MYELIN GENE EXPRESSION IN CULTURED RAT SCHWANN CELLS.

by

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DECLARATION

The contents of this thesis are the work of the author with the exception of Figures 3.9 and 3.10 which were produced by my supervisor, Professor I. R. Griffiths. The thesis has not been previously submitted for the award of a degree to any other university.

Sylvia A. Morrison

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I would like to dedicate this work to my Dad and especially to the memory of my Mum.

SUMMARY

This thesis examines expression of myelin protein genes, in particular, the major myelin protein Po, in cultured rat Schwann cells. Schwann cells from various sources, sciatic nerve, dorsal root ganglia, cervical sympathetic trunk, stellate ganglia and superior cervical ganglia, were studied with respect to their ability to form myelin, and their expression of Po. Schwann cell gene expression was studied in the presence and absence of neuronal influences, and in the presence and absence of serum components. Freshly dissociated myelinating Schwann cells from neonatal sciatic nerve express Po mRNA, however signal intensity falls markedly in the absence of neurons such that by day 7 *in vitro* only basal levels are detectable, and are negligible compared to the level *in vivo*. Dorsal root ganglia from day 16 embryos (E16) display no significant levels of Po mRNA. When cultured in full myelinating medium containing serum and chick embryo extract increasing expression is observed from 4 to 5 days and myelination occurs at about day 14 *in vitro*. Schwann cells in dorsal root ganglia maintained in serum-free defined medium express Po mRNA in similar levels to those observed in myelinating medium prior to myelin formation. However, no Po protein is detected and the Schwann cells do not assemble a basal lamina or ensheath or myelinate axons. Schwann cells in the presence of sensory neurons in myelinating medium and defined medium also express galactocerebroside and the sulphatide recognised by the O_4 antibody. Neurons from sympathetic ganglia also induce expression of Po mRNA in associated Schwann cells, although myelination does not occur in these cultures. Axonal signals appear to induce Po mRNA expression to a certain extent in defined medium and in myelinating medium, however several criteria appear to be required to initiate myelin formation. PC12 cells may also be capable of inducing Po mRNA in Schwann cells extracted from sciatic nerve. Induction of Po mRNA synthesis appears to be mediated through axonal contact signals since no expression was detected in Schwann cells exposed to DRG-conditioned medium. The results in chapters 3 - 9 suggest that Po gene expression may be regulated at several stages of synthesis.

INTRODUCTION

Peripheral myelination is a highly complex process encompassing many facets of topical interest. This being the case, my introduction will take the form of a comprehensive review of the literature relevant to areas including the structure and function of the peripheral nervous system, and peripheral myelin formation. Myelin specific molecules will be discussed in some detail. Neuronal biology will be examined and Schwann cells will be reviewed in some detail, from origin and morphology to function, development, proliferation and differentiation.

AIMS OF THE THESIS

This study aims to unravel some of the unsolved mysteries associated with peripheral myelination. In particular, I wished to elucidate events associated with up-regulation of myelin proteins, with particular interest in the major myelin protein Po. I wished to show if myelination events could be influenced by altering culture conditions, how neuronal type affected Schwann cell gene expression, and if external factors could be manipulated to influence myelination.

AIMS:

* To elucidate the time course of expression of the Po gene in myelinating cultures and compare with expression in non-myelin-forming cultures.

* To compare expression of Po with the appearance of other myelin markers, for example, galactocerebroside, myelin basic protein, myelin associated glycoprotein etc., and to monitor development of markers in myelin-forming Schwann cells and non-myelin-forming Schwann cells.

* To compare myelination events in the presence and absence of serum components in an attempt to unravel some of the mechanisms of myelination.

* To examine neuronal influence on Schwann cells by studying expression of Po mRNA in Schwann cells under the influence of different sources of neurons and in the absence of neurons.

* To attempt to induce myelin formation in cultures lacking neuronal contact.

* To examine the influence of NGF-differentiated PC12 cells on myelin gene expression in Schwann cells.

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ABBREVIATIONS

AraC	Cytosine arabinoside
AMP	Adenosine monophosphate
CNS	Central nervous system
CST	Cervical sympathetic trunk
cDNA	Complementary DNA
CNP	2',3'-cyclic nucleotide 3'-phosphodiesterase
CEE	Chick embryo extract
cAMP	Cyclic AMP
DEPC	Diethylpyrocarbonate
DRG	Dorsal root ganglion
DMEM	Dulbecco's MEM
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
GGF	Glial growth factor
GC	Galactocerebroside
GFAP	Glial fibrillary acidic protein
HBSS	Hank's balanced salt solution
HCl	Hydrochloric acid
H_2O	Water
ISH	In situ hybridisation
ICC	Immunocytochemistry
kDa	Kilodalton
kb	Kilobase
mRNA	Messenger RNA
MAG	Myelin-associated glycoprotein
MBP	Myelin basic protein
MEME	Eagle's modification of essential medium
NGF	Nerve growth factor
NGF-R	Nerve growth factor receptor
N-CAM	Neural cell adhesion molecule
PBS	Phosphate buffered saline
PC12	Pheochromocytoma cell line
PLL	Poly-L-lysine
PLP	Proteolipid protein
PDGF	Platelet derived growth factor
PAP	Peroxidase anti-peroxidase
RER	Rough endoplasmic reticulum
R-mAb	Ranscht monoclonal antibody
RNA	Ribonucleic acid
RPM	Revolutions per minute
SER	Smooth endoplasmic reticulum
SCG	Superior cervical ganglion
SDS	Sodium dodecylsulphate
TRIS	Tris[hydroxymethyl]-aminomethane hydrochloride
TRITC	Tetramethyl rhodamine-labelled isothiocyanate
TE	TRIS EDTA buffer

CHAPTER 1

LITERATURE REVIEW

1.1 PERIPHERAL NERVOUS SYSTEM

1.1.1 Development and Ontogeny

The nervous system is considered to be the most complex set of structures in the body. It contains a massive number of cells: 10^{12} neurons (human) with at least as many glial and ancillary cells.

Development of the nervous system may be described in several steps:

Genesis of nerve cells;	
	Proliferation, specification,
	migration and differentiation
Establishing connections;	
	Axon and dendritic growth
	Synapse formation
Modifying connections;	
	Nerve cell death
	Reorganisation of initial inputs
Adult plasticity;	
	Learning
	Nerve regeneration following injury

Figure 1.1 Developmental Steps in the Vertebrate Nervous System

Adapted from : Essentials of Neural Development. Ed., Brown, Hopkins and Keynes (1991).

The mammalian nervous system develops from embryonic ectodermal cells. The commitment of embryonic cells to neuronal development commences at the gastrula stage, when mesoderm, migrating under the ectoderm, induces it to become neural ectoderm. The first sign of neural development in human embryos is the formation of a longitudinal axis identified by an invagination termed the primitive streak. This primitive streak is formed by the migration of a sub-

population of ectodermal cells into the embryonic interior and immediately precedes neurulation, the first major phase of nervous system development. Neurulation involves the transfer of molecules between the mesoderm and ectodermal cells. During this time, a population of ectodermal cells condenses to form the neural plate from which three structures develop; the neural tube, the neural crest and the ectodermal placodes. These three structures are the origin of all the cells eventually comprising the nervous system. The study of cell lineages (especially in the CNS) is still incomplete and on occasion is subject to some conjecture. However, it is generally acknowledged that cells derived from the neural tube contribute to the CNS while the PNS is formed from neural crest cells and the placodes.

The second major phase of neural development is termed segmentation. This encompasses cell proliferation, migration and the structural organisation of the maturing nervous system into the brain and spinal cord. Spinal and cranial nerves mark the nervous system at specific sites, with sensory and autonomic ganglia organised segmentally within the system. Proliferation of nerve cells initially occurs only along the inner surface of the neural tube and latterly at some sites along the external brain surface.

Neurons mature, migrate and develop cellular processes termed neurites or dendrites. Migration of neurons (by now post-mitotic) and growth of neurites are influenced by interactions with other cells and with extracellular matrix. Neurons migrate radially through adjoining nerve tissue to their final destinations, guided by specific interactions with radially-orientated glial cell processes. Sensory, motor and autonomic neurons project long cell processes into the periphery. These are classified as axons rather than neurites and such processes terminate in specific organs such as muscle, skin and the endocrine system. The process whereby nerve cells form synaptic connections within, and externally to, the CNS is termed projection. The halting of axon extension and the start of chemical transmission are the first steps in synapse formation. Synaptogenesis involves transmitter receptors becoming localised to the developing post-synaptic junctional membrane, and release sites are induced in the pre-synaptic nerve terminal on instruction from the muscle. Studies on synaptogenesis have predominantly been focused on the neuromuscular junction. Full maturation of the mammalian neuromuscular junction requires firing of action potentials by the post-synaptic cell. The peripheral branch of sensory axons takes part in the development of specialised sensory end-organs. Motor axons cause aggregation of pre-existing acetylcholine receptors and synapse-specific molecules for the basal lamina (Brown, Hopkins and Keynes, 1991).

Following synaptic formations certain nervous system modifications occur, the first of which is neuronal cell death. A variable, but usually large proportion of neurons which have sent out axons to targets are involved. This cell death probably eliminates errors in the initial pattern of connections and matches pre- and post-synaptic cell numbers. The mechanism underlying neuronal cell death may depend upon electrical activity in the nerve-target pathways and involve uptake by the neurons of substances essential for survival eg., Nerve Growth Factor.

1.1.2 Structure and Function

1.1.2.1 The Axon: Myelin is not essential for the development and functioning of axons. Indeed, impulse conduction in axons commences, during development, before the formation of myelin sheaths (Jacobson, 1987).

The axon arises from a conical axon hillock protruding from the neuronal perikaryon or proximal dendrite of a motor neuron. The axon hillock contains sparse ribosomes and polysomes but no large Nissl granules. At the apex of the hillock, the axon emerges and becomes the initial segment, where the initiation of action potentials commences. The axon hillock and the initial segment receive synaptic input. The initial segment widens out to a normal internodal calibre, but narrows again at the nodes of Ranvier (Berthold, 1978). There are no ribosomes in the initial segment or the axon itself.

The length of axon surrounded or ensheathed by one Schwann cell is termed the internode, and the node of Ranvier is the highly specialised narrowing of the sheath where adjacent Schwann cells align. The paranode is lateral to the node. The axon is enclosed by a plasma membrane, termed the axolemma. The cytoskeletal matrix and organelles are termed the axoplasm.

The calibre of individual myelinated axons vary along their length. In cross-section, the initial segment and the nodes of Ranvier are narrow and rounded, the paranodal regions are fluted. Internodal segments are irregular, circular and have the greatest volume.

