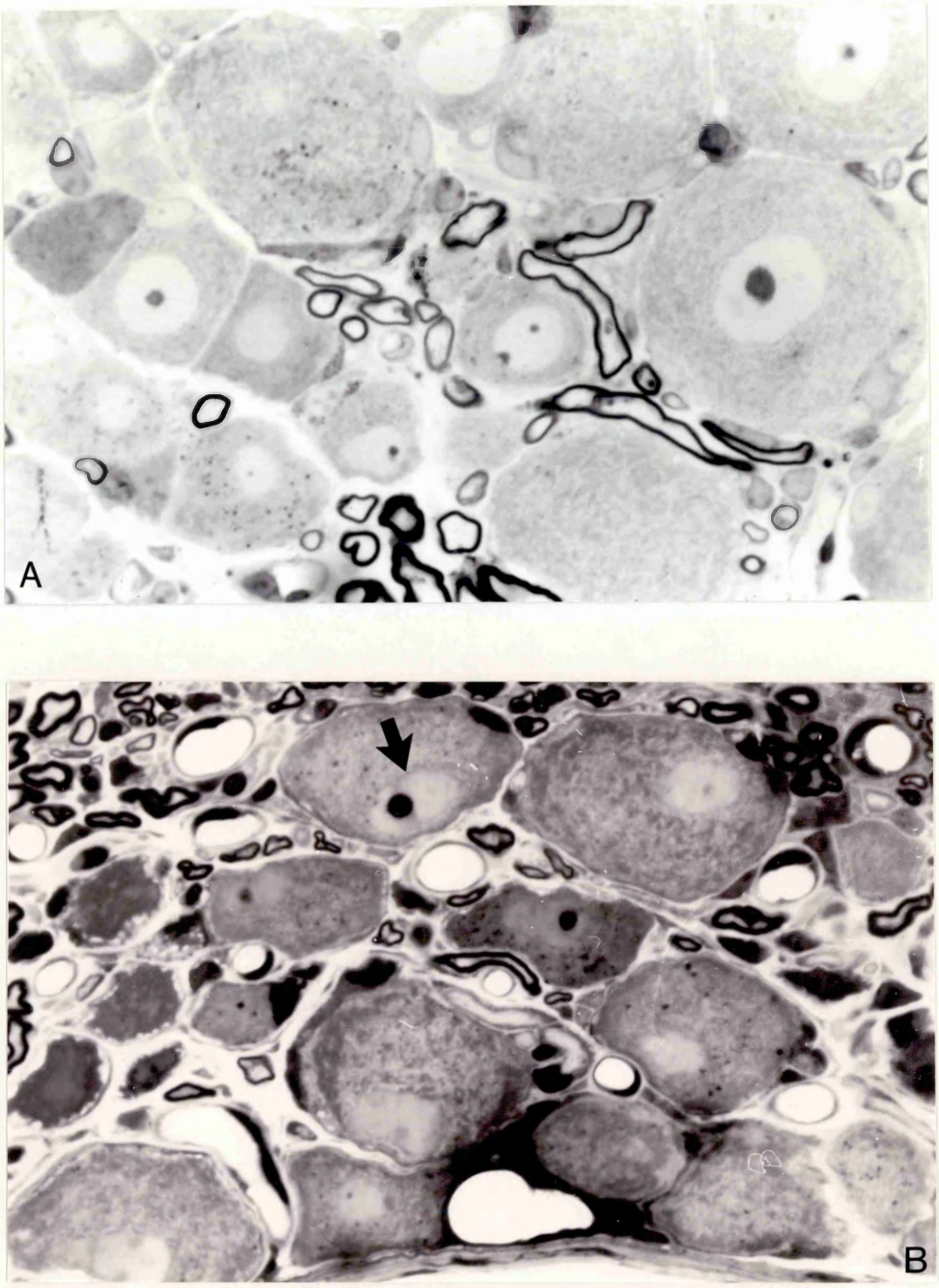


Figure 5.8: Axonal reaction in the spinal ganglia of C57Bl/Ola mice. (A) Normal (L4) spinal ganglia with centrally located nuclei. (B) Spinal ganglia 15 days after transection; many of the nuclei have become eccentrically located (arrow) (x 750). Cresyl violet stain.

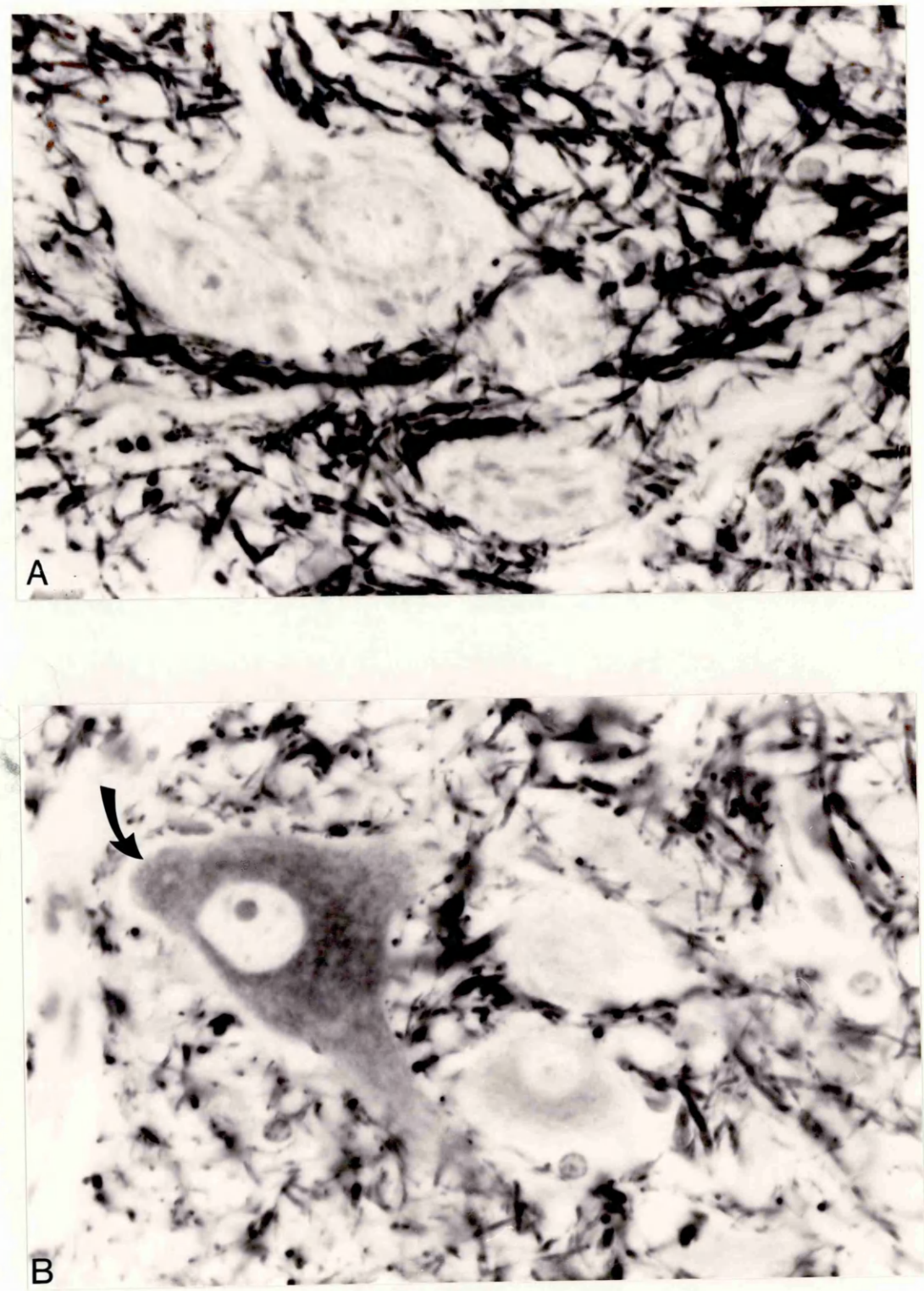


Figure 5.9: **Axonal reaction in the ventral horn cells of C57Bl/Ola mice.** Tissue sections were stained with RT 97, a monoclonal antibody against phosphorylated neurofilament, by the peroxidase-antiperoxidase method. (A) Normal, negatively staining ventral horn cells of the L4 spinal cord segment. (B) Ventral horn cells at 10 days after transection staining positively for the phosphorylated neurofilament (curved arrow) (x 750).

5.3.1.5 P_0 mRNA expression

Teased fibres

Single axotomy

The level of P_0 mRNA expression by Schwann cells in the teased fibres obtained from normal nerve was graded 8+ (fig. 5.10A). In the transected nerves of normal mice the signal was almost absent having been reduced to poorly defined, low level (1+) accumulations of silver grains. The fibres themselves were obviously degenerate with fragmentation of the sheath, and marked cellular proliferation as indicated by increased numbers of cell nuclei (fig. 5.10B). This was similar to that observed in transected rat nerve (see section 4.2.3). In contrast, the majority of fibres harvested from the mutant mice were not different from the positive controls taken from normal right intact sciatic nerve of the C57Bl/Ola mice (fig. 5.10C). Fibres were reasonably intact although early degeneration could be identified in occasional internodes. A strong perinuclear signal, similar to that expressed by the non-transected nerves, could be identified in the majority of cut C57Bl/Ola fibres. In those internodes in which degeneration was occurring, the signal intensity was reduced. In some fibres, this degeneration was observed to be intercalated between normal internodes.

Quantification of the intensity of P_0 mRNA expression using the Quantimet image analysis system identified strong preservation of the signal on the nerve fibres obtained from the transected mutant mice. The sampling system used identified the largest fibres with the strongest signal. Any low-expressing or negative cells would not be included. In the normal mice, the signal was reduced to less than 1/3 of the control nerves by 5 days and was virtually absent by 7 days (fig 5.11). However, a high level of expression was maintained in the C57Bl/Ola fibres.

Double axotomy

Five or 7 days after transection, the middle segment of the nerve which had been axotomized proximally and distally, was obtained and processed in a similar manner to the above. The normal right nerve, and the segments proximal and distal to the upper and lower axotomy site respectively, were also examined. Wallerian degeneration was observed in some of the nerve fibres in the middle segment, however many of the fibres were relatively normal in appearance and the level of expression of P_0 mRNA was similar to that in the normal nerve. The segment distal to the distal axotomy was similar to the middle segment and the section of nerve proximal to the proximal axotomy was like that of the normal right nerve.

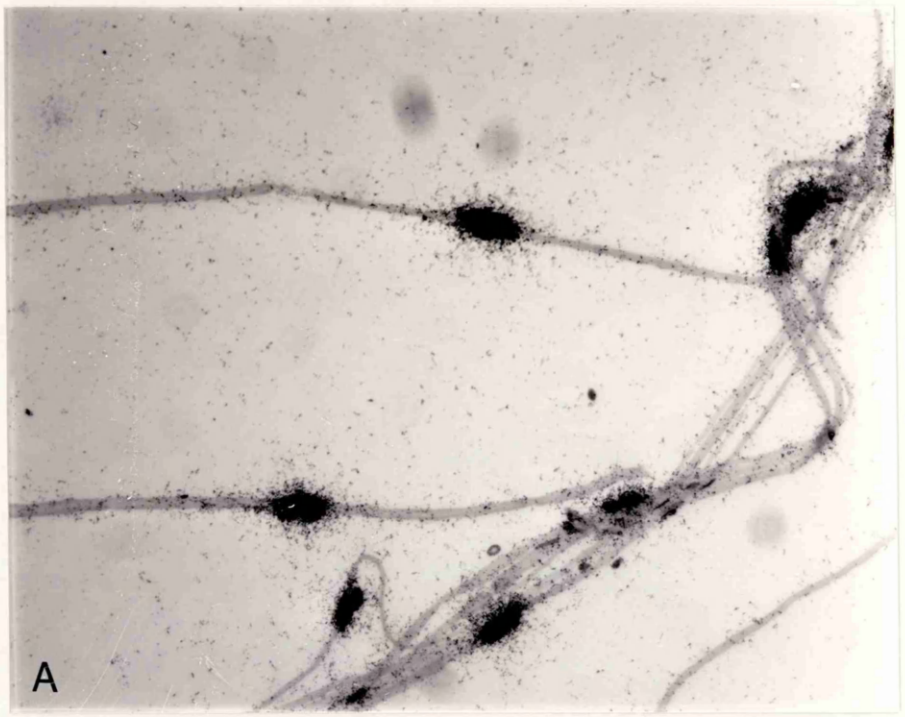


Figure 5-10. Expression of P_0 mRNA in teased fibres from C57Bl/Ola and normal mouse nerve. Intact right and transected left sciatic nerve was obtained from normal and C57Bl/Ola mice 5 days after axotomy. Bundles of fibres were teased and probed for P_0 mRNA expression using *in situ* hybridization methodology. After autoradiography, they were counterstained with haematoxylin and eosin. (A) Fibres from the right intact nerve of C57Bl/Ola mice. Perinuclear clusters of silver grains attest to the high level of expression of P_0 mRNA (x 170).

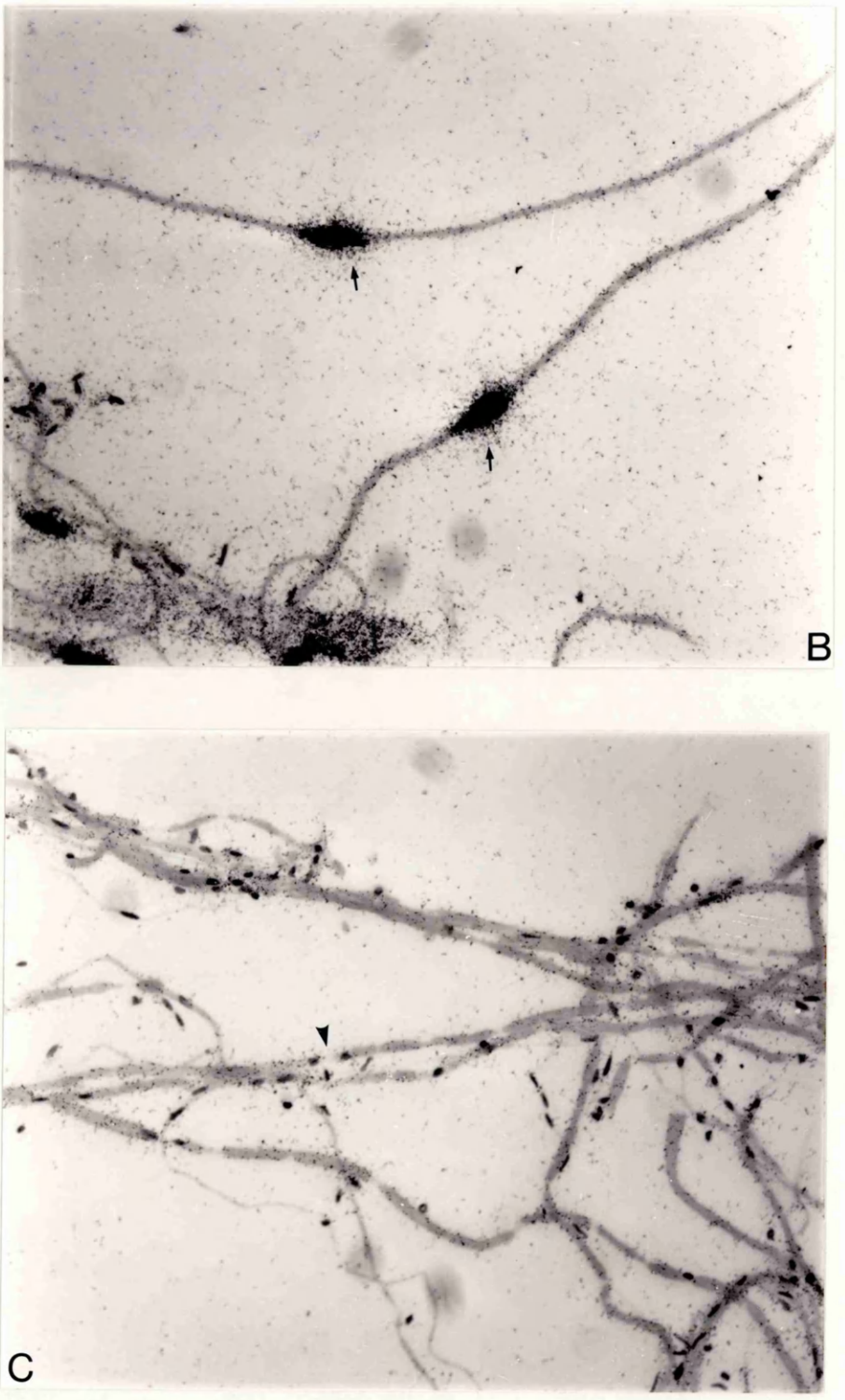


Figure 5-10 (cont.). Expression of P_0 mRNA in teased fibres from C57Bl/Ola and normal mouse nerve. (B) Fibres from transected left nerve of a C57Bl/Ola mouse. Perinuclear clusters of silver grains, indicating high levels of P_0 mRNA are still present in the majority of fibres (arrows). (C) Fibres from transected left nerve of a normal mouse. The expression of P_0 mRNA is essentially absent and a marked increase in cell density (arrow head) is apparent; this proliferation was not noted in the mutant mouse (x 170).

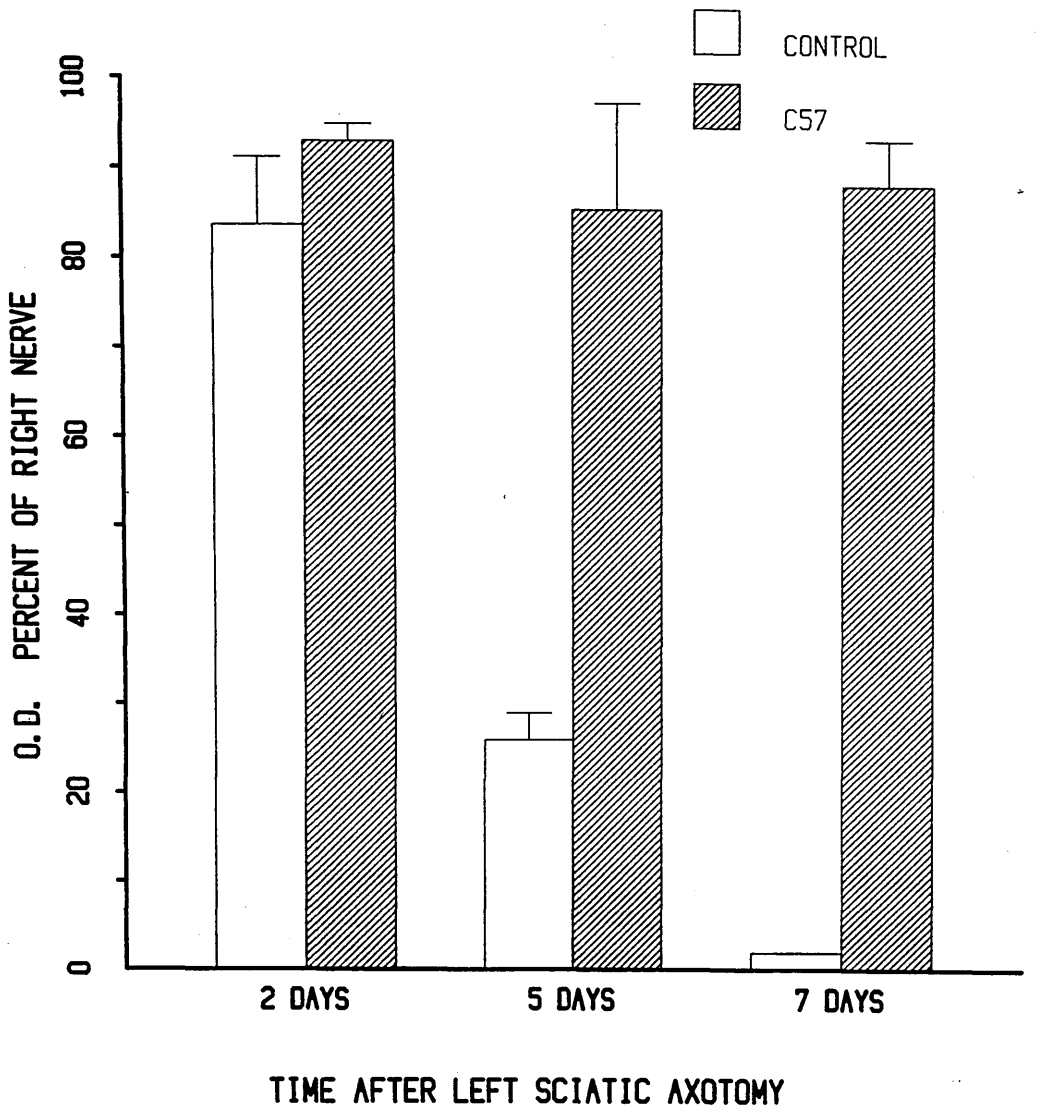


Figure 5.11: **P_0 mRNA expression after axotomy in C57Bl/Ola and normal mice.** The optical density of signal intensity was measured in 120 fibres from the intact right and transected left nerves in both the mutant and control mice at 2, 5 and 7 days after axotomy. The fibres with the greatest expression of the gene were measured and the optical density is presented as a percentage (mean +/- S.E.M.) in the axotomized nerve compared to the intact right nerve. The signal from the mutant mouse is not significantly decreased compared with the intact right nerve, whereas that from normal mice is greatly reduced at 5 days post-transection, and too low to measure accurately at 7 days.

Dissociated neonatal Schwann cells

Dissociated neonatal Schwann cells were cultured for 2, 5 and 7 days and assessed by *in situ* hybridisation for P_0 mRNA expression. The level of expression of these cells at day 0 was 8+, but by day 2 had declined to low levels (1+ expression) and was negative at 5 and 7 days.

Northern blotting

Based on the *in situ* hybridization results a 5 day post-transection period was chosen. By this time, the level of P_0 mRNA in the normal mice was essentially absent (fig. 5.12). In the C57Bl/Ola mice the signal was still very much present although reduced to 41% of the control. Equal loading of RNA in each lane was confirmed by probing for 7S using a [^{32}P] cDNA prior to stripping the filters (section 2.4.5.3) and probing for P_0 mRNA.

5.3.1.6 Southern blotting:

DNA of mutant mice was isolated and screened for gross differences of the P_0 gene compared with normal mice. No difference in the size of DNA fragments digested with PST I, EcoR I, Hin dIII and BamH I was noted between the two strains of mice.

5.4 DISCUSSION

The first paper by Lunn *et al.*, (1989) on the C57Bl/Ola mutant identified that these mice had delayed myelin and axonal degeneration after axotomy. This was associated with slow and sparse leucocyte invasion. A similar effect could be produced in normal mice by interfering with macrophage activity. The authors concluded that Wallerian degeneration requires rapid recruitment of myelomonocytic cells and degeneration is delayed in their absence. Concurrent failure of Schwann cell proliferation for the first 10 days was also noted. The mutation was thought to revolve around failure of myelomonocytic recruitment, but the reason for this failure was unclear (Lunn *et al.*, 1989). It was considered possible that the components of the degenerating nerve failed to deliver the right chemotactic signal for myelomonocytic cell recruitment, or that the cells were unable to enter the nerve (Lunn *et al.*, 1989; Perry *et al.*, 1990a). Subsequent studies using immunocytochemical methods to label macrophages confirmed the lack of macrophage recruitment into the distal stump of transected saphenous and sciatic nerve (Brown *et al.*, 1991). Although motor

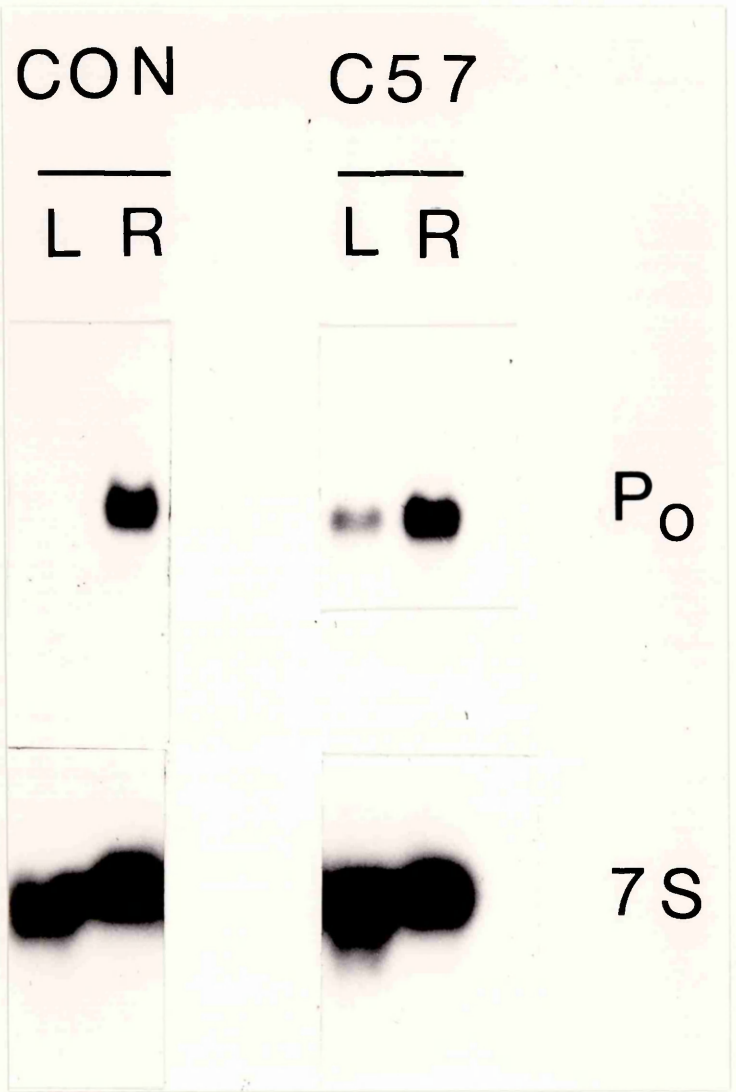


Figure 5-12. Northern blots of P_0 mRNA in transected and intact nerve of C57Bl/Ola and normal mice. 10 ug of total RNA was loaded into each lane, electrophoresed on a 1.5% denaturing agarose gel and blotted onto nitrocellulose paper. The filter was probed initially with a [^{32}P] labelled 7S cDNA probe to confirm equal loading of RNA, stripped and reprobed with a [^{32}P] P_0 cDNA probe. Lane 1: left axotomized nerve from normal (control) mice; lane 2: right intact nerve from normal mice; lane 3: left axotomized nerve from mutant mice; lane 4: right intact nerve from mutant mice. By 5 days post-transection, the signal was absent from the left nerve of the normal, control mice, but was present in the mutant mice, although reduced to 41% of the intact nerve (x 2).

regeneration was not impaired by the presence of predominantly intact axons in the distal stump (Lunn *et al.*, 1989), regeneration of the sensory nerves was slowed. Wallerian degeneration was thought to be a necessary prerequisite for normal sensory axon regeneration (Bisby and Chen, 1990).

It appears that the delayed degeneration is intrinsic to the nerve itself and not a function of the myelomonocytic population. This was ascertained by creating chimaeric mice using irradiation and bone marrow transplants such that the majority (> 90%) of the invading cells were of donor origin. The rate of degeneration of the transected sciatic nerve was found to be host-dependent rather than dependent on the immune system. The authors found that the delayed degeneration was also a property of the nerve fibre *in vitro*. Even at 5 days *in vitro*, the action potential could be elicited from C57Bl/Ola mice but was absent by 3 days *in vitro* for C57Bl/6J mice (Perry *et al.*, 1990a). Only a minimal increase in the mRNA levels for NGF and NGF receptor was documented in the distal stump of transected C57Bl/Ola mice; this was thought to be due to absence of the macrophages (Brown *et al.*, 1991; Perry *et al.*, 1990a). Lack of NGF and NGF receptor in these mice may account for the poor regeneration of the NGF-sensitive sensory nerve fibres (Brown *et al.*, 1991). Slowed Wallerian degeneration was also a property of axons in the CNS where there are no Schwann cells (Perry *et al.*, 1990a).

Heterozygotes of the mutation degenerate more rapidly than C57BL/Ola, but not as quickly as wild type mice. These experiments suggest the action of single dominant gene or genes. The housing environment also had an effect on the rate of Wallerian degeneration expressed in the mutant mice but not in the control mice. This environmental effect may have been mediated through the immune system possibly by affecting macrophage function (Perry *et al.*, 1990b).

The morphological studies performed here confirm those of Lunn and colleagues (1989) in detailing the delayed breakdown of both axons and myelin sheaths. By 5 days post-transection, the nerve fibres in the distal stump of normal mice had degenerated associated with a significant amount of phagocytosis, whereas in the C57Bl/Ola mice, there was little change from the control normal nerve. Even at 10 days post-axotomy, the nerve fibres from the latter mice were well preserved with maintained nodes of Ranvier and Schmidt-Lantermann clefts. By electron microscopy, preservation of all aspects of the nerve fibre was documented, although inter-fibre variation in the degree of fibre preservation was also noted. Of special interest in this study was the preservation of the axonal compartment with retention of cytoskeletal elements and the axolemma. Of the cytoskeletal components which were assessed by immunocytochemistry, actin, tubulin and the neurofilament 200 protein were retained

in non-degenerate fibres. Overall, neurofilament 200 was somewhat better preserved than actin or tubulin. The preservation of neurofilaments in these mutant mice was similar to that observed by Lunn *et al.*, (1989) and Perry *et al.*, (1990b). However, actin and tubulin was lost from degenerate axons by 5 days after axotomy in these mice. This is of interest considering their subaxolemmal location (Kobayashi *et al.*, 1986; Papasozemonos *et al.*, 1982) and the maintenance of the myelinating signal which is presumed to be axolemmal in location. Expression of P_0 protein in the myelin sheath was minimally affected, which was consistent with the morphological preservation of the sheath. The immunostaining for this protein highlighted where sheath fragmentation had occurred.

The teased fibres also were useful in assessing morphological changes in the nerve fibres. Maintained fibre integrity and lack of cellular proliferation were obvious on the transected nerves of the C57Bl/Ola mice. In the control mice, fibre fragmentation, myelin abnormalities and a marked increase in nuclear concentration were noted. This difference in cell numbers was also noted by Lunn and coworkers (1989), and it was only at 6 days post-sectioning that an increase, albeit minor, in nuclear density was observed in mutant mice (Lunn *et al.*, 1989).

Despite the delayed degeneration in these mice, the axonal reaction was not impaired and changes such as nuclear eccentricity and an increase in perikaryal phosphorylated neurofilaments were observed as discussed in section 1.5.2.4. The number of neurons in which axonal reaction was observed in the Ola mice was not different to the number in normal mice. These data imply that the signal that stimulates the axonal reaction, which is possibly retrogradely transported to the somata, is intact and does not rely upon degeneration in the distal stump for its presence and action.

The teased fibres and northern blots hybridized for P_0 provide interesting and conclusive information on the ability of the Schwann cell to maintain P_0 mRNA expression during delayed Wallerian degeneration in these mice. The Quantimet studies on the teased fibres had a selective bias as only the larger fibres with measurable signal were analysed. The northern blot is a representation of all fibres, including small and completely down-regulated ones and, therefore, demonstrates a lower level of P_0 mRNA in the transected C57Bl/Ola nerve. The mouse DNA was probed with a cDNA derived from rat P_0. However the gene encoding the P_0 protein has been well conserved throughout evolution and the rat and mouse P_0 gene are

highly homologous (You *et al.*, 1991). The rat cDNA was, therefore, suitable for probing the mouse nucleic acid.

Under normal circumstances, as described in sections 1.5.3.2, 1.5.3.3 and chapter 4, axotomy quickly induces down-regulation of myelin-specific molecule mRNA expression by the Schwann cells. Distal to the axotomy site, the axon is functionally and anatomically separated from its neuronal cell body and, hence, from the main supplier of its nutrients and the director of its function (Schlaepfer, 1974). The data given in this chapter can be considered in the light of the original hypothesis of this thesis which suggests that the putative axolemmal signal(s) is manufactured in the neuron and transported down the axon to be inserted in, or act near, the axolemma and thus interact with the adjacent Schwann cell to induce upregulation of myelin-specific molecules. If this is the case, then the results obtained with C57Bl/Ola mice suggest that the signal is fairly stable in the axolemma in the absence of degenerating factors, and continues to interact with the Schwann cell and direct myelin-specific molecule expression by that Schwann cell. This theory of a fairly stable signalling molecule(s) with a low turn over rate is supported by Rubinstein and Shrager, (1990) in which remyelination of transected axons occurred in a species with slowed Wallerian degeneration.

The double axotomy experiment effectively blocks retrograde axonal transport in the middle segment. If retrograde transport was involved in the maintenance of this myelinating signal(s) then, presumably, the expression of P_0 mRNA would have declined in the middle segment. However, as this was not the case, these data suggest that fast and retrograde axonal transport is not involved in maintaining the myelinating signal(s) in axons of both poikilothermic and homeothermic animals.

This mutation does not appear to revolve around increased autonomy of the Schwann cells such that they can maintain a high level of myelin-specific molecules in the absence of axonal contact. This is suggested by the fact that hitherto actively myelinating Schwann cells obtained from the sciatic nerve of 5 day old mice, rapidly down-regulated expression of P_0 mRNA upon dissociation from the axons.

This mutation also does not appear to be a gross deletion, insertion or rearrangement of the P_0 gene as indicated by the Southern blot. However, retrospectively, it would have been better to use four-cutter enzymes, instead of the six-cutters chosen. This would have markedly increased the probability of identifying a mutation of the gene.

5.5 CONCLUDING REMARKS

Delayed Wallerian degeneration is well documented in the C57Bl/Ola mouse mutant. The studies described in this chapter, identified that this delay was associated with preservation of P_0 mRNA expression in the Schwann cells. This is consistent with work done by Rubinstein and Shrager (1990) in which remyelination, and therefore P_0 mRNA expression, can occur on transected nerve if Wallerian degeneration is delayed. Schwann cells of these mice, dissociated from the axon, down-regulated the expression of this gene in the classical manner implying that maintained axolemmal-glia contact is necessary for maintained gene expression. The preservation of signal occurred simultaneously with maintained morphological integrity of the nerve fibre. This suggests a strong correlation between morphological integrity and signal expression. This concept is approached and discussed from other angles in chapters 6, 8 and 9.

Delayed Wallerian degeneration with preservation of P_0 mRNA expression suggests that the putative myelinating signal is still interacting with the Schwann cell in a biologically effective manner. This occurs in the absence of contact with the neuronal somata and implies that axonal transport (fast or retrograde) is not important in this interaction. Thus, the putative myelinating signal appears to be stable with a low turn over rate.

CHAPTER 6: DELAYED WALLERIAN DEGENERATION *IN VITRO*

6.1. INTRODUCTION

The results of *in vivo* studies performed on a mouse mutant which exhibits delayed Wallerian degeneration were discussed in the last chapter. Other investigators have attributed this delayed *in vivo* degeneration to failure of normal myelomonocytic cell recruitment (see section 5.4) (Brown *et al.*, 1991; Lunn *et al.*, 1989; Perry *et al.*, 1990a). In normal nerves *in vitro*, Wallerian degeneration is delayed partially due to decreased clearing of myelin debris by phagocytic activity (Crang and Blakemore, 1986). These data prompted the question as to whether nerve from normal mice and C57Bl/Ola mutants would degenerate *in vitro* at a similar rate. In both these situations decreased phagocytic activity would occur. In association with the studies described in chapter 5, preliminary studies were performed in which segments of desheathed C57Bl/Ola and normal mouse sciatic nerve were cultured for 2 or 5 days in SCGM at 37^{o}C. Electron microscopy of these nerves fragments identified that degeneration was advanced in both fibre types by 5 days *in vitro*. In contrast to this, Perry and coworkers (1990) found delayed degeneration of these mutant mouse fibres *in vitro* as well as *in vivo*. My findings suggested that, at least *in vitro*, degeneration was not delayed as it was *in vivo* and that factors other than myelomonocytic recruitment act during Wallerian degeneration. The contribution of other degrading factors, such as lysozymal activity, has been discussed in sections 1.5.2.1. and 1.5.2.2..

Survival of the transected axon is partially dependent upon its ability to maintain ionic homeostasis with retention of certain ions and exclusion of others (Schlaepfer and Bunge, 1973). The intra-axonal ionized calcium concentration is normally about 0.3 μM (Baker *et al.*, 1971) and this cation plays an important role in degeneration of the nerve fibre (Hodgkin and Katz, 1949; Schlaepfer, 1971; Schlaepfer and Bunge, 1973; Schlaepfer, 1974). Calcium, by itself, can induce a myelopathy when applied to exposed spinal cord (Balentine and Dean, 1982). *In vitro* studies have confirmed the role of this ion in nerve fibre degeneration. When rat sciatic nerve segments were placed in buffer containing calcium ions, extensive granular disruption of neurofilaments and neurotubules was observed within 4 hours. If the nerve was placed in calcium free buffer, then cytoskeletal components were preserved for 96 hours (Schlaepfer, 1971). Similarly, diffuse myelin degeneration was noted within 48 hours if the buffer contained calcium, whereas no degeneration was

observed at this time in calcium-free medium (Schlaepfer, 1974). The effect of calcium on the axoplasm appears to be specific and was not produced with other divalent cations such as magnesium, strontium and barium (Hodgkin and Katz, 1949). Thus, if Wallerian degeneration was delayed *in vitro* by chelating the calcium from the medium, or using a calcium-free medium, what effect would this have on expression of myelin-specific molecules such as P_0 mRNA?

Wallerian degeneration encompasses several different events which include degeneration of the axon and myelin sheath, phagocytosis and clearing of the debris, axonal reaction in the associated neuronal somata, Schwann cell proliferation and formation of bands of Bungner. Associated with these events is the down-regulation of the transcripts encoding myelin-specific molecules. Switching the Schwann cells from a myelin sheath-maintaining state to a dividing state during Wallerian degeneration would involve the upregulation of the battery of genes involved in cell proliferation and the down-regulation of genes involved in sheath maintenance. Recent work by Morgan and colleagues on P_0 expression by Schwann cells has identified that cells with the myelinating phenotype were a different population from the dividing cell population. This implied that DNA synthesis and expression of the myelinating phenotype was incompatable in the one Schwann cell (Morgan *et al.*, 1991). Addition of antimitotic agents appears to delay degeneration of the axotomized nerve both *in vivo* and *in vitro*. Treatment of the distal stump of transected nerve with mitomycin C was found to delay myelinolysis and preserve the axolemma somewhat. In untreated but cut nerves, an increase in [^{3}H] thymidine incorporation corresponded with a fall in [^{3}H] saxitoxin binding to axolemma sodium channels. This implied that, in the cut nerve not treated with antimitotics, Schwann cell proliferation was associated with a loss of nodal axolemmal molecules (Pellegrino *et al*, 1986). Myelin degradation was also much slower in cultured explants treated with cytosine arabinoside (Bonnekoh *et al.*, 1989; Crang and Blakemore, 1986) or colchicine (Bonnekoh *et al.*, 1989).