Larger axons tend to have a greater number of neurofilaments (see below), with the cross-sectional area of myelinated axons being directly proportional to neurofilament number, suggesting that neurofilament content is a determining factor in axonal diameter (Berthold, 1978). A constant ratio G exists between the axon diameter and overall nerve fibre diameter. This ratio reflects the optimal myelin thickness for achieving maximal conduction velocities (Price *et al.,* 1984). Axon diameter ranges from 0.1-16μm in myelinated mammalian peripheral nerve and in unmyelinated nerves ranges from 1-2μm (Berthold, 1978).

As the axon does not contain ribosomes, it is dependent on the neuronal cell body for the synthesis of cytoskeletal components, membranes and nutrients. Continuous delivery of these essential constituents is achieved by axonal transport.

Schwann cells influence the differentiation of axons during development. The excitable membrane of the axon is restricted to the nodal region of myelinated nerves by the Schwann cells. Saltatory conduction does not develop in axons if glial cells are absent, and saltatory conduction is replaced by continuous conduction if mature axons are demyelinated with diptheria toxin (Bostock and Sears, 1978).

1.1.2.2 Axoplasm: Axoplasm constitutes the fluid compartment within the axon, the viscosity of which in mammals is five times that of water (Berthold, 1978). Bi-directional axoplasmic flow takes place within the axoplasm at variable rates (Ochs and Worth, 1978). Axoplasmic elements include the cytoskeleton, matrix and membranous organelles such as mitochondria, smooth endoplasmic reticulum (SER), vesicles and inclusions. Axoplasm contains microtubule-rich channels, neurofilament-rich domains and sub-axolemmal domains where actin-like filaments are bound. Axoplasm contains no ribosomes therefore lacks the ability to synthesise protein. However, radiolabelled protein has been traced autoradiographically in the axoplasm after administration of labelled amino acids to the periaxonal glia of the giant squid axon, suggesting axonal interaction with glia, the glia taking up and incorporating amino acid into protein and transferring said protein to the axon.

The axonal cytoskeleton: Axons and dendrites derive their structural support from the cytoskeleton, which chiefly comprises microtubules, neurofilaments and their associated proteins. Individual cytoskeletal proteins are synthesised in the neuronal cell body and transported along axons.

Neurofilaments; are unbranched longitudinally-oriented structures approximately 10nm in diameter and are of variable length (Wuerker and Kirkpatrick, 1972). Groups of neurofilaments form a lattice-like structure within the axon (Thomas and Ochoa, 1984), forming a regular network traversing the length of the axon. In mammalian peripheral axons, the cross-sectional density of neurofilaments is 100-300/um^2. They first appear when developing neurons form axons, and are probably restricted to neural cells. Neurofilaments are abundant in internodal regions of large axons (Price *et al.*, 1984), and less prevalent in the nodal axoplasm. They are generally present in lower numbers in unmyelinated fibres than

myelinated fibres. Neurofilaments form the major cytoskeletal framework determining axon size and shape. As axonal calibre increases the neurofilament number increases proportionally, that is, axonal calibre is a function of neurofilament numbers (Friede and Samorajski, 1970). However, neurofilament density varies along a single axon, implying that local regulation of axonal cytoskeletal organisation must occur (Berthold, 1978). Neurofilaments are polymers of three independently synthesised peptides regulated by separate genes (de Waegh *et al.*, 1992). The three subunits have apparent molecular weights of 200, 150 and 68kDa, and all three polypeptides appear to be present in each neurofilament (Pleasure, 1984). Neurofilament peptides are synthesised in the neuronal perikaryon and transported through the axon (see **1.1.2.4** Axonal Transport). All three subunits of neurofilaments are subject to posttranslational modification, with the most significant modification being phosphorylation. Neurofilament 200 (NF200) and NF 150 have unusually high levels of phosphorylation (Jones and Williams, 1982). NF200 is important for the formation of fine processes protruding from the neurofilaments connecting with microtubules and membrane components.

Microtubules; are present in all eukaryotic cells, but are most abundant in nervous tissue. Microtubules are involved in cellular movement, morphological changes and mitotic phases (Quarles, 1988).

Microtubules are long, thin, tube-like structures with an outer diameter of 150Å, and may be 100*u*m or longer *in vivo* (Bunge *et al.*, 1986). They do not generally extend the whole length of an axon. Fine processes ranging from 20nm in length extend into the axoplasm from the microtubules, connecting with neurofilaments and membranes (Thomas and Ochoa, 1984). Microtubules possess polarity, unlike neurofilaments, that is, their fast growing ends are distant from the neuronal soma (Bunge *et al.*, 1986).

Microtubules are composed of tubulin dimers which align longitudinally. The tubulin sub-unit is an acidic, non-covalently bound heterodimer consisting of an alpha and a beta monomer. Assembly of microtubules is facilitated by microtubule-associated proteins (MAPs), which are a heterogeneous group of proteins. MAP1, MAP2 and Tau proteins stimulate the assembly of microtubules and stabilise the microtubule assembly. MAP2 stimulates polymerisation of tubulin (structural component of microtubules) (Matus, 1987). Tau proteins bind to microtubules, forming a fuzzy coat, and the fine processes extending from the microtubules. The assembly process is temperature and calcium-ion sensitive and is dependent on magnesium ions, GTP and free sulphydryl groups.

Isolated microtubule preparations consist of 80-90% tubulin and 10-20% MAPs. MAPs may be released from microtubules by elevating the salt concentrations.

More microtubules than neurofilaments are present in non-myelinated fibres. The ratio of tubules to filaments is reversed in myelinated fibres and changes in axon calibre are independent of presence of myelin. Axon calibre correlates with the number of neurofilaments and microtubules per fibre (Friede and Samorajski, 1970). Correlation is related to the uniform spacing of neurofilaments and microtubules. Mitochondrial density varies indirectly with neurofilament/microtubule presence.

There may be a higher density of microtubules in the branched extensions of an axon than in the parental axon, giving support to the branchings. Microtubules may play a a major role in fast anterograde and retrograde axonal transport.

1.1.2.2.3 *Other Organelles*

Accompanying the cytoskeleton are membranous organelles including mitochondria which vary in diameter from 0.1-0.3*u*m and 0.5-0.8*u*m in length and are oriented longitudinally (Berthold, 1978). They tend to occur in higher densities in smaller axons than larger axons. In smaller axons, the concentration of mitochondria varies between 2-5/um^2, and in larger axons, tends to be around 0.1/um^2 (Berthold, 1978). They are formed in the neuronal soma and transported along the axon, and are frequently to be observed in association with microtubules.

Endoplasmic reticulum forms a network extending from the axonal hillock to the nerve terminals and is composed mainly of SER although the occasional ribosome may be associated with it. Ribosomes are rarely observed distal to the axon hillock.

1.1.2.3 Axonal Plasma Membrane/Axolemma; Axonal organelles and cytoplasm are enclosed by a plasma membrane termed the axolemma. This is defined as the surface membrane of the axonal processes from the point at which it emerges from an axon hillock of the neuronal perikaryon to the axonal terminal or nerve ending. The membrane is an excitable trilaminar structure 7-8nm. thick. In myelinated nerves the axolemma is largely ensheathed by Schwann cell-derived myelin.

The axonal plasma membrane is actively involved in ion translocation as observed by analysing axolemmal enriched preparations. A number of various axolemmal preparations have elucidated several metabolic activities associated

with the membrane. DeVries (1984) summarises the main findings and discusses synthesis of ATP, GTP, and several glycolytic enzymes, activity of ATPase, acyltransferase, phospholipase and phosphatases.

Estridge and Bunge (1978) showed, using rat dorsal root ganglion cultures, that the axolemma is important in the incorporation of glucosamine into macromolecules. The majority of enzymatic activities which have been associated with the axolemma are concerned with lipid or glycolipid metabolism. Lipids constitute at least 50% of the dry weight of all axolemmal preparations with those derived from unmyelinated PNS axons of invertebrates having the greatest lipid content. Of this lipid, 50-75% is comprised of phospholipid with ethanolamine and choline phosphatides being present in the greatest concentrations and approximately equal levels. It is thought that the axonal membrane may be required to synthesise its own lipid from appropriate precursors to provide the correct molecular assembly at its surface for glial cell interaction. In support of this, Constantino-Ceccarini *et al.*, (1979) reported that the activity of UDP-galactose: ceramide galactosyltransferase in axolemmal enriched preparations is greatest during the initial stages of myelination with a sharp decrease in activity on completion of myelination. Glycolipids including cerebroside, sulphatides and gangliosides are found only in axolemmal fractions derived from myelinated axons.

Phospholipase D utilises its own endogenous lipid as a substrate and such axolemmal lipolytic activity may modulate enzymatic activities such as Na^+,K^+-ATPase and influence sodium channel function (DeVries, 1984).

Several proteins other than enzymes have been demonstrated in the axonal plasma membrane including tubulin, actin, tropomysin and myosin. Axolemma isolated from myelinated axons or cultured axons contains a membrane bound mitogenic signal that will cause a quiescent population of cultured Schwann cells to divide (see Section 1.4.4 on growth factors and mitogens).

The axolemma is rich in sodium channels (Peters, 1976).

1.1.2.4 Axonal/Axoplasmic Transport:

Components of the cytoskeleton such as microtubules, which are present in continuous tracks along the length of an axon, play a major role in axonal transport. The axon does not contain ribosomes and is dependent on the neuronal soma for the continuous supply of cytoskeletal components, membranes and material. Substances may be transported in both directions along the axon; distal-to-proximal flow is termed retrograde and proximal-to-distal referred to as either orthograde or anterograde transport. Axoplasmic flow occurs at varying rates; from 1-10mm/day to greater than 100mm/day.

Molecular motors drive axonal flow. A 120kDa vesicle-associated protein, kinesin, interacts with microtubules and vesicles. In an ATP-dependent mechanism, kinesin can drive the movement of one relative to the other. Microtubules are anchored by other cytoskeletal components and membranes, with the net effect of kinesin being movement of vesicles on an orthograde direction.

Retrograde transport is mediated by a microtubule-associated protein (MAP1), dynein.

Slow axonal transport conveys greater amounts of protein than fast transport, whereas relatively few polypeptides are carried by the slow system, predominantly those associated with the cytoskeleton.

1.2 BIOLOGY OF THE NEURON

In this section several neuronal types and ganglia will be discussed in detail according to their relevance to the thesis.

The neuron is the individual signalling element of the brain. The neuronal cell body is termed the soma. The most prominent organelle is the nucleus, containing the genomic material, DNA. The neuron is enclosed by a plasma membrane, which is a bi-layer of phospholipid molecules. Additional organelles include mitochondria, smooth endoplasmic reticulum (SER), Golgi complex, lysosomes and ribosomes located on the rough endoplasmic reticulum (RER) and in close proximity to the nucleus (therefore termed Nissl substance). Neuronal ultrastructure is illustrated in Figure 1.2.