Addition of antimitotics is associated with preservation of fibre morphology and Schwann cell proliferation may be linked to changes of the axolemma. These data raised the possibility that preventing Schwann cell proliferation during Wallerian degeneration may be linked to preservation of signal for myelin-specific molecule expression and delayed down-regulation of the P_0 transcript. Was the preserving effect of antimitotic addition due to prevention of the transition of Schwann cell activities from sheath maintenance and myelin-specific molecule expression, to mitosis? It seemed more likely that the preserving effect of antimitotic agents was

associated with decreased phagocytic cell numbers as described by Bonnekoh *et al.*, (1989).

The role of cyclic-AMP as a second messenger in the transduction of axolemmal-related mitogenic signals within the Schwann cell has been discussed in section 1.3.1.4. and 1.3.2.1. The expression of axonally-dependent molecules such as GalC (Sobue and Pleasure, 1984), O4 and AOO7 (Mirsky *et al.*, 1990) has been upregulated by exposing cultured Schwann cells to cyclic-AMP elevators or analogues. Down-regulation of P_0 gene expression in cultured neonatal Schwann cells has been also been reversed by agents such as forskolin, cyclic-AMP analogues and cholera toxin that raise intracellular levels of cyclic-AMP. Lemke and Chao (1988b) observed induction of the mRNAs for P_0 and MBP but not suppression of NGF receptor mRNA in response to forskolin. Whilst Morgan and coworkers (1991), noted that the cells became positive for P_0 protein and negative for N-CAM, NGF receptor, A5E3 and GFAP in response to these agents, but only in the absence of Schwann cell division. The expression of O4 was also induced, but this occurred in both dividing and non-dividing cells (Morgan *et al.*, 1991). The different response of NGF receptor protein and mRNA between the two studies may suggest that forskolin induces transcription of the NGF receptor mRNA but this is not subsequently translated.

Another study found that neither P_0 nor MBP was induced in response to 8-bromo cyclic-AMP or forskolin, whilst P_{170k} was. This glycoprotein is unique to the peripheral nervous system and its expression is axonally dependent (Shuman *et al.*, 1988; Shuman *et al.*, 1988). There are basic culturing differences between these three experiments which may partially account for the variable upregulation of P_0. Lemke and Chao used longer term cultures expanded with glial growth factor and forskolin; whilst both Morgan and Shuman used shorter term cultures but Morgan used a much richer medium.

These studies implied a role for cyclic-AMP analogues and activators of the cyclic-AMP pathway in the transduction of the putative axolemmal signal to the Schwann cell nucleus for stimulation of myelin-specific molecule expression. The role of other intermediate molecules, such as SCIP, in this pathway, has been discussed (see section 1.6.2) (Monuki *et al.*, 1989). Thus, if loss of the putative axolemmal signal for myelination could be bipassed by supplementing degenerating explants with these agents, would the expression of P_0 mRNA be maintained?

Corticosteroids are considered capable of stabilizing cell membranes by forming cross links between free radicals lying on the cell surface (Metz *et al.*, 1982).

In a similar fashion they are thought to stabilize lysosomal membranes against peroxidation (cited in Metz *et al.*, 1982). For these reasons, corticosteroids are used in shock states to help maintain vascular and lysosomal integrity (Haskins, 1983). Dexamethasone is a drug often used in such situations. As the release of lysosomal enzymes has been described in Wallerian degeneration (Lassman *et al.*, 1978) and was thought to lead to segmental axonal degeneration (Joseph, 1973), the question was raised as to whether administration of this drug during degeneration would have any beneficial effect on maintaining nerve integrity and hence P_0 mRNA signal expression.

It was the aim of this chapter to assess the effect of retarding nerve fibre degeneration *in vitro*, on fibre morphology and relate this to changes in the expression of various axonally controlled molecules such as P_0 mRNA, NGF, N-CAM and GFAP proteins. The retarding agents included removal/absence of calcium from the medium, use of antimitotics, and dexamethasone. Also, the question was asked as to whether in the absence of the axonal signal as occurs in Wallerian degeneration, could the expression of the P_0 mRNA be sustained using a potential second messenger?

6.2 MATERIALS AND METHOD

The sciatic nerve from the greater trochanter to the level of the stifle was obtained in an aseptic fashion immediately after death from adult Sprague-Dawley rats or C57/BL/6 mutant mice (see chapter 5) or the same strain of mice which were known to be non-mutant. All perineurial tissue was meticulously dissected from the nerve which was cut into 3 to 5 mm segments. Using the tips of fine forceps (Taab No. 5) the nerve was gently stroked longitudinally to separate the fibres and allow better diffusion of nutrients during culture. The segments were cultured in different media. The basal medium was Dulbecco's modification of Eagle's medium supplemented with 1.0% glutamine and 7.5% foetal calf serum supplement which is referred to as Schwann cell growth medium (SCGM). For experiments which were assessing the effect of calcium the following media were used: supplemented medium as described above with or without 0.01 M EGTA, DMEM only, or Hank's balanced salt solution (calcium and magnesium free). The EGTA-containing medium was buffered with 1.0 M NaOH until the medium returned to an appropriate colour. Mouse explants were cultured in SCGM with or without EGTA only, whilst rat explants were cultured in all four different media.

The medium used in the following experiments was supplemented SCGM without EGTA and only rat nerves were used. Cytosine arabinoside was added at 0.01 mM and colchicine at a final concentration of 1 μg/ml. 8-bromo cyclic-AMP and dibutryl cyclic-AMP were both added to achieve final concentrations of 0.1 mM which in some experiments was decreased to 0.01 mM after 48 hours. Dexamethasone (Sigma, 9-α-fluro-16-α methylprednisolone) was dissolved initially in 2 ml of ethanol and then diluted with L15 medium to 2.5 μg/ml. Explants were cultured in 25 and 50 ng/ml concentrations of dexamethasone.

All explants were cultured at 37^{o}C for either 2 or 5 days at the end of which time they were fixed in 4% paraformaldehyde, or used unfixed for immunostaining (see below) or for RNA extraction (2.4.1.2). RNA was also extracted from normal rat nerves and nerves which had been transected and allowed to degenerate for 5 days *in vivo*. Between 2 and 6 sciatic nerves were used for each RNA extraction. To assess the quality of RNA, 4.0 μg samples from normal nerve and nerve cultured in the presence and absence of EGTA, was electrophoresed in a 1.5% denaturing analytical gel, transferred to nitrocellulose paper (2.4.5.1) and stained with 0.04% methylene blue/0.5 M sodium acetate dye (A4.2.6). Dot blotting was used to assess the P_0 mRNA level in normal nerve, transected *in vivo* degenerated nerve, and explants cultured in the four different media for 5 days. After probing with P_0 cDNA, the filters were stripped (2.4.5.3) and reprobed with NGF receptor cDNA (2.4.2.3). Fixed fibres from cultures, and normal and *in vivo* degenerated nerve, were processed for electron microscopy (A2.3.3.2), or were teased onto poly-L lysine-coated, RNase-free slides (A4.1.4) and probed for P_0 mRNA by *in situ* hybridisation (2.4.4).

Teased fibres were prepared for immunofluorescence studies to assess the effect of transection and the various media on the expression of GFAP, N-CAM and NGF receptor protein. Fibres were teased onto poly-L lysine-coated slides and assessed at day 0 and after 5 days degeneration *in vivo*, or *in vitro*. Staining was performed as described in section 2.3.1.1 on both fixed and unfixed fibres for NGF receptor, N-CAM and GFAP. For GFAP staining, the fibres were first treated with 0.1% triton-X 100 for 10 minutes at room temperature. After staining with the primary antibody for 30 to 60 minutes, fibres were washed 3 times within 30 minutes and secondary antibodies were applied. For both NGF receptor and N-CAM the secondary antibody was a goat-anti-mouse IgG_1 linked to fluorescein, whilst for GFAP it was a goat-anti-rabbit-FITC. Both were applied for 30 minutes. The fibres were washed in distilled water, fixed in 4% paraformaldehyde if not previously fixed, and mounted in PPD mountant. Signal intensity was graded from 0 to 8+ for both immunofluorescence and *in situ* hybridisation.

A final experiment was performed in which fibres were cultured in the presence of both dibutryl and 8 bromo- cyclic-AMP (0.1 mM) with or without EGTA. These were assessed for P_0 mRNA and NGF receptor expression.

6.3 RESULTS

6.3.1 Morphology

6.3.1.1 Effect of calcium and its chelation

There was no obvious difference in fibre preservation between the two strains of mice when sciatic nerve explants were cultured *in vitro* in either medium. The delayed degeneration noted *in vivo* in the C57/BL6/Ola mice was not observed under *in vitro* conditions. There was a dramatic difference in the degree of preservation of fibres cultured in calcium-chelated medium rather than calcium-containing medium in both the mice and rats. This was obvious even during explant culture as those in calcium-free medium retained a loose fibrillar appearance, whereas those in calcium-containing medium condensed to form tight balls of tissue.

2 days *in vitro*

By light microscopy 50% of fibres were lost from the mouse explants cultured in SCGM compared with intact nerve; the axoplasm had condensed and pulled away from the edge of the myelin sheath. Occasional sheaths were irregular or, more commonly, fragmented and forming ovoids. In contrast, the explants cultured in SCGM with EGTA were far better preserved with fewer fibres exhibiting degeneration both of the myelin and the axon.

Similar findings were noted in the rat explants with the addition of EGTA to the medium being associated with increased preservation of fibre morphology. Similarly, better fibre preservation was noted in those fibres cultured in calcium and magnesium-free Hank's balanced salt solution (HBSS), but fibres were not preserved in DMEM or SCGM without EGTA. The most obvious changes were splitting and degeneration of the myelin sheath, and vesiculation of the axoplasm in the latter two media.

Teased fibres from explants with or without EGTA had a vesiculated and "bubbling" appearance to the myelin which seemed to be most pronounced around the node of Ranvier in some fibres although other internodes were equally affected along their whole length.

Electron microscopy, identified similar changes in explants cultured in SCGM without EGTA, HBSS or DMEM. On some fibres the myelin sheath was reasonably intact, whilst in others, the lamellae were beginning to separate. Very degenerate sheaths were also present. In some fibres, axoplasm was condensed occupying a smaller volume, but the cytoskeletal components were preserved. Periaxonal vesiculation was a common finding, and, not uncommonly, multiple vesicles formed giving a lacey appearance to the area originally occupied by the axon. Thus, most of the axoplasm was lost. Any remaining axoplasm often had a watery, smudged appearance associated with the dissolution of the cytoskeleton. Preservation of the axolemma was variable. Explants cultured in SCGM with EGTA were better preserved with many fibres appearing relatively normal in both the myelin sheath and the axon. Mitochondria and cytoskeletal elements such as neurofilaments and microtubules were identifiable.

5 days *in vitro*

By light microscopy, it could be seen that many of the fibres cultured in SCGM with EGTA were still reasonably well preserved although some lamellar and splitting and axoplasmic condensation had occurred. Those fibres cultured in the absence of EGTA had mostly degenerated with overt myelin degeneration and axonal loss.

Unlike the teased fibres at 2 days, there was a marked difference in the degree of fibre preservation between the two different media at 5 days. Without EGTA the fibres were very fragmented with formation of ovoids and proliferation of Schwann cells resulting in a marked increase in numbers of nuclei spread out along the degenerating fibres. In fibres cultured in the presence of EGTA, the myelin was much better preserved with nodes of Ranvier still being present on some fibres (fig 6.2). There was no obvious increase in the nuclear count in these explants. The order of media from most to least preserving was SCGM with EGTA, HBSS, DMEM and then SCGM without EGTA.

By electron microscopy, it could be seen that the degeneration in calcium-containing media had continued with intact cytoskeletal elements being only a rare legacy of the axoplasm (fig 6.1). Fibres cultured in the presence of EGTA were variably preserved. Some were predominantly intact in both the myelin sheath and the axon, whilst others had degenerated quite markedly with multiple periaxonal vesiculations and splitting of the myelin sheath.

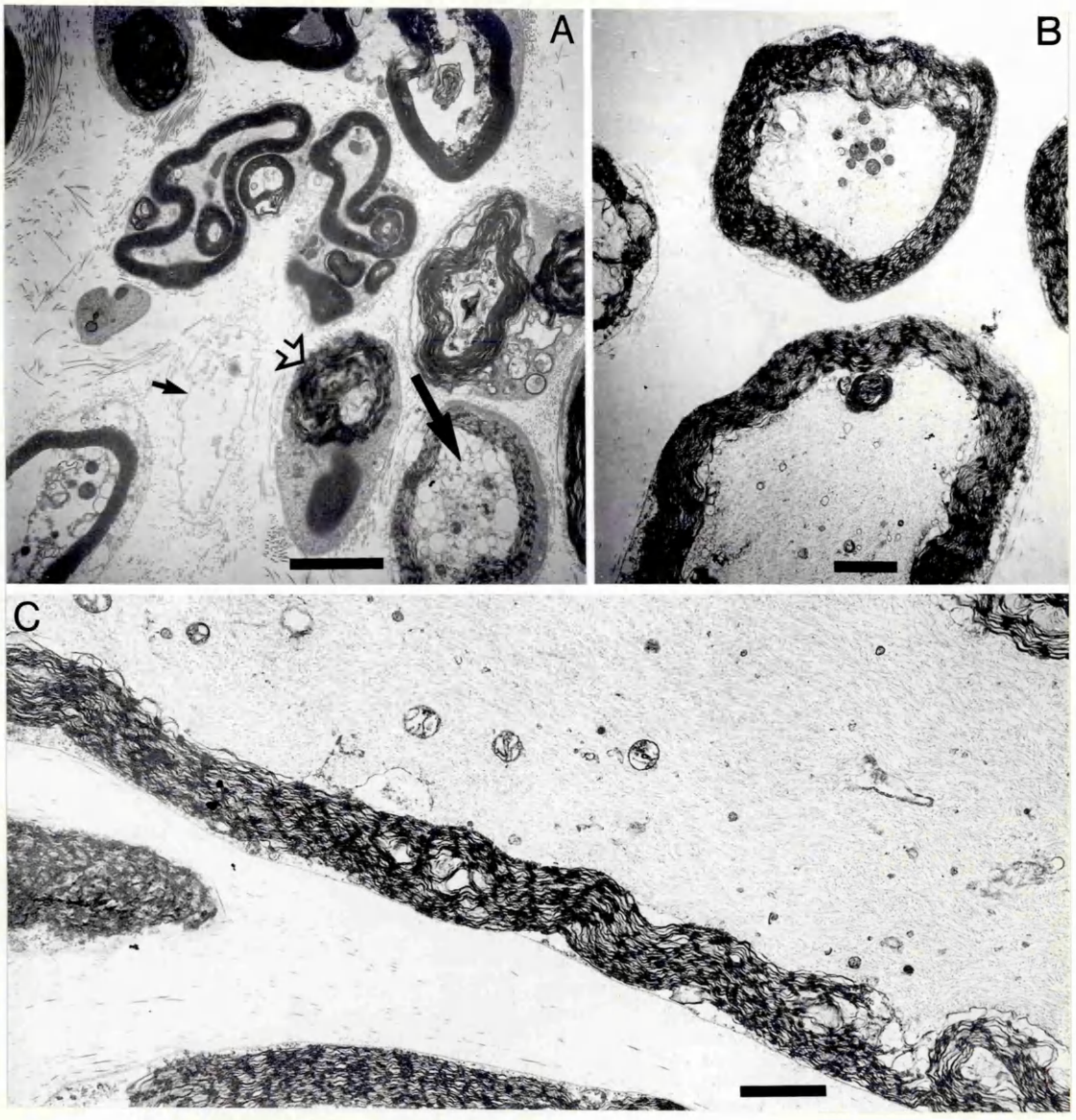


Figure 6.1: The effect of calcium on the preservation of myelinated fibres *in vitro* - EM. Small bundles of loosely teased fibres were cultured as free-floating explants in the presence or absence of calcium for 5 days. (A) With calcium present, the axons rapidly break down (large arrow) and may be absent (open arrow). An empty basal lamina tube (small arrow) is also evident. When calcium is chelated (B and C) the axonal preservation, especially the axonal cytoskeleton, is markedly improved. (Bar = 10 um in A and B; and 2 um in C).

7 days *in vitro*

Only the mouse explants were assessed at this time. By light microscopy, the discrepancy between those fibres cultured with, and those fibres cultured without EGTA, was still maintained as for 5 days *in vitro*.

Teasing of fibres cultured without EGTA resulted in marked fragmentation. In contrast, fibres cultured with EGTA were much less fragmented and better preserved even to the extent of retention of nodes of Ranvier.

Electron microscopic observations identified extensive degeneration of fibres cultured in SCGM. The axon was often unrecognizable. If it was present the axoplasm was condensed, had lost its cytoskeletal components and had retracted away from the myelin sheath. Multiple periaxonal vesiculations were frequent findings, whilst degenerating mitochondria and multivesicular bodies were observed in the axoplasmic remnants. Axolemma was difficult to discern amongst the debris. The Schwann cells contained large amounts of lamellar myelin debris and angular cavities were present in their cytoplasm which may have represented cholesterol clefts. The remaining sheaths were very fragmented and split. Those explants cultured in SCGM with EGTA were much better preserved both with respect to the myelin sheaths and the axons. Neurofilaments, microtubules and mitochondria were not uncommon. The axoplasm often filled the majority of the sheath and the axolemma was occasionally discernable. Some periaxonal vesiculation was present but this was greatly reduced compared to explants cultured in SCGM without EGTA. Although there was some loosening of the compaction of the myelin lamellae, many of the sheaths were still reasonably intact.

6.3.1.2 Effect of dexamethasone and cyclic-AMP analogues

The morphology of explants treated with these compounds was only assessed on teased fibre preparations. Addition of dexamethasone (fig. 6.3B) or cyclic-AMP analogues (fig 6.4) had no effect on fibre preservation at either 2 or 5 days *in vitro*.

6.3.1.3 Effect of antimitotics

Of the two antimitotics used, only colchicine appeared to have any effect on the preservation of fibre morphology but this was still not marked (fig 6.3C and D). However, this beneficial effect was constant throughout the 5 days.

6.3.2 Effect on P_0 and NGF receptor mRNA expression

6.3.2.1 *In situ* hybridisation

Only P_0 mRNA expression was assessed by ISH. The intensity of P_0 mRNA expression on teased fibres collected immediately from the animal was 7 to 8+ for all fibres tested regardless of their source (fig. 6.2A) and by 5 days *in vivo* only basal P_0 mRNA expression was still observed (fig. 6.2B). In test (cultured) fibres, there was some variation in fibre degeneration and signal expression within each sample. However, the overall appearance of the majority of fibres in a sample was very similar. In mice, whilst occasional fibres were expressing this transcript on day 2 at a level of 6 to 7+, most fibres were negative. So despite marked preservation of fibre morphology especially with EGTA, little retention of the P_0 signal was noted. At 5 and 7 days all mouse fibres were negative and there was no difference in the level of expression of this message in fibres cultured with or without EGTA. A similar situation was observed in rat teased fibres (fig 6.2) however, the decline in signal intensity on rat fibres was slower than on mouse fibres. There was a direct correlation in the level of P_0 mRNA expression and the degree of preservation of the fibre at 2 days *in vitro*. Fragmented and degenerated fibres were negative for expression, whilst positive expression was observed on relatively intact fibres. By 5 days *in vitro,* a faint legacy of expression was occasionally observed on rare fibres. It was notable that this was only on fibres that were less degenerated and not on severely degraded fibres. Similarly, no preservation of signal was noted if dexamethasone (fig 6.3B) or antimitotic agents (figs. 6.2, 6.3C and D) were added to the culture medium.

Little P_0 signal preservation occurred on fibres which had been cultured with cyclic-AMP analogues (fig 6.4), either with or without EGTA. Rare fibres cultured in their presence expressed a low level of P_0 mRNA at 3+ to 4+ at 5 days *in vitro*.

6.3.2.2 Dot blots

Despite adding similar amounts of nerve to each of the media at the start of the culture period, very different amounts of total RNA were extracted at the end of 5 days. The amount obtained was proportional to the degree of degeneration induced by the media. Thus, the quantity of RNA obtained from SCGM with EGTA was only 25% of that obtained from nerve cultured in SCGM without EGTA. Similarly, the amount obtained from the intact normal nerve was only 20% of that obtained from the transected nerve. However, the amount of total RNA obtained from the explants

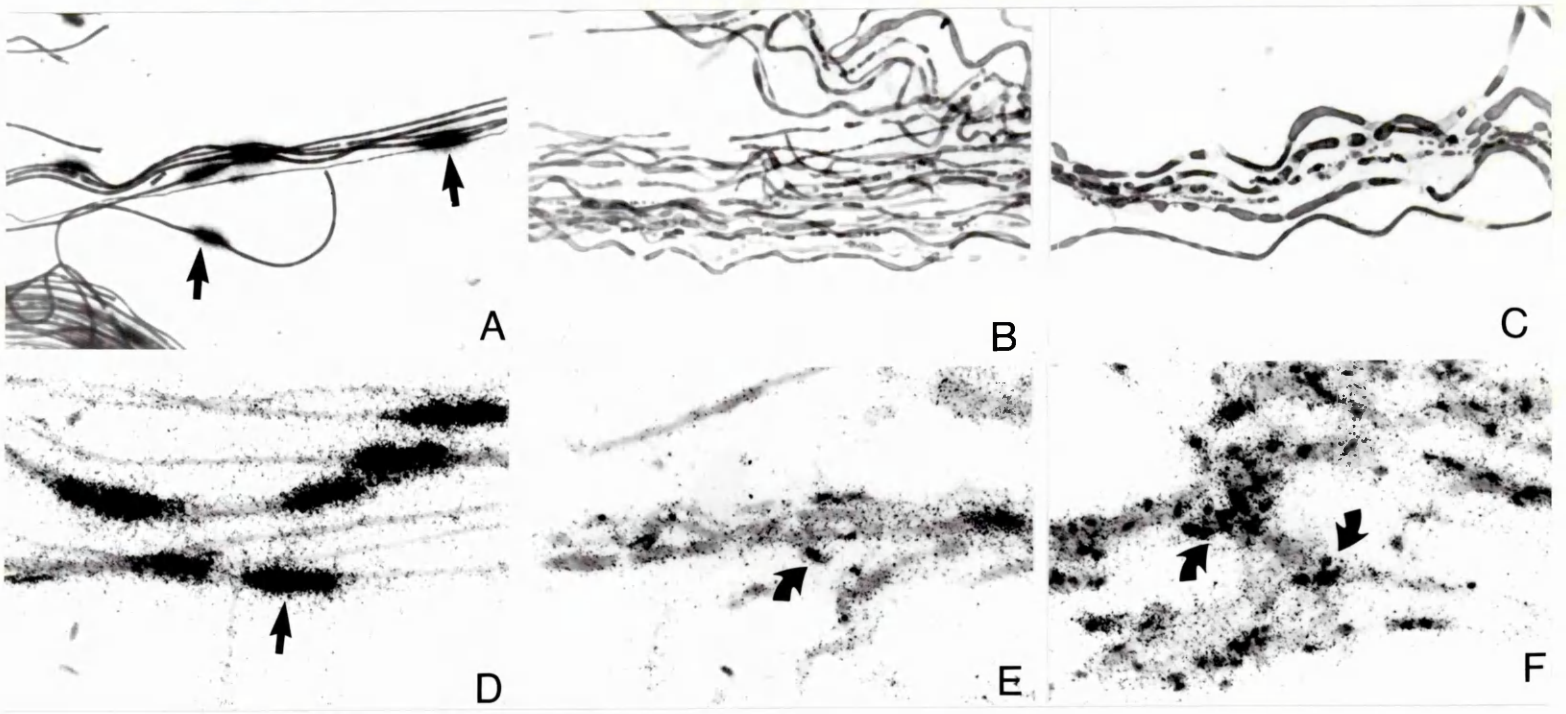
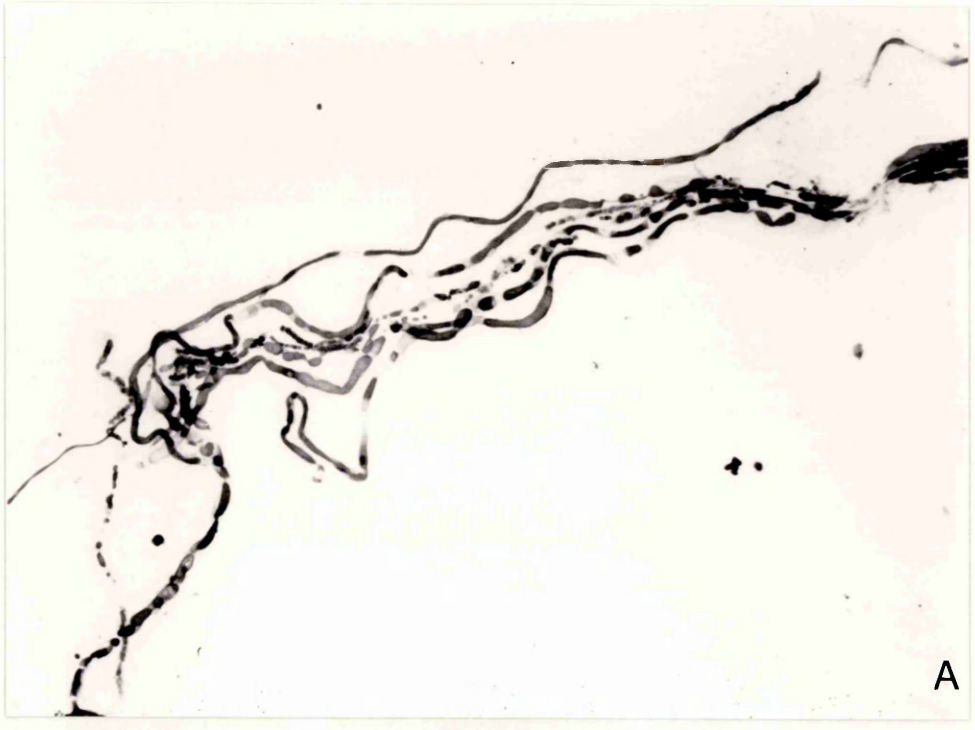


Figure 6.2: The effect of cytosine arabinoside on P_0 mRNA expression in cultured explants. A, B and C are stained with sudan black; D, E and F are stained with haematoxylin and eosin. Normal control nerve fibres are shown in A and D and exhibit strong expression of P_0 mRNA (arrows). Fibres in B, C, E and F have been cultured as free-floating explants for 5 days. The addition of cytosine arabinoside (0.01 mM) had no effect on the preservation of fibres or P_0 signal (B and E) compared with C and F to which no cytosine arabinoside was added. The addition of this agent did seem to affect the number of nuclei visible after 5 days in culture (E and F) (curved arrows). (A, B, C x 112; D, E, F x 200).



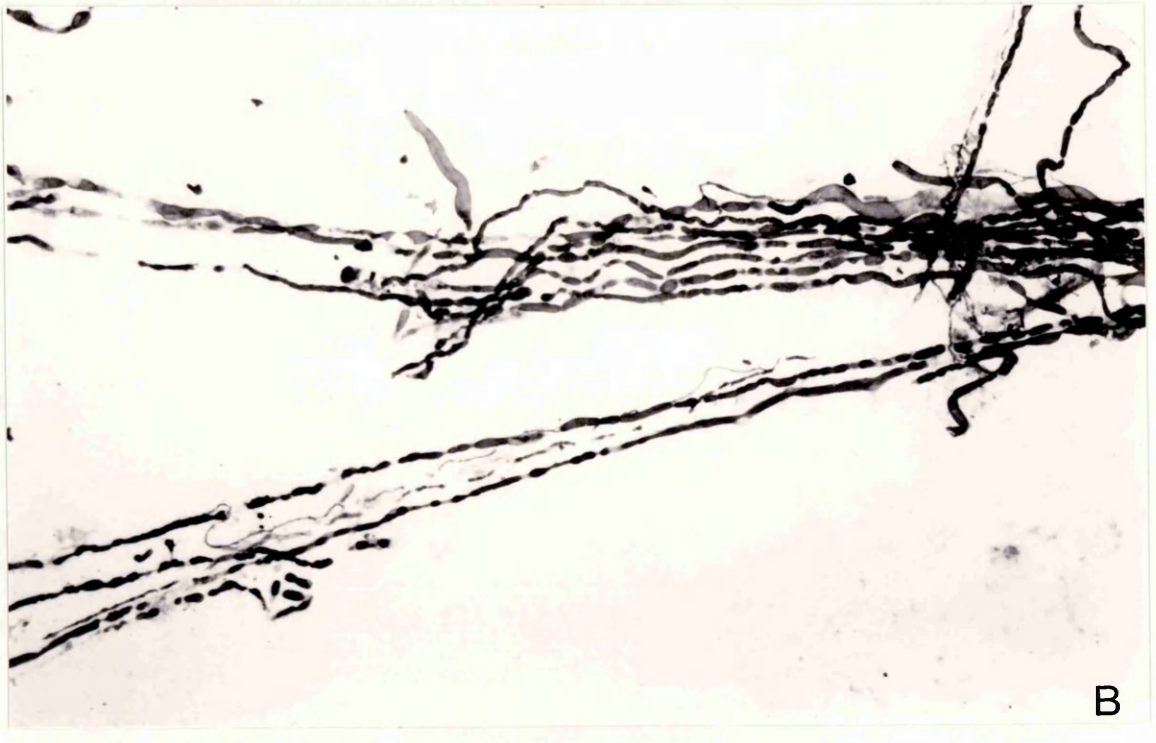


Figure 6.3: The effect of antimitotics or dexamethasone on preservation of myelinated fibres *in vitro*. Loosely teased bundles of sciatic nerve fibres were cultured as free floating explants for 5 days, then probed for P_0 mRNA expression by *in situ* hybridization. After autoradiography, the fibres were stained with sudan black. (A) Fibres cultured in Schwann cell growth medium containing 7.5% foetal calf serum and 1.0% glutamine. (B) Fibres cultured in the same medium to which dexamethasone (50 ng/ml) was added. The fibres have degenerated in both situations (x 150).

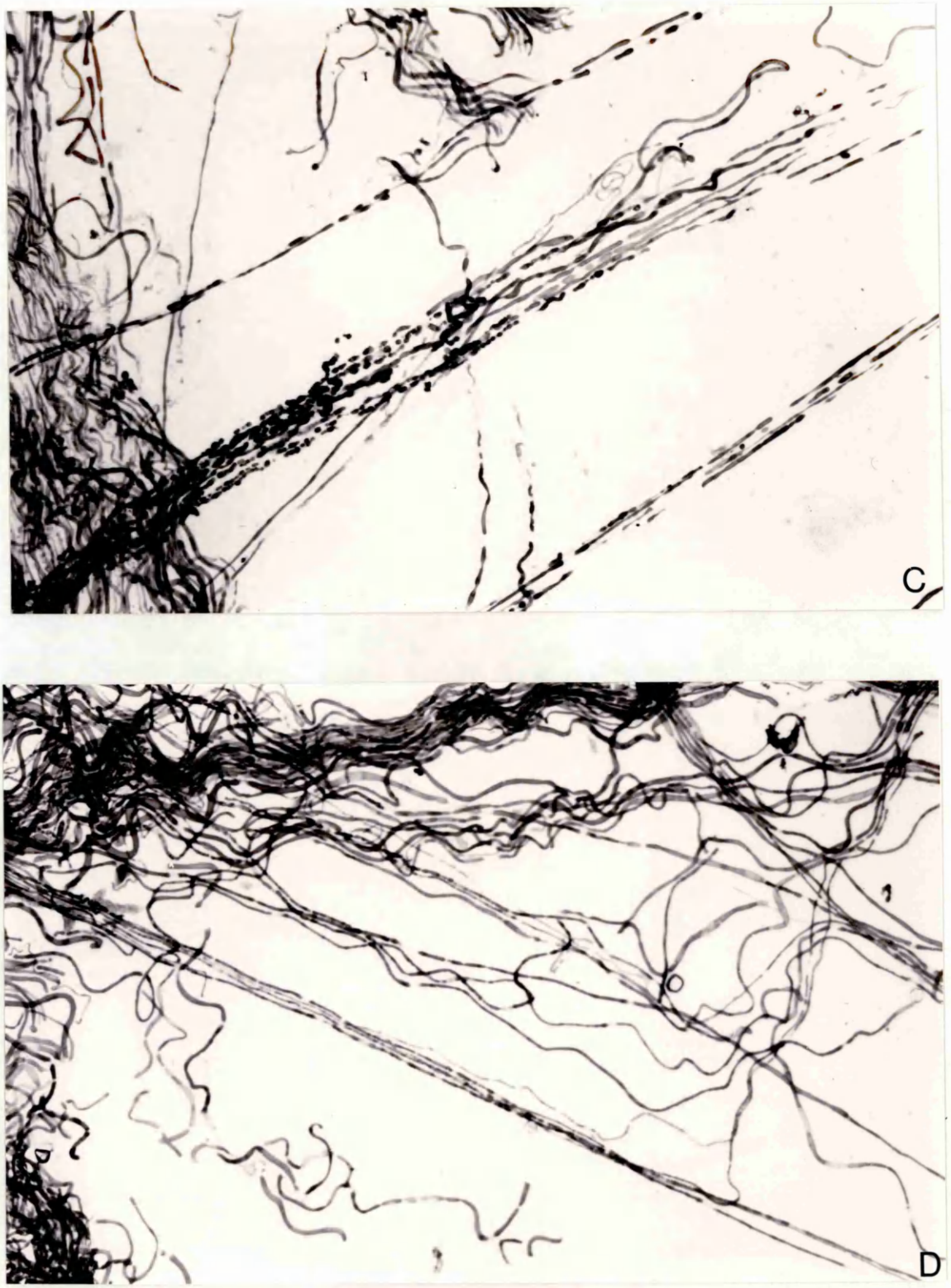


Figure 6.3 (cont.): The effect of antimitotics or dexamethasone on preservation of myelinated fibres *in vitro*.(C) Fibres cultured in Schwann cell growth medium containing 7.5% foetal calf serum and 1.0% glutamine and cytosine arabinoside (0.01 mM). (B) Fibres cultured in the same basic medium, but colchicine (1 ug/ml) was added instead of cytosine arabinoside. No preservation of P_0 message is noted in either situation, although the morphologic preservation is somewhat imporved in the medium containing colchicine (x 90).

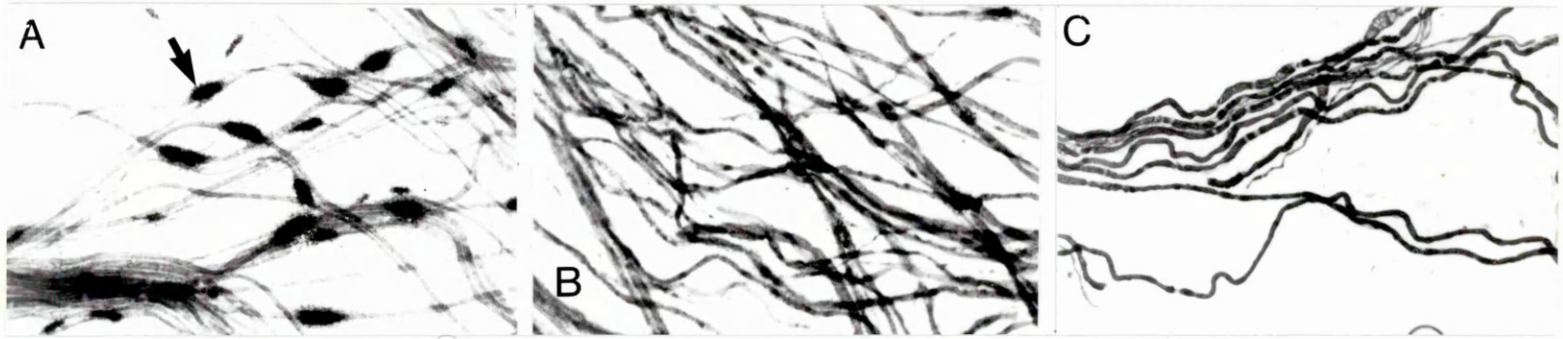


Figure 6.4: The effect of cyclic-AMP on preservation of myelinated fibres *in vitro*. Loosely teased bundles of sciatic nerve fibres were cultures as free floating explants for 5 days in Schwann cell growth medium containing cyclic-AMP analogues (0.1 mM), and probed for P_0 mRNA expression by *in situ* hybridization. After autoradiography, the fibres were stained with sudan black. (A) Teased fibres from normal control nerve with strong P_0 mRNA expression (arrow). (B) Fibres cultured in the presence of cyclic-AMP (either dibutryl- or 8-bromo cylic AMP). (C) Fibres cultured in the absence of cyclic-AMP. No preservation of P_0 message is seen (x 112).

cultured in SCGM without EGTA was only half that obtained from the nerve removed 5 days after transection *in vivo*.

The dot blots confirmed the data obtained by ISH depicting the marked down-regulation of P_0 mRNA expression in samples cultured in different media irrespective of the degree of fibre preservation (fig. 6.5B). The down-regulation was similar to that observed in transected nerve which underwent Wallerian degeneration *in vivo*. The dot blots also confirmed the results of immunostaining (see next section)for NGF receptor (fig. 6.5C). Only those fibres which degenerated markedly, expressed increased levels of NGF receptor mRNA. This increase was noted on dots from 5 day *in vivo* transected nerve, and nerve that had been cultured in DMEM and SCGM without EGTA.

6.3.3 Immunofluorescence studies

Schwann cell of normal fibres were assessed for the expression of GFAP, N-CAM and NGF receptor. In each case, only the unmyelinated fibres stained positively for these antigens. The staining intensity for GFAP and N-CAM was bright (5+) and the unmyelinated fibres appeared as fine, bright threads of positive stain. The intensity of the staining for NGF receptor was much less (1+) with fibres fixed prior to staining showing a greater reactivity than those surface stained. NGF receptor was strongly re-expressed by Schwann cells on axotomized fibres which had degenerated for 5 days *in vivo* (fig. 6.6A to D). The following results pertain to fibres after 5 days in culture.

6.3.3.1 NGF receptor

SCGM + EGTA, HBSS

Very little NGF receptor protein was identified by immunofluorescence on any fibres, although a low level (1+) was just seen on unmyelinated fibres (fig 6.7A and C).

DMEM

There was increased staining (4+) for NGF receptor on fibres cultured in this medium. This was found both on the surface and in the cytoplasm of degenerating myelinated fibres.

SCGM - EGTA

Many degenerating fibres were observed and were correspondingly brightly (5+) stained (fig. 6.7B and D).

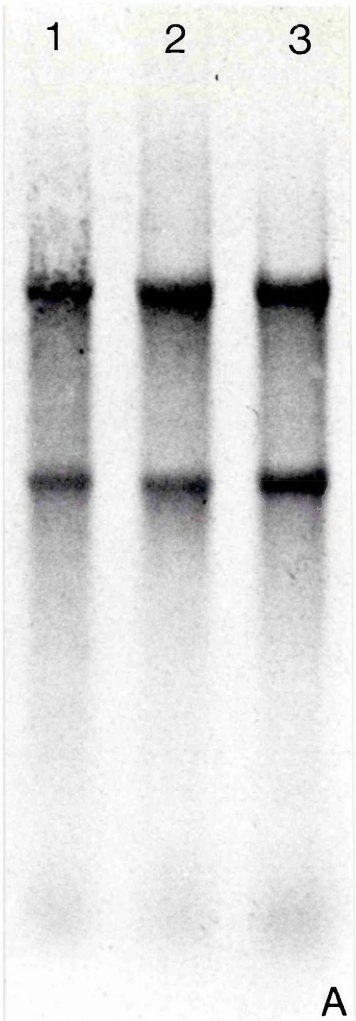
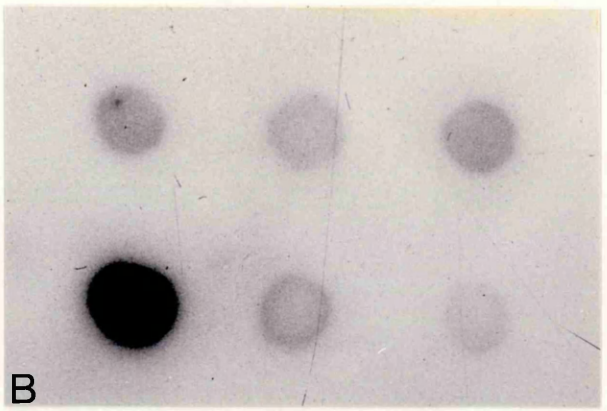


Figure 6.5: The effect of degeneration *in vivo* and *in vitro* on P_0 mRNA expression. RNA was extracted from normal nerve, transected nerve which had been allowed to degenerate *in vivo* for 5 days, and explants cultured in calcium-containing or calcium-free medium. (A) Prior to dot blotting, a northern blot was performed to assess the integrity of the RNA. 2.0 ug of total RNA from nerve cultured for 5 days in calcium-free medium (lane 1); calcium-containing medium (lane 2) and normal nerve (lane 3) were electrophoresed on a 1.5% denaturing agarose gel, blotted and stained with 0.04% methylene blue/sodium acetate dye. Two major bands are visible; 28S, 18S (top and middle) and a fainter 5S band is seen at the bottom (x 2).



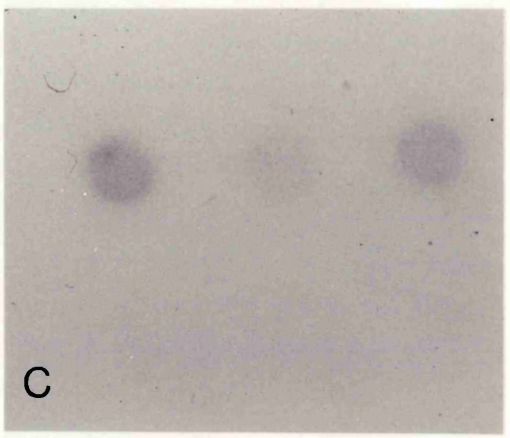


Figure 6.5 (cont.): The effect of degeneration *in vivo* and *in vitro* on P_0 mRNA expression. 2.0 ug of total RNA for each sample was dotted onto the filter: top row of dots from left to right - transected nerve which was allowed to undergo Wallerian degeneration *in vivo* for 5 days; nerve cultured in DMEM; nerve cultured in regular SCGM; bottom row of dots from left to right - normal, intact nerve; nerve cultured in HBSS; nerve cultured in calcium-chelated SCGM (please see text for details). (B) Filter probed with [^{32}P] labelled P_0 cDNA. (C) The filter was stripped and reprobed with [^{32}P] labelled cDNA for NGF receptor. There is no preservation of P_0 mRNA in any of the media, irrespective of morphologic preservation. However, in those media in which degeneration occurred (calcium-containing) and in the *in vivo* degenerated nerve, upregulation of NGF receptor mRNA has occurred (top three blots). The other three blots cannot be discriminated from the background but are located below the positive blots (x 3).

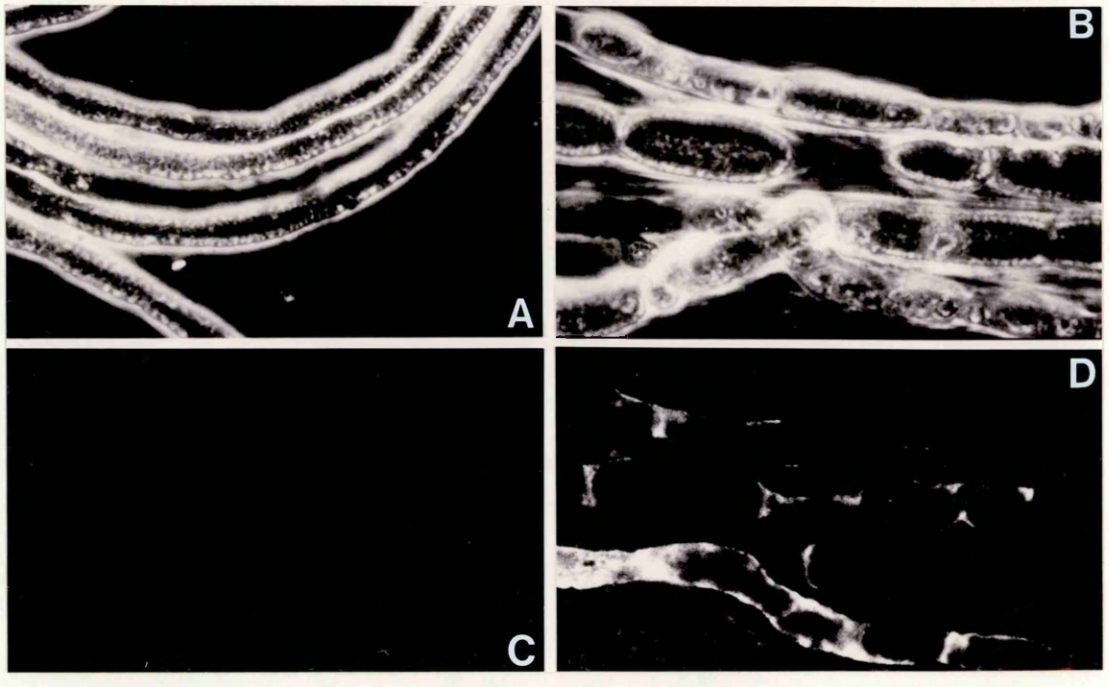


Figure 6.6: NGF receptor protein expression on normal and 5 day post-transected fibres. Phase optics are shown in A (normal nerve) and B (degenerating nerve) and their corresponding fluorescein optics are shown in C and D. Fibres are immunostained for NGF receptor protein. Normal intact nerve (C) is unstained, whilst there is marked upregulation of expression of this protein on degenerating fibres (D) (x 600).

6.3.3.2 GFAP

SCGM + EGTA, and HBSS

In both these media positive 5+ linear staining was observed on many fine fibres corresponding to unmyelinated fibres (fig 6.8A and C). Little staining was noted on myelinated fibres and if present was at a low level (2+) on the surface of the few degenerating myelinated fibres that were present.

DMEM

Teased fibres from these explants exhibited more degeneration and more staining than fibres from the first two media. This appeared to be associated with degenerating myelin. Unmyelinated fibres with fine linear staining were still present but were fragmented.

SCGM - EGTA

Unmyelinated fibres with the fine linear staining had mostly disappeared. Most fibres appeared to be very degenerated and fragmented and displayed patchy bright (3+) staining (fig 6.8B and D). Good correlation between the patchy, irregular staining and fibre degeneration was observed.

6.3.3.3 N-CAM

SCGM + EGTA, HBSS

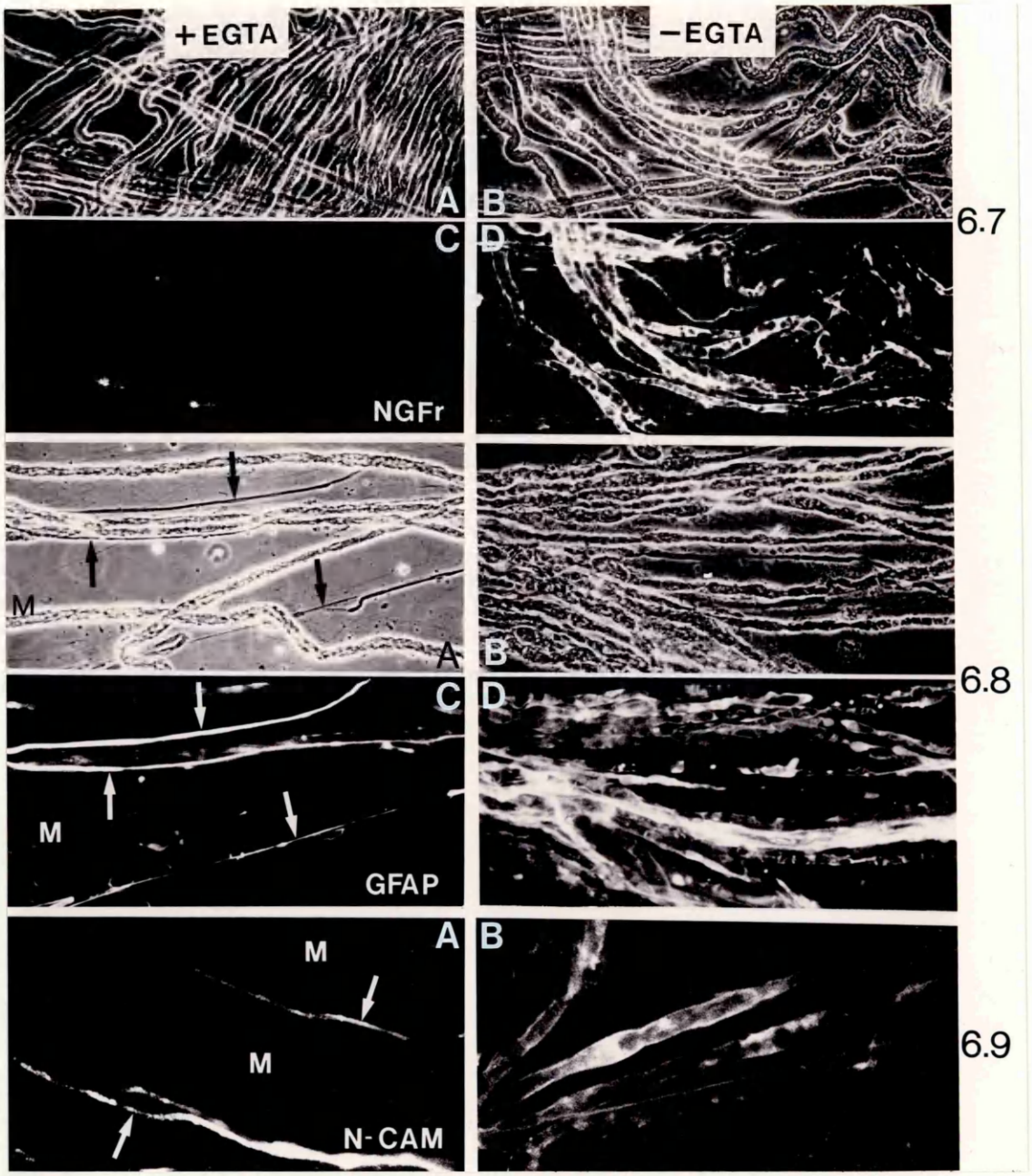
The results from fibres in this group were similar to the GFAP stained group with bright (5+) linear staining on very fine, non-degenerated, unmyelinated fibres (fig 6.9A). Few degenerating fibres and little surface staining were noted.

DMEM, SCGM - EGTA

The upregulation of N-CAM expression was only slight (1+) on degenerating fibres in this group but was clearly greater than the negative expression of day 0 intact fibres (fig. 6.9B).

SCGM + CYCLIC-AMP ANALOGUES, + EGTA

The expression of NGF receptor was determined by the calcium status of the medium and this was not changed by the addition of the cyclic-AMP analogues. Hence, bright staining was observed on the degenerating fibres of the SCGM - EGTA medium and very little NGR receptor was identified on fibres cultured in SCGM + EGTA irrespective of the presence or absence of cyclic-AMP analogues.



Figures 6.7-9: **The effect of calcium on the expression of cell markers.**

Figure 6.7 - NGF receptor: A and B are phase optics; C and D are fluorescein optics. Myelinated fibres cultured in the calcium-free medium (C) are negative for NGF receptor; whereas degenerating fibres cultured in the calcium-containing medium are immunopositive for NGF receptor (D) (x 168).

Figure 6.8 - GFAP: A and B are phase optics and C and D are fluorescein optics. (C) In calcium-free media, only non-myelinated fibres (arrows) express GFAP; myelinated fibres (M) are negative for GFAP. (D) But if calcium is present, degenerating myelinated fibres are also positive (x 168).

Figure 6.9 - N-CAM: A and B are fluoroscein optics. (A) In calcium-free medium, only non-myelinated fibres (arrows) stain with anti N-CAM; myelinated fibres (M) are negative for N-CAM. (B) In calcium-containing media, some upregulation of N-CAM expression occurs on degenerating fibres (x 336).

6.4 DISCUSSION

6.4.1 The role of calcium in Wallerian degeneration

6.4.1.1 The toxic nature of calcium

Calcium ions are known to cause precipitation of a number of cytoskeletal proteins (Wilson *et al.*, 1970). Studies on the axoplasm of the giant squid axon identified that the axoplasm was very susceptible to the effects of calcium ion in a dose-related manner. The gel-like axoplasm would dissolve in a NaCl solution containing 390 mM $CaCl_2$ in 0.5 minutes but survive up to 100 minutes in a 0.71 mM $CaCl_2$ solution. This effect seemed to be specific to calcium as solutions containing magnesium, strontium or barium chloride did not cause dispersal of the axoplasm (Hodgkin and Katz, 1949). The ion appears to be directly neurotoxic as it can induce a myelopathy when applied onto exposed spinal cord. Within 24 hours granular disintegration and spheroid formation of the axons occurred. The myelin sheaths exhibited vesicular degeneration and oedema (Balentine and Dean, 1982).

Further evidence for the toxic nature of this ion came from studying the effects of culturing explants in media containing different levels of calcium (Schlaepfer, 1971; Schlaepfer and Bunge, 1973; Schlaepfer, 1974). Rat sensory ganglia fibres were cultured in minimum essential medium containing 6 to 8 mg/100 ml calcium (0.15 to 0.2 M). Within 6 to 24 hours of transecting these fibres, fragmentation, swelling and collapse of the severed myelinated fibres occurred. The accompanying axoplasmic changes included granular transformation and disintegration of the microtubules and neurofilaments. Similar degenerative changes were observed in unmyelinated fibres. In low calcium medium (25 to 50 μM) the axoplasm remained relatively intact over a 48 hour period of observation with no granular transformation nor loss of neurofilaments, however, the microtubules were lost. In EGTA-chelated medium, both microtubules and neurofilaments were maintained and in their correct longitudinal orientation. It was only in calcium-chelated medium, in which the calcium was reduced to the intra-axonal resting level, that the microtubules were preserved. Clusters of neurofilaments tended to form away from the microtubules. These studies emphasized the role of calcium in nerve degeneration. It was noted that the axoplasmic degeneration observed *in vitro* in calcium-containing medium was the same as that observed during Wallerian degeneration *in vivo* (Schlaepfer and Bunge, 1973). Similar effects of the media were observed on the degeneration of the myelin

sheath. No myelin degeneration occurred during 48 hours of incubation in calcium-free or calcium-chelated media, compared with the diffuse myelin degeneration noted in calcium-containing media. The latter degeneration was similar to that observed *in vivo* 48 hours after nerve transection (Schlaepfer, 1974).

This effect was specific to calcium and not induced by high concentrations of other cations. As these changes were similar to those observed in Wallerian degeneration, the entry of calcium into the axoplasm during Wallerian degeneration may be responsible for its initiation (Schlaepfer, 1971). Incubation in Triton-X 100 detergent accelerated the axoplasmic degeneration in normal medium causing change within 1 hour, but the axon was well preserved in calcium-chelated medium. Similarly, freeze-thawing accelerated axonal degenerative changes in nerves in normal medium, but not in calcium-chelated medium. The calcium is presumed to enter through the open ends of the cut nerve fibre or through the axolemma. It was proposed that degeneration of the axolemma allows diffusion of the calcium into the axon and that this influx into nerve fibres after loss of contact with the neuron, initiates degeneration. This occurs naturally after 24 to 48 hours or is accelerated by membrane damaging procedures such as freeze-thawing or detergent extraction. Normal intra-axonal calcium is maintained at low levels possibly by the membrane itself, a calcium pump or intra-axonal calcium sequestration. Disruption of these calcium homeostatic mechanisms would initiate cytoskeletal damage, some degree of axonal degradation and damage to calcium-excluding mechanisms (Schlaepfer, 1974).

6.4.1.2 Nerve fibre degeneration and P_0 mRNA expression

In the experiments conducted here, the degree of Wallerian degeneration observed *in vitro* was dependent on the concentration of calcium within the medium. The least amount of degeneration was noted in the calcium-chelated, fully supplemented medium, and also in Hank's medium which was calcium-free. Dulbecco's modification of Eagle's medium, which also forms the basis of SCGM, contains 264.9 mg/l calcium. Marked degeneration was noted in fibres cultured in both these media. However, despite significant preservation of fibre morphology by chelating the calcium from the medium, this was not associated with preservation of P_0 mRNA expression.

The variation in the rate and degree of fibre degeneration within explants of cat sciatic nerve noted by Crang and Blakemore (1986) was also observed here. Differences in the degree of degradation between replicate cultures and within one culture were seen. Within a single teased fibre sample at 2 days, there was good

correlation between the degree of fibre preservation and the level of P_0 mRNA expression. But, because of the intra-sample variation between fibres, *in situ* hybridisation methodology only suggested the trend of P_0 mRNA expression; dot blots confirmed this trend. This data suggests that despite the preservation of morphology by calcium chelation, the putative myelinating signal was not likewise preserved.

6.4.1.3 Degeneration and mRNA levels

It was interesting to note that the amount of RNA extracted from the samples was affected by the degree of fibre degeneration. An increase in the DNA content of transected nerve has previously been recorded (McCaman and Robins, 1959a). The yield of total cellular RNA of sciatic nerve was noted to be significantly higher after both crush injury and nerve transection compared to normal adult nerve (Gupta *et al.*, 1988; Le Blanc *et al.*, 1987). Proliferation of Schwann cells, endothelial cells, pericytes and fibroblasts takes place, accompanied by an influx of macrophages after axotomy (Gupta *et al.*, 1988; McCaman and Robins, 1959a; Pellegrino *et al.*, 1986; Poduslo *et al.*, 1985a). This increased cellularity would result in increased gene products not involved with myelination or the maintenance of the myelin sheath (Gupta *et al*, 1988). If Wallerian degeneration is delayed then the resulting cellular proliferation would be decreased and the amount of extractable RNA would likewise be affected. The amount of RNA extracted from the most degenerating fibres *in vitro* was significantly less than that obtained from nerve that had been allowed to degenerate for 5 days *in vivo*. This may reflect a lesser concentration of cells per unit mass of nerve in the former, which would be consistent with the absence of haematogenous myelomonocytic cell recruitment observed *in vitro* (Crang and Blakemore, 1986). Macrophages have been implicated in Schwann cell division during Wallerian degeneration (Baichwal *et al.*, 1988) and potentially, the exclusion of haematogenously derived macrophages in culture, may limit the Schwann cell proliferation. The amount of RNA extracted may also be different because the activity of RNases may be different between the two situations.

6.4.1.4 N-CAM expression

As discussed in section 1.5.1.2, N-CAM is an integral membrane protein found on those adult Schwann cells not associated with myelin production (Daniloff *et al.*, 1986; Edelman, 1983; Jessen *et al.*, 1987b; Nieke and Schachner, 1985) It was

also detected on immature Schwann cells prior to the commitment to myelination (Jessen *et al.*, 1987b; Nieke and Schachner, 1985). Contact with an axon that expresses signals for myelination is associated with down-regulation of N-CAM expression. Axonal contact is essential for suppressing this expression and loss of contact by myelin-forming Schwann cells was associated with re-expression of N-CAM. *In vitro*, the majority of cultured Schwann cells from 10 day old rat nerves re-express N-CAM within 24 hours of dissociation from the axon. This re-expression is likely to require fresh protein synthesis, but not cell division (Jessen *et al.*, 1987b). L1 is a cell surface glycoprotein (reviewed by Rutishauser and Jessell, 1988) with an almost identical location to N-CAM; that is, on non-myelin-forming Schwann cells and unmyelinated axons (Martini and Schachner, 1988a; Nieke and Schachner, 1985; Seilheimer and Schachner, 1987). The re-expression of these molecules after nerve injury is thought to be a prerequisite for nerve regeneration. *In vivo* studies have identified that the N-CAM and L1 antigens reappear within 3 days of axotomy with peak expression after 14 days. Both molecules were observed on Schwann cells in bands of Bungner but not on myelin debris (Nieke and Schachner, 1985). It has been suggested that the expression of these CAMs on the surface of the Schwann cells is associated with the formation of the bands of Bungner (Daniloff *et al.*, 1986). The expression is lost with reinnervation but retained in the distal stump of the permanently transected nerve (Daniloff *et al.*, 1986; Jessen *et al.*, 1987b). In the experiments described here, N-CAM expression was lost from unmyelinated fibres cultured in SCGM without EGTA or in DMEM. This may represent fragmentation and dissolution of the unmyelinated fibres during this time. As expected, the expression of this molecule was up-regulated on the surface of Schwann cells associated with degenerating fibres, albeit at a low level.

6.4.1.5 NGF receptor expression

It is thought that Schwann cells isolated from axonal contact express NGF receptor and that suppression of this signal requires contact with the axon; that is, it is a non-diffusable signal. This molecule is expressed by immature Schwann cells and its expression is down-regulated as the Schwann cell-axon relationship matures and myelin-specific molecules are formed (Jessen *et al.*, 1990; Johnson *et al.*, 1988; Taniuchi *et al.*, 1988). This receptor was transiently expressed by myelin-forming Schwann cells during their earliest stage of myelination at birth but the expression was quickly lost (Jessen *et al.*, 1990). In sections of normal rat sciatic nerve there was no detectable immunostaining for NGF receptor (Tanuichi *et al.*, 1988). However, using

teased fibres from mature rat sciatic nerve, some positive staining for Ran-1/217c, which is the NGF receptor (Ferrari *et al.*, 1991), was observed on unmyelinated fibres. Positive staining was also noted on the non-myelin-forming Schwann cells of the cervico-sympathetic trunk. The intensity of staining of non-myelin-forming Schwann cells *in vivo* was less than that of denervated Schwann cells *in vitro* (Jessen *et al*, 1990).

Local synthesis of NGF receptor mRNA by Schwann cells of the distal stump has been observed within 24 hours of axotomy (Heumann *et al.*, 1987b) and an increase in the protein within 4 days. This was considered due to loss of axonal contact (Raivich and Kreutzberg., 1987). The protein was located on the Schwann cell surface and not on the myelin debris. Peak level of expression on transected nerve was at least 50 fold greater than normal. It was maintained for 2 weeks and then declined although was still elevated for the 10 weeks of the experiment (Taniuchi *et al.*, 1986; Taniuchi *et al*, 1988). This increase was observed on Schwann cells of many different types of nerves, not just NGF-sensitive ones suggesting that all Schwann cells respond to axotomy by upregulation of NGF receptor (Johnson *et al.*, 1988; Taniuchi *et al.*, 1986; 1988). Down-regulation of its expression correlates closely with ingrowth of regenerating axons (Heumann *et al.*, 1987b; Johnson *et al.*, 1988; Taniuchi *et al.*, 1988). The NGF receptor mRNA was observed to increase during the first 3 days in sciatic nerve explants placed in culture. Data beyond 72 hours was not given. Addition of activated macrophages to these cultures had no effect on the levels of NGF receptor mRNA (Heumann *et al.*, 1987b).

In experiments performed here, NGF receptor mRNA was upregulated in degenerating medium and in the 5 day *in vivo* degenerated nerve. Strong re-expression of the protein occurred on the surface of Schwann cells associated with degenerating myelin sheaths in culture. In the preserving-media, the mRNA and protein remained down-regulated. Similarly, the delayed degeneration observed in C57Bl/Ola mice was associated with only a poor upregulation of NGF receptor mRNA. This was attributed to the preserved axon-Schwann cell interaction (Brown *et al.*, 1991).

6.4.1.6 GFAP expression

Another protein expressed by non-myelin-forming Schwann cells in mature peripheral nerve is GFAP. It was also noted to be transiently expressed by GalC-positive Schwann cells perinatally. These cells are known to become myelin-forming cells. GFAP expression begins at embryonic day 18 to 19 and occurs on all Schwann cells even if they are in the initial stages of myelin formation. However its synthesis is

down-regulated soon after synthesis of myelin-specific molecule begins. The molecule is quickly re-expressed after axonal contact is lost both *in vitro* and *in vivo* (Jessen *et al.*, 1990). Likewise, media associated with fibre degeneration here were also associated with increased GFAP expression.

6.4.1.7 Unmyelinated fibres

The expression of these proteins by unmyelinated fibres decreased in the degenerating media. This was possibly associated with fragmentation and dissolution of these fibres. GFAP-stained fibres exhibited this best with good preservation of staining on unmyelinated fibres in SCGM with EGTA, some fragmentation of unmyelinated fibres in DMEM and virtual obliteration of these fibres in SCGM without EGTA. Similar findings were observed on unmyelinated fibres stained for N-CAM. Positively-stained fibres for NGF receptor were only poorly visible in day 0 normal fibres and were still just visible on fibres cultured in SCGM with EGTA or HBSS. Upregulation of this protein on degenerating fibres in the other media made it difficult to see the fate of unmyelinated fibres. Denervation was thought to have little effect on the expression of GFAP and 217c on non-myelin-forming Schwann cells (Jessen *et al.*, 1990).

6.4.2 The effect of EGTA on Wallerian degeneration *in vitro*

The effect of EGTA may be to inhibit activation of calcium-activated neutral proteases (Malik *et al.*, 1982; Simonson *et al.*, 1985; Zimmerman and Schlaepfer., 1984) and thus retard Wallerian degeneration in this manner. Several forms of calcium activated neutral proteases (CANP, calpains) have been purified from mammalian brain. They are activated by different levels of calcium (Malik *et al.*, 1982; Simonson *et al.*, 1985; Zimmerman and Schlaepfer., 1984). CANP-1 is maximally activated at 0.7 mM calcium whereas CANP-2 is activated by only 0.002 mM calcium. Calpains can extensively degrade fodrin, MAPs, tubulin and neurofilaments (Malik *et al.*, 1982, cited in Simonson *et al.*, 1985). Calcium is also required for removal of myelin debris by macrophages. Studies have identified that the calcium concentration outwith the range of 1.0 to 2.5 mM is associated with decreased cell viability (Bruck and Friede, 1990a). Inhibition of both macrophage activity and CANP activity could account for the dramatic morphological preservation observed in fibres cultured in the absence of calcium.

Myelin and axonal degeneration ultimately do occur in these fibres in the presence of calcium chelation. This, in part, reflects loss of axonal integrity associated with severance of the axon from its neuronal somata. Myelin degeneration would then reflect loss of contact with an intact axon.

> "*Excised and isolated nerve segments are, in fact, analogous to the distal segments of transected amputated nerve fibres. In both situations, a critical physical alteration of the tissues involves the dissociation of intact axoplasma with the biosynthetic centre and source of vital metabolites.*" (Schlaepfer, 1974)

Alternatively the effect of calcium chelation could have been mediated through its effect on Schwann cell mitosis. It has been demonstrated that the mitogenic signal of axolemma or myelin is profoundly decreased by chelating free calcium from the medium or interfering with the activity of the calcium-binding protein using trifluorperazine (Meador-Woodruff *et al.*, 1984). The mitogenic signal associated with degenerating myelin is thought to act during Wallerian degeneration (see sections 1.3.1.4 and 1.5.2.5). It was possible that chelation of calcium in the studies reported here precluded Schwann cell division and thus inhibited Schwann cells from leaving their myelin sheath maintaining role for a more active dividing and potentially phagocytic role (see section 1.5.2.1). This may have aided morphological preservation. However, in my studies, the addition of antimitotics to these explants was not associated with morphological preservation of the fibre, so this seems unlikely (see next page).

Lack of cell division in calcium-chelated media may be associated with failure of NGF receptor to increase in expression. This protein was observed to increase on Schwann cells at 4 days post transection (Raivich and Kreutzberg, 1987) and Schwann cell proliferation has been observed at 72 hours (Crang and Blakemore, 1986; Pellegrino *et al.*, 1986) with peak [^{3}H] thymidine incorporation occuring at 4 days (Pellegrino *et al.*, 1986). Thus Schwann cell division may be a prerequisite for re-expression of this signal. The failure of upregulation of this protein in calcium-chelated medium may thus either be due to preservation of the axon leading to its suppression, or because of failure of Schwann cell division due to lack of calcium. However, Schwann cells dissociated from 10 day old rat sciatic nerves were found to dramatically increase the expression of NGF receptor such that by 9 hours *in vitro*, greater than 80% of cells were positive for NGF receptor (Jessen *et al.*, 1990). This data makes it unlikely that suppression of this protein on Schwann cells in calcium-chelated medium was associated with failure of the cells to divide. Rather it would

appear that calcium-chelation was associated with the preservation of the signal, presumably axonal in origin, that inhibits expression of this protein.

Similarly, GFAP expression rapidly increased within the first 9 hours of culture on dissociated Schwann cells from 10 day old rat sciatic nerve (Jessen *et al.*, 1990). This also implies a dependence on axonal contact for molecule suppression and not a reliance on Schwann cell division for re-expression. With respect to N-CAM, Schwann cells which had hitherto been myelin-forming re-expressed it after 4 days in culture (Mirsky and Jessen, 1987) but data on its re-expression at earlier times *in vitro* was not found.

6.4.3 The effect of antimitotics on Wallerian degeneration *in vitro*

It is known that cytosine arabinoside is an effective blocker of Schwann cell division in neonatal rat pups (Aguayo *et al.*, 1975). Murine peripheral nerves cultured for 10 days *in vitro* were found to contain significant numbers of phagocytes which were actively phagocytosing myelin debris. The addition of the antimitotic agents cytosine arabinoside and colchicine to these cultures was associated with preservation of nerve fibre morphology. This was probably due to a reduction in the number of phagocytes and myelin phagocytosis as it was reversed by addition of peritoneal monocytes even in the presence of the antimitotics (Bonnekoh *et al.*, 1989). Thus preservation of fibre morphology in the presence of antimitotics was probably due to the suppression of phagocytic cell proliferation rather than by precluding Schwann cell division. Schwann cells were not thought to have a significant role in myelin phagocytosis in cultures of degenerating nerve (Bonnekoh *et al.*, 1989; Crang and Blakemore, 1986). Hence, preventing Schwann cell proliferation is probably not associated with retarded degeneration because of decreased Schwann cell phagocytosis.

Similarly, Wallerian degeneration was observed to be delayed in distal nerve stumps after treatment with Mitomycin C. Axolemmae were still present in about 50% of fibres at 5 days post-transection. By 7 days axoplasm was floccular but the axolemmae were present in 50% of fibres. At 16 days post-transection, many myelin sheaths were present in mitomycin C-treated nerves but not in control transected nerves. However, preservation of the nodal axolemma by preventing Schwann cell proliferation with mitomycin C was not associated with maintained production of P_0 although catabolism of P_0 was delayed (Pellegrino *et al.*, 1986).

Antimitotics were used in these experiments to attempt blockade of Schwann cell proliferation and delay Wallerian degeneration as described by other workers

(Crang and Blakemore, 1986; Bonnekoh *et al.*, 1989; Pellegrino *et al.*, 1986). The effect of retarding proliferation could then be assessed on the expression of myelin-specific molecules such as P_0. Cytosine arabinoside inhibits DNA polymerase with limited effects on RNA, whilst colchicine acts by inhibiting the formation of the mitotic spindle. Mitomycin C was not used because at high doses it also interferes with RNA and protein synthesis (American Society Hospital Pharmacists, 1989) and thus may preclude formation of P_0 mRNA. In the studies reported here, preservation of fibre morphology was minimal using antimitotics and, in support of Pellegrino *et al.*, (1987), no preservation of P_0 mRNA was found.

6.4.4 The effect of dexamethasone on Wallerian degeneration *in vitro*

With respect to dexamethasone, the failure of preservation of fibre morphology let alone myelin-specific molecule expression may be related to use of inappropriate concentrations. Alternatively, this drug may not have any effect on stabilizing lysosomal membranes and precluding lysozyme release. Or, dexamethasone may have precluded release of lysosomal enzymes, but if these enzymes only play a minor role in Wallerian degeneration, preventing their release does little to delay the degeneration. Certainly at the concentrations used here, no delaying of Wallerian degeneration was observed.

6.4.6 The effect of cyclic-AMP analogues on Wallerian degeneration *in vitro*

As reviewed in 1.3.1.4, the role of cyclic-AMP in transducing the myelinating signal is controversial as fractions of nervous tissue enriched in axolemma or myelin do not appreciably increase Schwann cell intracellular cyclic-AMP levels (Meador-Woodruff *et al.*, 1984). Preventing degradation of cyclic-AMP by inhibiting cyclic-AMP phosphodiesterase with isobutylmethyl xanthine did not increase the response of Schwann cells to the axolemmal mitogen (Salzer *et al.*, 1980c). These data suggest that cyclic-AMP is not involved in the transduction of axolemmal signals, at least for mitosis.

In Lemke's studies, reversible elevation of P_0 and MBP mRNAs and proteins was induced by treating 3 week cultures of dissociated neonatal Schwann cells with forskolin. These cultures had previously been expanded with a combination of the same concentration of forskolin and crude glial growth factor. The elevation became more irreversible with prolonged exposure to the drug (Lemke and Chao, 1988b). Forskolin acts by elevating adenylate cyclase ((Lemke and Chao, 1988b) which then

converts ATP to cyclic-AMP. Hence these authors used cyclic-AMP elevators in combination with glial growth factor to initially expand the Schwann cell population and then used the same cyclic-AMP elevator to stimulate development of the myelinating phenotype. This is consistent with the theory proposed by Morgan *et al.*, (1991) in which it is postulated that cyclic-AMP may have roles as second messenger for several aspects of Schwann cell development and differentiation. Thus it could be a second messenger for Schwann cell mitosis, and also for differentiation of the cell into a myelin-forming phenotype. The role that it undertakes is dependent upon other concomitant stimuli. Thus if a dividing cell has increased intracellular cyclic-AMP, and the cell leaves the mitotic cycle, then the cyclic-AMP may transduce the signal for myelin formation (Morgan *et al.*, 1991).

Morgan *et al.*, (1991) found that induction of P_0 expression with cyclic-AMP elevators was associated with down-regulation of NGF receptor expression, implying a converse co-regulation of these genes. In the experiments undertaken here, the addition of cyclic-AMP analogues to the Schwann cells in the cultured explants was not associated with maintained expression of P_0 mRNA nor suppression of NGF receptor. This was irrespective of the morphological state of the fibres which was determined by the presence or absence of EGTA. This is not consistent with Lemke and Chao's findings (1988b). There may be several reasons for this. Firstly, Schwann cell proliferation is part of Wallerian degeneration (see section 1.5.2.5) and dividing Schwann cells were found to respond to elevation of intracellular cyclic-AMP by DNA synthesis and not expression of the myelinating phenotype (Morgan *et al.*, 1991). Shuman *et al.*, (1988) found that the re-expression of P_{170k} induced by 8-bromo cyclic-AMP was optimized by initiating treatment with the analogue or forskolin 4 to 5 days after Schwann cell isolation. The effectiveness of induction was decreased if less than 4 days had elapsed between isolation of the Schwann cells and initiation of the treatment (Shuman *et al.*, 1988). Peak proliferation of Schwann cells as detected by [^{3}H] thymidine incorporation was found to occur on the fourth day of Wallerian degeneration (Pellegrino *et al.*, 1986). Thus, according to Morgan and colleagues' work, during the time from days 0 to 5 post-transection in which the study here was undertaken, the addition of these analogues to the medium may have just enhanced Schwann cell proliferation and Wallerian degeneration. Certainly Wallerian degeneration was well advanced in the explants cultured in calcium-containing medium with cyclic-AMP. However, in calcium-free conditions there was no obvious Schwann cell mitosis and yet the cyclic-AMP analogues did not maintain P_0 mRNA expression. A further reason for failure of the explants to respond to the cyclic-AMP

analogues may pertain to the concentration of the drugs used. Similar concentrations to those of Morgan and coworkers (1991) were used here. However, Shuman *et al.*, (1988) thought that concentrations of less than 0.1 mM were too low for stimulation.

6.4.7 Summary

Despite testing a number of different regimes *in vitro* on sciatic nerve explants, and successfully retarding Wallerian degeneration with some of them, P_0 mRNA expression was not preserved. However, suppression of molecules such as GFAP, N-CAM and NGF receptor was maintained, presumably because the axon (or sheath? - see chapter 8) was preserved in media with low/no calcium. At the beginning of these experiments, it seemed more likely that upregulation of these molecules in association with down-regulation of P_0 mRNA would occur. However, this was not the case. This implies that the positive and negative effect of the axon on Schwann cell gene expression is not due to the same stimulus. That is, the putative signalling mechanism(s) for myelin-specific gene induction and maintenance is different from that suppressing the above three proteins. This could be due to the signals having different susceptibility (possibly because of different molecular composition or structure) to calcium activated degradation. Alternatively, they may be at different locations on/in the axolemma and potentially one is more protected from degradation than the other. Finally, the two signals may totally unrelated and in very different locations; this theory is expanded upon in chapters 8 and 9.

6.5 CONCLUDING REMARKS

Whilst delayed Wallerian degeneration in the Olac mouse mutant was associated with both morphological preservation and maintained P_0 mRNA expression, delaying degeneration by removing calcium from cultured sciatic nerve was not as efficacious. Certainly the anatomic integrity of the nerve was maintained to a variable degree in calcium-free medium, however, despite this, the putative myelinating signal was not stabilized. Comparing the two forms of delayed degeneration by electron microscopic techniques, the preservation was superior in the nerves from the mouse mutant. This applied to both the integrity of the axon-glia interface and the basement membrane, as well as the axonal contents and the myelin sheath. Degradation of the periaxonal region may well be associated with destruction of the axon-to-glia signalling mechanisms. The loss of basement membrane, may also

have a role to play in down-regulation of the myelin-specific gene expression considering its role in the differentiation of the Schwann cell.

However, the axonally-suppressed molecules appear to be less sensitive to pertubations of the axon-glia interaction. Suppression of N-CAM, GFAP and NGF receptor proteins was maintained in the calcium-free media which failed to support preservation of P_0 mRNA expression. This lack of co-regulation implies some difference in the signalling molecule(s) directing suppression of some molecules and stimulation of others. This is either because the signals for the two sets of molecules are different, or have different sensitivities to degradation.

It appears that Schwann cell proliferation during Wallerian degeneration has a minimal role in the loss of P_0 mRNA expression as antimitotic agents had little effect on preservation of fibre morphology or gene expression. The addition of cyclic-AMP analogues potentially may circumvent a perturbed axon-glia relationship and stimulate the Schwann cell directly. However, the addition of analogues of these secondary messengers had a minimal effect on maintaining gene expression during the acute phase of Wallerian degeneration in my experiments. Attempting to delay degeneration by blocking release of lysosomal enzymes using dexamethasone had no preserving effect. Either this agent is inadequate, or lysozymal activity is not very significant in Wallerian degeneration.

CHAPTER 7: THE EFFECT OF AXOLEMMAL FRAGMENTS ON THE EXPRESSION OF MYELIN-SPECIFIC MOLECULES BY CULTURED SCHWANN CELLS.