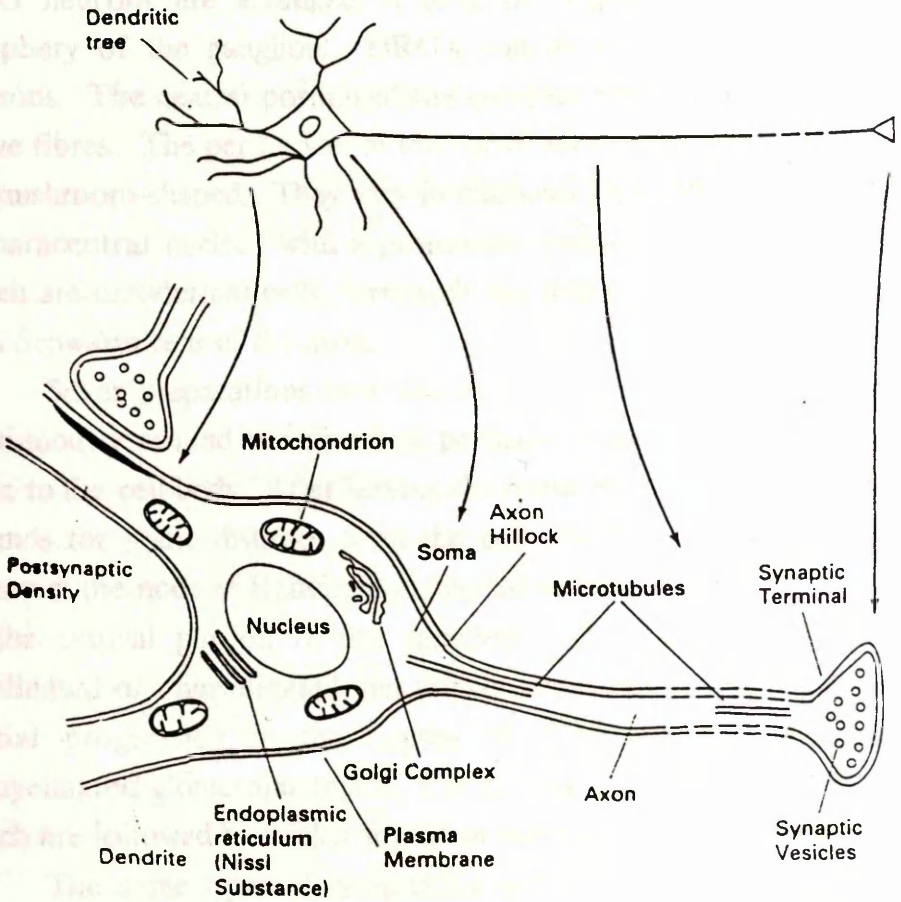


Figure 1.2 General Ultrastructure of a Neuron

(The Neuron: Cell and Molecular Biology. Levitan and Kaczmarek, 1991)

1.2.1 Dorsal Root Ganglion

Dorsal root ganglia (DRG), are part of the PNS. Dorsal root nerve cells and their satellite and sheath cells originate from the neural crest. The ganglion is a collection of around 10,000 somatic and visceral nerve cells and their processes, which form an ovoid enlargement of the dorsal root. The axons of the dorsal root originate from the cell bodies in the ganglion and carry sensory impulses to the spinal cord through the dorsal root. The nerve bundle which exits from the peripheral pole of the ganglion, joins the ventral roots of the spinal cord to form the spinal nerve trunk.

The ganglion has a thick connective tissue capsule, which is continuous with the epineurium and perineurium of the spinal nerves. The perineurium acts as a diffusion barrier to the passage of many substances into the nerve and ganglion. DRG neurons are arranged in rows or groups and are concentrated at the periphery of the ganglion. DRG s contain the cell bodies of around 10,000 neurons. The central portion of the ganglion contains primarily large bundles of nerve fibres. The perikaryon of the dorsal root neurons are globular, pear-shaped or mushroom-shaped. They vary in diameter from 20-100μm, and have a central or paracentral nucleus with a prominent nucleolus. A capsule of satellite cells, which are ectodermal cells, surrounds the individual neurons, and are continuous with Schwann cells of the axon.

Silver preparations have shown that the axon of larger neurons may wind continuously around the neuronal perikaryon, or may coil forming a glomerulus close to the cell body. After leaving the dorsal root neuron, the single axon usually extends for some distance from the cell and then bifurcates. This bifurcation occurs at the node of Ranvier and may take place in the vicinity of the cell body or in the central portion of the ganglion. The axons in the ganglion may be myelinated or unmyelinated, but thicker axons tend to be myelinated. There is a spatial progression in the degree of myelination as the axon leaves the unmyelinated glomerular region, that is, there are unusually thin layers of myelin which are followed by thicker myelin in segments close to the axonal bifurcation.

The same types of cytoplasmic organelles are present in large and small neurons but their number and distribution are different. Organelles include Nissl granules, a Golgi network, mitochondria, neurofibrils and pigment granules. These may all be observed in sensory neurons by light microscopy. The Nissl substance is basophilic, a property which allows it to be easily stained, for example, by toluidine blue or cresyl violet, to give better visualisation. The Golgi stains black or grey with silver or osmium, and neurofibrils can be distinguished by silver preparations or by immunofluorescence techniques. The Golgi complex consists of

closely packed, flattened saccules and vesicles and is commonly found as a small "C" shaped curve. Mitochondria appear as fine curved threads as long as 4μm in fixed preparations and can be seen by phase contrast microscopy in culture. They appear ellipsoidal or rod-like. Neuronal mitochondria have an outer limiting membrane and an inner membrane that is folded into plate-like cisternae.

Neurofilaments are common in the large light neurons and increase greatly in number in the axon hillock. Neurofilaments are the predominant organelle in the initial segment of the axon of the DRG neuron. Neurofilaments and microtubules are the major part of the cytoskeleton and microtubules are an essential component of the fast axonal transport system (see **1.1.2.1** and **1.1.2.4**).

The neuronal nucleus is usually spherical and chromatin is finely dispersed throughout. The nucleus is surrounded by a double layered nuclear envelope which fuses to form pores. The pores have a diaphragm with fibrous material on both sides and are thought to be the site of passage of substances between the nucleus and the cytoplasm. The inner nuclear membrane is fairly smooth, but the outer has slight undulations. Occasionally, ribosomes are attached to the outer nuclear membrane. The nucleolus is large and has a dense, coiled nucleolonema, which is formed of very fine filaments termed the pars fibrosa, and granules called the pars granulosa. The granules are smaller than cytoplasmic ribosomes. Both parts contain ribonucleoprotein. The less dense zone in the interstices of the coiled threads is karyoplasm and may contain some chromatin.

The Nissl substance consists of flattened ribosome-studded cisternae constituting the RER. Together with the clusters of ribosomes called polyribosomes they form the major sites of protein synthesis within the cell. Nissl substance is found not only in the perikaryon, but also in dendrites although not in the axon.

Dorsal root and sympathetic neurons are completely ensheathed by a capsule of satellite cells which have long thin processes which often interdigitate with each other. The sheath is usually directly apposed to the neuronal cell bodies and follows any irregularities in the neuronal surface. There is usually a space of about 20nm intervening between the surface of the neuron and its sheath. Occasionally an axon or a dendrite runs along the surface of the perikaryon before exiting from the capsule.

As the axons and dendrites of ganglionic neurons leave the capsule they are surrounded by a sheath of Schwann cells which are morphologically similar to the satellite cells and are continuous with them. The satellite and Schwann cells are coated with a basement membrane on their outer surface facing the extracellular space.

Peroxisomes have been found in the satellite and Schwann cells of rat DRG and contain catalase.

The neurons send out a single process which bifurcates within the ganglion, sending one branch to sensory receptors in the skin and muscle, and the other to central synapses on the spinal cord. The cells comprise a relatively homogeneous population subdivided into two classes on the basis of size and time of developmental origin (Lieberman, 1976). The cell bodies are enveloped by satellite cells which may be identical or closely related to Schwann cells, while the myelinated and unmyelinated processes are surrounded by Schwann cells and connective tissue (Fields *et al.*, 1978).

1.2.2 Superior Cervical Ganglion

The Superior Cervical Ganglion (SCG) is situated in the upper part of the neck, near the cranial base. This ganglion adjoins the nodose ganglion of the vagus nerve. The largest nerves exiting the ganglion are the cervical sympathetic nerve and the internal carotid nerve. Some postganglionic fibres of the SCG return to the cervical sympathetic nerve or enter the vagus nerve by anastomoses between the SCG and the nodose ganglion.

Sympathetic neurons are derived from the neural crest, which is a transient structure that gives rise to most peripheral neurons. Following migration from the dorsal margin of the neural tube, sympathetic precursors aggregate to form ganglia (Hawrot, 1980), and acquire a noradrenergic phenotype. The precursor cells undergo several rounds of cell division and then withdraw from the cell cycle. In the rat SCG, which is the most anterior ganglion in the paravertebral chain, the sympathetic neuron begins to differentiate as soon as it undergoes its final cell division. The neuron elaborates an axon that forms a plexus in an appropriate target region, constructs a dendritic arbor and receives afferent input.

Neurons of mammalian sympathetic ganglia are oval or stellar shaped, multipolar, and have short dendrites which do not leave the ganglion. The axons are long and leave the ganglion, as described above, by postganglionic fibres. Some superficially localised neurons of the SCG differ from other neurons in this ganglion by their high content of protein, RNA, and lipids, and in their more dense capillary net covering the cells, again suggesting functional differences within neurons in the SCG (Skok, 1973). As reviewed by Skok (1973), the SCG neuronal soma in 50% of human SCG neurons measures 25-32μm, in 27%, 35-55μm, and in 23%, 15-22μm. Several authors describe the neurons as varying between 15-60μm. Mammalian sympathetic ganglion neurons generate 1-10 dendrites and contain norepinephrine storage vesicles, which may be in transit from the perikaryon to the

axon terminals. In addition to cytoplasmic ribonucleoprotein, peculiar inclusion bodies termed nematosomes have been observed in the cytoplasm of rat sympathetic neurons. These have the appearance of the coiled fibrillary component of the nucleolus. Cytoplasm contains protein and some RNA but no ribosomes.

In the rat, virtually all neurons in the paravertebral chain are generated before birth. In contrast, most ganglionic non-neuronal cells are generated after birth. Continued differentiation and maturation of the immature sympathetic neurons depend on both retrograde and anterograde signals, for example, nerve growth factor (NGF), a trophic molecule produced by sympathetic target tissues that is specifically taken up and retrogradely transported by sympathetic neurons. A more complete discussion of the SCG and its neurons may be found in a review by Landis (1987).

The SCG receives preganglionic fibres from seven upper thoracic spinal nerves. Stimulation of the preganglionic fibres in the cervical sympathetic nerve has several effects including dilation of the pupil, contraction of the nictitating membrane and the muscle of the upper eyelid, and secretion of the salivary glands (Skok, 1973). Stimulation of sympathetic fibres also causes vasoconstriction in the conjunctiva and in the skin of the head and neck. Damage to the SCG results in ptosis (drooping of the upper eyelid) and enophthalmos (sinking of the eye into the orbit) (Kahle, 1986). Nicotine is a blocker of all SCG function.