7.1 INTRODUCTION

The importance of contact between the Schwann cells and axon for the expression of myelin-specific molecules (MSM) has been established (see section 1.5.3, chapter 4). In the presence of the appropriate axonal contact, Schwann cells will upregulate their expression of myelin-specific molecules and, given the correct circumstances, will myelinate the axon (see also section 1.3.3). If the axonal contact is removed, then the expression of these molecules is down-regulated by the Schwann cells and they ultimately adopt a quiescent (non-dividing, non-myelin sheath maintaining) role. Considering the anatomy of the axon-glial cell interface, it would be logical to presume that the most important part of the axon for stimulating myelin-specific molecule expression is the axolemma. Of all parts of the axon this part is in the closest contact with the Schwann cell and interaction is most likely mediated through it.

The hypothesis of this thesis regarding this interaction, was based on the presence of some specific axonally-associated molecule that interacts with the Schwann cell plasmalemma and triggers the complex process of myelination. It was reasonable to suggest that this putative signal was produced in the neuronal soma as this is where much of the neuronal synthetic machinery is located. It would then be transported down the axon and inserted in the axolemma. As myelinated and unmyelinated axons are in intimate association within mixed peripheral nerve it has been suggested that it is less likely that the signalling molecule is secreted by the axon, diffuses across the periaxonal space and interacts with the Schwann cell plasmalemma (Brockes *et al.*, 1981). Also during nerve regeneration, re-expression of myelin-specific molecules occurs only after contact with the axon (Mitchell *et al.*, 1990; Politis *et al.*, 1982). However, none of these studies rule out the possibility of a molecule which does dissociate from the axolemma but can cross only very small areas such as the 12 to 14 nm periaxonal space. From its position on or near the axolemma, the putative signalling molecule(s) could then interact with a receptor on the Schwann cell membrane and the signal is then transduced to the nucleus. The role of cyclic-AMP and other molecules as intermediary messengers in transduction of this signal to the Schwann cell nucleus has been discussed (chapter 6). The upregulation of expression of myelin-specific molecules is initiated and ultimately myelination may

ensue. This chapter reports the results of attempts to create an *in vitro* model of Schwann cell/axon interaction for use in studying the nature of the theoretical myelinating signal(s). The expression of the major myelin protein mRNA, P_0 was the marker used to detect "switching on" of the Schwann cells. It was hoped that the following simple equation would be accurate:

Axolemma + Schwann cell => increased P_0 mRNA.

However, the system appears to be more complex than that and much of the time spent on this project was used in developing "in house" methodology of Schwann cell culture. This included examining the effect of basement membrane substrates (chapters 3 and 4) on Schwann cells.

7.2 SCHWANN CELL CULTURE

The materials and method, and results of culture were given in chapter 3. In this section, I will discuss the rationale for establishing the culture methods described in chapter 3 which were used in the experimental work involving axolemmal-enriched fractions (AEF).

7.2.1 Dissociated neonatal sciatic nerve

Initially, dissociated neonatal rat Schwann cells were used as the test bed for addition of AEF. However, as these cells proved not to be very satisfactory (see section 7.5.1), other methods were tried. Previous workers have suggested that Schwann cells cultured without neurons, dedifferentiate. Much data was available in the literature regarding reversion of the Schwann cell phenotype after dissociation from the mature myelinating one to the non-myelin-forming one which is similar to the immature (prior to myelination) phenotype (sections 1.3.2.3, 1.5.3). The expression of the three markers N-CAM, NGF receptor and GFAP is pertinent to this and has been discussed in chapter 6. Perhaps, more significantly, Poduslo and Windebank (1985b) found that P_0 protein was still produced, albeit at low levels, in endoneurial slices cultured for 14 days. At the time of my initial studies (1988/89), it was thought that Schwann cells obtained from neonatal nerve stopped synthesising P_0 protein after 4 days *in vitro*. This difference in the ability of Schwann cells to continue synthesising P_0 protein in the absence of axonal contact was attributed to a dedifferentiation of the neonatal Schwann cells compared with the adult Schwann cells. It was thought that the adult Schwann cells did not require axonal contact to

express P_0 protein, but did require contact for amplification of gene expression (Poduslo and Windebank, 1985b). These data raised the question as to whether dissociated neonatal Schwann cells were differentiated enough to respond to the addition of AEF by upregulating their P_0 mRNA expression.

Another point of consideration that might affect the Schwann cells' ability to respond to the AEF was the heterogeneity of the neonatal Schwann cells population. These cells were obtained from animals in which myelination was still ongoing and so the population would be quite heterogenous with respect to the cells' state of myelination at the time of harvesting. The cells were obtained from 3 to 7 day old animals. Myelination begins perinatally and peaks in rats at about 2 weeks of age (Webster, 1971). Thus some of the cells dissociated from 3 to 7 day old rat pups would have formed a myelin sheath, but many would only have partially formed sheaths, or would not have started producing a sheath. The range of cell maturity would be quite considerable in such a cell population and it was a possibility that immature Schwann cells would not be able to respond to the AEF.

An alternative Schwann cell source was from mature animals which would give just two cell types - myelin-forming and non-myelin-forming. All of the former cells would be mature cells which had produced and maintained a myelin sheath and potentially might be better able to respond to AEF addition. Attempts were made to dissociate Schwann cells from mature nerve by the same method as used for neonatal nerve, but were unsuccessful. The very fact that many of these cells were associated with a myelin sheath made it virtually impossible to harvest Schwann cells from a mature nerve by dissociation techniques. Mechanical dissociation was too damaging to the cells presumably because the cell with its myelin sheath was too large, and structurally too complex, to survive the rigors of trituration. Also, older nerves were more difficult to dissociate because of their increased fibrous component. Even attempts to dissociate sciatic nerve from animals older than 8 to 9 days resulted in much lower cell yields than from younger animals. In an attempt to obtain mature Schwann cells by the dissociation method, I used intradural nerve roots. These fibres have less fibrous component than extradural and peripheral nerve fibres. However, the cells did not survive the preparation. A source of mature Schwann cells, which were structurally less complex, had to be utilized.

7.2.2 Adult explant cultures

The production of Schwann cell cultures by non-dissociative methods has been discussed in section 1.3.2.1. In 1979, Spencer *et al.*, described a technique of

harvesting, but not culturing, Schwann cells from adult rabbits. These workers utilized the phenomenon of Wallerian degeneration to free the Schwann cells from the myelin sheaths and increase the number of Schwann cells. Briefly, the sciatic nerve was transected just below the sciatic notch and the nerves were removed 5 to 12 weeks after the operation. Morphological studies identified that Schwann cells comprised 85 to 90% of the cellular portion of the nerve. The non-cellular portion of the nerve occupied approximately half the cross sectional area (Spencer *et al.*, 1979). I have modified this method to produce cultures of Schwann cells from adult rats. Adult rats were neurotomized as described in section 3.2.2 and sciatic nerves harvested aseptically at various subsequent times. The nerves were desheathed and minced using two scalpel blades in a scissor like fashion to create fragments of tissue, less than 2 mm diameter, which are referred to as explants.

Initial cultures were placed on poly-L lysine-coated 13 mm coverslips, but were mostly unsuccessful because of failure of explant attachment and poor outgrowth. To encourage both these factors, the next set of cultures were placed on rat tail collagen-coated or collagen/Matrigel-coated coverslips and attachment encouraged using a second coverslip. Attachment rates of the explants were much improved and within 4 days of preparation, reasonable cell outgrowth was observed. Both fibroblastic and spindle shaped cells were observed. Subsequent explants were cultured on a collagen/Matrigel mixture and "glued" down by gelling a few microlitres of Matrigel on top of the explant (see chapter 3.2.2 for details of methodology).

7.2.3 Neonatal explants

Further experiments adding AEF to Schwann cells made me reconsider the possibility of using neonatal Schwann cells in the test bed. It was possible that the failure of these cells to respond previously was not due to the age of the donor animal, and hence the maturation state of the cells, but rather the method by which they were prepared. The persisting differentiated state of neonatal explants was later verified in a paper by Brunden *et al.*, (1990b) in which it was shown that P_0 protein was still produced. Trypsin is known to affect expression of the Schwann cell surface molecule Ran-1, (Mirsky *et al.*, 1980), the response of Schwann cells to AEF addition in mitotic studies (Ratner *et al.*, 1987) and expression of neuronal surface molecules such as the Schwann cell mitogen (Ratner *et al*, 1987, Salzer *et al*, 1980c). It has been demonstrated that adhesion of CNS axolemmal fragments to Schwann cells is largely mediated by cell surface components of Schwann cells and that brief

trypsinization interferes with this binding (Sobue and Pleasure, 1985). Thus Schwann cell-AEF interaction is vulnerable to damage by proteases. Enzymatic dissociation could, therefore, potentially effect expression of receptors on the Schwann cell surface and thus affect their interaction with AEF. Alternatively failure of dissociated neonatal Schwann cells to respond to AEF initially may have been due lack of other "permissive factors" such as basement membrane components (see section 1.3.3)

Explants were thus prepared from non-transected, neonatal sciatic nerve in much the same way as from denervated adult rat. Outgrowth of Schwann cells was good, usually beginning within 12 to 24 hours of culture initiation.

7.2.4 Culture of the cervico-sympathetic trunk

In an attempt to find Schwann cells that were naive for expression of MSMs, cervico-sympathetic trunks (CST) were harvested from adult rats, and from these, explant cultures were initiated (section 3.3). The methodology was the same as for other explants, except the nerves were not previously transected.

7.3 ROLE OF BASEMENT MEMBRANE IN SCHWANN CELL CULTURES

7.3.1 Introduction

Most studies with dissociated neonatal Schwann cells have been performed using poly-L lysine as the substrate for cell growth. Cationic poly-L lysine has been widely used as a substrate for cell culture and markedly increases the degree and stability of cell attachment to the culture surface. Whether it was efficacious because it decreased the overall net negative charge of the substrate surface or because it actually rendered the surface charge positive was not known (McKeehan and Ham, 1976). However as discussed in section 1.3.3, the requirement for a more complex substrate comprised of basement membrane components appears to be absolute for permitting full expression of Schwann cell functional potential. Two important points pertaining to the experiments described in this chapter arise from previous work on basal lamina in the PNS.

1) Cultured Schwann cells are dependent upon axonal contact for the production of basement membrane.

2) Exogenous complex basement membrane can be added to cocultures and will adequately replace endogenously-formed basement membrane.

It has also been noted that, generally, the polarity of cultured epithelial cells is much greater on complex basal lamina then if they are cultured on plastic, poly-L lysine or even individual basal lamina components (Kleinman *et al.*, 1986). Polarization of the Schwann cell is considered necessary for ensheathment and myelination. It is thought that absence of basal lamina inhibits polarization upon which further differentiation of the Schwann cell is dependent (Bunge *et al.*, 1986; Eldridge *et al.*, 1987; Owens and Bunge, 1989).

Schwann cells when isolated from neuronal contact will adopt a spindle-shaped morphology with long processes when grown only on type 1 collagen. However, when these same cells were grown on a complex basement membrane they had a broader, more sheet like appearance. Also, in cocultures grown in defined (non-myelinating) medium, myelination occurred if the complex basement membrane was added (Carey *et al.*, 1986a; Eldridge *et al.*, 1989), but did not occur in cultures to which just type I collagen was added (Eldridge *et al.*, 1989). Similarly, in areas where the Schwann cell did not contact the basement membrane, myelination was not observed (Carey *et al.*, 1986a). This evidence suggested that complex basement membrane is required for terminal differentiation of the Schwann cell. The association of basement membrane with Schwann cell maturation is also noted *in vivo*. Conversion of the proliferating, migrating Schwann cell to the non-proliferating, ensheathing state may occur at the same time as the appearance of the basement membrane (Billings-Gagliardi *et al.*, 1974; Carey *et al.*, 1983). The absence of basement membrane is associated with abnormalities of myelination as observed in the dystrophic mouse (Bray *et al.*, 1983; Copio and Bunge, 1980; Madrid *et al*, 1975). Similarly, basement membrane appears to be required for Schwann cell remyelination of demyelinated central axons (Blakemore, 1984; Blakemore and Crang, 1985). In both the *in vivo* and *in vitro* situations, Schwann cell maturation is associated with the formation of the basement membrane (Armati-Gulson, 1980). Both the extracellular matrix components (collagen type I) and basal lamina components appear to be required for Schwann cell maturation (Bunge *et al.*, 1978a; Bunge *et al.*, 1978b; Carey *et al.*, 1986a; Eldridge *et al.*, 1989).

The murine Engelbreth-Holm-Swarm (EHS) sarcoma is a source of artificial basement membrane. Type IV collagen, laminin, heparan sulphate proteoglycan, entactin and nidogen have been extracted from it (Kleinman *et al.*, 1986). Matrigel (R) (Collaborative Research Incorporated) is a commercial preparation derived from

this sarcoma. As well as containing the above compounds (Collaborative Research Incorporated, 1991; Kramer *et al.*, 1986) it also contains transforming growth factor-ß, fibroblast growth factor, tissue plasminogen activator and other growth factors which occur in the tumour. It was developed for *in vitro* use to create a basement membrane which promotes:

> "*attachment and differentiation of normal and transformed anchorage-dependent epithelioid and other cell types*" (Collaborative Research Incorporated, 1991).

It was the source of complex basement membrane used in the experiments of this thesis.

By electron microscopy, this basement membrane forms a dense, anastomosing network of fine laminae; granular material, that may be proteoglycan, was interspersed amongst the network (Kramer *et al.*, 1986).

7.3.2 Materials and Method

The materials and method of utilizing basement membrane substrates are given in chapter 3.2.2. Briefly, either rat tail collagen (type I collagen) or Matrigel or a 1:1 mixture of both substrates were used with an average total volume of 12 µl/13 mm coverslip. In latter experiments the coverslips were first coated with poly-L lysine and then washed 3 times with sterile distilled water. Laminin distribution was examined to assess the efficacy of addition of the exogenous basement membrane (section 3.2.3).

7.3.3 Results

Initial explant experiments were carried out without using a substrate, however the poor attachment and outgrowth led me to experiment with different substrates (see chapter 3.2.2). Collagen alone was the first substrate used and good outgrowth was achieved on this within 4 to 6 days. Attachment was improved by using a second coverslip to press the explant onto the substrate (see chapter 3). However, addition of AEF to the Schwann cells still did not elicit a strong upregulation of P_0 mRNA expression. Matrigel alone was then tried as the substrate and good outgrowth was obtained from explants grown on this. In section 1.3.3.5 and the introduction to this section, the role of extracellular matrix components such as collagen was discussed. The literature suggested that extracellular matrix components such as type I collagen may also be a requirement for complete Schwann cell maturation. Therefore, this was also added to the culture substrate. Most experiments were then carried out on a

mixture of rat tail collagen and Matrigel as described in chapter 3.2.

The effect of various culture techniques on cell surface distribution of basement membrane is detailed in chapter 3. Briefly, dissociated neonatal Schwann cells cultured on poly-L lysine expressed surface laminin as very fine patchy staining at day 0 and were essentially negative for it at day 5 and 10 *in vitro*. The surface coating of laminin was greatly improved if the dissociated cells were cultured on a substrate containing Matrigel. Schwann cells cultured from explants were evaluated only between days 5 and 10. Cells grown on collagen were also virtually negative for laminin. However, cells grown on Matrigel-containing substrate had a good, although sometimes patchy, coating of laminin. In all samples, fibroblasts were negative for surface laminin staining.

7.3.4 Discussion

The decision to use the complex basement membrane, Matrigel, in Schwann cell culture was based on the demonstrated requirement for this substrate for the terminal differentiation of Schwann cells (Carey *et al.*, 1986a; Carey and Todd, 1987a; Eldridge *et al.*, 1987; Eldridge *et al.*, 1989; Moya *et al.*, 1980). If deposition of basement membrane by the Schwann cells was inhibited, then myelination was also inhibited. This was observed when the proteoglycan inhibitor (4-methylumbelliferyl-ß-D xyloside) was added to cocultures (Carey *et al.*, 1987b) or if defined medium was used (Eldridge *et al.*, 1987; Eldridge *et al.*, 1989; Moya *et al.*, 1980). There is an interdependence of both neurons and Schwann cells on each other for basement membrane formation. Schwann cells require the presence of neurons for the formation, but not the persistence of basal lamina (Bunge *et al.*, 1980; Bunge *et al.*, 1982; Carey *et al.*, 1983; Cornbrooks *et al.*, 1983; Dubois-Dalq *et al.*, 1981). Likewise, sensory neurons require Schwann cells for the formation of basal lamina and collagen fibrils (Bunge *et al.*, 1980).

The requirement for contact of the Schwann cells with collagen appeared necessary both *in vitro* (Bunge *et al.*, 1978a; Bunge *et al.*, 1978b; Copio and Bunge, 1980) and *in vivo* (Blakemore, 1984; Blakemore and Crang, 1985; Bray *et al.*, 1983) for normal Schwann cell-axon interaction. Thus, some rat tail collagen was incorporated into the substrate upon which the explants in this thesis were cultured.

Growing dissociated Schwann cells on a complex basement membrane provided the cells with a basal lamina, or at least, a coating of laminin (sections

3.1.3; 3.2.3). Bunge *et al.*, (1980 & 1982) identified that tryspin treatment of Schwann cells cocultured with neurons causes thinning or loss of the basal lamina. Removing neuronal contact also causes loss of the basal lamina in culture (Bunge *et al.*, 1982). During dissociation of Schwann cells by the method of Brockes and colleagues, (1979) both trypsin treatment and isolation of Schwann cells from axonal contact occurs. The cells are also exposed to collagenase and vigorous mechanical trituration which would increase degradation of the basement membrane. Not surprisingly, these cells are not associated with adequate amounts of basement membrane. This dissolution of basement membrane may compound the postulated neonatal Schwann cell immaturity discussed earlier in this chapter (7.2.1)

A different situation exists with the adult explants. After axotomy, the myelin sheath is broken down, the Schwann cells proliferate and finally become quiescent and aligned within basal lamina tubes in bands of Bungner (Weinberg and Spencer, 1978). Antilaminin staining on cells grown on collagen only, identified that these "predegenerated" Schwann cells migrating from the explant, did not remain enveloped in basement membrane and, as they were not in contact with axons, no new basement membrane was formed. This corroborated previous work by Bunge *et al.*, (1982). However, if the explants were grown on a Matrigel containing substrate, then laminin, at least, became associated with the surface of the Schwann cell.

7.4 PREPARATION OF AXOLEMMA ENRICHED FRACTIONS

7.4.1 Introduction

The aim of these preparations was to create a fraction of nervous tissue that was rich in axolemma and hence, the putative signal for myelin stimulation. If such an axolemmal-enriched fraction (AEF) was prepared, it could be added to cultured Schwann cells which were not expressing significant amounts of P_0 mRNA. Upregulation of P_0 mRNA would indicate the presence of the putative myelinating signal in a form utilizable by the cells. The preparation could then be used to investigate the nature of the theoretical myelinating signal. The idea of preparing an AEF from nervous tissue and adding it to cultured Schwann cells is not new. DeVries and coworkers have been using such methodology to study the Schwann cell mitogen (DeCoster and DeVries, 1989; DeVries *et al.*, 1982; DeVries *et al.*, 1983b; Yoshino *et al.*, 1984). Other workers have also been using CNS preparations enriched in axonal membrane to stimulate Schwann cell proliferation (Cassel *et al.*, 1982; Ratner

et al., 1988).

In 1978, DeVries and colleagues prepared AEF from adult rat brain taking advantage of the different densities of the major cellular components of the myelinated axons to separate them. Briefly, the methodology involved homogenization and centrifugation of crudely dissected brain stem to obtain a floating layer of myelinated axons and a pellet of nuclei, capillaries and debris. The isotonic salt buffer helped to maintain the axon-myelin relationship at the expense of other white matter components. The floating layer was osmotically shocked in a hypotonic medium to strip the myelin sheath from the axon. The density of the AEF is intermediate between that of the axonal components and the myelin. The axolemma-containing fraction was separated from the other constituents on a discontinous sucrose density gradient. Centrifugation of the gradient resulted in a pellet of axonal material, AEF at the 1.0/1.2 and 0.8/1/0 M sucrose interfaces and a floating layer of myelin. The two membrane layers were harvested and the percent yield of the starting weight from each interface was 0.4%. By EM, the interfaces were composed mainly of unilamellar membrane vesicles of up to 1 μm diameter, occasional linear and mitochondrial membranes, and multilamellar myelin figures. Biochemically, these fractions were enriched with enzymes generally associated with surface membranes such as Na^+,K^+-ATPase, 5' nucleotidase and acetylcholinesterase. The level of mitochondrial and endoplasmic reticulum markers (cytochrome C oxidase and NADPH cytochrome C reductase) and microsomal membranes were depleted in the AEF. The authors thought that the level of contamination of the AEF by other CNS components (myelin or subcellular organelles) was minimal (DeVries *et al.*, 1978). Later studies used a continuous sucrose density gradient and the AEF was isopycnic with the 28 to 32% sucrose density (DeVries *et al.*, 1983a). As discussed in section 1.3.1.4, these AEF were found to still be biologically potent, with respect to stimulating Schwann cell proliferation, despite the extensive preparative procedures. In this work, a modification of this methodology has been used.

Molecules expressed in the central nervous system can act upon peripheral nervous system glia not just as mitogens, but also as stimulators of myelination. Axons of the central nervous system that have been experimentally demyelinated by various methods, were remyelinated by Schwann cells invading, or transplanted, from the peripheral nervous system (Blakemore, 1975; Blakemore, 1982b; Blakemore, 1984; Blakemore and Crang, 1985, reviewed by Blakemore and Franklin, 1991). Schwann cells have also been reported to myelinate CNS multiple sclerosis plaques. Also, axons of ventral horn cells are proximally myelinated by oligodendrocytes and

distally by Schwann cells. Therefore the same axon can stimulate myelination by both glial cell types (cited in Weinberg and Spencer, 1979). Myelination of peripheral fibres by oligodendrocytes has been reported (Weinberg and Spencer, 1979). These data suggest the presence of a myelinating signal which can stimulate the myelinating glia of the PNS and CNS. Therefore, the CNS could be used as a source of the putative myelinating signal for the Schwann cell. Tissue from the CNS instead of the PNS was chosen because of its greater amount and availability, and lack of fibrous component.

There were two significant differences between the AEF prepared here on the sucrose density gradient and those prepared by DeVries *et al.*, (1978). These are the starting material, and the actual procedure itself which is given in the next section. With respect to the starting material, the signal that I wished to obtain was one that stimulates myelin-specific molecule expression by Schwann cells. It was logical to use a starting material presumably rich in this signal. Maximal MBP mRNA expression occurred in the medulla oblongata of 5 to 7 day old rats and this had declined by 12 days (Kristensson *et al.*, 1986) Up-regulation of the mRNA precedes translation of MSMs (Mitchell *et al.*, 1990) and expression of the myelinating signal must precede that. Hence, presumably expression of the myelinating signal must be intense within the first post-natal week. It, therefore, seemed logical to mainly use neonatal CNS tissue to prepare a fraction of tissue enriched in the myelinating signal.

7.4.2 Materials and Method

The main source of nervous tissue was rat brain with most of the tissue being harvested from neonatal rats, although adult animals were also used. Tissue was harvested under aseptic conditions and all procedures were performed at 0 to 4^{O}C. Two methods of preparation were utilized; both used a hypotonic buffer to osmotically lyse the cells, but the centrifugation schedule was less complex in the simple preparation. In both methods the tissue was minced and then homogenized in a glass hand held homogenizer for 10 strokes in a 0.32 M sucrose buffer. Centrifugation details and buffer compositions are given in the appendix (A6 and A2.1.4 respectively).

7.4.2.1 Simple preparation

The homogenate (A) was centrifuged at 600 rpm for 5 minutes on a Wifug

bench top centrifuge, and the pellet suspended by trituration in the hypotonic shock buffer. After 10 minutes, the solution was centrifuged as before to pellet the heavier debris (B) (capillaries, connective tissue). The supernatant was centrifuged at 2000 rpm for 30 minutes and pellet (C) resuspended in 0.25 M sucrose, 0.01 M TES, pH 7.5. Protein concentration was assessed using the Pierce protein assay (chapter 2.5.1).

7.4.2.2 Discontinuous sucrose gradient preparation

The homogenized suspension was centrifuged at 600 rpm for 10 minutes to remove the heavier debris. The supernatant was then centrifuged at 20,000 rpm for 15 minutes to pellet the tissue. The pellet was resuspended by trituration in the hypotonic shock buffer and this solution was then centrifuged at 20,000 rpm for 15 minutes. The pellet was resuspended by trituration through a 21G needle in 0.75M sucrose buffer, and overlaid over 1.5 x the volume of 1.2 M sucrose buffer. The gradient was centrifuged at 28,000 rpm for 2.5 hours. Tissue was obviously concentrated at the interface between the two sucrose solutions whilst debris, more dense than 1.2 M sucrose, was pelleted and any myelin remained floating on the top of the 0.75 M solution. The interface was removed by aspiration with a pasteur pipette and resuspended by trituration in L15 tissue culture medium. Leupeptin (R) (Peptide Marketing Institute) protease inhibitor was added to most experiments to limit protease degradation. Samples were taken at various times during this preparation, stained with haematoxylin and assessed by light microscopy for efficacy of purification. Some of the AEF was resuspended in 2.5% glutaraldehyde in isotonic cacocylate buffer, pelleted in an eppendorf tube by microcentrifugation for 20 minutes at 13000 rpm and the sample processed for electron microscopy (A3.3).

In some experiments, half of the AEF was left at this point ("native AEF") and the other half was incubated with an equal volume of 0.2 M $NaCO_3$ and 0.002 M dithiothrietol on ice for 1 hour and then frozen overnight. The alkali was removed by washing the pellet with large volumes of L15 or Hanks buffered salt solution. This washing procedure was performed until the medium was no longer cyan pink (indicating a basic pH) but had returned to the orange/pink colour of physiologically neutral tissue culture solution. Protein concentration was assessed as before with the calibrating sample and the protein standards being made up with the resuspension buffer (L15).

7.4.2.3 Biochemical assessment of AEF

SDS-polyacrylamide gel electrophoresis was performed as described in chapter 2.5.2, using a stacking gel of 2.0% and a resolving gel of 12.5% for the simple preparation. A 5 to 17.5% gradient gel was used for the sucrose gradient preparation and 60 μg samples of protein were loaded into each well for electrophoresis. The methodology for western blotting is also described in chapter 2.5.3. Proteins that were assayed by western blotting were actin, tubulin, fodrin, neurofilament 160, MAG, MBP, GFAP and PLP. The primary antibodies were all raised in mouse except fodrin which was produced in the rabbit. All primary antibodies were used at 1:1000 except neurofilament (1:100) and PLP (1:2000). Secondary antibodies, used at 1:1000, were raised in goats and conjugated to peroxidase.

7.4.3 Results

7.4.3.1 AEF preparations

The percentage yield of the starting weight was 0.002 to 0.01% from the simple preparation and 0.01 to 0.07% from the sucrose gradient preparation.

Morphological assessment of the AEF

Microscopic examination of the simple preparation identified that sample B was rich in nuclei and cytosolic components such as mitochondria and endoplasmic reticulum, and neurofilaments. The final sample C had far fewer nuclei, and increased amounts of membranous structures. However, large numbers of mitochondria, clumps of neurofilaments, and some rough endoplasmic reticulum were also present along with many unidentifiable membranous structures (see fig 7.1).

From the sucrose gradient preparation, samples for light microscopy were taken from the sediment created by the first centrifugation after homogenization (S1), and from the sediment created by centrifugation after hypotonic shock of the cells (S2). S1 was densely packed with nuclei and some intact capillary lengths and connective tissue sheets were observed. In contrast, S2 contained very few nuclei, no capillaries or connective tissue and was, instead, composed mainly of finely granular amorphous debris. Electron microscopy, confirmed the absence of nuclei in the final sample obtained at the end of the procedure (fig.7.2). The majority of the fragments was composed of varying diameter vesicles (0.01 to 0.12 μm) with one or a few surrounding membranes although occasional multilamellar structures were noted. The

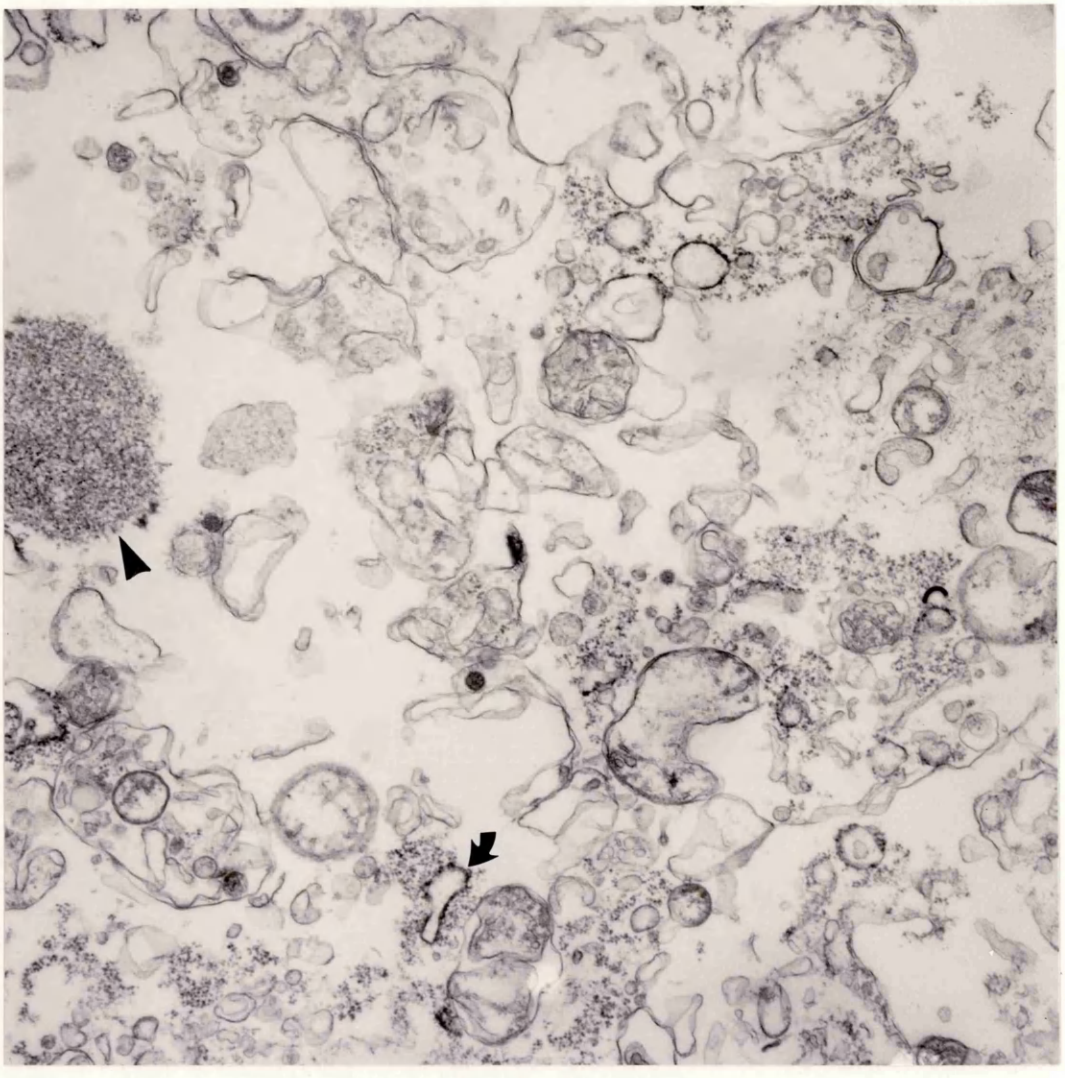


Figure 7.1: EM of axolemmal-enriched fragments - simple preparation. Degenerate subcellular organelles are visible by EM of AEF prepared by the simple method (please see text for details). Cytoskeletal elements (arrow head) and endoplasmic reticulum (curved arrow) are present, along with many unidentifiable membranous structures (x 15,000).

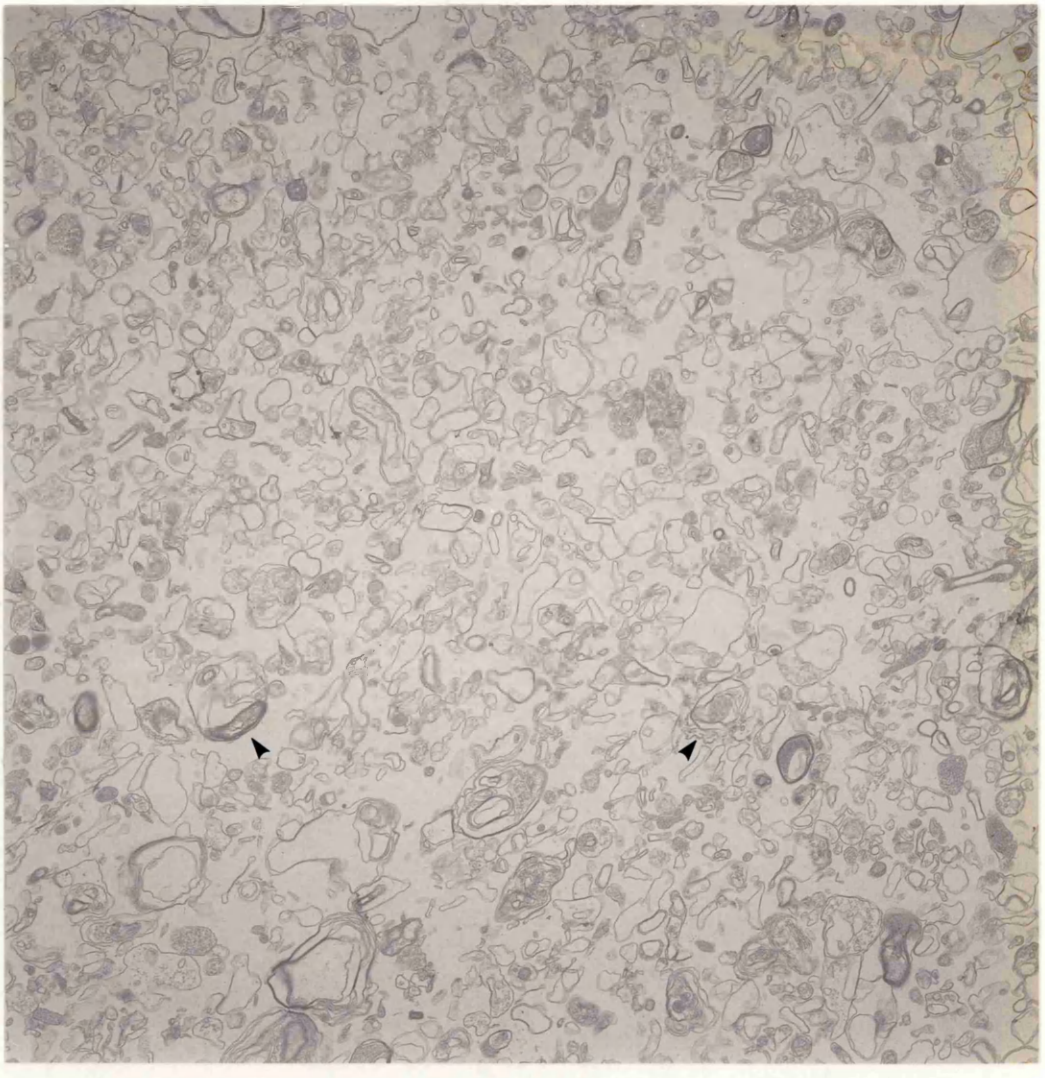


Figure 7.2: EM of axolemmal-enriched fragments- sucrose gradient preparation. Compared with the simple preparation, this one is greatly enriched in membranous structures of varying sizes, some of which are unilamellar whilst others are multilamellar and appear to enclose axonal remnants (arrow heads) (x 10,000).

latter often enveloped axonal remnants containing recognizable cytoskeleton. Mitochondria were also noted but these were far fewer than in final fragments of the simple preparation.

7.4.3.2 SDS-PAGE and western blots of AEF preparations

An SDS-PAGE of the samples obtained at different stages of the simple preparation identified a trend of progressive enrichment for proteins at approximately 18.4 kDa, 17 kDa and 14.3 kDa. The first and third proteins coincided with the proteins P_1 and P_2. There was also some lesser enrichment of protein at the 50 kDa and 60 kDa bands. There was a decrease in the staining intensity of bands at the 20 kDa and 51 kDa regions. The 51 kDa protein comigrated with GFAP. However, the pattern of protein enrichment was not consistent between separate preparations. Western blotting positively identified fodrin, actin, MAG and MBP on the S3 sample and was negative for neurofilament 160, and tubulin (table 7.1). Blotting for PLP and GFAP was not performed.

SDS-PAGE of different sucrose gradient preparations, prepared by the same method, identified some differences in the pattern of separated proteins (fig 7.3). Compared with whole neonatal brain homogenate there was decreased intensity of bands in the 18 to 22 kDA region (possibly MBPs); 43 and 51 kDa region (possibly actin and GFAP); 160 kDa region (possibly neurofilament 160) and 240 kDa (possibly fodrin).

Both native and sodium carbonate treated fractions from the sucrose gradient AEF preparations were assessed by Western blotting. The native fragments were positive for actin, tubulin, fodrin, PLP and GFAP. They were negative for MAG, neurofilament 160 and MBP. The sodium carbonate treated fragments were negative for all proteins tested (table 7.1).

7.4.4 Discussion

Manufacture of AEF by sucrose gradients has been used to elucidate characteristics of the Schwann cell mitogen (see section 1.3.1.4) (DeCoster and DeVries, 1989; DeVries *et al.*, 1982; DeVries *et al.*, 1983; Yoshino *et al.*, 1984). Biochemical evaluation of the AEF identified that there was some contamination by mitochondrial membranes, but there was little contamination by myelin. The AEF was enriched in enzymes associated with the axolemma (DeVries *et al.*, 1978; DeVries *et al.*, 1983a). Similarly, Sobue and Pleasure (1985) found enrichment of

ANTIGEN	SIMPLE PREPARATIONS	SUCROSE PREPARATIONS	
		NATIVE	SOD. CARBONATE
Actin	+	+	-
Fodrin	+	+	-
GFAP	ND	+	-
MAG	+	-	-
MBP	+	-	-
NF 160	-	-	-
PLP	ND	+	-
Tubulin	-	+	-

Table 7.1: **Results of western blotting of AEF prepared by the simple method, or on a sucrose gradient.** The axolemmal-enriched fragments (AEF) prepared on the sucrose gradient were either treated with sodium carbonate (sod. carbonate) or left untreated (native). ND = not done; GFAP = glial fibrillary acidic protein; MAG = myelin associated glycoprotein; MBP = myelin basic protein; NF 160 = the 160 kDa neurofilament protein; PLP = proteolipid protein.

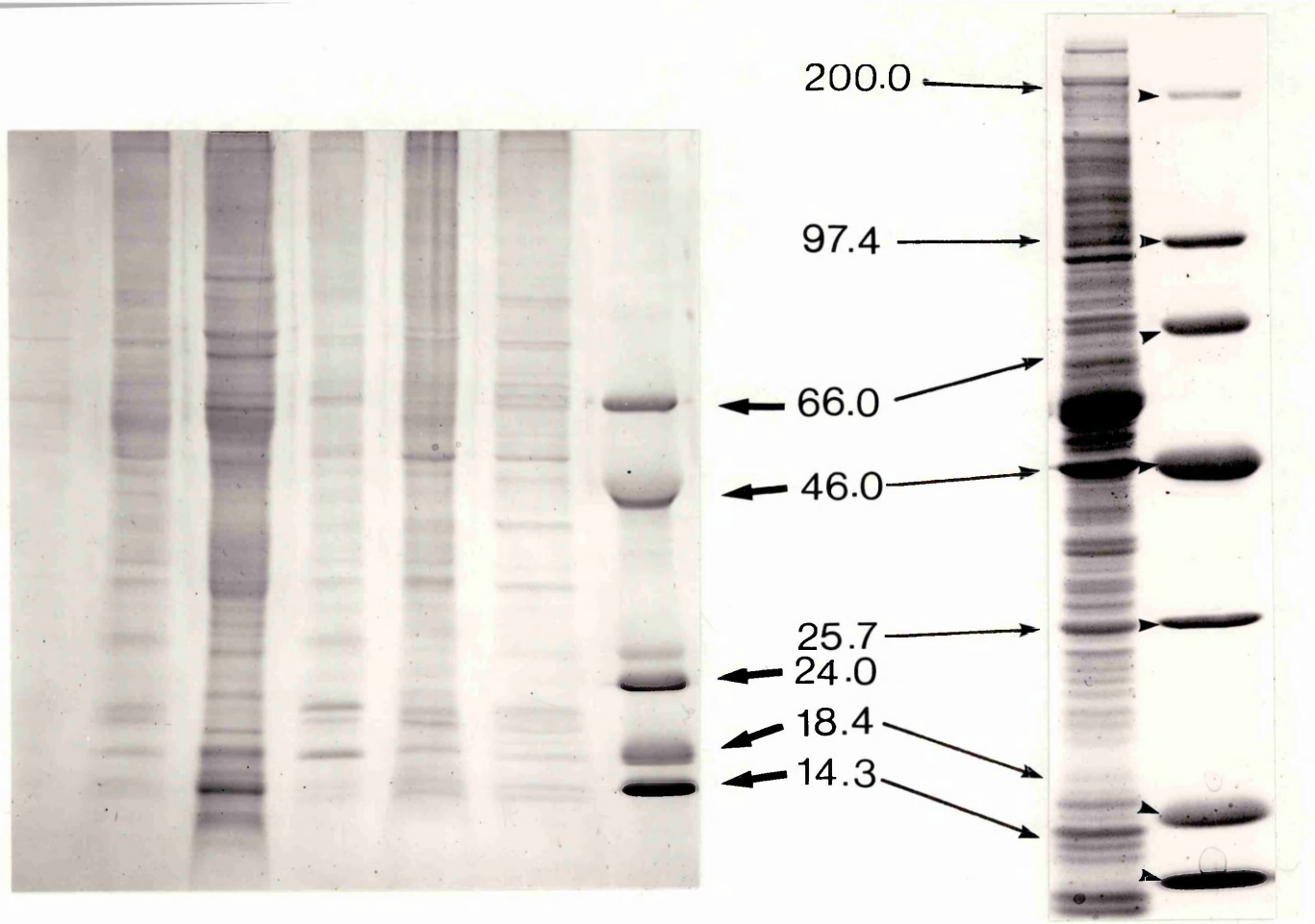


Figure 7.3: SDS-PAGE gels of axolemmal-enriched fragments and whole neonatal rat brain. 60 ug of protein was loaded into each lane. After electrophoresis on a 5 to 17.5% gradient gel, the gel was stained with coomassie brilliant blue. (A) Axolemmal-enriched fragments from sucrose gradient preparations - 4 different experiments are represented in this gel. Lane 1 = sodium carbonate treated fragments; lane 2 = native fragments, lane 3 = liver fragments , lane 4 = native fragments, lane 5 = native fragments, lane 6 = native fragments, lane 7 = low molecular weight standards (x 1) (B) Whole neonatal rat brain. Compared with the sucrose gradient preparations, there is decreased intensity of staining in the regions of 18 to 22 kDa, 43 kDa, 51 kDa, 160 and 240 kDa (x 1.4)

Na^+,K^+-ATPase activity and low levels of mitochondrial-associated enzyme activity in the fractions richest in mitogenic activity. This corresponded morphologically with EM pictures of 0.1-1.5 μm diameter vesicles without visible contents. Some mitochondrial and myelin fragments were observed.

In another study, the binding of radiolabelled AEF to neonatal dissociated Schwann cells was found to be specific with only low levels of labelling of fibroblasts, and only 1/5 of the amount of sarcolemma and erythrocyte membranes bound to the Schwann cells. Maximum adhesion of the AEF to the Schwann cells was achieved at 4 hours incubation of the AEF with the cells. At this time, most of the AEF was still surface bound and was not sequestered within the cell. Adhesion was not dependent upon the presence of calcium ions (Sobue and Pleasure, 1986).

There are four described proteolytic systems that can act on membrane proteins and these include mitochondrial proteases, lysosomal enzymes, extracellular proteases and soluble cytosolic proteases. Transmembrane proteins can be degraded by several different classes of proteases; the intracellular domain by the cytosolic proteases, the transmembrane domain by lysosomal enzymes and the extracellular domain by extracellular enzymes. Lysosomal enzymes are inhibited by leupeptin (reviewed by Hare, 1990). Leupeptin inhibits serine, thiol and aspartic proteases to varying degrees and has been shown to be effective in inhibiting calcium activated neutral proteases within the brain. These proteases are efficient degraders of neural proteins (see section 6.4) (Malik *et al.*, 1983; cited in Simonson *et al.*, 1985). At < 5.0 μM concentration, leupeptin almost completely inhibited protease activity in brain tissue (Nilsson and Karlsson, 1986). For these reasons, leupeptin was added to the AEF preparations.

The aim of the studies detailed in this section was the production of an *in vitro* model of myelination so that the characteristics of the myelinating signal(s) could be studied. Initial experiments in this work used a modification of DeVries' technique (DeVries *et al*, 1978) in an attempt to create a fraction of nerve tissue that would stimulate P_0 mRNA expression in cultured neonatal dissociated Schwann cells. However, results suggesting upregulation of expression of this gene were tantalizing but not conclusive (see next section). It was possible that the lack of upregulation may have been because this method of producing AEF led to the loss of the theoretical myelinating signal. A simpler preparation using hypotonic shock and slow centrifugation to pellet the heavier tissue debris was investigated. This procedure was similar to that used by Ratner *et al.*, (1983) which involved homogenization of brains and then slow centrifugation to pellet nuclei. I added a hypotonic shock stage, adapted

from DeVries *et al.*, (1978), to my method to rupture the cells and sheaths and potentially release the axolemma. Again the results of addition of this simple preparation to dissociated Schwann cell cultures were suggestive, but equivocal. All the different fractions (A,B,C) obtained within this preparation were assessed for their effect on the Schwann cell P_0 mRNA expression. The first fraction, A, had a similar effect as the third fraction, C. This suggested that the simple method of preparation was doing little to enrich the biological activity of the tissue. Also, as it was considered that the lack of significant P_0 upregulation might be due to the method of Schwann cell preparation, the preparation of AEF by the sucrose gradient method was again re-instituted.

Electron microscopy of AEF has identified empty 0.1-1.5 μm diameter vesicles. Less than 10% of these were recognizable as membranes derived from organelles other than axolemma (Sobue and Pleasure, 1985). Although contamination of the unilamellar membranes by other membranes occurred, this was at a low level (DeVries *et al.*, 1978). In my studies, EM assessment of the AEF prepared by both methods identified better enrichment of membranous structures in the sucrose gradient preparation. The AEF prepared here on the sucrose gradient had more mitochondrial and cytosolic remnants than that of De Vries *et al.*, (1983), or Sobue and Pleasure (1985). However, it was still greatly enriched in membranous structures compared with the starting tissue. The vesicle diameter was smaller in my preparations compared with that observed by other investigators. This may reflect the age of the animal from which the tissue originated as axonal diameter increases with age and maturation (Visozo and Young, 1948).

Treatment of AEF with sodium carbonate/bicarbonate buffer (pH 9.0) has been shown to enrich the fraction with respect to mitogenic activity. The pellet represented 50% +/- 5% of the starting protein and contained 95% +/- 10% of the mitogenic activity. This treatment is thought to remove other proteins from the preparation but leave the axolemmal mitogen unchanged (DeCoster and DeVries, 1989). In my experience, treatment of AEF with sodium carbonate was associated with a return of only 25% of the starting amount of protein.

In both preparations performed here, western blotting identified contamination of the native fragments with myelin debris. Both PLP and MBP were identifed in the preparations. Neurofilament 160 was not detected in either preparation suggesting that some of the axonal cytoskeleton was lost during the procedure. However, actin and fodrin were present in both preparations and tubulin was identified in the sucrose

preparation. Fodrin (brain spectrin) was noted to copurify with crude membrane perparations of CNS tissue and is associated with the axolemma (Anderson, 1984; Goodman and Zagon, 1986; Zagon *et al.*, 1986). However, it is also interconnected with other structural elements within the cell such as mitochondria, endoplasmic reticulum, filaments and tubules (Zagon *et al.*, 1986). Actin and α- and ß-tubulin were found to be major components of the subaxolemmal cytoskeleton of the giant squid axon (Kobayashi *et al.*, 1986). Actin has also been localized to the central region of the axon as well as the subaxolemmal region (Papasozomenos and Payne, 1986; Zagon *et al.*, 1986). Thus the presence of actin, tubulin and spectrin may be expected in axolemmal rich preparations of the CNS considering their subaxolemmal locations. However, because they are also found in the cytoskeleton of the neural tissue, their presence does not rule out contamination by cytosolic components of the axon. Considering the ultrastructure of the AEF in which only small amounts of cytoskeletal elements can be identified, and the lack of neurofilament on western blotting, it is likely that molecules identified by western blotting were subaxolemmal in location. GFAP is found in astrocytes (Jessen *et al.*, 1984) and its presence in the sucrose preparation suggests that some glial cell contamination was still present in the preparation. The lack of myelin components, and axonal cytoskeletal components in the sodium carbonate treated fragments suggests that the alkali may have removed molecules present on either side of the axolemma.

7.5 THE EFFECT OF AXOLEMMA ADDITION ON MSM EXPRESSION IN SCHWANN CELL CULTURE:

7.5.1 Introduction

The studies described in this section were aimed at testing the theory that the putative myelinating signal is associated with the axolemma. If competent Schwann cells were presented with AEF, then they should respond by upregulating the expression of myelin-specific molecules, specifically P_0 mRNA.

In chapters 3, 4, and the beginning of this chapter, the preliminary studies to this section are discussed. These were performed to develop potentially competent Schwann cells and to assess their inherent level of P_0 mRNA expression under different circumstances.

In the last section, the methodology for developing the protocol of AEF preparation was considered. The aim was to produce a fraction of the nervous system

which was enriched in the axolemma, and, theoretically, the myelinating signal. However, because the exact nature and location of this signal is not clear, a simpler method of preparation was also employed. This was in case the sucrose gradient procedure was, in fact, extracting the signal from the preparation. Subsequent studies suggested that the AEF prepared on the sucrose gradient were better than the simple preparation.

Other factors that had to be considered in the planning of these studies included storage of the AEF after preparation, the specificity of stimulation, the dosage regime (timing and amount) as well as the competency of the *in vitro* system used for culturing Schwann cells (as previously discussed).

Freezing and thawing of AEF prepared by the method of DeVries *et al.*, did not seem to affect the mitogenicity of the axolemma (Cassel *et al.*, 1982; Sobue *et al.*, 1983). This was an important consideration as, because of the length of the procedure, the AEF could not be easily prepared fresh for each addition. However, I attempted to make a fresh batch of AEF for each new experiment and only thaw it once prior to addition. This was because the effect of freezing and thawing on the myelinating signal was not known.

The frequency of dosage had to be considered. Maximum adhesion of the AEF to the Schwann cells was achieved at 4 hours incubation of the AEF with the cells. At this time, it was determined that most of the AEF was still surface bound and was not sequestered within the cell. The full mitogenic response of the Schwann cells required 24 hours exposure to the AEF (Sobue and Pleasure, 1985). These data suggested that the minimum time of exposure of the Schwann cells to the AEF was 24 hours. Whilst Sobue and Pleasure (1985) only pulsed cells for 24 hours, DeVries *et al.*, (1982) pulsed each 24 hours for 48 hours. Although Salzer *et al.*, (1980b) were using a different method of preparing axolemma (neuritic membranes harvested from cultured spinal ganglia) they found that 2 or 3 x 24 hour pulses gave the optimal mitogenic response to stimulation. Thus, the most frequently used dosage regime employed in the studies undertaken here was 3 x 24 hour pulses. The dose size of AEF was based on that used in mitotic studies by DeVries *et al.*, (1982 & 1983). In my studies, a range of 5 to 100 μg/ well (of a 24 well tissue culture plate) was used; a saturating dose was thought to be 25 μg (DeVries *et al.*, 1983).

7.5.2 Neonatal dissociated Schwann cells

7.5.2.1 Introduction

The simplest test bed to assess the effect of AEF addition on P_0 mRNA expression was that of dissociated neonatal Schwann cells cultured on poly-L lysine. The methodology for culturing Schwann cells in this manner was well established and seemed effective in producing an enriched population of Schwann cells (see section 1.3.2.1) which could respond to the addition of AEF by proliferating (see section 1.3.1.4). Whether these Schwann cells would be able to respond to AEF by upregulation of P_0 mRNA was unknown. These cells were being cultured in a nutrient-rich medium which seemed to support their growth but in the absence of axonal contact they were down-regulating the expression of P_0 mRNA (see section 1.3.2.3 and chapter 4). If contact with a fraction of nervous tissue enriched in axolemma was reinstated, could these cells re-express the mRNA? It was not known whether all the factors required by Schwann cells for myelin-specific molecule expression subsequent to axolemmal contact were present. However, the consideration of other factors did not become important until subsequent studies.

7.5.2.2 Materials and method

The method of dissociated Schwann cell preparation, modified from Brockes *et al* (1979), is given in chapter 2.1.1. The cells were plated onto poly-L lysine coated 13mm glass coverslips (A4.1.4), fed and treated with cytosine arabinoside as previously described. In the last experiments in this series, neonatal dissociated Schwann cells were plated onto poly-L lysine, collagen, Matrigel or collagen/Matrigel mixture. In some experiments AEF were added for 2 to 4 daily doses beginning at 5 to 6 days in culture. In other experiments, the AEF were not added until the Schwann cells had been cultured for 2 weeks. The simple preparation was used in the first and second time schedules of addition, but the discontinuous sucrose gradient preparation was only added after 5 to 6 days in culture. The A, B and C components of the simple preparation were all assessed for their effect on the Schwann cells. Doses of AEF in the range of 10 to 200 μg/ml of culture medium were used. The old medium was aspirated from the cultures and fresh medium added prior to each addition of AEF. For each experiment, the positive controls were dissociated Schwann cells that were fixed within 24 hours of dissociation, and the negative controls were Schwann cells which were dissociated and cultured under identical conditions to the test cells, but

had only the medium changed when the AEF was added to the test cells. For some experiments, liver fractions were prepared in the same way as the AEF. Also pure fibroblast cultures were prepared by scraping the dermis of the rat pups and subjecting this tissue to the same enzymatic and mechanical dissociation as the Schwann cells. Twenty-four hours after the final addition of AEF, the cells were fixed and processed by *in situ* hybridisation for detection of P_0 mRNA. The methodology is given in chapter 2.4.1. Both antisense and sense probes were used. The identity of the samples was hidden and the results scored in a "blind" fashion. The intensity of the P_0 mRNA expression was graded from 0 to 10+ as in chapter 4.3.

7.5.2.3 Results

The methodology using the discontinuous sucrose gradient was tried first. It was known to be successful, morphologically and biochemically, in producing a fraction of CNS tissue enriched in axolemma (see section 7.4.1). The results of AEF addition on P_0 mRNA expression are given in table 7.2.

Discontinuous sucrose gradient AEF preparation

The AEF could be observed in the culture medium as a fine granular, amorphous debris. During the treatment schedule, the cells became plumper with shorter processes and, within 48 hours, appeared to be forming clusters, or clumps, of cells. The clustering of the Schwann cells made interpretation of the signal strength difficult and the results were suggestive, but not conclusive, of P_0 mRNA stimulation.

Simple AEF preparation

Under the inverted microscope, the fragments of the simple preparation of AEF seemed to adhere to the cells. Many of the Schwann cells formed clusters during these experiments, and the number of Schwann cells in the higher dose wells (> 200 μg/ml) decreased over the time course of AEF addition. Again cell clustering made it difficult to assess the effect of AEF on P_0 mRNA expression. There was no obvious difference in the level of stimulation by the different fractions of this simple AEF preparation. The liver fraction failed to stimulate either the Schwann cells or the fibroblasts, and the AEF failed to stimulate fibroblast colonies. Fibroblasts in AEF treated cultures, and the background were only associated with silver grains at 1+ intensity. The response of the AEF stimulated cells was variable between experiments ranging from no apparent stimulation, to expressing the P_0 mRNA up to 3+.

CELL TYPE	SUBST-RATE	AEF PREP	DAY 0	SC	SC - AEF	SC + AEF	FB
			antisense	sense			
Dissoc. SC	PL	simple	8	1	1-2	2-3	1
	PL	simple	7-8	1	1	1	1
	PL	S,N	5	1	1	2-3	1
	PL	S,N	7	1	1	1	1
	C/M	S,N	7	1	1	1	1
	C/M	S,N	8	1	2-5	2-5	1
	PL	liver	7-8	1	1	1	1
Sciatic explant							
adult chronic	collagen	S,N	7	1	1	3-4	1
	C/M	S,N	8	1	1-2	3-5	1
	C/M	S,N,C	8	1	1-2	4-7	1
	C/M	S,A	8	1	1-2	2-3	1
	C/M	S,A,C	8	1	1-2	3	1
adult acute	C/M	S,N	7	1	3-5	3-5	1
neonatal	C/M	S,N	7	0	1	5	1
	C/M	S,N	8	0	5	5	1
	C/M	S,N	10	0	2-4	3-4	1
	C/M	S,N	10	0	2-4	2-3	1
	C/M	S,N	10	0	2-4	1-2	1
	C/M	S,N,C	10	0	2-4	2-4	1
CST explant	C/M	S,N	8	0	1-3	1-3	1
	C/M	S,N	8	0	1-3	1-3	1

Table 7.2: The effect on P_0 mRNA expression of adding AEF to cultured Schwann cells. Schwann cells were cultured from neonatal and adult animals. From neonatal animals they were dissociated (dissoc.) or cultured as explants. From adult animals, only explants were produced; these were either produced from the sciatic nerve which had been neurotomized and left to degenerate *in vivo* for 4 to 6 weeks (adult chronic) or for 4 days (adult acute). Explants were also created from the cervico-sympathetic trunk (CST) of adult animals. Neonatal dissociated and explant cells were cultured for 5 to 6 days; adult CST and chronic sciatic explants were cultured for 8 to 12 days and acute adult explants were cultured for 7 to 8 days prior to addition of AEF. Cells were cultured on either poly-L lysine (PL), collagen or collagen plus Matrigel (C/M) substrate. The AEF was prepared in a simple fashion, or on a sucrose gradient (S). The latter was prepared from either neonatal CNS tissue (N) or adult tissue (A) and left as "native" fragments or the AEF were further treated with sodium carbonate (C). Dissociated Schwann cells which had been cultured for less than 24 hours were used as positive controls (antisense) or negative controls (sense) for *in situ* hybridisation. AEF = axolemmal-enriched fraction, SC = Schwann cells, FB = fibroblasts. The numbers (1-10) refer to the intensity of silver grain (and hence mRNA) expression by the Schwann cells., where 0 = no expression, 5 = 50% of the cells' nucleus being covered by silver grains, and 10 = a density of silver grains so heavy, that the grains totally overlap and occlude the nucleus.

Growing these cells on collagen or collagen plus Matrigel substrate did not alter their level of response to AEF.

7.5.2.4 Discussion

From these experiments, it was concluded that stimulation of the Schwann cells by the axolemmal fragments may occur, but was at a low and inconsistent level. As stimulation of the cells by the interface fraction of the discontinuous gradient AEF preparation was not great, it was considered that this more elaborate preparation may not be concentrating the putative myelinating signal. A simpler preparation of brain tissue with fewer procedures was devised. Ratner *et al.*, (1988) attempted a purification of the Schwann cell mitogen from whole neonatal rat brain by simple homogenization in a sucrose/salt buffer and subsequent centrifugation to pellet the nuclei. I used a modification of this procedure combined with the idea of hypotonic shock from DeVries *et al.*, (1978) to create a simple method of preparing AEF. Because the efficacy of this procedure was unknown, all fractions of this preparation were assessed for their potency. However, there was no obvious difference in the activity of the various fractions of this simple AEF preparation. In fact, doses of greater than 200 μg/ml appeared toxic resulting in Schwann cells losing their processes, becoming spherical and forming clusters. Eventually the cell number decreased in these colonies.

The stimulation of the Schwann cells that did occur, seemed to be specific for the target cell and for the membrane preparation. This was demonstrated by the failure of the sucrose gradient AEF, or liver fraction, to stimulate fibroblasts and the failure of the liver fraction to stimulate the Schwann cells.

The addition of neuritic membranes to Schwann cell cultures in high doses caused the cells to become more flattened and irregular in appearance with loss of their spindle shape. The cells migrated into clusters several cells thick. This response was thought to be due to neuritic membranes inducing Schwann cell migration (Salzer *et al.*, 1980b). It is not unreasonable to suspect that the alterations in Schwann cell arrangement and appearance in my cultures occurred for similar reasons.

Basement membrane was stripped off during dissociation of Schwann cells (section 3.1.3) and lack of basement membrane is associated with failure of terminal differentiation of Schwann cells. However, whilst addition of exogenous basement membrane to the dissociated cells in my studies appeared to be effective in coating the cells with laminin, it did not significantly affect the cells' response to the addition of AEF.

7.5.3 Peripheral nerve explants

7.5.3.1 Introduction

The results of AEF addition to neonatal dissociated Schwann cells were suggestive, but not conclusive, of upregulation of P_0 mRNA expression in response to AEF. A more convincing test model was required. It had been suggested that neonatal Schwann cells in culture dedifferentiate or revert back to a neonatal progenitor state and are no longer capable of P_0 gene expression (Poduslo and Windebank, 1985b). Certainly when myelin-forming Schwann cells are separated from axonal contact, down-regulation of MSM expression is observed and the cells adopt a more primitive state with respect to cell marker expression (see sections 1.3.2.3, 1.5.3.2, 1.5.3.3).

The problems of Schwann cell differentiation in the absence of contact with appropriate matrix components have been discussed both in section 1.3.3 and in section 7.3.1. Thus there were at least two possible reasons for the poor response of the neonatal dissociated Schwann cells to the AEF. The first was lack of axonally-induced differentiation of the target cell and secondly, absence of specific essential factors for differentiation. Hence a new source of Schwann cells were sought, which were potentially more differentiated and a new means of culturing them, which would provide them with access to extracellular matrix components and to basement membrane.

As there was little difference in the Schwann cell response to the different fractions of the simple AEF preparation, it seemed likely that the simple method of enriching the preparation was unsuccessful. It was strongly suspected that the putative myelinating signal was located on the axolemma and DeVries and coworkers (1978) had shown by a number of criteria that the discontinuous sucrose density gradient was effective in producing an axolemmal-enriched fraction from central nervous system tissue. Thus, this method of AEF preparation was again tried with a slight modification.

7.5.3.2 Materials and method

The method of preparing Schwann cells from adult rat sciatic nerve is given in chapter 3.2.2. Most explants were obtained from sciatic nerve that had been transected 4 to 8 weeks previously and allowed to undergo Wallerian degeneration *in vivo*. In one experiment, the explants were prepared from nerve that had been

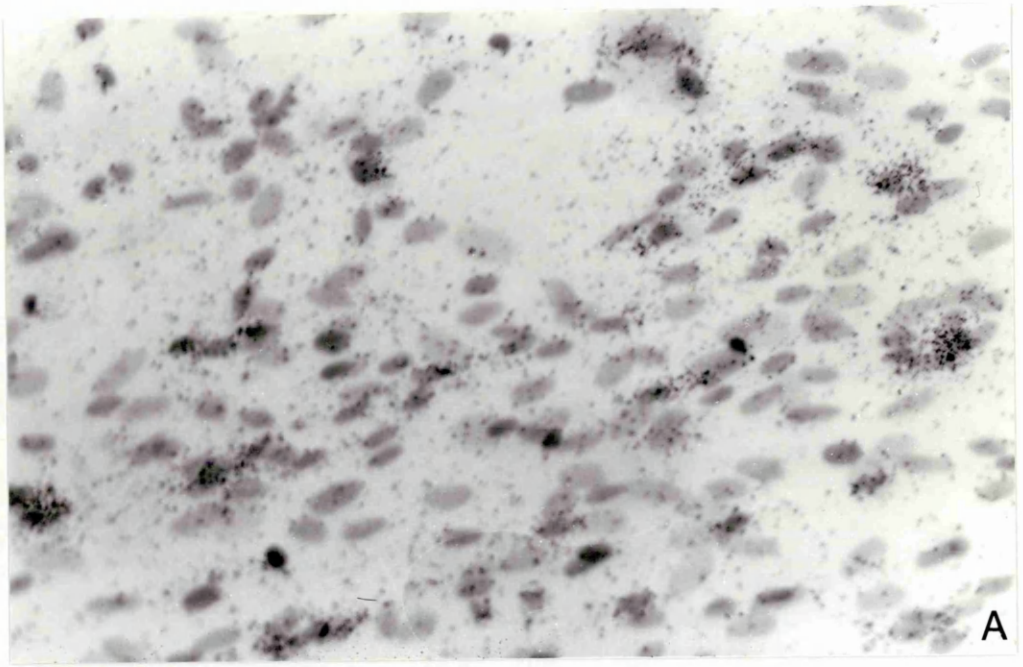
transected only 4 days prior to culture establishment, and in another, cultures were attempted from non-neurotomized nerves. Explants were also produced from neonatal sciatic nerve (section 3.2.2) and adult CST (section 3.3.2) obtained without enzymatic or mechanical dissociation. Dissociated Schwann cells were prepared and cultured on poly-L lysine, collagen or collagen plus Matrigel.

The AEF were prepared from either neonatal brain or adult rat brainstem using a modification of De Vries' method. In several experiments, some of the AEF was treated with an equal volume of 0.2 M $NaCO_3$ in 0.002 M dithiothrietol on ice and frozen overnight.

Because of the low copy number of P_0 mRNA within the cultured Schwann cells, dot blotting was only successfully performed on neonatal explants as these were the only cultures in which sufficient cells could be produced. The quality of the RNA was assessed by northern blotting (section 2.4.5.1) and staining with methylene blue (section 2.4.5.2) (fig. 7.5A). Approximately 15 and 45 μg of RNA extracted from AEF treated Schwann cells and 30 μg from the negative control Schwann cells (section 2.4.1.2), were heat denatured, dotted onto nitrocellulose paper (section 2.4.5.2) and probed with [^{32}P] labelled P_0 cDNA (section 2.4.3.1). After autoradiography, the filter was stripped (section 2.4.5.3) and reprobed with [^{32}P] labelled S100 cDNA (section 2.4.3.1) prepared as described in section 2.4.2.1. The ODs of the two autoradiograms were measured on a Quantimet 970 and the intensity of P_0 mRNA expression was expressed after adjusting for the amount of S100 mRNA present, thereby correcting for variations in the amounts of Schwann cell RNA loaded onto the filter.

7.5.3.3 Results

The results of addition of AEF to chronically neurotomized sciatic nerve explants is given in the table 7.2. There was no alteration in the cell morphology in response to the addition of AEF unlike that observed using a simple AEF preparation on neonatal dissociated Schwann cells. Chronically neurotomized adult rat sciatic nerve explants appeared to be stimulated by both adult and neonatal AEF preparations (fig. 7.4), which were treated by sodium carbonate or left untreated (native). The sodium carbonate-treated neonatal-derived AEF seemed most efficacious. However, there was some overlap in the level of P_0 mRNA expression of the untreated controls and some of the test cells. The level of response by Schwann cells within one sample was variable with some cells not expressing the gene whilst others were strongly expressing it. Most commonly, the Schwann cells closest to the explant were



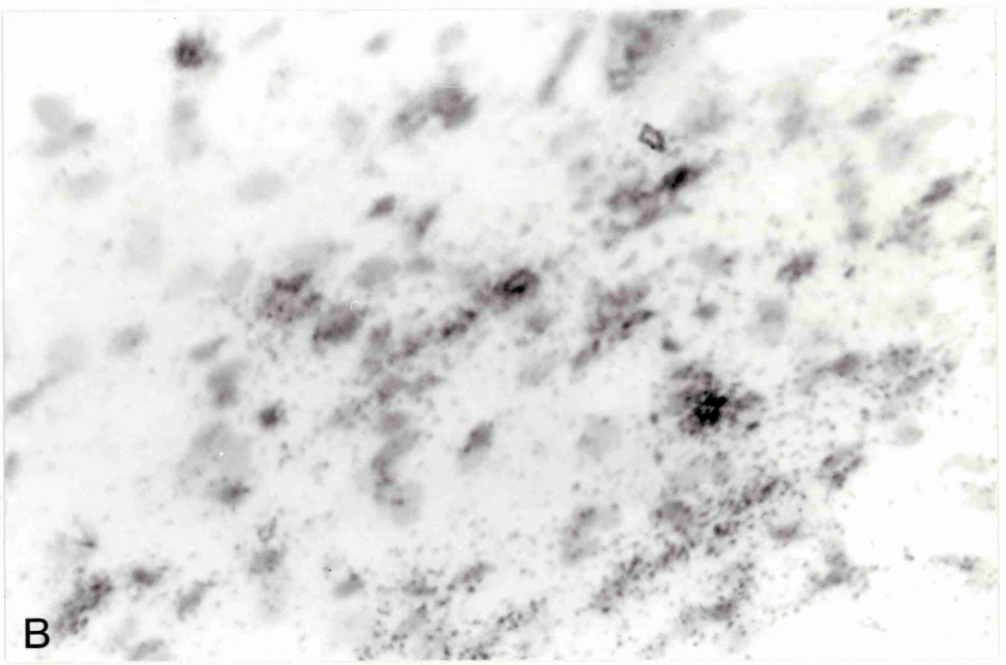


Figure 7.4: The effect on P_0 mRNA expression of adding AEF to cultured Schwann cells. Schwann cell explant cultures were established from adult sciatic nerve which had been transected and allowed to degenerate for 6 weeks *in vivo* prior to harvesting the distal stump. Explants were established on collagen plus Matrigel and cultured for 12 days prior to the addition of AEF. AEF were prepared by the sucrose gradient method, from adult rat brain stem, or neonatal rat whole brain. Some AEF were left untreated (native) whilst others were treated with sodium carbonate (please see text). 25 ug of AEF was added to the cultures for 3 successive days, the cultures were fixed 24 hours after the third AEF pulse and processed by *in situ* hybridization methodology to assess the level of expression of P_0 mRNA. After autoradiography, the nuclei were stained with haematoxylin. Other explants from the same sources were cultured simultaneously, but did not have AEF added. These served as negative controls. (A and B) Adult explant cells treated with AEF prepared from adult rat brain stem; (A) native AEF; (B) sodium carbonate treated AEF (x 170).

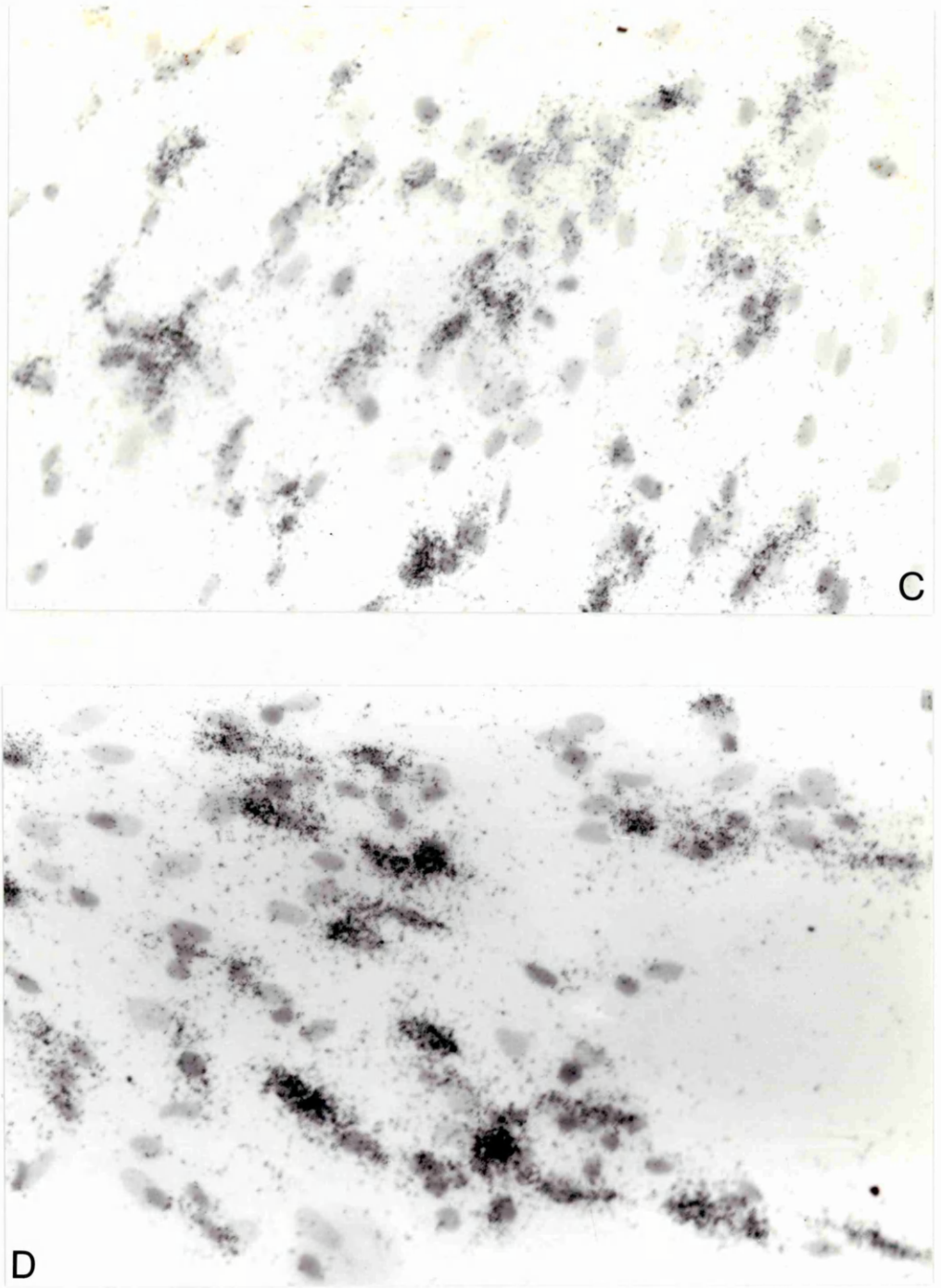


Figure 7.4 (cont.): The effect on P_0 mRNA expression of adding AEF to cultured Schwann cells. (C and D) Adult explant cells treated with AEF prepared from rat brain stem; (C) native AEF; (D) sodium carbonate treated AEF. The upregulation of P_0 mRNA appears to be most intense by the AEF prepared from neonatal rat brain and treated with sodium carbonate (x 170).

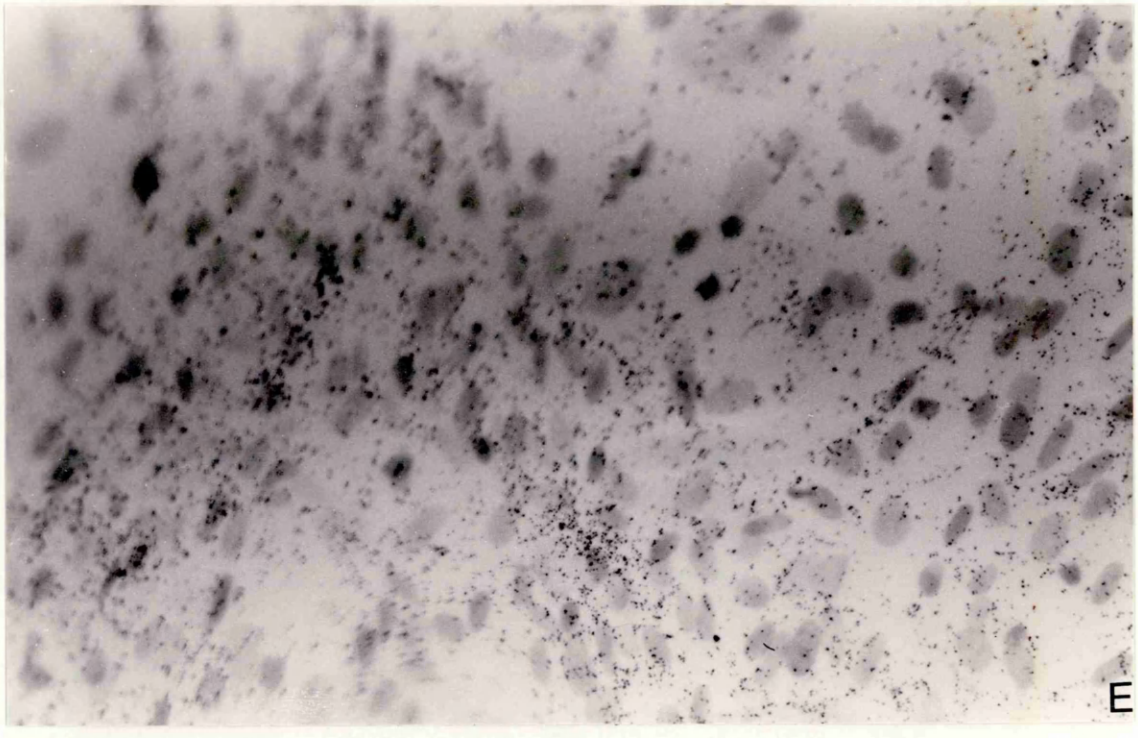


Figure 7.4 (cont.): The effect on P_0 mRNA expression of adding AEF to cultured Schwann cells. (E) Adult explant cells cultured from the same source as A to D under identical conditions, but without AEF addition, forming negative controls. No significant expression of P_0 mRNA is visible (x 170).

expressing P_0 mRNA with the more distant cells being negative. No binding of riboprobe was ever observed over fibroblast nuclei, nor was any binding of the sense probe observed over the Schwann cells or fibroblasts.

The situation of the explants cultured from adult nerve that had been cut only 4 days previously was interesting for the cells which were not exposed to the AEF were expressing P_0 mRNA to the same level as the cells to which AEF had been added. A similar situation was observed in neonatal explants.

Attempts to quantitate the effect of AEF addition on Schwann cell P_0 mRNA by dot blotting were initially unsuccessful. This was due to the low copy number of the P_0 gene and hence relatively large amounts of mRNA were required. Several attempts were made to produce dot blots from both the chronically neurotomized adult sciatic nerve and the neonatal nerve before sufficient cells were generated from which adequate amounts of RNA could be extracted (fig. 7.5B and C). Loading variations in the amount of RNA were corrected relative to the amount of S100 present. S100 is a Schwann cell specific marker within the system that was used. The corrected amount of P_0 mRNA present in the two AEF treated dots was 1.63 and 2.7 times the amount of P_0 mRNA present in the non-AEF treated dot. However, because of overlap of an area of non-specific binding into the area of the second AEF treated blot, only the first one was considered to be an accurate representation of the effect of AEF treatment on P_0 mRNA on cultured neonatal explant Schwann cells.