Dissociated sympathetic neurons, if cultured long-term in the absence of non-neuronal cells will develop many of the properties of normal adrenergic neurons (Hawrot, 1980).

Sympathetic ganglia contain several neuronal subpopulations that differ in their transmitter phenotype. Most sympathetic neurons are noradrenergic, but some are functionally cholinergic. These properties can be influenced in vitro, for example, cholinergic differentiation of these neurons has been demonstrated under the influence of factors present in human placental serum or chick embryo extract, both of which may be constituents of myelinating medium (Saadat *et al.*, 1989).

The SCG plays an important role in the development of the cervical sympathetic trunk (CST). The CST is a unifascicular autonomic nerve composed mainly of preganglionic unmyelinated nerve fibres which ascend and synapse in the SCG. The nerve extends within the carotid sheath from the upper mediastinum to the carotid bifurcation. In some rats, a small descending branch of the vagus nerve joins the CST for a variable distance. This can be distinguished from the CST due to the vagus fibres being myelinated.

At birth, the CST is composed of large bundles of neurite axons enclosed by Schwann cell processes and basal lamina. During the first week following birth, there is a marked decrease in the number of axons. In normal developing CST, the Schwann cells undergo rapid multiplication during this first post-natal week but after P10 the rate of division is negligible. However, Schwann cell units continue to increase due to formation of processes (Aguayo *et al.*, 1976). More than 99% of all nerve fibres in the CST are unmyelinated (Mirsky and Jessen, 1984).

Post-natal Schwann cell proliferation is influenced by axonal populations. Removal of SCG ganglionic cells causes a retrograde loss of preganglionic fibres and in adult rats, postganglionic axotomy causes neuronal loss in the SCG. (Aguayo *et al.*, 1976c). Post-ganglionic fibres form a plexus around the internal and external carotid arteries (Kahle, 1986).

1.2.3 Stellate Ganglion

The stellate ganglion is located near the bases of the II-III ribs and lies on the lateral surface of *m. longus coli*. The stellate is formed from the fusion of two or three upper thoracic ganglia of the terminal sympathetic trunk and by the inferior cervical ganglion. Stimulation of the stellate ganglion causes the contraction of cardiac muscle fibres to be more synchronous, increases their conduction velocity and dilates the coronary blood vessels. The stellate ganglion sends bronchodilatory fibres to the lungs, fibres to the oesophagus and the stomach and some fibres enter the vagus nerve. The stellate receives preganglionic fibres from some thoracic spinal nerves and the ganglion is crossed by preganglionic fibres to the middle, accessory and superior cervical ganglia. All fibres of the vertebral nerve are axons of nerve cells located in the stellate ganglion. More detailed information may be found in Skok (1973), and in work published early this century by Langley (1904).

1.2.4 PC12 Cells

The rat pheochromocytoma clone PC12 is a neural crest-derived adrenergic tumour cell line and is particularly useful in cell culture as a model for the study of neuronal development.

The cell line has a homogeneous and near-diploid chromosome number of 40 (Greene and Tischler, 1976). Doubling time in culture is 92 hours in the correct conditions. Such cells have a rounded or polygonal appearance and have a tendency to clump together. PC12 cells attach well to a collagen substrate, but do not have a good affinity for Poly-L-lysine or tissue culture-treated plastic.

PC12 cells contain dense core granules which may be up to 350nm in diameter and are similar to those seen in adrenal chromaffin cells and sympathetic neurons (Greene and Tischler, 1976). They contain catecholamines and have more dopamine than norepinephrine (in contrast to adrenal cells). They also synthesise and release acetylcholine. PC12 cells do not synthesise epinephrine and cannot be induced to do so (Greene and Tischler, 1976).

On exposure to the polypeptide hormone NGF, PC12 cells hypertrophy, cease division and begin to extend neurite-like processes resembling sympathetic neurons *in vitro*. Processes reach 500-1000*u*m in length and are fine, profusely branched, with growth cones (Greene and Tischler, 1976). PC12 cells also express a number of differentiated neuronal properties, morphologically, and biochemically. Cells in culture become electrically excitable and more sensitive to depolarisation. These NGF-induced changes take place almost immediately and neurite outgrowth can be observed within 48 hours (Guroff, 1985). NGF-differentiated PC12 cells contain increased levels of transmitter-synthesizing enzymes (Greene and Shooter, 1980, Huff *et al.,* 1981) and exhibit many properties of mature terminally-differentiated sympathetic neurons. Differentiation is entirely reversible on withdrawal of NGF. Within 24 hours, most cells have lost their processes and cell division recommences within 72 hours (Greene and Tischler, 1976).

PC12 as a model for sympathetic neurons.

Sympathetic neurons have nicotinic acetylcholine receptors which are indistinguishable from those found on PC12 cells (Guroff, 1985). PC12 cells also have NGF receptors located on the plasma membrane and are of two types, classified according to their enzymic dissociation rate (Landreth and Shooter, 1980).

When NGF-differentiated PC12 cells extend neurites, they also express large quantities of neurofilaments. All three neurofilament proteins may be identified (Trojanowski and Lee, 1985). PC12 cells maintained in the absence of NGF, contain 3 times more vimentin than NF68, and only traces of NF150 and NF200 (Lee and Page, 1984).

Addition of NGF to PC12 cells induces an approximate doubling in the cell surface expression of both Thy-1 and N-CAM (neural cell adhesion molecule) after 24 hours *in vitro* (Doherty *et al.,* 1988). The addition of NGF to cultured PC12 cells produces increases in mRNA's coding for NF68 protein and Thy-1 glycoprotein within 24 hours with maximal effects for NF68 seen at 12 days following addition (90-fold stimulation) and maximal effects for Thy-1 4 days

following treatment (45-fold stimulation) (Dickson *et al.*, 1986). Changes in N-CAM levels induced by NGF are accompanied by changes in N-CAM isoforms.

Studies on the effects of NGF in conjunction with factors which elevate cAMP show that forskolin and/or cAMP analogues potentiate the action of NGF, but establishment of neurite networks cannot be induced by forskolin, cAMP or cAMP analogues alone.

PC12 cells also respond to Epidermal Growth Factor (EGF) (Huff *et al.*, 1981). EGF enhances cell proliferation and does not cause hypertrophy nor stimulate formation of neurites.

PC12 cells stimulate Schwann cell division and membranes derived from PC12 cells have been shown to have mitogenic effects similar to those of sensory neuron membranes (Ratner *et al.*, 1984). In cell culture, PC12 neurites in contact with Schwann cells induced proliferation of Schwann cells. Intact PC12 cells are mitogenic for Schwann cells, but to a lesser degree than DRG neurons. The PC12 mitogen is thought to be a surface mitogen, as is the neurite mitogen, and both are trypsin-sensitive (Ratner *et al.*, 1984). The PC12 mitogen is more characteristic of the neurite mitogen than of axolemmal mitogens.

PC12 stimulation of Schwann cells *in vitro* occurs independently of serum components. PC12 cells grown in defined media (SATO N_2) are mitogenic to Schwann cells (Ratner *et al.*, 1984). The mitogen is a constitutive feature of the PC12 cell, and occurs regardless of NGF presence (Ratner *et al.*, 1984).

1.3 PERIPHERAL MYELIN AND MYELIN FORMATION

Myelin is a membranous structure characteristic of nervous tissue and in the vertebrate nervous system exists in two distinct but comparable forms, central and peripheral. Myelin is morphologically unique, consisting of a lipid bi-layer sandwiched between two layers of intrinsic and extrinsic protein and wrapped spirally around a segment of axon. This portion of myelinated axon is referred to as the internode, at each end of which are the nodes of Ranvier. Using electron microscopy, myelin appears as a set of concentric spirally-arranged lamellae wrapping the axon.

The peripheral myelin sheath arises from Schwann cells and central myelin from oligodendrocytes. The formation of mature myelin in developing peripheral nerve is preceded by a complex array of sequential events during which Schwann cells become associated with axons and culminating in those which will form myelin and those which will become non-myelinating Schwann cells.

1.3.1 Myelinogenesis

Initially, in the formation of peripheral nerve fibres, a pioneer axon extends from a neuron and advances by means of an amoeboid growth cone (Speidel, 1964; Webster, 1975). The pioneer axon is closely followed by more axons and by migrating Schwann cells derived from the neural crest. These migrating glial cells can move at rates of up to 115 microns per day, but may remain stationary for long periods. At this stage, Schwann cells are ovoid in shape, have blunted processes and no basal lamina is evident. The Schwann cells move by means of process extension, displacement of cytoplasm and retraction of the trailing processes. Schwann cells align themselves alongside large nerve bundles and begin to migrate towards the interior of the bundle, proliferating rapidly in response to the axons (Wood and Bunge, 1975). Axons become partially or completely enveloped by invaginations of the Schwann cell. Further proximally, where the nerve is more mature, the migrating Schwann cells have increased their number mitotically and form a complete layer on the outside of the nerve bundle, separating them from surrounding tissue.

The axis of mitosis is parallel to the long axis of the Schwann cell and of the axons, so that once division is complete, daughter cells are arranged in series along the length of the nerve fibre. Each daughter cell extends a slender cytoplasmic process from near the nucleus.

Larger axons become segregated and invaginate the Schwann cell surface, adopting a 1:1 relationship. Axons at this stage are about 1μm in diameter. Smaller axons do not seem to stimulate myelin formation. It seems to be peculiar

to the peripheral nervous system that larger diameter axons are more likely to become myelinated. Once this relationship has been achieved, the myelinating Schwann cell ceases to divide, and goes on to form a mesaxon.

At this stage a basal lamina is formed by the Schwann cells which envelopes the myelinating structure. Schwann cells are now characteristically bi-polar and spindle shaped. During prophase, Schwann cells retract their sheet-like processes in which they originally enclosed groups of axons (Martin and Webster, 1973).

The outer faces of Schwann cell plasma membrane which come together to form the mesaxon are separated by a gap of 12-14nm. Only at the outer end of the mesaxon do the membrane surfaces come closer to form a tight junction. The mesaxon elongates in a spiral manner around the axon, but this does not occur at the same rate along the length of the internode (Bunge *et al.*, 1989). The number of turns of mesaxon is usually greater in the paranuclear regions than at the ends of the Schwann cell where the nodes of Ranvier form. The direction of turns may also vary even within the same internode. Further elongation of the mesaxon leads to a more regular spiral and when 3-4 turns are complete, the cytoplasm between them is extruded and compact myelin begins to form. As myelination proceeds, the only pockets of cytoplasm which persist are contained in a series of small helical pockets, termed the Schmidt-Lanterman incisures.

From now on, the rate of compaction of the myelin sheath appears to be relatively constant. Compaction of the turns of the mesaxon has two effects: loss of cytoplasm from between the turns brings the cytoplasmic faces of the plasma membrane forming the mesaxon into opposition fusing to form the major dense line. Simultaneously, the 12-14nm gap in the mesaxon closes to only 2-2.5nm so that the intraperiod line forms due to close apposition of the outer leaflets.