7.5.3.4 Discussion

The response of the adult Schwann cells derived from chronically denervated sciatic nerve to AEF prepared on a discontinuous sucrose gradient was much more promising than that observed with neonatal dissociated Schwann cells. Qualitatively, these Schwann cells did seem to respond to AEF addition by upregulating the expression of P_0 mRNA as measured by ISH. However, the response was variable across one coverslip. Where the Schwann cells were in high density, the vast majority expressed P_0 mRNA after addition of the AEF. The high density of cells occurred most commonly around the explants. Studies by Morgan *et al.*, (1991) have identified that dividing Schwann cells are not capable of expressing the myelinating phenotype. Also, these workers noted that Schwann cells exhibit a strong density-dependent inhibition of cell division. It is possible that the high density of cells near the explant had inhibited further Schwann cell division in the studies performed here, whilst those further away from the explant were still dividing. Thus only a certain proportion of cells could respond to the AEF by up-regulating the expression of P_0 mRNA. It is

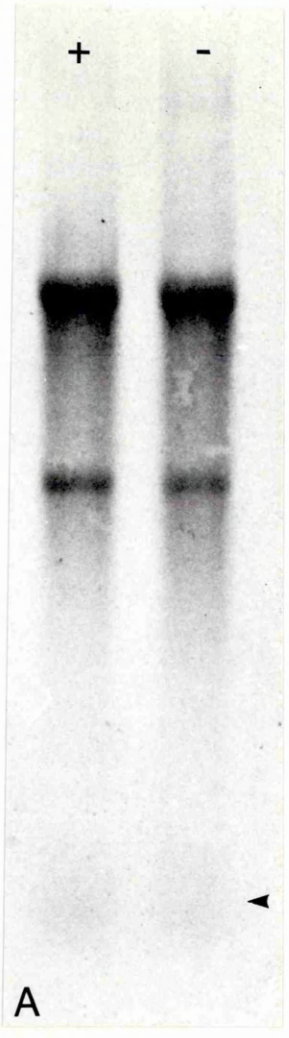


Figure 7.5 The effect on P_0 mRNA expression of adding AEF to cultured Schwann cells - northern and dot blots. Schwann cells were cultured from neonatal explants on collagen plus Matrigel for 6 days prior to adding native AEF prepared from neonatal rat brain. This was added for 3 consecutive days to some of the explants, and not to the rest which served as negative controls. At the end of the culture time, the body of the explant was excised leaving behind only the outgrown Schwann cells from which RNA was extracted. (A) The integrity of the RNA was checked by electrophoresing 4 ug of each sample through a 1.5% denaturing gel, blotting onto nitrocellulose paper and staining with 0.04% methylene blue/sodium acetate dye. The two major bands recognized are 28S (top), and 18S (middle) and a fainter band, 5S (arrow head), is seen at the bottom. (+) = AEF treated cultures; (-) = AEF negative cultures (x 2).

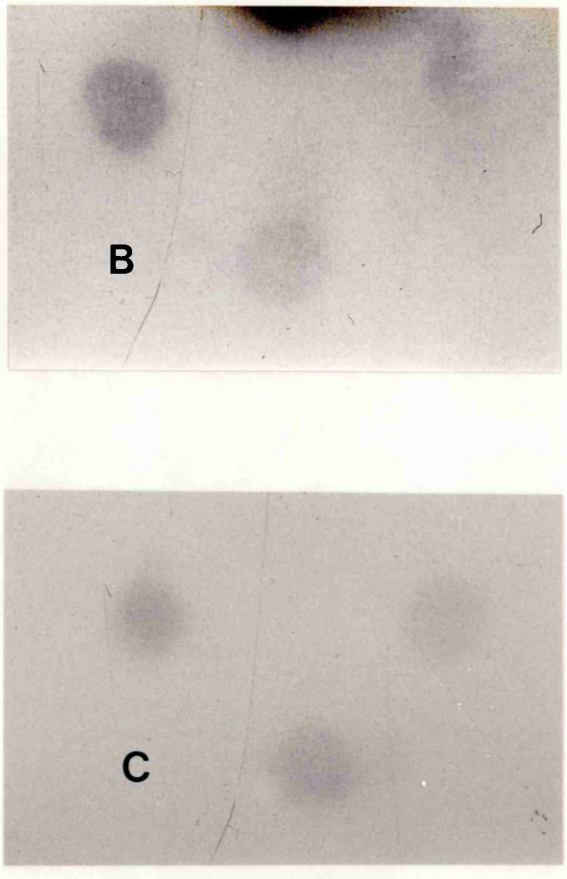


Figure 7.5 (cont.) **The effect on P_0 mRNA expression of adding AEF to cultured Schwann cells - northern and dot blots.** (B) RNA from both the positive and negative cell cultures was dotted onto nitrocellulose paper and probed with a [^{32}P] labelled P_0 cDNA probe. Top dots are from RNA extracted from cultures treated with AEF (approximately 45 ug (left) and 15 ug (right)), whereas the bottom central dot is from non-treated cultures (approximately 30 ug of RNA). An area of non-specific probe binding is seen interfering with the right dot. (C) The filter was stripped and reprobed with a [^{32}P] labelled cDNA probe for S100, a Schwann cell specific marker, to assess the amount of Schwann cell RNA loaded on each dot. This allowed for correction in loading variations of the RNA. The corrected amount of P_0 mRNA present in the top left dot (AEF treated) was 1.63 x that present in the bottom dot (non-AEF treated). This confirmed the upregulation of P_0 mRNA due to treatment with AEF (x 2.5).

also quite probable that the AEF prepared on the sucrose gradient contained the Schwann cell mitogen and that many of the Schwann cells in these cultures actually responded to the addition of the AEF by proliferating instead of upregulating the expression of P_0 mRNA. Mitotic index studies on cultures treated with AEF could have been used to ascertain this.

Ideally, the Schwann cells to which these fragments were being added should be rendered quiescent with respect to division. The presence of serum may be required for Schwann cell division (Morgan *et al.*, 1991), therefore, given more time, it would have been interesting to see if adding AEF to these cells in the absence of serum was associated with an increased number of cells expressing P_0 mRNA. The mitotic index studies described in chapter 3 were undertaken to assess the proportion of dividing Schwann cells within cultures not treated with AEF. Such dividing cells would be unable to respond to AEF addition by upregulating the expression of MSMs, and this would partially explain the variability in cells' response. A significant proportion (1/3 to 1/4) of cells were found to incorporate [^{3}H] thymidine during a 24 hour pulse (see table 3.2), which would, according to Morgan and coworkers (1991), render them incapable of responding to the putative myelinating signal.

The maximum level of expression of P_0 mRNA by these Schwann cells as detected by ISH was 5+, and was often less than this. This is compared with the expression of (8+) by freshly dissociated Schwann cells, or teased fibres. A quantitative measure of the upregulation was required. Dot blotting substantiated the trend observed by ISH and the Schwann cells did appear to respond to AEF addition by upregulating the level of P_0 mRNA expression by 160%. An unspecified increase in the expression of P_0 mRNA and protein was observed after addition of bovine AEF to cultured Schwann cells (Tennekoon *et al.*, 1985). AEF were thought to cause a two fold increase in the level of P_0 mRNA expression in transfected and primary Schwann cells (DeVries *et al.*, 1990). However, I have not come across reports of any other such experiments in the literature other than the two abstracts described above.

The rationale for using acutely neurotomized/non-neurotomized nerve for creating explants was based on early explant experiments in which AEF addition had stimulated P_0 mRNA expression but only to a low level. It was considered possible that Schwann cells chronically lacking in axonal contact may not be able to respond to AEF *in vitro*. *In vivo*, after Wallerian degeneration and prior to myelination of regenerating axons, the Schwann cells undergo division, alignment and adhesion to regenerating neurites. During this pre-myelinating period a number of complex Schwann cell-axon interactions occur, many of which we may not yet know. These

interactions may be a prerequisite to stimulation of Schwann cell MSM expression and may not occur in the chronically neurotomized explants used here. It was also considered that theoretical Schwann cell receptors for the putative myelinating signal may be down-regulated on cells chronically deprived of axonal contact, thereby decreasing their response. Thus, non-axotomized Schwann cells and Schwann cells only recently deprived of axonal contact were studied for their ability to respond to AEF. The response of the adult explants derived from sciatic nerve that had been transected only 4 days prior to culturing was curious in that both wells with and without AEF added to them were expressing P_0 mRNA. By the time this experiment was terminated, these cells had been separated from intact axon for 12 days. Down-regulation of MSM gene expression to almost zero should have occurred by this time (section 1.5.3). A similar situation was observed with some of the neonatal explants. However, I still had the impression was still that increased levels of P_0 mRNA were expressed in AEF treated cultures and that non-treated wells had fewer cells expressing the gene. This was substantiated by dot blots. Insufficient outgrowth occurred from the non-neurotomized adult explants to permit their utilization in AEF experiments.

The upregulation of P_0 mRNA on explanted Schwann cells was more obvious than on dissociated cells. This may pertain to the method of dissociation adversely affecting a Schwann cell membrane receptor for the putative myelinating signal. In another study, Schwann cells were treated with trypsin or scraped off the culture dish and added to DRG cultures. Those cells treated with trypsin lagged behind those scraped off the dish in their ability to respond to the neurones suggesting that the receptor on the Schwann cell surface for the mitogen has a trypsin sensitive component (Ratner *et al.*, 1988).

The results described in this chapter suggest that addition of AEF does increase P_0 mRNA expression in sciatic nerve explants, but not really in CST explants nor in dissociated neonatal Schwann cells. Further experiments need to be undertaken to complete these initial studies. Such experiments would include physical and chemical manipulation of the AEF, especially further studies on the effect of sodium carbonate extraction of the AEF. Unfortunately time did not permit dot blotting to assess the effect of such extraction on the potency of the AEF, however, the ISH studies suggested that such an extraction may be valuable. The optimum dose frequency and amount has not been established. One of the problems with this system was the length of time for one experiment. A minimum of 2 to 3 weeks was required for one experiment. The other major problems with this system was the number of

known permutations to the experiment, which involved AEF source and preparation, Schwann cell source and preparation and their culture conditions. Even such minor variations as the length of drying of collagen substrate prior to plating out of cells have been found to affect subsequent Schwann cell behaviour (Cassel *et al.*, 1982).

7.6 CONCLUDING REMARKS

7.6.1 Theory

The original hypothesis behind much of this thesis was that the neuron produces a myelinating signal, probably in the somata as this is where the majority of the cell's synthetic machinery is located. This signal is transported down the axon and inserted into, or is in close association with, the axolemma. From this point it interacts with the Schwann cells, probably via a receptor located on its plasmalemma. The myelin stimulating signal is then transduced to the Schwann cell nucleus, possibly via a secondary messenger system, where it initiates the upregulation of gene expression directing myelination. (see fig. 1.1.1).

To study this myelinating signal, it seemed logical to attempt preparation of nervous tissue enriched in axolemma, as, if the above hypothesis is correct, the signal should be associated, with and possibly concentrated on, the axolemma. Previous work on the Schwann cell mitogen by DeVries and coworkers, had offered a method by which an axolemmal-enriched fraction of the nervous tissue could be prepared.

7.6.2 Aim

The aim of these experiments was to attempt to create an *in vitro* model of the upregulation of myelin-specific molecule expression by cultured Schwann cells using an axolemmal-enriched fraction from the nervous system. This preparation would provide a starting point from which the nature of the myelinating signal could be investigated.

As previously stated, it was hoped that the following simple equation would be accurate:

axolemma + Schwann cells => increased P_0 mRNA.

However, I suspect that a number of other factors are also required for this equation to hold true. Given the right technique of AEF preparation, and appropriately responsive Schwann cells, then increased P_0 mRNA may result.

7.6.3 Source of AEF

In the many studies undertaken in this section, it seemed that AEF prepared from the neonatal tissue was more effective than that prepared from the adult tissue in producing a biologically active stimulus for myelination. It is also likely that protease inhibitors, such as leupeptin, are required to help maintain biological efficacy.

However, the actual efficacy of "enrichment" was an unknown factor. Given the time and the expertise, biochemical assessment of the fractions should have been undertaken. However, as the methodology on the discontinuous sucrose gradient was not greatly different to that of the instigator, Dr. DeVries, it was reasonable to assume that the fraction was appropriately enriched. One aspect of the experimental methodology used here that may have been adjusted was the concentrations of sucrose in the interface. The interface between 0.75 M and 1.2 M sucrose retains a fraction of tissue with a broader range of densities than that used by DeVries and colleagues. This would result in more contamination of the AEF by other components (myelin and subaxonal membranes) in my studies. This appeared likely considering the EM evaluation of the AEF in my studies compared with DeVries. However, the nature of the myelinating signal is not known, nor is the density of sucrose with which it would be isopycnic. By using sucrose solutions more similar in concentration (and concentrating a narrower band of material) the actual molecule(s) may have been excluded from the preparation.

It is also possible that the physical structure of the axolemma determined whether or not the Schwann cell can be stimulated to express MSMs. This aspect is considered further in chapter 9.

7.6.4 Schwann cells

The results of AEF stimulation of different Schwann cell cultures were not very consistent making interpretation of the effect of various experimental permutations difficult. However, it would seem that neonatal cells which had been enzymatically and mechanically dissociated were less responsive than sciatic explant cells. The Schwann cells from the neonatal explants were more vigorous in their growth characteristics than those from the adult animal and may respond more to the addition of AEF. However, the disadvantage to these cells was the inconsistency of basal level of P_0 mRNA expression. They took longer to down-regulate the expression of this mRNA than expected. Even by 9 to 12 days *in vitro*, it was not

unusual for these cell to still be expressing this mRNA above basal level (see fig. 4.5). However, longer term cultures of these cells were associated with lower cell viability and vigor. This became a problem when attempting to culture sufficient cells to produce enough RNA for dot blotting. Because of the low copy number of the P_0 transcript, relatively large masses of RNA (> 15 μg) were required for a single dot blot.

For ISH studies, the Schwann cells derived from the chronically neurotomized sciatic nerve, were more reliable. If obtained 4 to 6 weeks after axotomy, they expressed basal levels of the gene. However, it was difficult to achieve sufficient cell numbers for dot blotting, so although quantitative studies were attempted, they were not successful. The use of the polymerase chain reaction, to increase the number of copies of the gene, may have been of benefit in this situation.

The cells from CST explants did not show any upregulation in response to the AEF. This is despite the knowledge that non-myelin-forming Schwann cells can be stimulated to myelinate if contacted by the appropriate axon (see section 1.5.3.2). The reason for this lack of CST response in my experiments is not known. The basal level of expression by the CST cells was slightly greater than expected though.

The interaction between Schwann cells and axons is obviously very complex with the characteristics of each component being affected by the other. The behaviour of the Schwann cell, both morphologically and biochemically, is very much determined by the presence or absence of the axon, and the type of axon, with which it interacts (myelin- or non-myelin-forming). The supportive nature of the axon for the Schwann cell is only hinted at by the formation of the basement membrane, expression of various molecules and ultimately the formation of the myelin sheath. It is quite possible that many other trophic interactions occur in this partnership and in attempting to culture one without the other, not all factors required for true development of the Schwann cells, or axon, are supplied under artificial conditions. It is quite possible that the AEF prepared from the CNS are enriched in the putative myelinating signal, but complete upregulation of Schwann cell gene expression is not possible because cultured Schwann cells are deficient in some factor(s) essential for strong myelin-specific molecule expression.

7.6.5 Basement membrane

Within the first year of these studies, the ability of neonatal dissociated Schwann cells to respond to the AEF was questioned as upregulation of the P_0 mRNA in response to AEF addition was only suggested but not confirmed. It was considered

that because the cells were obtained from a juvenile animal they may not be mature enough to respond. That is, many of the cells would not have been associated with a sheath at the time of dissociation. Also, much evidence was present in the literature detailing the requirement for the presence of basement membrane for complete Schwann cell differentiation, as assessed by the formation of a myelin sheath. Finally, I was suspicious that the rigorous method of dissociation and treatment with antimitotic agents was too harsh and may affect the cells' ability to interact with axolemma, or worse, the cells' viability.

Thus I began culturing the cells from explants and using basement membrane components as substrates. Matrigel seemed to effectively coat the cells with at least laminin, and probably other components of basement membrane. Type I collagen was also added to the preparation as the literature suggested that it was also necessary for complete differentiation. The combined substrate was associated with improved cell growth and viability. It was also interesting to note that the addition of Matrigel was associated with increased expression of the P_0 mRNA in freshly dissociated cells, although these cells were negative for expression by day 10 *in vitro*. However, explant derived neonatal cells grown on this substrate had a prolonged expression of the gene which implied that the method of preparation (explant versus dissociation) had an effect of gene expression. The cells derived from an acutely neurotomized adult explant, also had prolonged expression of this gene.

The addition of basement membrane to Schwann cell cultures was associated with better growth, altered cell morphology, increased and prolonged expression of the P_0 gene in the absence of axonal contact.

The lack of normal Schwann cell function when basal lamina is absent has been interpreted as due to failure of development of "sidedness" or polarity of the Schwann cells (Bunge and Bunge, 1983). The basal lamina is thought to have a role in polarizing Schwann cells into adaxonal and abaxonal regions. The former is MAG^+ and basal lamina free and can interact with the axon, whilst the latter has basal lamina and is MAG^- and does not interact with the axons (Trapp *et al*, 1984). Schwann cells were cultured on a complex basement membrane substrate in the experiments performed here in an attempt to develop this polarization. It appeared to be successful in imbuing the cells with at least a coating of laminin (see chapter 3). However, EM studies performed by Baron-van Evercooren and colleagues (1986) have identified that Schwann cells plated <u>onto</u> basement membrane substrate, were <u>covered</u> in a dense fibrillar matrix, which was sometimes organized into a basement membrane-like structure. I have noticed that the Matrigel coating the coverslip becomes patchy with

time in culture, possibly because components may be dissolving into the media. Eldridge and coworkers (1989) noted that addition of Matrigel at a 1:50 or 1:100 dilution with the media still resulted in deposition of laminin, type IV collagen and heparan sulphate proteoglycan on the cell surface. This suggests that if components are dissolved in the media, then they will eventually settle out onto the Schwann cells. Thus, although the Schwann cells may initially be polarized, with time *in vitro*, it is probable that the polarization is lost and all surfaces of the cell become coated with basement membrane. This lack of polarization may be associated with the low level of response of the cells to AEF addition.

The "development of sidedness" was one reason that I was initially reluctant to "glue" the explants onto the coverslip by gelling Matrigel on top. However, poor attachment rates of explants during culture initiation and the requirement for masses of Schwann cells for dot blotting, inspired me to try this method of attachment, knowing that it may compound the lack of polarization. However, it was also possible that even if Matrigel was dropped on top of the explant to increase attachment, the actual thickness of coverage may be no greater than if it was just used under the explant due to redistribution of the substrate with time in the medium.

7.6.6 Schwann cell proliferation

According to Morgan and colleagues (1991) the dividing Schwann cell does not express the myelinating phenotype. Therefore if there is significant cell division during the period of AEF addition then, presumably, these cells could not respond to AEF addition by upregulation of the myelinating phenotype. The mitotic index studies described in chapter 3 were undertaken to establish whether proliferation of Schwann cells was occurring and could preclude their responding to AEF. Certainly, almost 1/3 of neonatal explant Schwann cells and 1/5 of chronically neurotomized adult Schwann cells were dividing over a 24 hour period and this too, would have decreased the number of Schwann cells that could respond to AEF. Schwann cell proliferation may also have been stimulated by addition of AEF.

Schwann cell division could have been inhibited by the addition of antimitotic agents to the cultures, which would also have had a beneficial effect on inhibiting fibroblastic proliferation. However, the rationale for not using them, and also the reasonably dramatic effect of adding them to cultures, has been discussed (section 3.5.4)

CHAPTER 8 THE EFFECT OF DEMYELINATION ON SCHWANN CELL GENE EXPRESSION

8.1 INTRODUCTION

Two forms of demyelination occur. These are primary, in which the demyelination occurs due to a primary lesion of the Schwann cell or its sheath; and secondary, in which the main lesion is directed against the axon (Blakemore, 1982a). In the latter, the myelin sheath is lost purely as a consequence of the axonal damage. Axotomy is an example of secondary demyelination. As observed in the experiments of chapter 4, nerve crush, or transection, causes axonal damage distal to the site of the injury, with loss of the myelin sheath occurring as a result. Secondary demyelination was reduced when *in vivo* breakdown of the axon was delayed as noted in the C57Bl/Ola mouse mutant studies described in chapter 5. This was associated with slowed down-regulation of the myelin-specific genes. The upregulation of axonally-suppressed molecules such as NGF receptor, GFAP and N-CAM subsequent to axon-glia damage *in vitro* was described in chapter 6. Delaying Wallerian degeneration by removing calcium from the culture medium was associated with morphological preservation of the nerves and continued suppression of the above three molecules. Therefore, in situations associated with degeneration of the axon and the myelin sheath, molecules associated with the non-myelinating phenotype may be re-expressed. Studies on an *in vitro* model of axolemmal-induced upregulation in cultured Schwannn cells are presented in chapter 7 and supported a role for the axolemma in P_0 mRNA expression.

To approach Schwann cell gene expression from another angle, the effect of primary demyelination was studied using the experimental model of tellurium-induced neuropathy. When this element is fed to actively myelinating rats (third week postnatal), it causes primary demyelination in the peripheral nerves. If fed to pregnant rats, it causes hydrocephalus of the pups; and if fed to adult rats it is associated with cerebral lipofuscinosis. It does not cause paresis/paralysis if fed to rats less than about 2 weeks of age (cited in Duckett *et al.*, 1979). The toxicity is short-lived and specific, affecting primarily the hind limb nerves with minimal changes in the brachial plexus. Demyelination starts within 2 days of initiation of treatment and remyelination begins at the start of the second week and is complete by the end of that week (Bouldin *et al.*, 1988; Duckett *et al.*, 1979). On teased fibres, the demyelination is seen to be

variable ranging from irregularities of the myelin sheath, to paranodal demyelination, to complete segmental demyelination (Duckett *et al.*, 1979).

The demyelinating phase of the toxicity is associated with decreased motor nerve conduction velocities and clinical signs of paresis and paralysis. Both these abnormalities resolve with remyelination (Duckett *et al.*, 1979). Minimal changes in the axon occur and no changes are noted in unmyelinated cells (Bouldin *et al.*, 1988).

Northern blots performed at various times during the intoxication have identified decreased intensity of expression of P_0 and MBP mRNAs and a coincident increase in NGF receptor mRNA during the period of demyelination. These levels return to near normal during remyelination (Toews *et al.*, 1991). Collaborative work between Professor Griffiths' laboratory and that of Dr. Toews and colleagues' is ongoing. The studies detailed in this chapter form part of this work and were directed towards investigating the effect of tellurium at a molecular level on teased fibres.

8.2 MATERIALS AND METHODS

Weanling Sprague-Dawley rats had tellurium added to their diet from postnatal day 20. The diet consisted of 87% ground rat chow, 1.5% tellurium and 11.5% Mazola corn oil to bind. The animals were killed by an overdose of halothane anaesthetic and cervical dislocation at 3, 5, 7 and 9 days after beginning tellurium treatment. For immunocytochemistry studies, the nerves were removed and utilized fresh; for ISH, the animals were perfused with 4% paraformaldehyde/0.1% glutaraldehyde.

8.2.1 *In situ* hybridisation

Rats exposed to 3, 5, 7 and 9 days of tellurium were used for mRNA assessment. Bundles of nerve fibres were teased onto RNase-free poly-L lysine-coated slides and processed for ISH (section 2.4.4) for P_0 or NGF receptor mRNA (section 2.4.2.2 & 2.4.2.3). After autoradiography (section 2.4.6.3), the fibres were stained with sudan black (section A.1.2) to visualize myelin.

8.2.2 Immunochemistry

Immunochemistry was performed on nerves obtained from rats at 6 and 7 days exposure to tellurium. Teased fibres were processed for immunofluorescence (section

2.3.1.1) and stained with one of the following antibodies: N-CAM, NGF receptor or GFAP. Anti-N-CAM and anti-NGF receptor were applied to unfixed nerve for 30 and 60 minutes respectively, at room temperature (RT). After secondary antibody staining, fibres were fixed in 3% paraformaldehyde for 10 minutes at RT. Fibres pre-fixed in 3% paraformaldehyde for 30 minutes, 4°C were also stained with NGF receptor antibodies. Some fibres double stained for NGF receptor MBP. After NGF receptor staining, these nerves were fixed in 3% paraformaldehyde, extracted with 0.1% Triton X-100/0.1% deoxycholate for 30 minutes, RT, and stained overnight with the MBP antibody at 4°C.

For GFAP staining, unfixed nerves were extracted with 0.1% Triton X-100 for 10 minutes, RT; fixed for 20 minutes in paraformaldehyde and incubated overnight with anti-GFAP at 4°C. All immunofluorescence preparations were mounted in 0.1% PPD in PBS/glycerol (A3.1.3).

Teased fibres were fixed for 30 minutes on the slide in 4% paraformaldehyde, and stained for NGF receptor by the PAP technique (section 2.3.1.2), counter stained with sudan black for myelin (A3.1.2) and some were also stained with propidium iodide (2.3.1.1) to demonstrate nuclei.

8.3 RESULTS

8.3.1 *In situ* hybridisation

The normal control nerves displayed strong (8 to 9+), focal intensity of silver grain clusters in the perinuclear region when probed for P_0 mRNA. They were negative for NGF receptor mRNA.

Nerves obtained from tellurium treated animals were first noted to be affected 5 days after beginning the intoxication. The extent of demyelination was variable with some fibres just displaying an irregular myelin sheath; others exhibited varying degrees of partial demyelination; and occasionally internodes were observed which were completely demyelinated. The signal for P_0 mRNA was intact on minimally affected internodes, and decreased on extensively demyelinated internodes (figs. 8.1A and B). Signal retention was partial to complete on partially demyelinated internodes. Remyelinating internodes had increased foci of P_0 mRNA expression associated with Schwann cell proliferation and the formation of short internodes as described in section 4.2.3.2 (fig 8.1C).

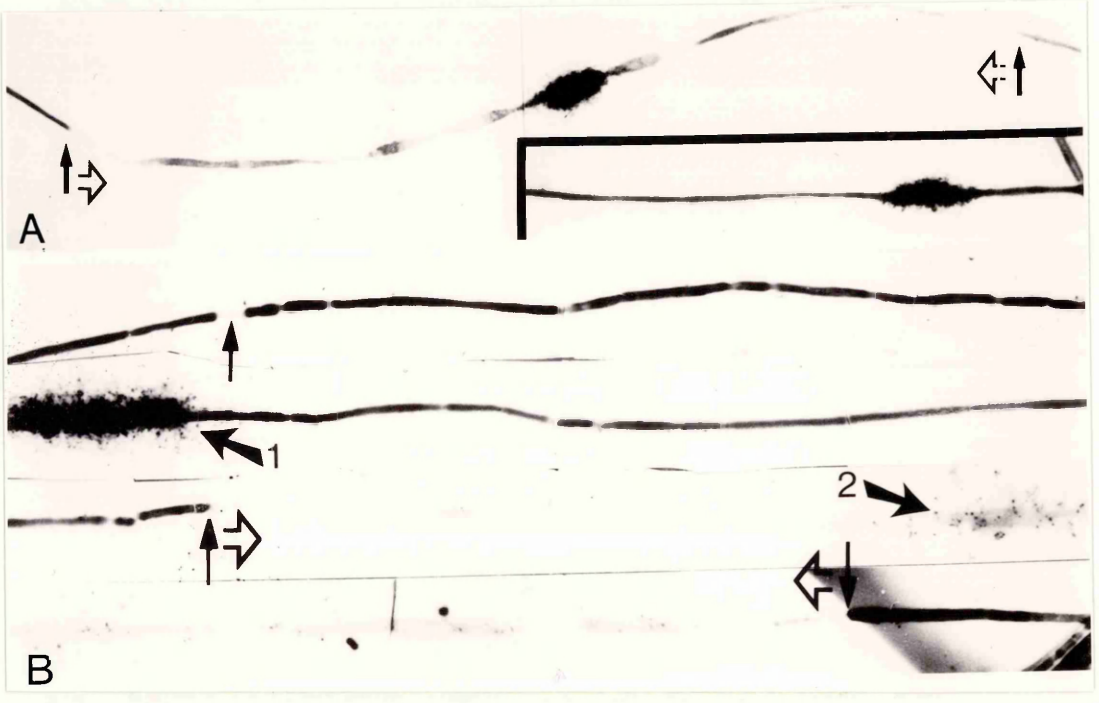


Figure 8.1: Tellurium neuropathy - effect on P_0 mRNA expression. After exposing rats to dietary tellurium (1.5%) for various periods, fibres were teased and probed for P_0 mRNA expression by *in situ* hybridization methodology. After autoradiography, they were stained with sudan black. In all fibres, nodes are indicated by vertical arrows and affected internodes lie within open arrows. (A) A single internode in the early stages of demyelination, after 5 days exposure to tellurium. Patchy loss of myelin and focal swellings of the sheath are visible. The intensity of the P_0 signal (located in the middle of the internode, in the perinuclear position) is similar to normal. The inset shows a fibre from an age-matched control rat (x 300). (B) A single fibre runs continuously from the top left to the bottom right of the fourth strip. A normal intact internode with strong P_0 expression (oblique arrow 1) can be compared to the adjacent demyelinated internode where P_0 intensity (oblique arrow 2) is virtually absent. This animal was exposed to tellurium for 7 days (x 550).

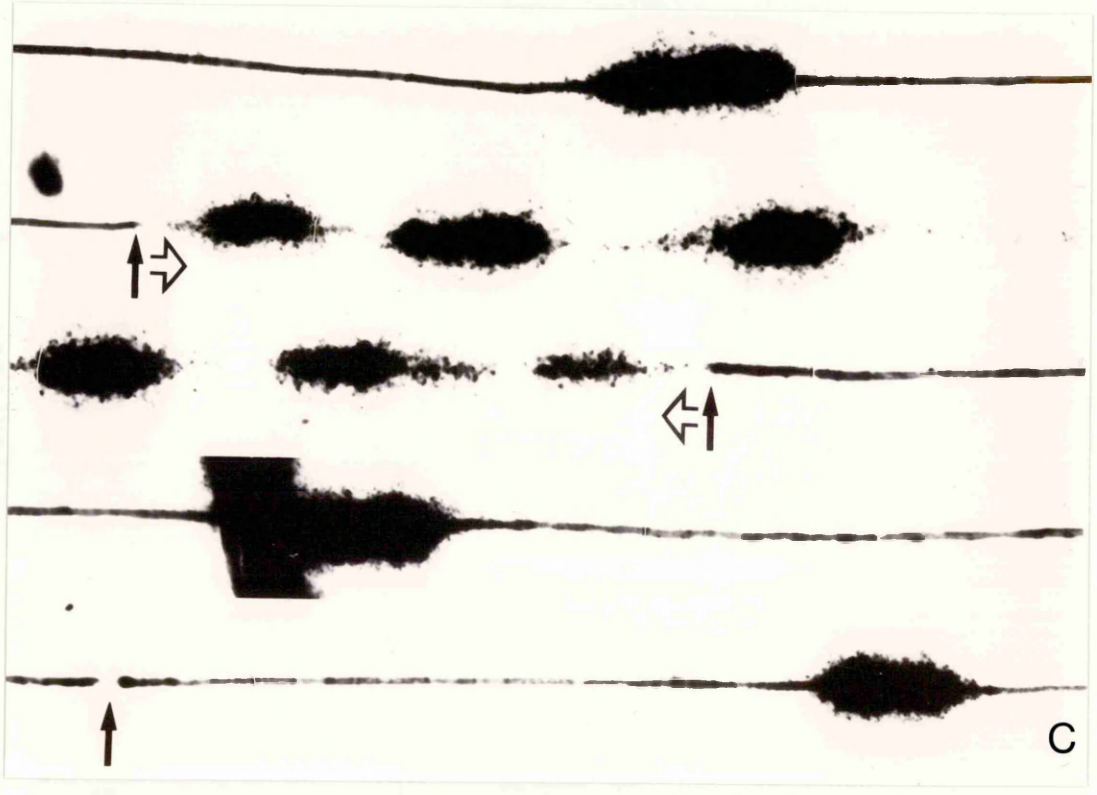


Figure 8.1 (cont.): Tellurium neuropathy - effect on P_0 mRNA expression. (C) A continuous fibre runs from the top left to the bottom right, from a rat exposed to tellurium for 9 days. The original internode, contained within the open arrows, has been demyelinated; Schwann cell proliferation has occurred with the formation of at least 6 intercalated segments which are associated with strong P_0 mRNA re-expression. This constitutes early remyelination (x 550). As different batches of P_0 riboprobe were used, and the fibres were exposed for different times, the message intensity cannot be compared between the different fibres in A, B and C.

The reverse was observed for NGF receptor mRNA with no expression over intact internodes (fig 8.2A); marked upregulation of expression on extensively demyelinated internodes; and variable degrees of upregulation on partially demyelinated internodes (fig. 8.2B and C). An increased frequency of postive foci were observed in regions of proliferated, remyelinating Schwann cells (fig. 8.2D).

8.3.2 Immunocytochemistry

Normal myelinated control nerves were positive for MBP and negative for GFAP, N-CAM and NGF receptor proteins (fig. 8.3). The staining pattern was reversed on the occasional normal unmyelinated fibres observed amongst the myelinated fibres.

Positive staining for NGF receptor was observed on both fixed and unfixed fibres where demyelination had occurred (figs. 8.4 and 8.5). In partially demyelinated internodes, there was some staining overlapping from a demyelinated area onto an intact area. Non-remyelinated areas, had no increase in Schwann cell nuclei as identified by propidium iodide staining. Within a single internode in such areas, a Schwann cell could express NGF receptor in the demyelinated zone, but not in the zone of intact myelin sheath. Adjacent intact internodes were negative for the protein. Remyelinating Schwann cells were also positive for the protein, and unmyelinated fibres were unaffected (i.e. still positive).

The pattern of GFAP staining was similar to that of NGF receptor (fig. 8.6). Whilst N-CAM upregulation also occurred in demyelinated areas, this was much more subtle than for GFAP or NGF receptor.

8.4 DISCUSSION

For a more complete discussion of the circumstances in which NGF receptor, GFAP, N-CAM and P_0 are expressed, please see the discussion in chapter 6. Briefly, the expression of N-CAM, GFAP and NGF receptor is thought to be supressed by initiation of myelination after contact with a competent axon, whereas P_0 expression is upregulated by such contact. It is thought that loss of axonal contact, both *in vivo* after axotomy and *in vitro* during cell culture, is associated with a reversion to the non-myelinating phenotype (P_0^-, NGF recptor$^+$, GFAP$^+$, N-CAM$^+$).

In tellurium neuropathy, axonal damage is rare (Bouldin *et al.*, 1988); it is the myelin sheath that is damaged. Demyelinated areas in the studies performed here were

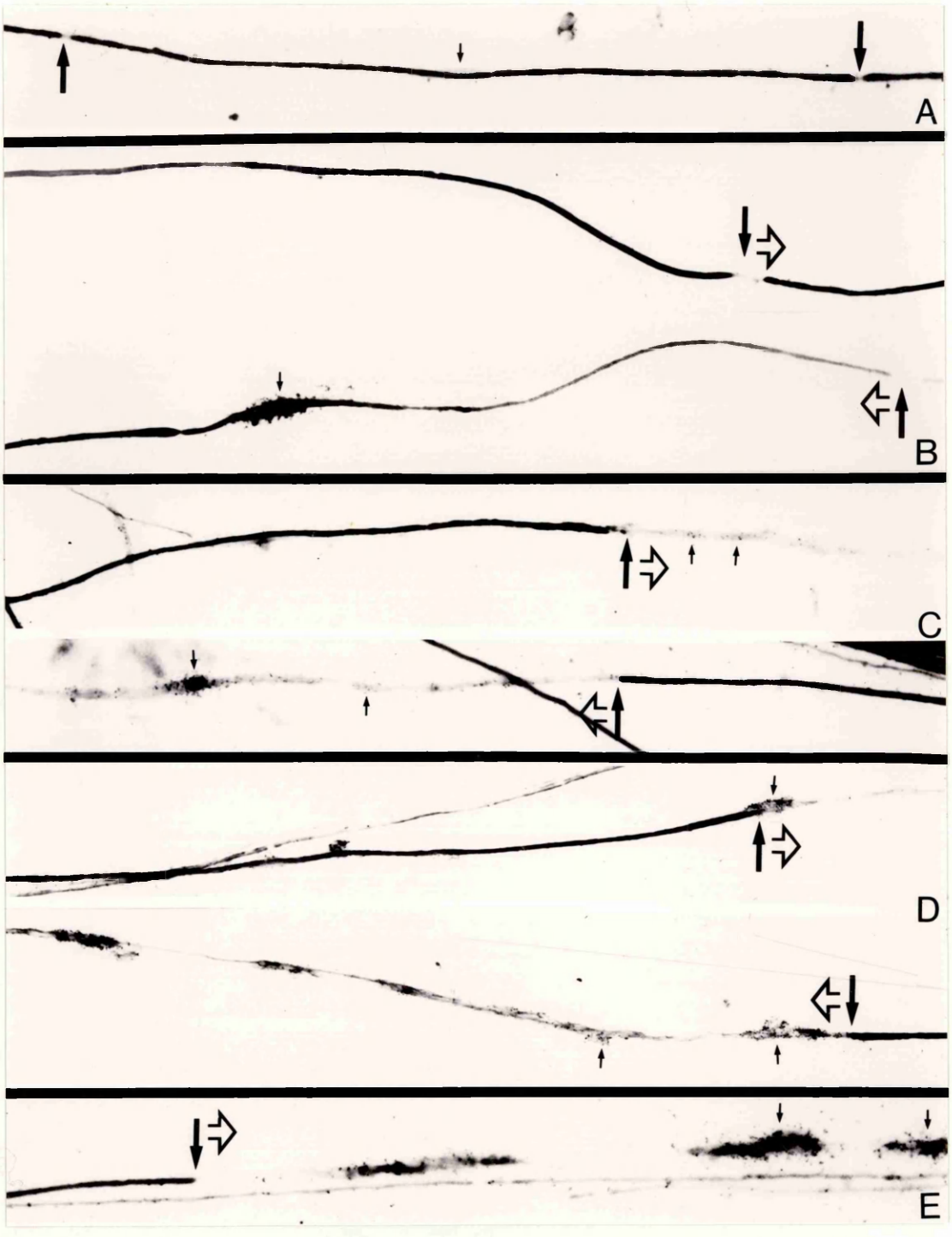


Figure 8.2: Teased fibres probed for NGF receptor mRNA. Animals were exposed to tellurium for 5 days, the fibres teased and probed for NGF receptor mRNA by *in situ* hybridization methodology, and stained with sudan black after autoradiography. In all fibres, nodes are indicated by the larger vertical arrows and affected internodes are shown by open arrows. (A) Teased fibre from a normal rat; the perinuclear area is visible (small arrow) and no signal is present (x 390). (B) Single fibre from top left to bottom right. Slight swelling of the fibre associated with myelin vacuolation just to the left of the nuclear area and small areas of paranodal myelin loss at each node are visible. The expression of mRNA for NGF receptor is increased in the nuclear area as indicated by the cluster of silver grains (small arrow) (x 320). (C) A single demyelinated internode is depicted in this teased fibe. There are several foci of silver grains (some indicated by small arrows), with a larger one in the centre. These foci correspond to nuclei as detected by propidium iodide staining. They probably represent a post-mitotic Schwann cell population in which NGF receptor gene expression is upregulated (x 250). (D) Similar to C, with a demyelinated internode and increased NGF receptor mRNA expression (small arrows). These foci are associated with nuclei, as detected by propidium iodide staining, and probably represent new Schwann cells (x 250). (E) Higher magnification of a portion of a demyelinated internode in which two foci of silver grains are visible (small arrows). Parts of the original sheath are visible as darkly stained areas (x 400).

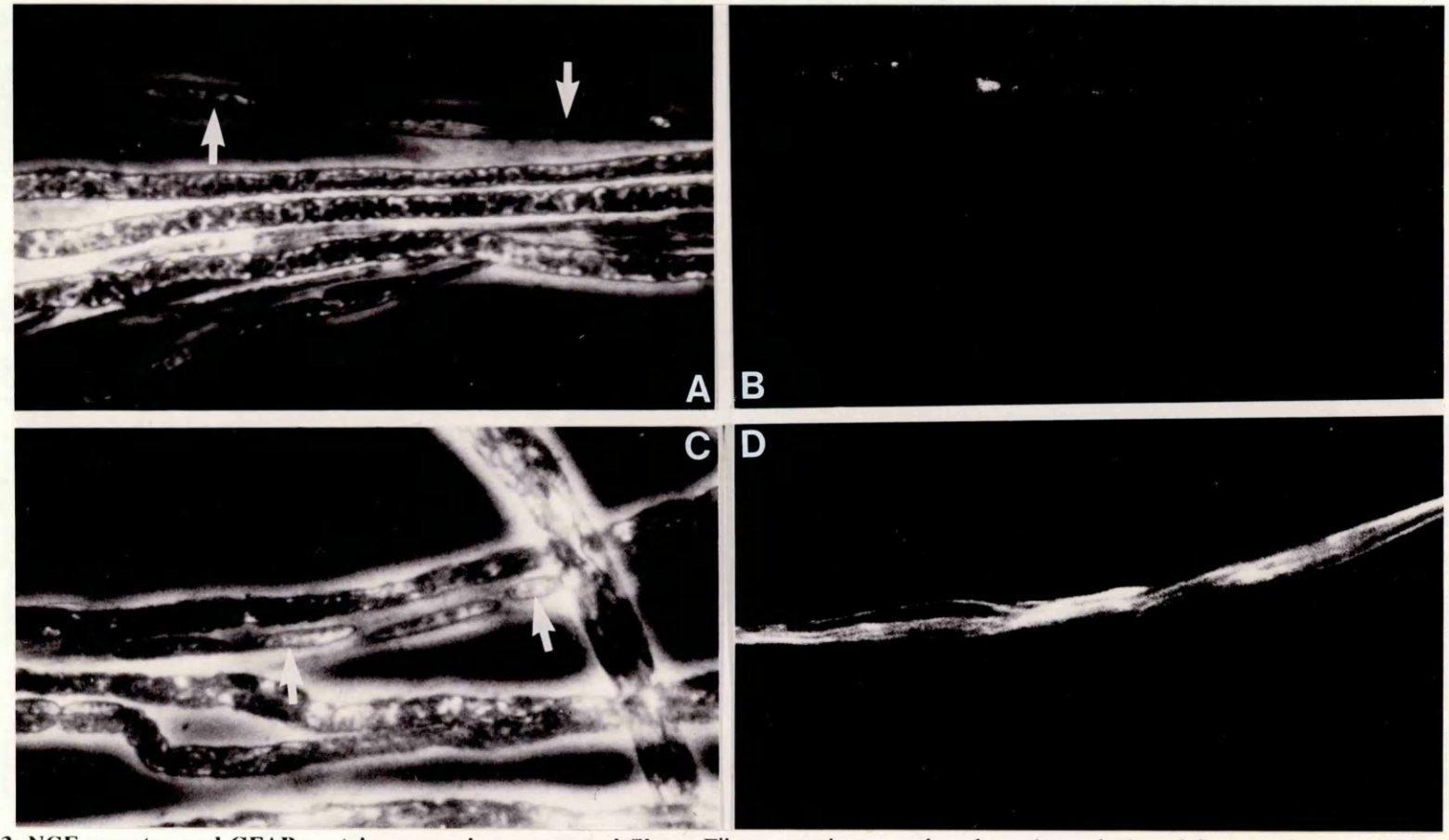


Figure 8.3: NGF receptor and GFAP protein expression on normal fibres. Fibres are shown under phase (A and C) and fluorescein (B and D) optics. The fibres are immunostained for NGF receptor (B) and GFAP (D). Only the unmyelinated fibres (arrows) are positive in either preparation; no expression of these proteins is observed on the myelinated fibres (x 670).

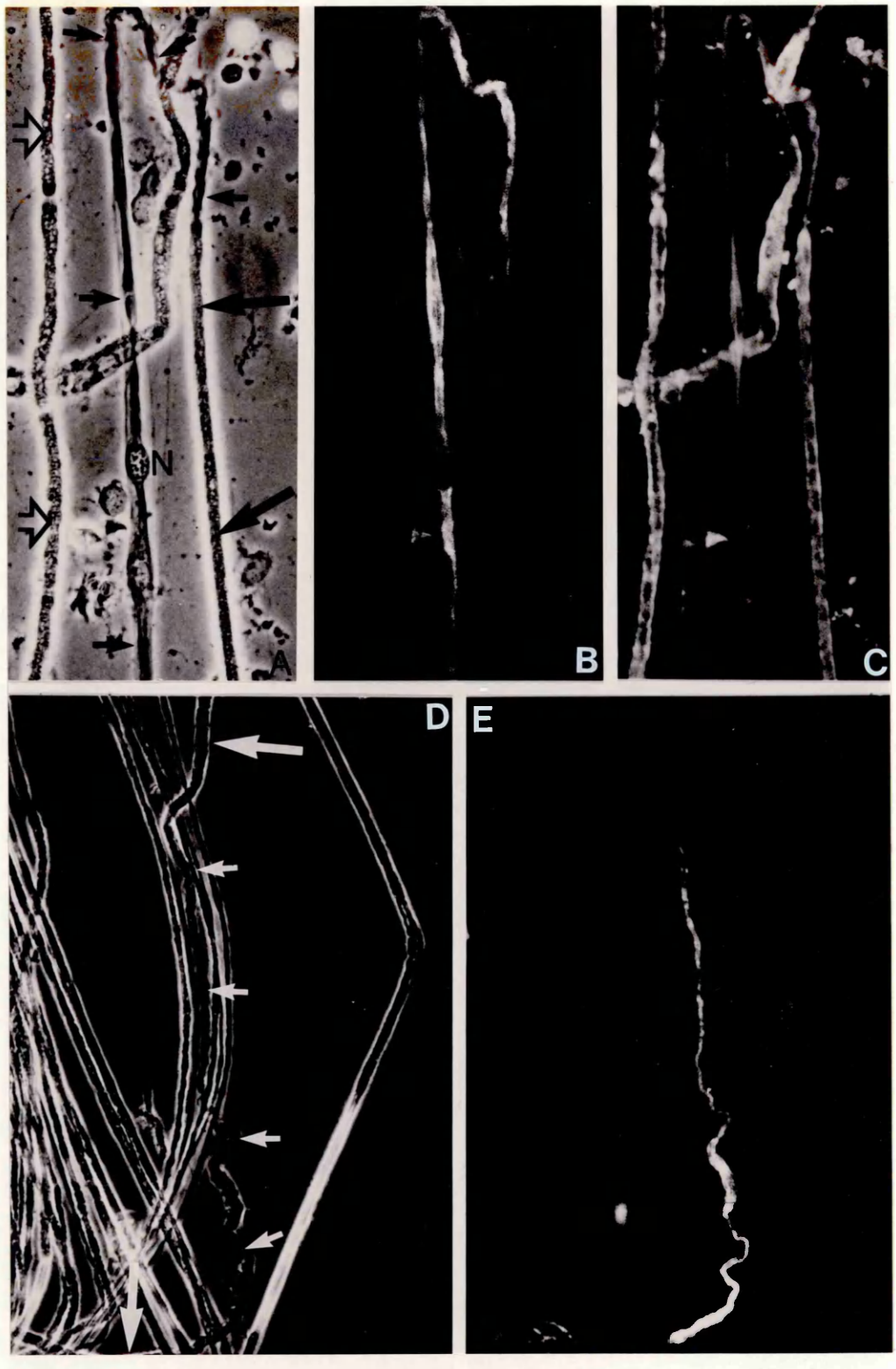


Figure 8.4: Teased fibres from a rat exposed to tellurium for 7 days. The fibres are shown under phase optics (A), immunostained for NGF receptor under fluorescein optics (B) and stained for MBP under rhodamine optics (C). A normal fibre (open arrows) is positively stained for MBP but not NGF receptor. Whereas a partially demyelinated fibre (small arrows) is positive for NGF receptor, but has reduced staining for MBP. A nucleus (N), possibly from a Schwann cell, is visible in the demyelinated area. The fibre continues (large arrows) to an intact internode in which the staining pattern returns to that of normal fibres (NGF receptor negative, MBP positive) (x 360). Other fibres from the same animal are shown under phase optics (D) and immunostained for NGF receptor (E). The majority of fibres are normal and fail to stain, but one fibre shows an area of demyelination (small arrows) which is positive for NGF receptor. Either side of this area (large arrows) the fibre has normal morphology and is negative for this protein (x 170).

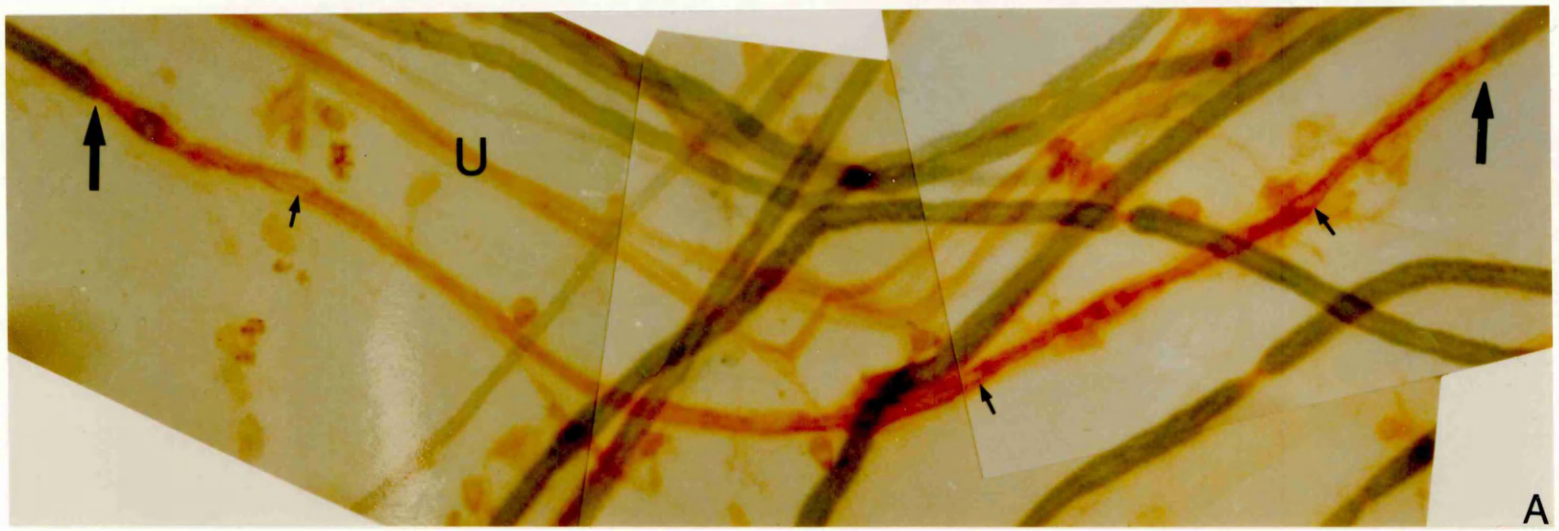


Figure 8.5: NGF receptor expression on teased fibres from a rat exposed to tellurium for 7 days. These fibres were immunostained for NGF receptor (brown colour) by the peroxidase-antiperoxidase technique. The normal myelin is stained by sudan black and appears as an olive colour. (A) A demyelinated internode is visible between the vertical arrows and is strongly positive for NGF receptor. Small arrows highlight several nuclear outlines which are visible along the length of the fibre. Unmyelinated fibres (U) are also normally positive for the protein. The small isolated brown cells are probably supernumerary Schwann cells expressing NGF receptor, which have become detached from their association with the fibres during the teasing process (x 270).
Original in colour.

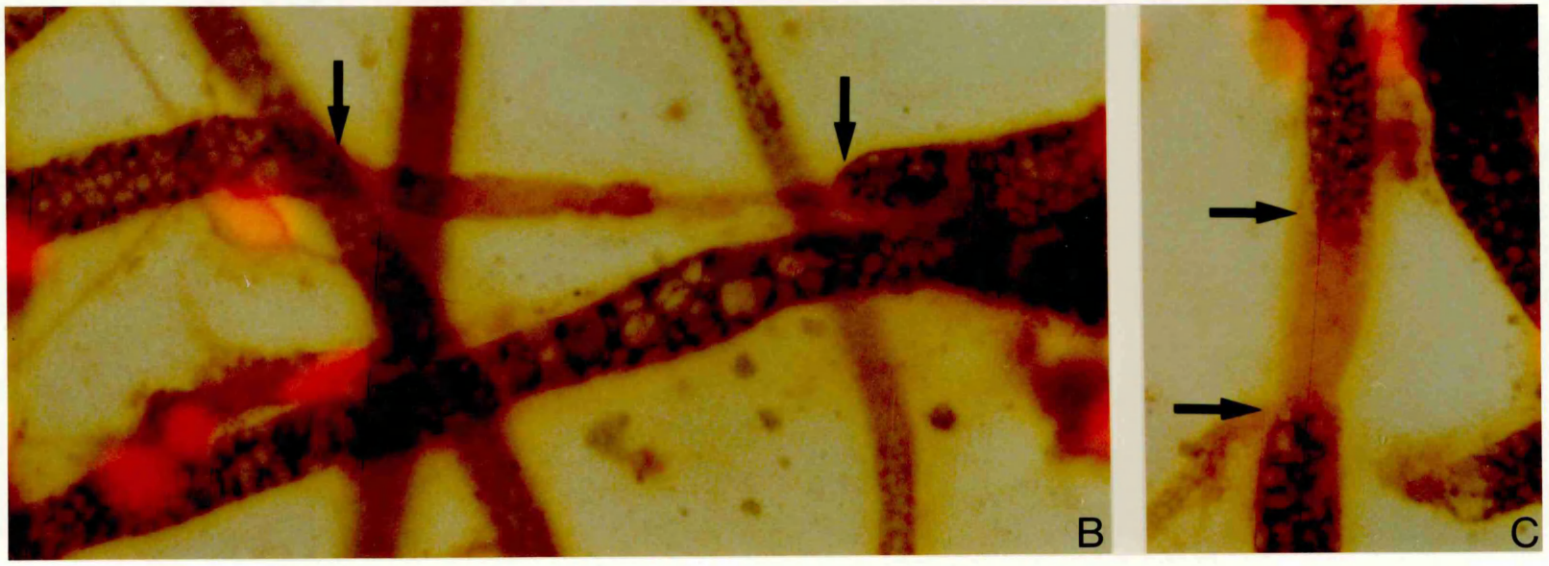


Figure 8.5 (cont.): NGF receptor expression on teased fibres from a rat exposed to tellurium for 7 days. (B and C) These fibres are from the same rat, as (A) stained for NGF receptor (brown), myelin with sudan black (dark brown), and propidium iodide (red) for the nuclei. The photographs were double exposed; firstly in brightfield and then under rhodamine optics to fluoresce the nuclei. This resulted in the slight colour change compared with (A). Partial demyelination of an internode (between arrows) is seen in both pictures, with corresponding expression of NGF receptor. The red nuclei are not associated with these areas, indicating that they are derived from the original internode and are not newly-formed, intercalated internodes (x 800). Original in colour.

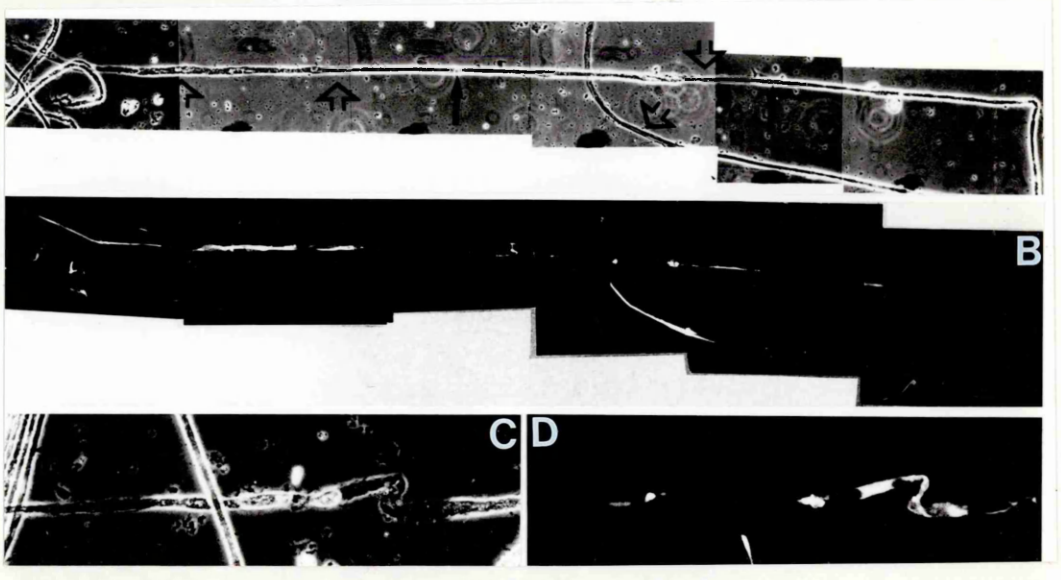


Figure 8.6: GFAP expression on fibres from a rat treated for 7 days with tellurium. (A) Fibres are shown under phase optics; (B) fibres are shown under fluoresence. The nodes are indicated by solid vertical arrows and demyelinated areas by open arows. The fibre continues out of the field at the top right and then curves down and around. This area to the upper right is normal, and GFAP expression is only visible associated with the demyelinated internodes (x 120). (C and D) Fibres from the same animal showing a demyelinated fibre under phase optics (C) and under fluoresence (D) expressing GFAP. A small bundle of normal fibres (arrow) are unstained (x 200).

associated with upregulation of proteins associated with non-myelin-forming Schwann cells. The demyelination could be focal - partially involving one internode, or segmental - involving the whole internode. However, the "non-myelin-forming" phenotype could co-exist with the myelinating phenotype (P_0 mRNA and MBP protein positive) within the same partially demyelinated internode. The absence of increased nuclear numbers (as determined by propidium iodide staining) suggested that this variable expression was occurring within the one internode. This implies very local, and focal, control on the phenotype expressed within the Schwann cell.

The effect of tellurium on the axon-glia interface needs to be ascertained in these partially demyelinated internodes. If this area of contact appears undamaged, then this would imply that the expression of GFAP and NGF receptor is determined, in these circumstances, by the presence of a morphologically healthy myelin sheath rather than by axonal contact. If axonal contact is impaired it implies a very precise focal inter-relationship between the axon and the adjacent part of the Schwann cell. The level of immunostaining for N-CAM was low and drawing conclusions from it was difficult.

P_0 mRNA expression was lost on internodes that were markedly damaged, but its expression was variable on partially demyelinated internodes. In the situations in which it was down-regulated, dramatic changes in both the axon-glia interface and the myelin sheath would have occurred. Further studies need to be performed in order to evaluate the significance of each of these changes on the expression of this mRNA.

As remyelination readily occurs, and the axon is rarely affected by the intoxication, then presumably, the axolemmal signal for myelination remains intact throughout the period of demyelination.

Morgan and coworkers (1991) have identified that NGF receptor, GFAP and N-CAM are expressed on P_0-negative dividing cells. However, the results presented here of partially demyelinated internodes in non-proliferated areas, suggest that cell division is not a necessary prerequisite for expression of this phenotype. As observed in the studies of Morgan and colleagues, the NGF receptor, GFAP and N-CAM positive, P_0 negative phenotype was observed on segmentally demyelinated fibres in the studies performed here. No change in molecule expression was observed on unaffected nodes.

8.5 CONCLUDING REMARKS

Tellurium neuropathy is an interesting example of primary demyelination. Within a partially demyelinated internode, Schwann cell phenotypic expression can be focally changed. If the axon-glial interface and interaction is undamaged by tellurium in these areas, then these data imply that the regulation of expression of some Schwann cell molecules is local within the Schwann cell and not necessarily determined by the axon.

In previous chapters, delayed Wallerian degeneration both *in vivo* and i*n vitro*, have been described. Only a minimal increase in the NGF receptor mRNA levels in the distal stump of the transected C57Bl/Ola mouse sciatic nerve was described (Brown *et al.*, 1991; Perry *et al.*, 1990a). Calcium chelation was associated with maintained suppression of NGF receptor, GFAP and N-CAM expression *in vitro* (see chapter 6). In both these circumstances the axon-glial contact was preserved, albeit better in the mouse mutant, than in the calcium-chelated studies. However, also in both these situations, the myelin sheath was preserved. Further investigations into the derangement of the axon-glia interface in this neuropathy need to be performed. Pending these, the above findings from the tellurium neuropathy may suggest that, in fact, it may not be the maintenance of the axon-glia contact that determines the expression of GFAP and NGF receptor by the Schwann cell, but rather, it is the viability of the myelin sheath. Alternatively if the axon-glia interface is focally deranged in the area of partial demyelination, but preserved along the rest of the internode then this still implies a very focal control over Schwann cell molecule expression; this control would still be axonally regulated.

In chapter 6, although calcium chelation of the medium was associated with preservation of fibre morphology, the maintenance of the axon-glia interface was not as good as in the C57Bl/Ola mouse mutant. P_0 mRNA expression was only maintained in the latter. It is possible that the poorer preservation of the axon-glia interface in the calcium-chelated studies of chapter 6, caused the loss of P_0 mRNA expression in these fibres. However, the myelin sheath was reasonably well maintained on many fibres in the calcium-chelated medium. If the control of NGF receptor and GFAP expression is associated with an intact myelin sheath, then this may account for maintained suppression of these molecules despite the down-regulation of P_0.

CHAPTER 9: GENERAL DISCUSSION AND CONCLUSIONS

9.1 INTRODUCTION

There is quite a body of literature to suggest that the expression of myelin-specific molecules by Schwann cells is not an all-or-none phenomenon. That is, in the absence of axonal contact, Schwann cell expression of the P_0 gene (for example) is not lost, but is just down-regulated. Studies suggesting this have been performed on cultured cells (Halligan and Chen, 1985; Rutkowski *et al.*, 1990) and endoneurial slices *in vitro* (Poduslo *et al.*, 1985a; Poduslo and Windebank, 1985b) and in transected nerve *in vivo* (LeBlanc *et al.*, 1987). Initially, it was thought that only endoneurial slices from adult rat sciatic nerve continued P_0 protein production, whilst neonatal rat Schwann cells dedifferentiated by 4 days *in vitro* and were no longer capable of P_0 gene expression (Poduslo *et al.*, 1985b). Continued production of this protein by neonatal explants was identified in subsequent studies (Brunden *et al.*, 1990b). After loss of axonal contact, both P_0 mRNA and P_0 protein levels decline, but are not lost. The mRNA for this glycoprotein, and MBP, were markedly reduced in the distal stump of the transected rat sciatic nerve, but were still being expressed at 31% and 40%, respectively, of the control nerve (LeBlanc *et al.*, 1987). However, whilst the mRNA is still being transcribed in both adult and neonatal tissue, most of the protein is rerouted to the lysosomes for degradation (Brunden and Poduslo, 1987; Brunden *et al.*, 1990b). The post-translational processing of the protein is also changed (Poduslo *et al.*, 1985a). It has been suggested that the axonal control over P_0 expression by murine Schwann cells is "relaxed" as these cells continue to incorporate [^{14}C] into P_0 protein at 2 weeks *in vitro*. However, this may just be a similar situation to that described above in rats, in that the expression is just down-regulated, but not lost.

The production of other "axonally-dependent" molecules, such as sulphatide and GalC, in the absence of axonal contact has also been recorded (Fryxell, 1980; Rutkowski *et al.*, 1990).

Considering the above literature, it seems likely that the Schwann cell is somewhat autonomous with respect to myelin-specific molecule expression such that in the absence of axonal contact, the molecules are expressed, but at a basal level. After axonal contact is made, this basal level is stimulated by some factor(s), and gene expression is amplified. Around the time of axonal contact, a number of

potentially significant events occur which may have a role in amplifying MSM expression. These events may include: Schwann cell proliferation (Pellegrino and Spencer, 1985; Peters, 1976; Salzer *et al.*, 1980b; Wood and Bunge, 1975), acquisition of a basal lamina (Billings-Gagliardi *et al.*, 1974; Carey *et al.*, 1983), three-dimensional structural changes in the shape of the Schwann cell and the contacts that it makes including achieving a 1:1 relationship with an axon, membrane expansion and ensheathment (Bunge *et al.*, 1989; Peters and Muir, 1959; Peters, 1976; Ranscht *et al.*, 1987). The Schwann cell undergoes very specific structural alterations upon contacting the axon. Within 5 hours of contacting a growing neurite, the Schwann cell realigns itself on the ingrowing neurites and between 5 and 13 hours:

> "*significant alteration in Schwann cell orientation was observed*" (Ratner *et al.*, 1987).

Much has been published suggesting that axonal contact is the prime determinant in dictating MSM expression (sections 1.3.2.3 and 1.5.3). It is considered most likely that some molecule(s) on the axolemmal surface interacts with the Schwann cell membrane and directs MSM upregulation/amplification. Loss of axonal contact results in a reversion of the Schwann cell phenotype to the non-myelinating one. As stated by Heath *et al.*, 1991:

> *"However, because it has been difficult to separate the effects of disrupted axonal contact from the cascade of cellular events which accompany Wallerian degeneration, this hypothesis has never been tested directly."* (Heath *et al.*, 1991).

The role of the physical structure of the Schwann cell in myelin-specific molecule expression.

The results of both chapters 6 and 8 raise the possibility that some of the molecules expressed by the Schwann cell may be regulated by the physical state of the Schwann cell and its sheath, rather than by the state of the axon-glial interaction. In chapter 6 (Delayed Wallerian degeneration *in vitro*), despite down-regulation of P_0 mRNA expression, the other indicators of the non-myelin forming phenotype, (NGF receptor, GFAP and N-CAM) were not re-expressed in situations where the tissue morphology was preserved. Alternatively, in the tellurium neuropathy described in the last chapter, these molecules were re-expressed in fibres still expressing P_0 mRNA, in situations in which the structure of the Schwann cell (myelin sheath) was altered.

Delayed Wallerian degeneration *in vivo*, was observed in the mouse mutant C57Bl/Ola described in chapter 5, and also in the transected frog nerves (Rubinstein and Shrager, 1990; Waller, 1850; Waller, 1852; Wang, 1985). The structural integrity of these nerves was maintained - both with respect to the axon-glia interface and the myelin sheath, and associated with this, the function and expression of MSMs were preserved (Rubenstein and Shrager, 1990, Thomson *et al.*, 1991; Waller 1850; Waller, 1852; Wang, 1985).

The putative myelinating signal has been postulated to be associated with the axolemma (see section 1.5.3.2) (Griffiths *et al.*, 1991) therefore it would seem logical that addition of a fraction of nervous tissue enriched in axolemma, may stimulate the expression of the myelinating phenotype by the Schwann cells. This formed the basis of the work described in chapter 7, and also by DeVries *et al.*, 1990. Addition of AEF to cultured Schwann cells does seem to be associated with upregulation of P_0 mRNA, but the results were somewhat variable. This could be due to the AEF not being chemically potent, or in the right physical form, or some other variability which occurs at the level of the Schwann cells and affects their ability to respond.

The ensheathing, and potentially myelinating, Schwann cell has a very specific structure and set of associations. It forms a cylinder enveloping the axon, and develops a very precise physical relationship with it. For myelinating Schwann cells the axon-glia interface is very regulated at 12 to 14 nm width (Trapp *et al.*, 1984b) and this periaxonal space is reduced at the paranode to 3 nm and at the node to 5 nm (Peters, 1976; Rosenbluth, 1983; Thomas and Ochoa, 1984). On the abaxonal surface of the Schwann cell, the basement membrane is formed and without this, myelination does not occur (see section 1.3.3). The basal lamina does not appear to be required for P_0 mRNA expression by Schwann cells as when they are cocultured with spinal ganglia in non-myelinating medium, this gene is still expressed (Brunden and Brown, 1990c; Morrison *et al.*, 1991). However, the expression of P_0 protein was very reduced (Brunden and Brown, 1990c) or absent (Morrison *et al.*, 1991). As myelination does not occur in the absence of basement membrane, myelin protein gene expression and myelin sheath synthesis may be separate events (Brunden and Brown, 1990c). It is possible that the presence of basement membrane may be essential for maturation of the Schwann cell by polarizing them and this may be essential for the complex mechanics of myelin sheath formation (Bunge *et al.*, 1986). Bunge and colleagues referred back to previous studies (Bunge and Bunge, 1978a) in which "guy-roping" was noted which was associated with lack of ensheathment and myelination in cocultures of Schwann cells and spinal ganglia. Polymerization of

collagen onto these areas corrected the lack of Schwann cell function. They suggested that this correction was not so much due to the chemical effect of adding the collagen, as it was due to the physical presence of a second surface which helped polarize the Schwann cells (Bunge *et al.*, 1986). It has also been suggested, that although MAG is upregulated when Schwann cells contact axons in defined medium, MAG may not be able to function until a basal lamina is formed. The polarization that this induces may allow a critical number of MAG molecules to interact with the axonal surface and ensheathment to ensue (Owens and Bunge, 1989).

The possibility of a molecule that spans from the extracellular space, across the Schwann cell plasmalemma and interacts with the cytosol has been raised. Such a molecule could communicate with the extracellular environment and matrix, and the intracellular one. The appearance of the basement membrane coincides with the cessation of proliferation, radical alterations in the Schwann cell morphology and up-regulation of the expression of myelin-specific molecules. A trans-membrane molecule could transduce the effect of extracellular activities, such as the appearance of the basement membrane, on Schwann cell metabolism (Carey and Todd, 1986b).

One of the "myelinating signals" that was first considered was based on structure rather than chemical stimulation. It was suggested that the axonal calibre was a possible determinant of whether or not the Schwann cell would myelinate (section 1.5.3.1). For many years, an axonal diameter of 1 to 2 μm was considered the determining size for myelination (Duncan, 1934; Matthews, 1968; Voydovic, 1989). Above this diameter, the majority of fibres are myelinated, and below this value, most fibres are unmyelinated. However, the dilemma concerning the fibres that are larger than this and remain unmyelinated, or vice versa, remains unsolved. For example, Hahn and colleagues (1987) noted that myelination in the developing sixth cranial nerve of the rat began on axons with diameters of 0.6 to 1.0 μm. Voydovic's elegant experiments consisting of altering the ratio of target size to the number of innervating axons yielded some very interesting information. Increasing the axonal diameter resulted in a 12 fold increase in the number of myelinated fibres within the nerve. The critical axonal diameter for myelination was found to be 1.6 μm. These data imply that there is no significant qualititative difference between myelinated and unmyelinated fibres (Voydovic, 1989). However, subsequently it has been suggested that if the axonal diameter exceeds a certain size, than this:

> *"could itself trigger the expression of myelination signalling molecules on the cell membrane"* (Mirsky and Jessen, 1990b).

Two other situations involving Schwann cell function with respect to ensheathment and myelination have been described which may help clarify, or confuse (!), the issue. The first involves sympathetic neurons grown with Schwann cells under myelinating conditions on collagen substratum. The glial cells failed to ensheath and myelinate the neurons, and a continuous basal lamina was lacking, yet these Schwann cells could myelinate normally when transplanted to spinal ganglia cultures. It was concluded that these Schwann cells, when associated with superior cervical ganglia neurons, were less "driven" by the neurons and depended more on environmental factors for differentiation (Roufa *et al.*, 1985).

This suggests that the final phenotype expressed by Schwann cells is determined by both neuronal or environmental influences. The second situation involves the superior cervical ganglion of normal rodents in which double myelination has been demonstrated. This phenomenon consists of a myelinating Schwann cell being displaced from the axon due to the invasion of a second Schwann cell. Axons enclosed by three or more myelinating Schwann cells were also observed. The axon becomes invested by the invading (inner) Schwann cell which, itself, is covered by the myelin sheath of the first (outer) Schwann cell (Kidd and Heath, 1988a; Kidd and Heath, 1988b). Axotomy resulted in the loss of 75% of the outer sheaths along with the inner sheath, but this was thought to occur because these outer cells still had some contact with the axon. However, the rest of the outer sheaths were extant and thus myelin ensheathed bands of Bungner were formed. The authors thought that in this situation, the maintenance of myelin did not depend on axonal contact, nor upon diffusable axonal factors (Heath *et al.*, 1991). The authors also proposed that myelin sheath break-down occurred during Wallerian degeneration because they are in physical contact with a degenerating axon (Heath *et al.*, 1991).

Based on all the above information, I asked the question as to whether MSM expression is dependent upon the integrity of the myelin sheath and basement membrane, as much as axon-glia contact? If only axonal breakdown occurred, and the other components of Wallerian degeneration were prevented, would Schwann cell molecule expression alter? This was considered in one way in chapter 6; i.e. if Schwann cell proliferation was prevented, would this be associated with preservation of MSM expression? It did not seem to be, but retrospectively, no measures were simultaneously taken to preserve the myelin sheath or basement membrane. In the delayed *in vitro* Wallerian degeneration experiments of chapter 6, P_0 mRNA was down-regulated despite good morphological preservation of the fibres. In assessing the electron micrographs of explants cultured in various media compared with transected

C57Bl/Ola nerve from chapter 5, it was noted that the basement membrane in the cultured explants was markedly deranged even in fibres cultured in SCGM + EGTA. Was this basement membrane damage associated with the myelin sheath disruption and potentially MSM down-regulation? The following experiment was instigated to investigate this question. For this, the experiments of chapter 6 were extended with the aim of increasing the basement membrane present on the explants to assess the role that loss of this structure has in MSM down-regulation.

9.2 MATERIALS AND METHOD

Explants of sciatic nerve were created from adult Sprague-Dawley rats as described in section 6.2. Cervico-sympathetic trunk was also harvested. The sciatic nerve fragments were cultured for 5 days at 37°C., 5% CO_2, in calcium-chelated or calcium-containing Schwann cell growth medium as in chapter 6. (Studies using HBSS or DMEM were not performed). To overcome the loss of basement membrane during culture, half the explants were placed on a collagen/Matrigel substrate coated coverslip (section 3.2.2) and 6 μl of Matrigel polymerized on top of them prior to feeding. Half-way through the experiment, the medium was removed, and retained, and a further 6 μl of matrigel gelled onto these explants. The same medium was replaced on the explants.

Both CST and sciatic fibres were teased onto RNase-free, poly-L lysine-coated slides (A4.1.4) and assessed for basement membrane using antilaminin immunofluorescence (2.3.1.1) and the sciatic fibres were evaluated for P_0 mRNA expression by ISH (section 2.4.4). Antilaminin staining was performed on unfixed teased fibres from the CST and the sciatic nerve at day 0 (positive control) and from the sciatic nerve explants on day 5 *in vitro*. After air-drying the teased fibres onto the slides for 30 minutes, they were equilibrated with PBS for 2 minutes, blotted dry and incubated with antilaminin (1:300 diluted with PBS) for 40 minutes, washed 3 times with PBS and incubated with the secondary (GAR-FITC) diluted 1:130 with PBS for 30 minutes. The fibres were fixed in 4.0% paraformaldehyde and mounted in 0.1% PPD/90% glycerol in PBS.

9.3 RESULTS

9.3.1 Anti-laminin staining

The normal, day 0 sciatic nerve fibres had a well-defined coating of basement membrane as determined by the presence of laminin. This stained reasonably brightly (5+) on all fibres, was slightly thicker at the nodes, and was obvious even at 20 x objective magnification. The basement membrane coating CST fibres was very fine and only visible by viewing under oil (50 to 100 x magnification).

After 5 days culturing, anti-laminin staining identified patchiness of the basement membrane coating within explants cultured in either calcium-free or calcium-containing medium, without added basement membrane components. The loss of basement membrane appeared slightly worse in the calcium-containing medium. The residual basement membrane stained moderately brightly (5+). In contrast, most fibres of the explants which had been cultured in the presence of added basement membrane stained very brightly (8 to 9+), although there were a few fibres in the calcium-containing medium that were less bright (6 to 7+). Addition of Matrigel had successfully coated these fibres in a thick coating of laminin-containing basement membrane.

9.3.2 P_0 mRNA expression

Whilst the addition of Matrigel to the explants was successful in imbuing them with a basement membrane coating, it did not affect the expression of P_0 mRNA as assessed by ISH. The day 0 positive controls were expressing the mRNA at a level of 6 to 7+, but the *in vitro* explants were down-regulated with only rare fibres expressing the mRNA at 2 to 3+.

9.4 DISCUSSION

The study described in this chapter was undertaken to further investigate the role played by the physical structure of basement membrane in supporting P_0 mRNA expression during Wallerian degeneration. Maintaining an artificial coating of Schwann cell basement membrane on the abaxonal aspect of the myelin sheath, was not sufficient to maintain the upregulated state of P_0 mRNA expression. However, the exogenous basement membrane was not derived from neural tissue and may thus not

be adequate for the requirement of isolated Schwann cells to maintain MSM expression. Some other factor(s) may be necessary for isolated Schwann cell, which the culture conditions alone do not provide, yet is provided when neurons are cocultured with the Schwann cells.

As previously stated, the question of axonal calibre in determining whether or not the fibre will be myelinated, has not been settled. It is known that the internodal myelin volume and the surface area of the axon beneath the myelin sheath (internodal axon surface area) are directly related. This holds for normal internodes and also remyelinated ones in which there appears to be an inappropriately thin sheath for the size of the axon. Similarly, the unmyelinated fibre would be predicted to be unmyelinated as the surface area beneath the ensheathing Schwann cell would be too small. Thus myelin volume, and hence sheath thickness, may be regulated by the area of the axolemma/Schwann cell contact beneath the sheath (Smith *et al.*, 1982).

The level of expression of the P_0 gene is related to fibre size. It has been speculated that if the myelinating signal is axolemmal associated then the abundance of the signal is related to the axolemmal surface area of the internode. This may account for the relationship between fibre size and the level of P_0 gene expression (Griffiths *et al.*, 1991).