When compactions of the lamellae are complete the cytoplasm is lost from throughout the sheath, however it is retained in the inside and the outside of the myelin. Schwann cell cytoplasm forms a narrow layer outside the myelin sheath, the abaxonal layer, containing an elongated nucleus approximately at the centre of each internodal length of myelin. A narrow zone of Schwann cell cytoplasm is visible inside the myelin sheath. The axolemma is separated from the surface membrane of the adaxonal portion of the Schwann cell by the periaxonal space. The only free parts of mesaxon are the inner and outer mesaxons.

1.3.2 Structure

Viewed in cross-section, the peripheral myelin sheath is compacted and spiral, and outer and inner mesaxons are at all times present (Figure 1.3). The period between major dense lines varies from 11.5 to 12.7nm in electron

microscope specimens, being fairly constant between axons and between animal species (Ribchester, 1986). The single major dense lines are derived from the fusion of the inner leaflets of the myelinating cell process and the double intraperiod line from the closely apposed outer leaflets. The cell surface is covered by a basal lamina. A moderate amount of cytoplasm is associated with the outer layer of the PNS myelin sheath.

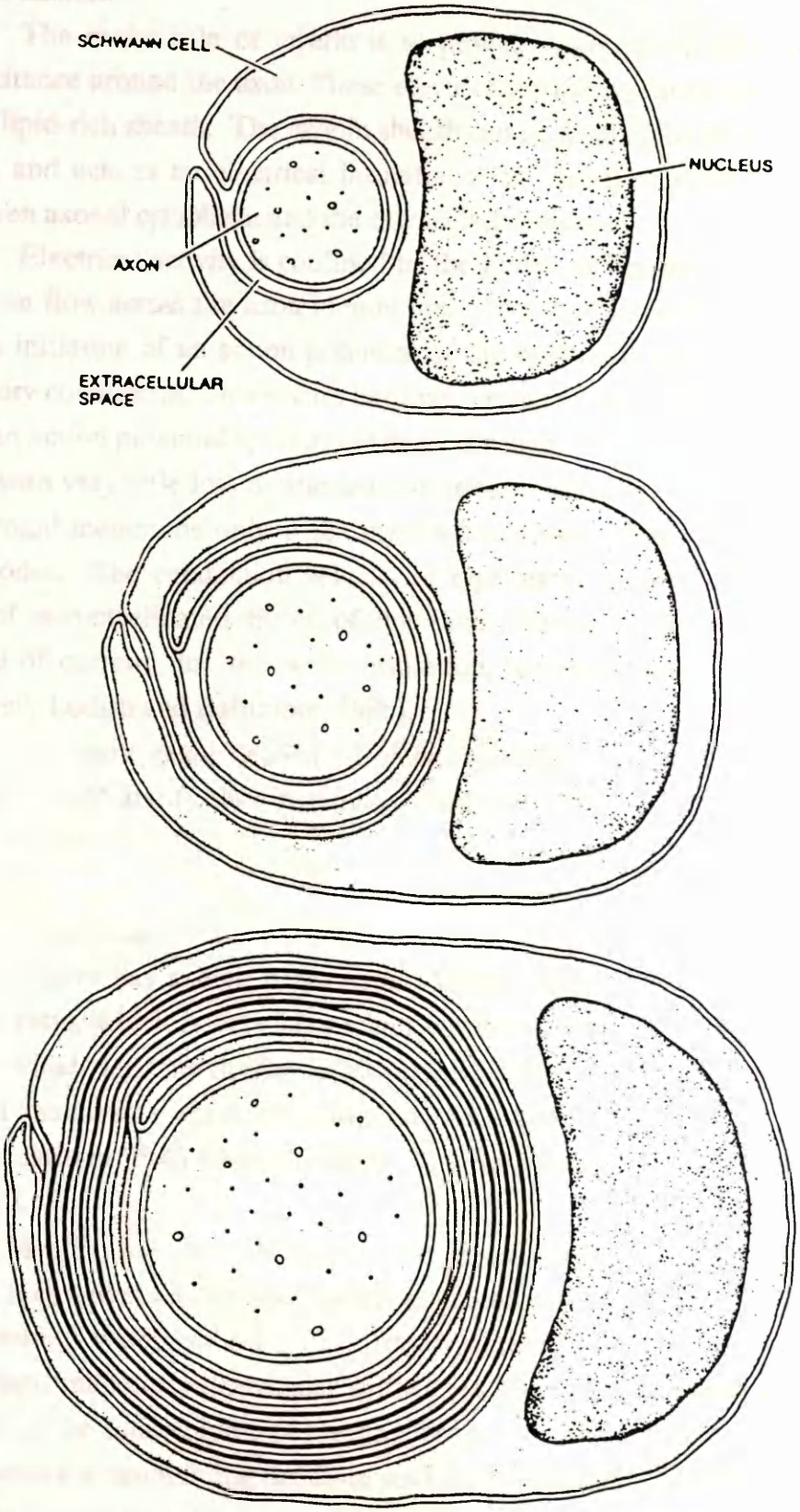


Figure 1.3 Compaction of Myelin Viewed in Cross-section

1.3.3 Function

The major role of myelin is to provide segments of high-resistance and low-capacitance around the axon. These electrical properties arise from myelin being a thick lipid-rich sheath. The myelin sheath can be as many as 50 or 100 membranes thick, and acts as an electrical insulator of the axon, preventing transfer of ions between axonal cytoplasm and the extracellular fluids.

Electrical activity is confined to the nodes of Ranvier, the only sites where ions can flow across the axon membrane. An action potential at one node results in the initiation of an action potential at the next node and so on, and is termed saltatory conduction. This occurs because the membrane depolarisation associated with an action potential spreads passively through the axonal cytoplasm to the next node with very little loss or attenuation; sodium ions are capable of moving across the axonal membrane only at the myelin-free nodes. Potassium ions can move at paranodes. The conduction velocity of myelinated nerves is much greater than that of non-myelinated fibres of the same diameter. The greater longitudinal spread of current, due to myelin insulation, increases the velocity of conduction (Darnell, Lodish and Baltimore, 1986).

The most rapid deposition of myelin occurs at 5 days postnatally in rats (Morell, 1984) and further myelination does not normally occur after the first few weeks of life.

1.3.4 Composition

Myelin has a high percentage of lipid content; an 80:20 lipid to protein weight ratio, which is very high when compared to other membranes (Guidotti, 1972), which tend to display a 50:50 ratio. For example, the oligodendrocyte plasma membrane which is continuous with myelin in the CNS, has a lipid to protein ratio of 55:45 (Poduslo 1975). The lipid composition of myelin is shown in Figure 1.4.

Myelin has an abundance of glycolipids such as cerebrosides and sulphatides which account for 15-20% of the myelin (Yao, 1984). Lamellar myelin also contains water and salts. The water, which is present in the spaces between the cytoplasmic and extracellular surfaces of the apposed membranes, constitutes 35-45% of the total volume of myelin. Water provides a milieu for the salts which are crucial for maintaining the close packing of membrane surfaces. These salts in lamellar myelin may also be involved in the ionic events which occur at the node of Ranvier during nerve impulse conduction (Padron and Mateu, 1981).

Reorganisation of the glial membrane components occurs during myelination. For example, the ratio of galactocerebroside (GC) to cholesterol increases as myelin is compacted.

LIPID COMPOSITION OF CNS MYELIN MEMBRANE %

Cholesterol	22
Phosphatidylcholine	11
Sphingomyelin	6
Phosphatidylethanolamine	14
Phosphatidylserine	7
Glycolipids	12

(From Molecular Cell Biology: Darnell, Lodish and Baltimore, 1986)

LIPID COMPOSITION OF PNS MYELIN MEMBRANE (RAT) %

Cholesterol	27.2
Phosphatidylcholine	19
Sphingomyelin	17
Phosphatidylethanolamine	38
Phosphatidylserine	22
Glycolipids	21.5

(From Myelin: Morell, 1984)

Figure 1.4 Lipid Composition of Myelin

1.3.5 Myelin Specific Molecules

1.3.5.1 Po

The glycoprotein Po is the major structural protein of peripheral myelin accounting for over 50% of the total protein found in the myelin sheath (Lees and Brostoff, 1984). Po is an integral membrane protein of molecular weight 28-30kDa (Lees and Brostoff, 1984) and is expressed only by myelinating Schwann cells. Po is confined to the PNS in mammals, but has been found in the CNS of some fish (Saavedra *et al.*, 1989).

Po is first expressed on Schwann cells at the time of Schwann cell associations with axons on a 1:1 basis and remains detectable during myelin formation and compaction (Martini *et al.*, 1988; Trapp *et al.*, 1981). Significant levels of protein may be detected after two or more compact lamellae are formed (Lamperth, 1990).

After myelination ceases, Po protein is no longer detectable in uncompacted areas eg. Schmidt-Lanterman incisures, paranodal loops and outer and inner mesaxons, nor is it found on basement membranes, interstitial collagen or non-myelin-forming Schwann cells (Martini *et al.*, 1988).

Immunocytochemical studies have shown the specificity of Po for peripheral myelin and its distribution in compact myelin. Po is synthesised by the Schwann cell in contact with axons, Po protein being most abundant in cytoplasmic areas which are rich in Golgi complex membranes (Trapp *et al.*, 1981), however, Po being a transmembrane protein is also expressed at the extracellular membrane surfaces of myelin lamellae. Application of the agent monensin (a fungal metabolite from *Streptomyces cinnamonensis*) has provided indirect evidence that Po glycoprotein passes through the Golgi apparatus en route to myelin (Rapaport *et al.*, 1982). Monensin disrupts trafficking of membrane receptors through the Golgi and inhibits processing of glycoproteins (Tartakoff, 1983).

Po protein is completely insoluble in aqueous form. The amino acid composition contains a high proportion of non-polar amino acids while the carbohydrate content is relatively low (approx. 6% by weight) (Morell, 1984). Po is glycosylated and contains galactose and mannose and can be phosphorylated and sulphated. Po is trypsin sensitive; it removes a hydrophilic portion of the molecule that represents approximately 30% of the total protein. The remainder of the molecule is hydrophobic and is probably buried within the lipid bi-layer. The extracellular domain of Po contains a region structurally similar to immunoglobulin and accordingly Po has been classed in the immunoglobulin "superfamily" alongside major histocompatability antigens and the neural cell

adhesion molecule N-CAM (Lemke *et al.*, 1988; Lai *et al.*, 1987). Figure 1.5 demonstrates the position of Po within the myelin membrane.

The mouse Po gene is located on chromosome 1 (You *et al.*, 1991; Kuhn *et al.*, 1990). Northern blot analyses indicate the Po gene is transcribed as a single mRNA species approximately 1.9kb long (Lemke and Axel, 1985). The Po gene is relatively small and, unlike MBP, does not contain large introns. Lemke and Axel (1985), have cloned a 1.85kb cDNA which hybridises with a single species of RNA 1.9-2.0kb in length. The gene, in both the rat and mouse, is split into 6 exons distributed over 7kb of DNA, interrupted by 5 introns (the first of which is much larger than the remaining four). The segregation of these exons is consistent with the functional segregation of Po protein into the extracellular, membrane spanning and cytoplasmic domains (Fig. 1.6) (Lemke *et al.*, 1988). Exon I encodes 5'-untranslated mRNA sequences together with most of the protein's amino-terminal signal sequence. Exons II and III together encode the Po extracellular domain. Exon IV encodes the transmembrane domain of the protein. Exon V encodes a portion of the cytoplasmic domain and exon VI the remainder of this domain, as well as the complete 3'-untranslated region (Lemke *et al.*, 1988). The boundary between the extracellular and membrane-spanning regions is delimited by the intron separating exons III and IV. The junctions between exons II and III and exons V and VI fall between codons (Figure 1.6). A full length cDNA of Po has recently been isolated from foetal human spinal cord (Hayasaka *et al.*, 1991). This clone is 1948bp and contains a 744bp open reading frame encoding a polypeptide of 248 residues. The deduced amino acid sequence is highly homologous to rat Po protein.

Several features of Po primary structure are relevant to its localisation in the myelin membrane. The initiator methionine at nucleotide 32 is followed by 28 uncharged and/or non-polar amino acids which precede the N-terminal isoleucine of the mature protein. This domain almost certainly comprises the signal sequence necessary for the translocation and insertion of nascent Po into the Schwann cell membrane (Lemke and Axel, 1985). The 5'-untranslated region of the Po gene contains an identical sequence of nucleotides as the MBP gene explaining previous speculations that these two genes are co-ordinately regulated (Lemke *et al.*, 1988). The Po transcript represents around 10% of the total mRNA present in the sciatic nerve during periods of active myelination in developing rat (Stahl *et al.*, 1990).

Mouse Po gene disruption leads to hypomyelination, abnormal expression of recognition molecules, and degeneration of myelin and axons (Giese *et al.*, 1992).

Extracted from: **Lemke G.** (1988). Unwrapping the genes for myelin. In: *Neuron.* **1,** 535-543.

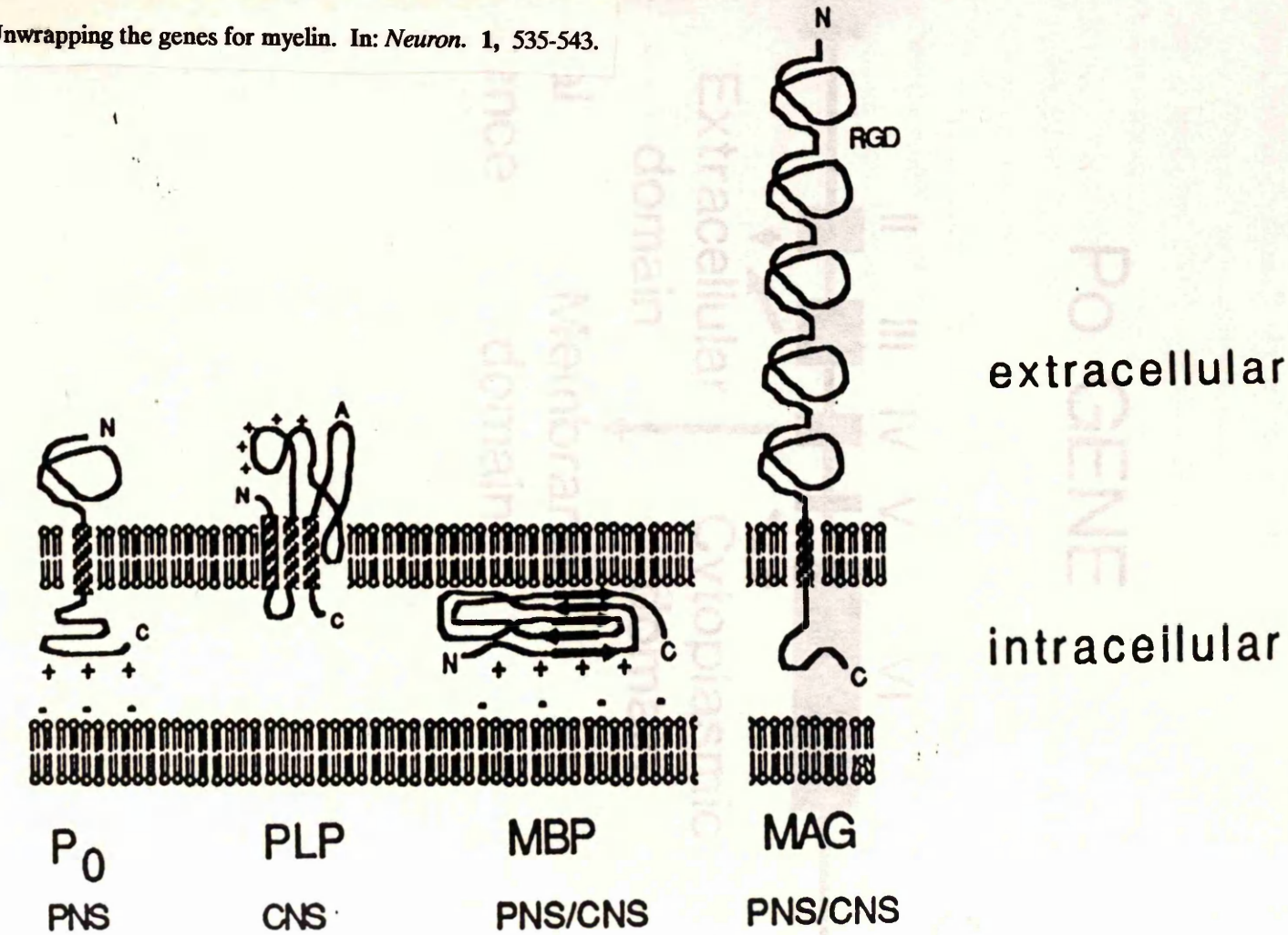


Figure 1.5 The position of Po within the myelin membrane in relation to other myelin proteins.

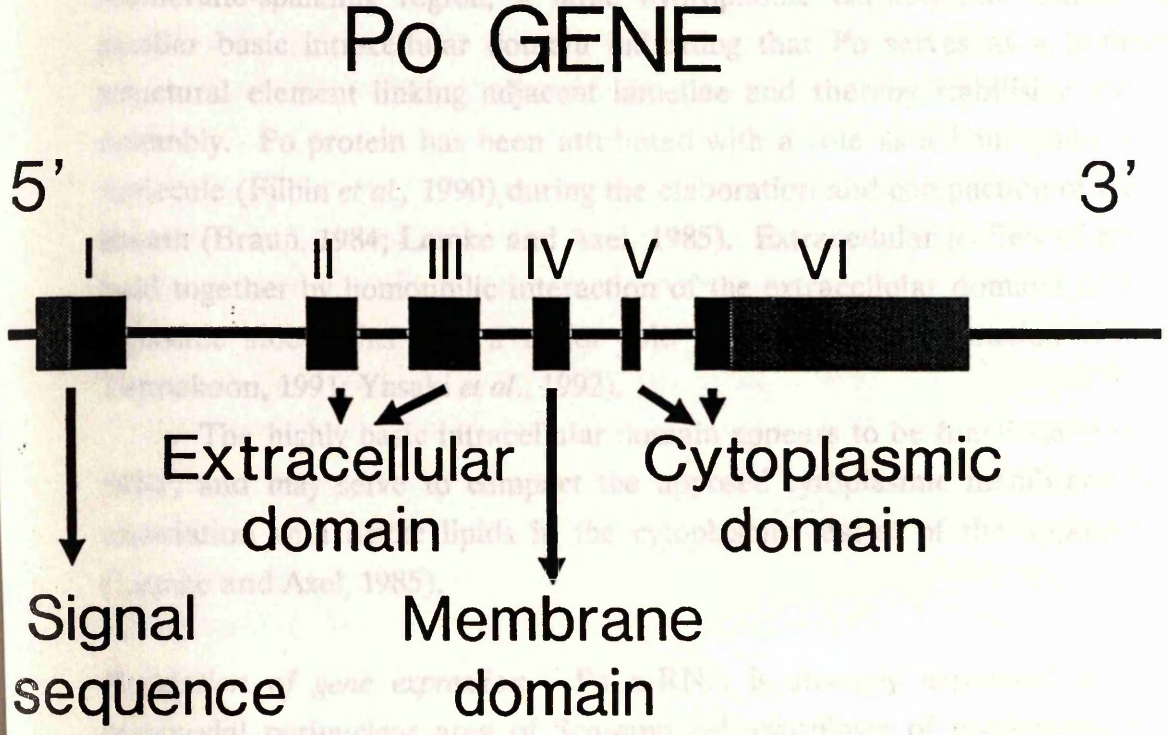


Figure 1.6 The Po Gene

Function of Po protein. The amino acid sequence has been defined for bovine Po (Sakamoto *et al.,* 1987), and has been deduced from cDNAs for rat (Lemke and Axel, 1985), chicken (Barbu, 1990), and shark (Saavedra *et al.,* 1989) Po.

The deduced amino acid sequence of mammalian Po (Lemke and Axel, 1985), confirms that Po is an integral membrane protein comprising a single membrane-spanning region, a large hydrophobic extracellular domain and a smaller basic intracellular domain indicating that Po serves as a bi-functional structural element linking adjacent lamellae and thereby stabilising the myelin assembly. Po protein has been attributed with a role as a homophilic adhesion molecule (Filbin *et al.,* 1990) during the elaboration and compaction of the myelin sheath (Braun, 1984; Lemke and Axel, 1985). Extracellular leaflets of myelin are held together by homophilic interaction of the extracellular domains of Po. The glycoside side-chains play a major role in this adhesive function (Filbin and Tennekoon, 1991; Yasaki *et al.*, 1992).

The highly basic intracellular domain appears to be functionally related to MBP, and may serve to compact the apposed cytoplasmic membrane faces by association with acidic lipids in the cytoplasmic leaflet of the apposed bilayer (Lemke and Axel, 1985).