Perhaps there is not one type of Schwann cell and two types of axons as has been previously suggested (Aguayo *et al.*, 1976a; Brockes *et al.*, 1981; Thomas and Ochoa, 1984; Weinberg and Spencer, 1976) but only one type of Schwann cell and one type of axon.

The results of Voydovic's experiments in which hitherto non-myelinated axons may become myelinated if their axonal diameter is increased (Voydovic, 1991), imply that that this is a possibility. Perhaps the decision to myelinate, or not to myelinate, is determined by the area of contact made by the ensheathing Schwann cell with the underlying axon. This would be an extension of the idea of Smith and colleagues (1982) and be consistent with the findings of Griffiths *et al.*, 1991. Schwann cells associated with unmyelinated fibres are known to be shorter, and the diameter of the unmyelinated axon tends to be smaller. Possibly, if the length of the internode multiplied by the circumference of the axon exceeds a certain value, then this may affect the nature of ensheathment of the axon. A direct relationship between fibre diameter and internodal length has been observed (Gutrecht and Dyck, 1976; Vizoso and Young, 1948). Visozo and Young (1948) suggested that the critical diameter for myelination of 1.0 μm was associated with a uniform initial internodal length of 250

μm, and that regardless of their ultimate dimensions, the uniform initial internodal length

> "*strongly suggests that there is a physical factor determining the segmentation*" (Visozo and Young, 1948).

It was also stated that during reinnervation, invading neurites were thin and non-myelinated at first and only later became thickened and acquired myelin sheaths (cited in Visozo and Young, 1948).

If the data available in the literature on the dimensions of unmyelinated fibre are considered, a very crude notion of the surface area of contact between the non-myelin-forming Schwann cell and the axon can be assessed. It must be noted however, that the diameter of the axon is not uniform, and the data is not drawn from the one source, and so, the following figures can only be rough estimates.

The internuclear distance between nuclei of the non-myelin-forming Schwann cell ranged from 40 to 130 μm, with an average value of 60.5 μm (Aguayo *et al.*, 1976). The critical diameter for myelination in Voydovic's study was 1.6 μm (Voydovic, 1989). Based on these data, the surface area of contact the unmyelinated Schwann cell makes with the axon (axonal circumference x Schwann cell length) is 201 to 653 μm^2. Myelinated fibres range in diameter from 2 to 22 μm with an internodal length of 100 to 1800 μm (Hildebrand, 1989; Thomas and Ochoa, 1984). Therefore the minimum surface area of contact between the myelinating Schwann cell and the axon, would be roughly estimated at 614 μm^2. The length of the internode at the onset of myelination during development would be a more important value to use, but was not obvious from the literature. Could this mean that there is a value for area of axon-glia contact of approximately 600 μm^2 above which the Schwann cell forms a myelin sheath and below which the Schwann cell just ensheaths the axon in a non-myelinating manner? The presumption behind this theory is that there is only one type of axon and the required "myelinating signal" is present on its surface. However, until the surface area of Schwann cell contact exceeds a certain value then there is an insufficient amount of "myelinating signal" in contact with the Schwann cell plasmalemma to upregulate myelin-specific molecule expression. Considering the evidence that suggests some degree of autonomy by the Schwann cell with respect to MSM expression (see section 9.1), this axonal contact then only has to amplify the expression.

If the surface area of contact between the axon and the Schwann cell is important in determining the nature of the ensheathment, then all mammalian axons have the potential to become myelinated if they exceed a certain diameter, as the

contact surface area between the axon and the Schwann cell is increased. Hahn and coworkers (1987) noted that the largest diameter axons were first to become myelinated in the developing sixth cranial nerve. Matthews' studies on nerves from identical locations in animals of very different sizes showed increasing myelination frequency of axons associated with increasing axonal diameter in the larger animals (Matthews, 1978). Voydovic (1989) manipulated axonal diameter and showed similar results. The nature of ensheathment in non-myelin-forming Schwann cells may also be affected by the surface area of axon-glia contact. The number of unmyelinated axons ensheathed by a non-myelin-forming Schwann cell is inversely proportional to axonal diameter (Matthews, 1968; Voydovic, 1989).

Alternatively, is it possible that the upregulation of the basal level of MSM expression by the Schwann cell is determined by structural factors, such as polarization and the area of the Schwann cell membrane (as discussed previously), and is not associated with a chemical signal from the axolemma at all? This would be consistent with the acquisition of the myelinating phenotype during development and regeneration of the nervous system, and the down-regulation of this phenotype after axotomy or dissociation for culture (see section 1.5.3). Lack of a specific myelinating signal may explain the scenario of double myelination which has been observed in which myelinating Schwann cells were observed which had no axonal contact (Kidd and Heath, 1988a; Kidd and Heath, 1988b; Heath *et al.*, 1991). It may also be an explanation for the prolonged, moderately-high level of P_0 mRNA expression observed in Schwann cells cultured from neonatal sciatic explants and from acutely neurotomized adult sciatic nerve explants, described in chapter 4. That is, these cells may have achieved the required physical limits and orientation, with respect to surface area and basement membrane, for maintained P_0 mRNA expression. Schwann cells grown on complex basement membrane have been noted to have longer and more processes, whilst those grown on type I collagen had long, thin processes extending from a small, central region of perinuclear cytoplasm (Carey *et al.*, 1986a; McGarvey *et al.*, 1984). It has been suggested that basal lamina components affect Schwann cell shape and, as a consequence, their behaviour (Mc Garvey *et al.*, 1984). The alteration in Schwann cell morphology implies a larger surface area for the Schwann cell grown on complex substrate which, within the theoretical possibilities suggested here, may potentially may affect its MSM expression.

The upregulation of MSM expression by AEF described in chapter 7, may be associated with the physical nature of the membranes interacting with a polarized Schwann cell, possibly by increasing the degree of polarization, or perhaps they

physically change the structure of the Schwann cell plasmalemma. It has been noted that the physical interaction of AEF and Schwann cells is a surface based phenomenon located on the Schwann cell plasmalemma (Sobue and Pleasure, 1985). This type of interaction would permit just a physical relationship to determine Schwann cell molecular expression. In mitotic studies using AEF, sonication, homogenization, trypsin treatment and heat decreased the potency of the fragments (DeVries *et al.*, 1982; DeVries *et al.*, 1983b; Sobue *et al.*, 1983). This is probably due to damage to proteinaceous compounds on the axolemma, however, the physical nature of the AEF would also be damaged especially with the homogenization and sonication; the physical damage may be significant. Hepatocyte membranes had no effect on Schwann cells MSM expression as described in chapter 7, perhaps rendering this purely structural theory of myelin stimulation less likely. However, further experiments would be necessary to verify this fact as a constant.

The role of second messengers in transducing the stimulus for myelination to the Schwann cell nucleus is still not clear. As discussed in chapter 6, some workers have found that cyclic-AMP analogues increased the expression of MSMs, whilst other investigators have not. It is interesting to note, that Schwann cells stimulated with cyclic-AMP elevators, or analogues, have a more flattened fibroblastic appearance with broad, extended cell membranes (Mirsky and Jessen, 1990b; Morgan *et al.*, 1990; Porter *et al.*, 1986; Raff *et al.*, 1978a). Could the upregulation of P_0 mRNA expression associated with cyclic-AMP analogues be due to a change in the shape of the Schwann cell? The shape of the cell may also be determined by other, unknown factors which vary between the different laboratories and may partially explain the vagaries in the response of the Schwann cell to these analogues. However, whilst the same morphological change was observed by Shuman and colleagues (1988), P_0 and MBP protein were not expressed, but the effect on the mRNAs was not investigated in this study.

Some final intriguing data comes from the studies of Kidd and Heath (1988b) and DeVries *et al.*, (1990). Firstly, in Kidd and Heath's work on double myelination, they noted that some axons were not continuously myelinated. Myelinated internodes were observed to abut non-myelinated portions of the same axon. The Schwann cells ensheathing the non-myelinated portion of the axon were sometimes in a 1:1 relationship with the axon, but often additionally ensheathed other axons in the manner of Remak fibres. The axolemma was affected by the type of ensheathment having an axolemmal undercoating in the paranodal region of the myelinated portion,

but not in the non-myelinated area. The abaxonal aspect of both myelinated and non-myelinated portions were mostly covered with basal lamina (Kidd and Heath, 1988b). Whether these non-myelinated portions were just transient, and would subsequently have become unmyelinated, was not clear. But the fact that one axon could be myelinated in one area and then be ensheathed in the manner of Remak fibres in another area, may be used in putting forward the case that there is only one type of axon, and myelination may be decreed by structure and surface area of contact. Alternatively, this data may be suggestive of there not being a chemical myelinating signal *per se*, but the structure and physical arrangement of the Schwann cell, its polarization and basement membrane, and most importantly, the expanse of its plasmalemma, may be the factors determining the expression of MSMs.

Secondly, DeVries and coworkers have also been attempting to prepare AEF for the stimulation of MSM expression by cultured Schwann cells. Dr. DeVries records that AEF prepared from

> "*unmyelinated splenic nerve were as potent as AEF derived from myelinated axons in increasing the expression of P_0 mRNA*" (DeVries *et al.*, 1990).

9.5 CONCLUDING REMARKS

This thesis has revolved around the influence of the axon on Schwann cell gene expression. It is widely suggested that the axon decrees the molecular and physical behaviour of the Schwann cell. This may indeed be so. However, there is still a great deal which is not known about the triggers of myelination within the PNS and I suspect that what is known represents only the tip of the proverbial iceberg.

In my mind, I cannot rule out the possibility that there is not a specific molecular signal that induces myelination, although this may well still be the case. I think that further studies into the physical nature of the ensheathing Schwann cell, including its 3-dimensional structure, and its orientation with respect to other components of the PNS such as the axon, and the role of the extracellular matrix molecules, warrants further investigation. Potentially, myelination or ensheathment may be decreed on the surface area of the Schwann cell plasmalemma and the polarization of the Schwann cell by basement membrane and an axon. These may be all that is required to determine the role played by the Schwann cell.

Referring to the effect of axonal loss on Schwann cell P_0 mRNA expression, I stated previously that:

> *"the intimate dependence of P_0 mRNA on healthy axon-glia relationship is demonstrated by these data"* (see section 4.2.4).

The role of the axon in this relationship may be secondarily mediated via its physical presence, rather than being primarily mediated through its molecular composition.

In the situations of delayed Wallerian degeneration *in vivo* in frogs (Rubinstein and Shrager, 1990; Wang, 1985) and C57Bl/Ola mice (Thomson *et al.*, 1991) the retained myelinating ability of the axon and P_0 mRNA expression respectively, may not be due to a maintained molecular interaction, but rather the preserved physical presence of the axon and basement membrane, and the physical shape and size of the Schwann cell.

In the tellurium studies, P_0 mRNA expression was maintained despite fragmentation of the myelin sheath. Pending further investigations into the status of axon-glia contact, it may be that the expression of MSMs by the Schwann cell is more determined by the Schwann cell's adaxonal surface than its abaxonal surface. These data are consistent with the role of the basement membrane in assembly of the myelin sheath, rather than P_0 mRNA expression (Brunden *et al.*, 1990a; Morrison *et al.*, 1991). Further studies into demyelinating neuropathies may help elucidate the role of the physical structure of the myelin sheath and basement membrane in determining expression of Schwann cell molecules. Studies with tellurium, or extensions of the work by Rubinstein and Shrager (1990) would be possibile avenues to pursue.

Myelination did not occur in cultures in which the Schwann cell was offered artificial fibres in lieu of axons (Field *et al.*, 1968) although it was thought that it did occur in another (Ernyei and Young, 1966). In view of the current knowledge on the requirement of a complex basement membrane for the elaboration of a myelin sheath, even if the Schwann cells used in these studies did associate with the artificial fibres, and, due to the physical nature of the association, upregulated the expression of MSM mRNAs, myelination could not have occurred. The effect of adding basement membrane to these Schwann cell-artificial fibre cultures would make an interesting study. This could be studied morphologically and using MSM mRNA probes.

A less extreme view of the myelinating trigger could still require the presence of basement membrane and axon for polarization and molecular signalling from the axon. Myelination or ensheathment is then determined by the area of contact between the Schwann cell plasmalemma and the axonal surface. Thereby, molecular interaction

between the Schwann cell and axon occur, but myelination only ensues if the surface area of contact exceeds a certain value (?600 μm^2) and therefore, the amount of axolemmal-associated "myelinating signal" contacted by the Schwann cell exceeds a certain required value. Further morphological investigations into this theory and evaluation of the dimensions of the myelin-forming and non-myelin forming Schwann cell, could prove illuminating.

Upregulation of P_0 mRNA expression was produced under *in vitro* conditions described in chapter 7. However in the context of the surface area theory, this upregulation could not be very strong, because the AEF membrane interaction with the Schwann cell surface is patchy. Therefore, if the degree of MSM expression is determined by the surface area of axon-glia contact, a high level of expression would not be possible. I suspect that variation in the particle size of AEF does occur between preparations, and this may also account for some of the variability in the intensity of the response.

I think that a quote from Drs. Mirsky and Jessen neatly understates the level of our knowledge on this complex subject.....

> *"The importance of axon diameter, polarization of Schwann cells and specific signalling molecules from neurons in this process needs investigation"* (Mirsky and Jessen, 1990b).

APPENDICES

All distilled water was also deionized.

A1 TISSUE CULTURE

A1.1 SD solution

- 0.52 mg/ml soy bean trypsin inhibitor; 0.04 mg/ml bovine pancreas DNase; 3.00 mg/ml bovine serum albumin (fraction V); Tissue culture medium such as L15, or DMEM was used as the carrier.

A2 ANIMAL STUDIES

A2.1 Buffers

A2.1.1 General buffers

- phosphate buffer: $\approx$80% Na_2PO_4, $\approx$20% KH_2PO_4 added to achieve a pH of 7.4
- phosphate buffered saline (PBS): 8.006 g NaCl, 0.2012 g KCl, 0.2042 g KH_2PO_4, 1.1356 g Na_2PO_4 (pH 7.3) in 1 litre distilled deionized water.
- 20 x SSC: 3.0 M NaCl, 0.3 M trisodium citrate (pH 7.0)
- isotonic sodium cacodylate buffer: 16.05 g sodium cacodylate, 3.8 g NaCl, 0.055 g $CaCl_2$, 0.102 g MgCl, made up to 1000 ml with distilled water and buffered to pH 7.2 to 7.3 with 1.0 M HCl.
- 0.08 M sodium cacodylate buffer: 17.1224 g sodium cacodylate/l.

A2.1.2 Buffers for preparing AEF

- Homogenizing buffer: 0.3 M sucrose, 0.003 M $MgCl_2$, 0.001 M EGTA, pH 7.4. The tissue was added at 20% homogenate w/v.
- Hypotonic shock buffer: 0.01 M TES, 0.003 M $MgCl_2$, 0.001 M EGTA, pH 7.4.
- Sucrose gradient buffers:
  0.75 M sucrose, 0.001 M EGTA, 0.001 M TES, pH 7.4.
  1.2 M sucrose, 0.001 M EGTA, 0.001 M TES, pH 7.4.
- To all buffers were added gentamicin (2.0 μg/ml) and leupeptin (0.1 mg/ml)

A2.2 Strong Fixative

This fixative was used for tissues destined for electron microscopy. The volume required for an adult mouse was 150-200 mls, and for an adult rat was 350-400 mls.

For 500 ml of fixative: 8.0% paraformaldehyde in 250 ml of 0.2 M phosphate buffer was heated to 70°C and 1.0 M NaOH added until the solution cleared. When cool, 100 ml of 25 % EM grade glutaraldehyde was added and the solution was made up to 500 ml with 0.08 M sodium cacodylate. 250 mg of $CaCl_2$ was added and the solution filtered.

A2.3 4% paraformaldehyde/0.1% glutaraldehyde

4.0% paraformaldehyde solutions were made up in 0.1 M phosphate buffer (A2.1.1) and heated to 70°C. The solution was cleared with 1.0 M NaOH cooled to room temperature and sufficient 25 % EM grade glutaraldehyde was added to make a 0.1 % solution.

A3 MORPHOLOGIC STUDIES

A3.1 Light microscopy

A3.1.1 Routine processing for light microscopy

The following procedure is for processing tissue for embedding in paraffin wax, and used a Shandon Elliot automatic tissue processor (Histokinette).

Fixed tissues were placed in cassettes and processed through 70% spirit/5% phenol for 2 hours; 90% spirit/5% phenol for 2 hours; methylated spririts for 2 hours; absolute alcohol/5% phenol for 2, 1 and 1 hours; 1% celloidin in methyl benzoate for 4 hours; 3 x 1 hour immersions in xylene; paraffin wax for 5 hours and 2 hours and then blocked out in fresh paraffin wax.

NB: Celloidin was obtained from Merck "Necoloidine" (R) as an 8% solution. This was treated as a 100% stock solution when made up with methyl benzoate.

A3.1.2 Staining procedures

Haematoxylin and eosin

Haematoxylin was used to stain for nuclei, and eosin for cytoplasm. The following procedure was used:

The slides were dipped in fresh water; stained in haematoxylin for 30 seconds; washed in gently running water for 2 minutes; Scotts tap water for 15 seconds; washed in gently running tap water for 2 minutes.

At this point the slides were either dehydrated if they were not counterstained with eosin (see below) or were placed in methylated spirits for 10 seconds followed by 25 seconds in saturated alcoholic eosin.

For dehydration after either type of staining, slides were dehydrated through methylated spirits, 2 x absolute ethanol and 2 x xylene.

The slides were removed from the last xylene only just prior to mounting with DPX mountant. The top coverslip was gently pressed onto the section to squeeze out air bubbles.

NB: Mayer's haematoxylin was used. This contained 1.0 g haematoxylin, 50.0 g potassium alum, and 0.2 g sodium iodate in 1 litre of water, which were brought to boiling point and left overnight. 1.0 g citric acid and 50.0 g chloral hydrate were subsequently added.

Scott's tap water substitute: 3.5 g sodium bicarbonate and 20.0 g magnesium sulphate in 1 litre of water.

Sudan black

Sudan black was used to stain the lipid in myelin of teased fibres. The following procedure was used:

Sections were fixed in formal calcium for 5 minutes; rinsed in distilled water and 70% methylated spirits; stained in freshly filtered saturated Sudan Black B in 70% methylated spirits for 30 minutes; rinsed briefly in 70% methylated spirits; washed well in running water; mounted in 90% glycerol/PBS.

NB: formol calcium is: 10 ml of 40% formaldehyde solution, 1 g $CaCl_2$, 90 ml distilled water).

Methylene blue/azure II

1.0% methylene blue, 1.0% azure II, 1.0% borax in distilled water. Slides were placed on a 60°C. hot plate, flooded with stain for 10-30 seconds and rinsed in water.

Cresyl violet

0.1% cresyl violet powder was dissolved in distilled water and heated to 60°C. in a staining dish. The dish was removed from the heat, the slides were added and stained for 6 minutes; rinsed in water, and the tissue differentiated with 600 mls of methylated spirits to which several drops of concentrated acetic acid were added; dehydrated through two changes of absolute alcohol, and 2 changes of "Histoclear" (B.S. & S. Services, Edinburgh); and mounted in DPX mountant.

A3.1.3 PPD mountant for immunofluorescence

For 40 mls, 40 mg PPD (P-phenylenediamine free base) was added to 4 ml PBS and mixed with 36 ml glycerol. The final pH was 8.6, adjusted with 0.1 M NaOH.

A3.2 Peroxidase-antiperoxidase immunocytochemistry

Peroxidase-antiperoxidase immunocytochemistry was performed on paraffin sections of 6 μm thickness. The methodology was as follows:

- The endogenous peroxidase activity of the tissue was blocked by washing sections in 3% hydrogen peroxide in phosphate buffered saline (PBS) (A2.1.1) for 30 minutes with occasional gentle tapping of the sections to release air bubbles formed by the hydrogen peroxide.
- The sections were washed in running water for 30 minutes.
- Non-specific binding sites were blocked with 10% normal goat serum in PBS for 2 hours and the sections were blotted dry with a tissue.
- The sections were incubated at 4°C overnight with the primary antibody diluted in 1% normal goat serum/PBS
- The sections were allowed to warm to room temperature and then washed in 5 changes of PBS over a 30 minute period.
- The link (secondary) antisera diluted with 1% normal goat serum/PBS was added to the sections for one hour.
- The sections were washed with 6 changes of PBS over a 20 minute period.
- The sections were covered with the peroxidase-antiperoxidase complex diluted in 1% normal goat serum/PBS for 30 minutes. The mouse complex was diluted 1:1000 and the rabbit complex was diluted 1:40.

- The sections were washed with 6 changes of PBS over a 20 minute period.
- The sections were placed in 0.2 M phosphate buffer (pH 7.3.) (A2.1.1) for 1 minute.
- The sections were transferred for 20 minutes to a filtered solution of 50 mg 3,4,3',4',-tetraminobiphenyl hydrochloride (DAB) in 50 ml distilled water which was then made up to 100 ml with the 0.2 M phosphate buffer.
- 330 μl of hydrogen peroxide solution was added to the DAB solution and the colour allowed to develop over 3-5 minutes.
- The sections were washed well in water.

A3.3 Processing for electron microscopy

All stages of this procedure were performed on a Taab rotator in a fume hood.
- The tissue was rinsed in isotonic cacodylate buffer (A2.1.1) for 10-30 minutes depending upon the length of time that the tissues had been in fixative.
- The buffer was replaced with 1.0% osmium tetroxide in isotonic cacodylate buffer for 2 hours and the tissue was washed in the same buffer for 1 hour.
- The tissue was dehydrated for 15 minutes each in alcohols of increasing concentration (50%, 70%, 80%, 90%) and rinsed twice in absolute alcohol for 20 minutes each.
- The samples were immersed twice for 15 minutes in propylene oxide and left in a 1:1 mixture of propylene oxide and resin overnight. The resin was composed of 30 g araldite, 25.2 g of DDSA hardener, 1.2 ml of DMP 30 accelerator, and 1.0 ml of di-butyl phthalate plasticizer.
- The tops of the bijoux were removed to allow the propylene oxide to evaporate for 2 hours.
- The tissue was then removed and embedded in resin which was polymerized overnight at 60^{o}C

Semithin sections (1 μm) were cut for light microscopy on a Reichardt-Jung Ultracut E ultratome and stained with new methylene blue/azure II (A3.1.2).

For electron microscopy, sections were cut at 70 nm thickness and mounted on 200 mesh copper grids, 3.05 mm diameter. Occasionally, small sections were mounted on 50 or 100 mesh copper grids coated with parlodion. Parlodion was made up as a 3.0% stock solution in amyl acetate and diluted to 0.6 % for coating the grids.

After air-drying, the grids were stained with uranyl acetate in 50% ethanol for 5 to 15 minutes at room temperature; rinsed in 50% and 75% ethanol; rinsed twice in distilled water and air-dried. The grids were secondarily stained with Reynold's lead citrate for 5-10 minutes.

NB: Reynold's lead citrate: 1.33 g lead nitrate, 1.76 g sodium citrate each dissolved in 15 ml distilled water; mixed together and shaken vigorously for 1 minute and then occasionally over a 30 minute period. 8.0 ml 1.0 M NaOH was added to clear the solution and the volume made up to 50 ml with water; final pH 12.

A3.4 Electron microscopic photography

Electron microscopic photographs were taken on Ilford EM grade film and processed at 20°C. in PQ Universal developer (1 in 10 dilution) for 5 minutes; washed in 0.1 % acetic acid for 1 minute; fixed in Hypam fixer (1 in 10 dilution) for 5 minutes and washed in gently running water for 30 minutes.

A4 MOLECULAR BIOLOGY TECHNIQUES

A4.1 *In situ* hybridisation methodology

A4.1.1 Prehybridisation treatment

All *in situ* hybridisation techniques were performed on either cultured cells or teased fibres. The following procedure was used: Sections were placed in 0.85% saline for 5 minutes; PBS (A2.1.1) for 5 minutes; 4% freshly made paraformaldehyde/PBS for 20 minutes; 2 x 5 minute washes in PBS; 0.002% proteinase K in 0.05 M Tris-HCl (pH 7.6), 0.005 M EDTA for 7.5 minutes; PBS for 5 minutes; 4% freshly made paraformaldehyde/PBS for 5 minutes; 0.1 M triethanolamine in distilled water for 10 minutes on a magnetic stirrer in a fume hood, to which was added 625 μl of acetic anhydride followed by another 625 μl 5 minutes later; PBS for 5 minutes; 0.85% saline for 5 minutes; sections were dehydrated for 5 minutes each through methylated spirits and 2 x absolute ethanol; and air-dried for 1 hour.

A4.1.2 Hybridisation

- hybridisation buffer: 50% formamide, 10% dextran sulphate, 1 x Denhardt's buffer

(A4.2.4), 0.02 M Tris-HCl (pH 8.0), 0.3 M NaCl, 0.005 M EDTA, 0.01 M sodium phosphate (pH 8.0), 0.5 mg/ml yeast tRNA and stored at -20°C until required.

Hybridisation

The [^{35}S] labelled P_0 riboprobe was diluted 1 in 10 with the hybridisation buffer and 1.0% 1.0 M di-thiothrietol diluted with DEPC-treated water. The mixture was heated at 80°C for 2 minutes, to denature the RNA and held on ice whilst being added to the sections. 3-6 μl of probe was used for each 13 mm coverslip or bundle of teased fibres. This was covered with a second, silicone treated coverslip (A4.1.4) and placed in a horizontal position in a slide holding box. At the bottom of the box was placed a tissue soaked in a mixture of 2.5 ml formamide, 1.25 ml 20 x SSC (A2.1.1), 1.25 ml distilled water. The box was sealed with tape, placed inside 3 sealed plastic bags and immersed in a 50°C waterbath for hybridisation overnight.

A4.1.3 Post hybridisation treatment and washing

The following procedure was used: the sections were removed from the box and placed in wash 1 (5 x SSC in distilled water, 0.01 M DTT) at 50°C for 30 minutes; wash 2 (2 x SSC in 50% formamide, 0.1 M DTT) at 65°C for 20 minutes; wash 3 (0.5 M NaCl, 0.01 M Tris-HCl (pH 7.5), 0.005 M EDTA) 3 x 10 minute washes at 37°C; 0.02 mg/ml RNase A diluted in wash 3 for 30 minutes at 37°C; wash 3 at 37°C for 15 minutes; wash 2 for 20 minutes at 65°C; 2 x SSC at room temperature for 15 minutes; 0.1 x SSC (A2.1.1) at room temperature for 15 minutes. The sections were sequentially dehydrated for 30 seconds each in the following solutions:

- D1: 75 ml ethanol, 162.5 ml water, 12.5 ml 6 M ammonium acetate
- D2: 150 ml ethanol, 87.5 ml water, 12.5 ml 6 M ammonium acetate
- D3: 200 ml ethanol, 37.5 ml water, 12.5 ml 6 M ammonium acetate
- D4: 237.5 ml ethanol, 12.5 ml 6 M ammonium acetate
- Sections were finally dehydrated through 2 x 2 minute immersions in absolute ethanol and allowed to air dry for 1 hour before autoradiography.

A4.1.4 Preparation of glassware

The following procedure was used to ensure cleanliness and absence of RNAse contamination of the glassware used in ISH. Glassware was soaked in 6.0% sulphuric acid, 6.0% potassium dichromate overnight; rinsed for 2 to 4 hours in tap water;

rinsed in distilled water; immersed in 0.01% DEPC-treated distilled water and dried in a 60°C oven. It was wrapped loosely in foil and baked at 180°C for 4 hours. RNAse-free slides were produced in the same manner.

Plastic eppendorfs and Pasteur pipettes were immersed overnight in DEPC-treated water, rinsed in distilled water, autoclaved and dried at 80°C for 90 minutes. Plastic pipette tips were autoclaved and dried at 80°C for 90 minutes.

Coverslips used to cover sections during hybridisation were siliconized to decrease probe binding. They were soaked in 1.0 M HCl for 30 minutes; washed in distilled water 3 times; air-dried; immersed in Repelcot(TM) for 20 minutes; rinsed in distilled water and baked for 90 minutes at 130°C.

Poly-L lysine-coated slides were prepared for ISH, and morphological studies, by covering RNase-free slides with 0.01% poly-L lysine in DEPC-treated water for 10 minutes at room temperature. The excess poly-L lysine was removed and the slides allowed to air dry, rinsed in distilled water and air-dried. They were kept at -70°C. if not used immediately.

A4.2 Blotting techniques

A4.2.1 Pre/hybridisation buffers

- prehybridisation buffer: 6 x SSC, 5 x Denhardt's (A4.2.4) buffer, 0.05 M phosphate, 100 μg/ml salmon sperm DNA which was heat denatured at 90 to 100°C for 5 minutes, and 50% deionized formamide. The calculated volume of buffer was 0.2 ml for each square centimetre of filter.
- hybridisation buffer: 6 x SSC, 1 x Denhardt's buffer, 20 mM phosphate, 100 μg/ml heat-denatured salmon sperm DNA and 50% formamide.

A4.2.3 Agarose gels

Analytical gels for DNA size fractionation were composed of 1.0% regular agarose in 1 x TAE buffer (A4.2.4). Preparative DNA gels were either 1.0% low melting point agarose, or regular agarose. The solution was boiled for 2 minutes and cooled to 60°C before pouring. After setting, the gel was covered in 1 x TAE to prevent dessication. The running buffer for electrophoresis was 1 x TAE/0.05 μg ethidium bromide.

Denaturing gels for RNA electrophoresis contained 1.5% agarose, 1 x MOPS buffer and 2.2 M formaldehyde. The agarose was dissolved in water by boiling for 2

minutes, cooled to 60°C, the other two components were added and the gel poured in a fume hood. 1.0 μl each of ethidium bromide (1 mg/ml) and northern gel dye were added to 10-15 μl RNA samples at the time of loading. 1 x MOPs buffer was used as the electrophoresis buffer. TAE gel dye was added to the sample prior to electrophoresis.

A4.2.4 Buffers

- TAE (Tris acetate EDTA) buffer: 0.04 M Tris-acetate, 0.001 M EDTA.
- T/A (Tris acetate) buffer: 0.1 M sodium acetate, 0.05 M Tris-HCl, pH 8.0.
- T/E buffer: 0.01 M Tris, 0.001 M EDTA, pH 8.0.
- denaturation buffer: 1.0 x MOPS, 2.2 M formaldehyde, 50% formamide.
- 10 x MOPS buffer (0.2 M (3-[N-Morpolino] propane sulphuric acid) sodium salt (MOPS), 0.05 M sodium acetate, 0.01 M EDTA) buffered to pH 7.0 with acetic acid, autoclaved and stored in light tight bottles at 4°C.
- elution buffer: 1.0 M lithium chloride, 0.1 M NaCl, 10% ethanol.
- Denhardt's buffer (100x): 2.0% ficoll, 2.0% polyvinyl pyrrolidone, 2.0% bovine serum albumin (Denhardt, 1966).
- DEPC-treated water: 0.1% DEPC in distilled water, suspended overnight and then autoclaved.
- Tris-saturated phenol chloroform buffer: Phenol was melted at 65°C. and 500 ml of phenol and 500 ml of Tris (pH 8.0) were mixed to form an emulsion in a 1.0 litre separating funnel. The mixture was allowed to stand, at room temperature, until phase separation occurred and the phenolic phases drained off. This was mixed with fresh Tris 2 to 3 times more until pH 8.0 was obtained. Chloroform was mixed with iso-amyl alcohol at a ratio of 24:1. The Tris-saturated phenol was added to the chloroform/iso-amyl alcohol in a 1:1 ratio, 0.1% hydroxyquinolone was added, and the buffer stored at 4°C. in a light-tight bottle.

A4.2.5 Dyes

- gel dye: 0.01% bromophonol blue, 0.01% xylene cyanol, 30% sucrose, in 1x TAE buffer
- northern gel dye: 0.01% bromophonol blue, 0.01% xylene cyanol, 30% sucrose, in 1 x MOPS buffer
- sephadex column dye: 0.1% blue dextran and 0.1% phenol red in 0.1 x SSC.

A4.2.6 Transfer of RNA to nitrocellulose paper

Capillary action was used to transfer RNA from the electrophoresis gel to the nitrocellulose paper (Thomas, 1983). An inverted gel mold was placed in a plastic dish containing 20 x SSC (A2.1.1). 2 x 2 sheets of 3M filter paper were soaked in 20 x SSC and overlaid across each other on top the gel mold ensuring that their ends were bathing in the bath of SSC. The gel was immersed in 50 mM NaOH for 20 minutes, rinsed in DEPC-treated water, and equilibrated in 20 x SSC for 45 minutes. The nitrocellulose paper was cut to the same size as the gel, washed in DEPC-treated water for 5 minutes, and then equilibrated in 20 x SSC for at least 5 minutes. The equilibrated gel was placed on top of the wet 3M paper and air bubbles gently removed from beneath. The nitrocellulose paper was laid on top of the gel and air bubbles removed. Four pieces of 3M filter paper, pre-wetted in 2 x SSC, were placed on top of the nitrocellulose paper, and 2 dry pieces of 3M paper placed on top of that. An 8 cm thick bundle of dry paper towels was overlaid on top. The filter "wicks" under the gel were covered with Parafilm to limit evaporation. A 0.5 kg weight was placed on top of the paper towels to create an even pressure for transfer of the RNA from the gel onto the nitrocellulose paper. The transfer was allowed to occur overnight at room temperature. After transfer, the nitrocellulose paper was rinsed in 6 x SSC to remove any agarose, air dried and baked for 2 hours at 80^{o}C under vacuum.

A4.3 Qiagen column

The manufacturer's instructions were followed for this procedure: The bacterial pellet was resuspended in 10 ml buffer P1; 10 ml buffer P2 was mixed into the solution and incubated at room temperature for 5 minutes; 10 ml of buffer P3 was gently mixed into the solution, centrifuged at 6,500 rpm, 4^{o}C. for 30 minutes and the supernatant removed; the supernatant was centrifuged at 16,500 rpm, 4^{o}C for 10 minutes; and applied to a Qiagen tip 500 column previously equilibrated with buffer QBT; the column was washed with 3 x 10.0 ml of buffer QC; the supernatant was eluted with 15 ml of buffer QF; the DNA was precipitated with 0.7 volumes of isopropanol and pelleted by centrifugation at 16,500 rpm, 4^{o}C for 10 minutes; the DNA was washed with 70% ethanol and resuspended in distilled water or T/E buffer.

- buffer P1: RNase A (100 μg/ml), 0.05 M Tris-HCl, 0.01 M EDTA, pH 8.0.
- buffer P2: 0.2 M NaOH, 1.0% SDS.

- buffer, P3: 2.55 M potassium acetate, pH 4.8.
- buffer QBT: 0.75 M NaCl, 0.05 M MOPs, 15 % ethanol, pH7.0, 0.15% Triton-X 100.
- buffer QC: 1.0 M NaCl, 0.05 M MOPS, 15% ethanol, pH 7.0.
- buffer QF: 1.25 M NaCl, 0.05 M MOPS, 15 % ethanol, pH 8.2.

A5 PROTEIN ANALYSIS TECHNIQUES

A5.1 Pierce protein assay

The standard protocol was employed which measured protein in the range of 100 to 1200 μg/ml and used BCA (bicinchoninic acid) protein assay reagent. 40.0 μl of reagent B was mixed to 2.0 ml of reagent A and 100 μl of the sample was added. The mixture was incubated at 37^{o}C. for 30 minutes and the optical density measured at 562 nm.
- reagent A: Sodium carbonate, sodium bicarbonate, BCA detection reagent and sodium tartarate in 0.1 M NaOH (exact concentrations not given).
- reagent B: 4.0% copper sulphate.5H_2O.

A5.2 Polyacrylamide gel compostions

For both resolving gels 15 μl of TEMED was added just before pouring the gel.

A5.2.1 5% resolving gel

5.0 ml acrylamide-bisacrylamide (30:0.8)
3.75 ml resolving gel buffer stock (3.0 M Tris-HCl (pH 8.8))
0.3 ml 10% SDS
0.7 ml 1.5% ammonium persulphate
20.25 ml water.

A5.2.2 20% resolving gel

20.0 ml acrylamide-bisacrylamide (30:0.8)
3.75 ml resolving gel buffer stock
0.3 ml 10% SDS
0.7 ml 1.5% ammonium persulphate
2.75 ml water.

A5.2.3 2.5% stacking gel

2.5 ml acrylamide-bisacrylamide (30:0.8)
5.0 ml stacking gel buffer stock (0.125 M Tris-HCl (pH 6.8))
0.2 ml SDS
1.0 ml 1.5% ammonium persulphate
11.3 ml water
0.015 ml TEMED

A5.3 SDS-PAGE buffers

A5.3.1 Discontinuous buffers

A discontinuous buffer system was used during the running of polyacrylamide gels.

- Gel buffer stock: 3.0 M Tris-HCl (pH 8.0)
- Stacking gel buffer: 0.125 M Tris-HCl (pH 6.8)
- Resolving gel buffer: 0.375 M Tris-HCl, (pH 8.0).
- Reservoir buffer: 0.025 M Tris-HCl, 0.192 M glycine, 0.1% SDS (pH 8.3)

A5.3.2 Sample buffer

- 0.0625 M Tris-HCl (pH 6.8), 2.0% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.002% bromophenol blue.

A5.4 Staining and destaining polyacrylamide gels

- gels were stained with 0.2% Coomassie blue, 10% acetic acid, 30% ethanol in distilled water.
- destaining was in 10% acetic acid, 30 % ethanol in distilled water.

A5.5 Western blot buffers

- western blot transfer buffer: 0.025 M Tris-HCl, 0.192 M glycine, 20% v/v methanol, (pH 8.3)
- blocking buffer: 0.2% gelatin, 0.1% Triton-X 100, in 10 mM Tris-HCl pH 7.4

A6 CENTRIFUGATION

For centrifugations less than 2000 rpm a Wifug bench top, free swinging centrifuge was used.

For centrifugations between 2000 and 20,000 rpm a Beckman J2-21 M/E fixed angle centrifuge.

For centrifugations greater than 20,000 a Beckman L2-62B free swinging ultracentrifuge.

Microfugations were performed on an MSE microfuge.

A7 ABBREVIATIONS

$CaCl_2$ - calcium chloride
CAM - cell adhesion molecule
CANP - calcium activated neutral proteases
CNS - central nervous system
CST - cervico-sympathetic trunk
DAB - 3'-diaminobenzidine tetrachloride
DEPC - diethyl pyrocarbonate
DMEM - Dulbecco's modification of Eagle's medium
DNA - deoxyribonucleic acid
EHS - Engelbreth-Holm-Swarm (murine sarcoma)
EM - electron microscopy
FCS - foetal calf serum
GalC - galactocerebroside
GGF - glial growth factor
H_20_2 - hydrogen peroxide
HBSS - Hank's balanced salt solution
ISH - *in situ* hybridisation
L15 - Liebowitz medium
LM - light microscopy
MAP - microtubule-associated protein
$MgCl_2$- magnesium chloride
MSM - myelin-specific molecule
NaCl - sodium chloride
NaOH - sodium hydroxide

NGF - nerve growth factor
OD - optical density
PAP - peroxidase anti-peroxidase
PNS - peripheral nervous system
rER - rough endoplasmic reticulum
RNA - ribonucleic acid
RT - room temperature
SCGM - Schwann cell growth medium
SDW - sterile distilled water
sER - smooth endoplasmic reticulum

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