Regulation of gene expression. Po mRNA is strongly expressed in the mid-internodal perinuclear area of Schwann cell cytoplasm of myelinating Schwann cells (Griffiths *et al.,* 1989). Po mRNA is readily detectable in rat sciatic nerve at birth, increases approximately 10-fold by P14, and then falls to a steady state basal level in the adult (Lemke and Axel, 1985). Expression of this gene is influenced by the presence or absence of myelin-competent axons. Axonal effects observed by several workers have indicated mediation at transcriptional, post-transcriptional, and protein processing and sorting steps (LeBlanc *et al.,* 1987; Poduslo *et al.*, 1985; Brunden and Poduslo, 1987). Following transection of peripheral nerve in P35 rats, a time when myelin deposition is just below peak levels observed at P21, axons distal to the transection degenerate and myelin-forming Schwann cells become markedly down-regulated with respect to expression of certain myelin-specific molecules including Po (Trapp *et al.,* 1988). In actively myelinating Schwann cells the down-regulation of the Po gene occurs at the level of mRNA expression and the decrease occurs simultaneously along the distal stump of a transected nerve. Trapp and colleagues (1988) also showed, using *in situ* hybridisation techniques, that the distribution of Schwann cells expressing Po mRNA in the distal stump following nerve transection is similar to that observed in

intact nerve, however the number of grains around any particular Schwann cell was markedly reduced to basal levels. Therefore Po message levels are down-regulated generally in all myelin-forming Schwann cells rather than differentially in a select sub-population. Northern blot analysis of the lowered levels of Po mRNA extracted from transected nerves showed the Po mRNA to be intact and not degraded in any way (LeBlanc *et al.*, 1987). The down-regulation of Po protein in transected nerve may also result from lysosomal degradation of the protein shortly after its translation (Brunden and Poduslo, 1987).

Following sciatic nerve crush and regeneration, mRNA levels for Po, MBP and MAG increase in a proximal to distal direction from the crush site (Mitchell *et al.*, 1990; Gupta *et al.*, 1988). There appears to be a close spatial relationship between the regenerating axonal tip and the induction of myelin protein genes and only a limited axon-Schwann cell contact is required for upregulation. MAG is upregulated earlier than Po, and Po and MBP appear to be co-ordinately expressed (Mitchell *et al.*, 1990; Gupta *et al.*, 1988). A temporal lag in the appearance of Po protein following Po gene expression in regenerating nerve supports the hypothesis for post-transcriptional regulation (Gupta *et al.*, 1988). The regulation of the expression of Po in peripheral nerve myelin occurs predominantly at the transcriptional level in neonatal animals, whereas in adults the control of Po gene expression is thought to be mainly at the post-translational level (Poduslo, 1987).

Po glycoprotein has been shown to undergo several post-translational modifications (Brunden and Poduslo, 1987; Poduslo, 1990). During development, and following crush injury, Po sulphation has been shown to occur within the Golgi apparatus as a post-translational modification of the oligosaccharide chain which is dependent on processing beyond the action of mannosidase I. This does not, however, occur following nerve transection (Poduslo, 1990). Golgi sulphation of the oligosaccharide chain occurs in the presence of myelin assembly, but not in its absence. Po phosphorylation is not dependent on active myelination nor protein translation (Brunden and Poduslo, 1987). Neither sulphation nor phosphorylation are observed in the absence of myelin assembly, therefore these modifications probably play a critical role during myelin assembly, compaction and maintenance. Po sulphation is associated with active myelination suggesting that this Golgi modification of the oligosaccharide chain may function in targeting the protein to the site of myelin assembly. Sulphation of Po can alter its macromolecular shape, and promote intramolecular associations critical to the adherence of the intraperiod line of myelin. The intraperiod line is the site where the Po oligosaccharide exists (Poduslo, 1990). Since Po is not phosphorylated in

transected nerve, this modification cannot be associated with the biosynthesis or processing of Po in the endoplasmic reticulum or Golgi apparatus, therefore it must occur after insertion of Po into the myelin membrane.

1.3.5.2. P_1 and P_2

P_2 is a soluble basic protein of molecular weight 14-15kDa (Brostoff, 1984), and first appears developmentally in rat sciatic nerve and DRG on the first day after birth (about the same time as Po and P_1), after the appearance of galactocerebroside and sulphatide. P_2 is not detectable prior to the onset of myelination (Winter *et al.*, 1982). In adult sciatic nerve and dorsal and ventral roots P_2 shows an uneven distribution and is absent from some myelinated axons. P2 has high homology to fatty acid binding proteins and may be important in interacting with lipids in myelin (Narayanan *et al.*, 1991).

P1 is identical to the large molecular weight form of CNS MBP and is discussed below.

1.3.5.3 Myelin Basic Protein (MBP)

MBP is common to both the PNS and the CNS, but is present in greater quantities in the CNS. It is one of the major structural proteins in myelin.

MBP consists of 6 closely related isoforms and are relatively small, ranging from 14-21.5kDa. MBP is an extrinsic membrane protein and is localised in myelin at the major dense line and confined to the cytoplasmic region of the membrane. The MBP gene is located on chromosome 18. MBP mRNA is detected early in myelination. MBP mRNA is concentrated around the Schwann cell nuclei during the initial stages of myelination (Trapp *et al.*, 1987), then becomes distributed diffusely over myelinated fibres, but with higher concentrations at paranodes (Griffiths *et al.*, 1989). This contrasts with PLP and Po which remain concentrated around the oligodendrocyte and Schwann cell nuclei respectively. The localisation of MBP mRNA within the cytoplasmic domains of myelin indicates that protein sorting during myelination involves transportation of mRNA to specific subcellular sites (Trapp *et al.*, 1987) and indicates that MBP is synthesised near the site of its insertion into the myelin membrane. Some myelin proteins are synthesised in the perikaryon and are subsequently transported to myelin membranes for insertion. MBP enters myelin within a few minutes following protein synthesis while PLP and Po enter myelin approximately 30 minutes following their synthesis (Colman *et al.*, 1982).

> The difference in sorting between PLP, Po and MBP is due to the requirement for post-translational modification of the former two proteins.

1.3.5.4 Myelin Associated Glycoprotein (MAG)

MAG is a cell adhesion molecule, and a member of the immunoglobulin gene superfamily. It is one of the first myelin-specific molecules to be expressed by Schwann cells during early myelination, and declines thereafter (Sternberger *et al.*, 1979). MAG is expressed solely on myelin-committed Schwann cells and is absent on non-myelin-forming Schwann cells (Jessen *et al.*, 1987a). MAG is present in membranes of Schmidt-Lanterman incisures, paranodal loops, and the inner and outer mesaxon and at the periaxonal space, where it possibly maintains the periodicity of these membranes (Trapp and Quarles, 1982). MAG is absent in compact myelin.

The molecule is an integral membrane protein and has a molecular weight of 100kDa, of which 30% is carbohydrate (Trapp, 1990). It occurs in two developmentally regulated forms with different carboxyterminal cytoplasmic domains: L-MAG and S-MAG (Salzer *et al.*, 1987). Sequencing of MAG cDNA has shown that the two forms of molecule arise from a single gene by alternative splicing (Lai *et al.*, 1987; Salzer *et al.*, 1987). L-MAG is a 72kDa polypeptide, (S-MAG is 67kDa), and is the predominant form of MAG during early and active stages of myelination in the CNS, whereas S-MAG predominates in the mature CNS. In the PNS, S-MAG represents around 95% of the total MAG during all stages of myelination (Trapp, 1990).

Recent elucidation of MAG cDNA sequences have shown that the extracellular region of MAG shares homology with N-CAM and other members of the immunoglobulin gene superfamily (Lai *et al.*, 1987; Salzer *et al.*, 1987; Arquint *et al.*, 1987). The amino acid sequence of MAG (CNS) deduced from cDNA clones demonstrates a single transmembrane protein, a large extracellular domain containing five immunoglobulin-like regions and eight potential N-linked glycosylation sites and one of the two possible cytoplasmic domains contains phosphorylation sites (Trapp, 1990).

MAG mRNA, when studied with *in situ* hybridisation techniques exhibits a focal signal associated with the Schwann cell perinuclear cytoplasm, similar to Po, however MAG is expressed at a much lower abundance than Po (Mitchell *et al.*, 1990). The MAG mRNA transcript isolated from rat sciatic nerve is 2.5kb. MAG is upregulated in Schwann cells by axon-Schwann cell contact and occurs prior to the 1:1 alignment of axon:Schwann cell. The molecule is located at the forming internode and therefore must have a role in the early stages of interaction between Schwann cell and axon. MAG seems to be critical for the segregation of large axons destined to be myelinated and for linear extension of myelinating Schwann cells along axons (Owens and Bunge, 1989). It has also been demonstrated that

liposomes containing purified MAG exhibit MAG-dependent binding to neurites (Johnson *et al.*, 1989; Poltorak *et al.*, 1987).

1.3.5.5 Proteolipid protein (PLP)

PLP is the major structural protein of the CNS and is expressed in oligodendrocytes. PLP comprises almost 50% of adult CNS myelin protein and is an integral membrane protein which may play a role in linking the apposed outer membrane surfaces within the myelin sheath (Braun, 1984).

PLP mRNA is also expressed on myelinating Schwann cells in the PNS, and PLP protein is synthesised by Schwann cells, although it is not incorporated into the peripheral myelin sheath (Puckett *et al.*, 1987). However, a more recent study (Agrawal and Agrawal, 1991) has provided evidence that PLP and DM20 (see below), are incorporated into the myelin membrane following their synthesis in peripheral nerve Schwann cells. This study also observed that PNS PLP differs from CNS PLP in that neither PLP nor DM20 in the PNS are acylated as they are in the CNS, possibly indicating different functional roles. The autoacylation of PLP in the CNS is thought to be important in the incorporation of PLP into the CNS myelin sheath, but this seems to be inhibited in the peripheral nerve (Bizzozero *et al.*, 1987).

PLP mRNA has been found in human acoustic neuromas and in rat and rabbit sciatic nerves using a human cDNA probe. PLP mRNA is present in the perinuclear cytoplasm of Schwann cells (Griffiths *et al.*, 1989). PLP transcript levels in rat sciatic nerve are very low in P21 animals and remain unchanged with adults, which contrasts with levels of Po and MAG mRNA (Gupta *et al.*, 1991). PLP function in the PNS is not known, however DM20 predominates over PLP in the PNS (by 2-fold in P14 rats) (Pham-Dinh *et al.*, 1991). PLP transcription in the peripheral nerve does not seem to be closely associated with axonal influences (Gupta *et al.*, 1991). This contrasts with observations for the other myelin proteins Po and MAG which are controlled by Schwann cell/axon interactions at the transcriptional level. PLP mRNA remains fairly constant following peripheral nerve transection or crush injury whereas Po and MAG levels drop dramatically under the same conditions (Gupta *et al.*, 1991).

1.3.6 Regulation of Myelin Gene Expression and Myelination

Myelinogenesis is a highly co-ordinated phenomenon in which a series of genes is transcribed at a particular stage of PNS development. Regulation of transcription is achieved either by induction of a myelin enzyme operon or by

sequential transcription, where the synthesis of one enzyme or its products regulates the transcription of the following genes.

PNS myelination commences at birth, increases and peaks at around 2 weeks post-natally in the rat, decreasing to a basal level by 4-5 weeks post-natally (Webster, 1971). In 2-3 week-old rats, around 10% of the mRNA in sciatic nerve encodes myelin proteins, whereas the corresponding value in adult rats is 2% (Stahl *et al.*, 1990). In the rat sciatic nerve, the level of expression of the MAG gene occurs maximally at P13, with peak expression of MBP and Po at P21. The genes encoding PLP and CNP are not developmentally expressed in correlation with sciatic nerve myelination. Po and MBP mRNA levels during development are very high, whereas MAG, PLP and CNP are much lower (Stahl *et al.*, 1990).

PLP expression rises from P1-P5 and remains elevated in 8-9 month-old animals. CNP message is found in maximal quantities at P1 and subsequently decreases gradually. Expression of PLP and CNP correlate well with myelination in the CNS (but not PNS). PLP and CNP proteins are not incorporated into peripheral myelin (Puckett *et al.*, 1987).

Axonal Regulation: Schwann cell expression of myelin proteins including Po and MBP genes depends on neuronal signals (Baron *et al.*, 1990). The neuron plays a dominant role in regulating the function and metabolic activity of axon-ensheathing Schwann cells (Spencer and Weinberg, 1978). Myelination by a Schwann cell is regulated by the axon with which it is associated (Aguayo *et al.*, 1976a; Weinberg and Spencer, 1976). Po and MBP have a similar developmental profile although relative increase in MBP transcription between P1 and P10 is considerably less than that for Po. Po and MBP messages are also modulated uniformly along the nerve throughout development suggesting signals regulating Po and MBP do not display a proximal-distal gradient.

Several hypotheses have been proposed as to the signalling mechanism between Schwann cells, and axons. Early impressions were that myelination is initiated when a critical axonal diameter is reached (Duncan, 1934; Matthews, 1968). This seems unlikely to be the sole factor involved (Spencer and Weinberg, 1978). Myelination *in vitro* is dependent on neuronal maturation, and Schwann cells *in vivo* become associated with the growing tips of myelin-competent axons in preference to those of unmyelinating axons, suggesting the signal switching on Schwann cells to form myelin is some form of chemical messenger derived by the neuronal perikaryon and transported via the axon to the Schwann cell.

Only axons greater than a certain diameter seem destined to be myelinated by Schwann cells and it has been suggested that alterations in axonal size mediate

the neuronal regulation. Friede (1972) theorised a 3-stage process involving stretching, elongation of the Schwann cell plasmalemma at the mesaxon to accommodate axonal expansion, the transformation of each unit of plasmalemma into units of myelin to produce new lamellae, and slippage between existing lamellae to adjust the sheath to further axonal enlargement. This theory encompasses the concept of a critical axonal diameter at which myelination will proceed, and the existence of a linear relationship between axon circumference and myelin thickness in the adult animal.

Axonal control and regulation may be demonstrated using transection or crush injury experiments and cross-anastomosis experiments.

Cross-anastomosis evidence for axonal regulation: Observations of cross-anastomosis in myelinated and non-myelinated nerve trunks shows, for example, that Schwann cells of a previously non-myelinated distal nerve segment or graft are induced to form myelin sheaths when the axons of a myelinated nerve sprout into that segment and vice versa (Aguayo *et al.,* 1976a; Aguayo *et al.*, 1976b).

Transection nerve injury; evidence for axonal regulation. Schwann cells require contact with axons both for initial induction of myelin-specific genes and for maintenance of expression (Bray *et al.*, 1981). Transection of myelinated peripheral nerves at a time when myelination is well advanced causes a dramatic down-regulation of mRNAs encoding Po and MBP proteins distal to the transection site (Lemke *et al.*, 1990). By 5 days post-transection, Po and MBP mRNA levels have fallen 40-fold to a basal level (Trapp *et al.,* 1988). The decrease occurs simultaneously along the entire distal stump. A similar down-regulation occurs in sciatic nerve Schwann cells cultured without neurons, which is essentially another method of transection or nerve injury (Mirsky *et al.,* 1980; Lemke *et al.,* 1990). Basal levels of mRNA are reached by 5 days *in vitro*. Lemke and Chao (1988) demonstrated that the low levels of Po and MBP mRNAs expressed by Schwann cells in culture in the absence of axons can be dramatically elevated by forskolin-induced increases in intracellular cyclic AMP. This second messenger strongly potentiates the transcriptional activity of the cloned promoter region of the rat Po gene. This promoter is active only when cells are cultured in the presence of an agent which elevates cAMP. Cyclic AMP is a potential mediator of axonal influences on myelin gene expression *in vivo* (Lemke and Chao, 1988). Poduslo *et al.* (1992), suggested cAMP acts via the axon and may require intact axons for full effect. Axonal dependence of Schwann cell gene expression *in vivo* and *in vitro* reflects changes in the instantaneous rate of transcription of the

major myelin genes, rather than alteration in the turnover rate of the mRNAs transcribed from them (Lemke *et al.*, 1990).

Permanent transection of adult myelinated nerves compared to early post-natal or young adult nerves results in a much more modest decrease (down by 3-fold) of Po mRNA (LeBlanc *et al.*, 1987), an alteration in the glycosylation pattern of Po protein (Poduslo, 1987), and an augmentation of the rate at which Po protein is delivered to lysosomes (Brunden and Poduslo, 1987). Taken together, these studies indicate that axonal effects are mediated at transcriptional, post-transcriptional, protein processing and protein sorting steps. Po protein expression on older adult animals is less sensitive to axon withdrawal than expression in neonates (Poduslo and Windebank, 1985).

Permanent sciatic nerve transection studies have shown that two species of Po occur (28.5 and 27.7kDa). By 14 days following transection only the 27.7kDa species is detectable (Poduslo *et al.*, 1985). The biosynthesis of complex oligosaccharide chains on glycoproteins involves high-mannose type intermediates not present in the 27.7kDa species, therefore this seems to be the mechanism by which Po is down-regulated, and myelin maintenance suspended (Poduslo *et al.*, 1985).

Axons are capable of both positive and negative regulation of Schwann cell gene expression, for example, NGF receptors and NGF-R mRNA increase markedly on withdrawal of axonal contact (Taniuchi *et al.*, 1986; Lemke and Chao, 1988).

1.4 BIOLOGY OF THE SCHWANN CELL

1.4.1 Origin and morphology

Ontogenetically, Schwann cells are believed to be of ectodermal origin, migrating from the neural crest into the PNS, during development (Harrison, 1924). As axons egress from the CNS, and enter the PNS they are followed by migrating Schwann cells.

The Schwann cell, named after Theodore Schwann (1810-1882) who first identified the cell in 1839, is of flattened appearance with an elongated, spindle-shaped nucleus located approximately in the centre of each myelinated PNS internode. The Schwann cell nucleus possesses clumped peripheral chromatin and is elongated in the length of the axon. The perinuclear accumulation of cytoplasm contains a profusion of organelles including RER, SER, Golgi membrane, mitochondria, centrioles, lipid vacuoles and Reich granules which accumulate with age and may be classed as lysosomes. Lipofuscin accumulates with age in Schwann cells associated with unmyelinated axons but not in those associated with myelinated axons.

The myelinating Schwann cell soma lies within a concavity of the myelin sheath and is circumferentially covered by a basal lamina that is continuous with that of the adjacent internode. The Schwann cell plasmalemma is a triple-layered membrane possessing caveolae especially in the perinuclear region and adjacent to incisures. These may increase the surface area of the Schwann cell thus compensating for stretching of the fibre during movement.

The size of Schwann cells varies greatly between Schwann cell types. For example, in human peripheral nerve, Schwann cells from myelinated fibres may range in length from 100-1800*u*m whilst unmyelinated Schwann cells vary between 20-30*u*m. An ultrastructural study by Fields and Raine (1982) estimated neonatal rat sciatic nerve elongated Schwann cells to be 40-50*u*m, bi-polar in shape and easily distinguishable morphologically from fibroblasts in culture. The diameter of myelinated fibres is much greater (2-22*u*m) than non-myelinated fibres (0.5-3.5*u*m). Internodal length is usually 100-200 times the axonal diameter. Axonal diameter will be discussed later as it is a critical factor in the initiation of myelination.

1.4.1.1.Cultured Schwann cells

Morphology and characteristics:

Typical Schwann cells in culture are small, spindle-shaped, with an oval nucleus, a bi-polar configuration and narrow tapering processes extending up to 100*u*m in opposing directions. They tend to aggregate from side to side or

longitudinally and have a tendency to align themselves alongside neurons, if present. Occasionally Schwann cells may adopt a flatter, more amoeboid shape and may be confused with fibroblasts, hence the need for specific markers. This flatter type of Schwann cell is more often associated with secondary than primary dissociated sciatic nerve cultures (Brockes *et al.,* 1979). The choice of culture medium also has an effect on morphology, which will be shown in the results section.

In DRG cultures, Schwann cells associate with single or fasciculated neurites and in the presence of serum and ascorbic acid (vitamin C) will develop a basal lamina and acquire extracellular fibrils oriented in parallel to the axis of both Schwann cells and neurites. These Schwann cells display similar ultrastructural features to sciatic nerve (neuron-free) cultures, including highly developed Golgi complexes and RER.

1.4.2 Function

The principle function of the myelinating Schwann cell is the formation and maintenance of the myelin sheath. Other functions include providing structural support and elasticity for axons along with endoneurial collagen. Schwann cells affect the differentiation and molecular expression of the neuron and help support neurons metabolically during activity. They may also produce collagen. Schwann cells may also be involved in the regulation of the extracellular environment and the synthesis of trophic and adhesion molecules (see Seminars in the Neurosciences, 2:1990 for several reviews by Bevan; Bunge and Hopkins; Hudson; Lemke; and Schachner). Schwann cells also have the ability to present antigens to activated T cells of the immune system thus having a potential role in the response of peripheral nerves to infection and injury (Mirsky and Jessen, 1990).

The transfer of molecules such as Lucifer Yellow from axon to glial cells has been demonstrated in crayfish (Viancour *et al.,* 1981). In the squid, transfer of proteins from periaxonal glia to the axon has been demonstrated (Gainer *et al.,* 1977; Tytell and Lasek, 1984). These results suggest a two-way exchange of molecules between axon and Schwann cell. The relationship between Schwann cells and axons appears therefore to have mutually beneficial effects.

It may also be possible for Schwann cells to phagocytose organelles and debris from both normal and injured or diseased axons, however this will be discussed later.

1.4.3 Development; *Myelin-forming and non-myelin-forming Schwann cells*

During development, Schwann cells undergo dramatic alterations in morphology and phenotype resulting in the formation of myelin sheaths around larger diameter axons or the surrounding of smaller axons to form enveloped but unmyelinated fibres.

Schwann cells associated with myelin differ in molecular expression from those which merely encompass the smaller diameter axons. Myelin-forming and non-myelinating-forming Schwann cells exist as two populations exhibiting distinctly dissimilar antigenic phenotypes and may be characterised by various cellular markers such as NGF-R and Po protein. It is probable, however that the two types of Schwann cell derive from a common precursor glial cell (Jessen *et al.,* 1989), and when removed from axonal contact, both types revert to an identical naive phenotype comparable to that of Schwann cells in early embryonic nerves prior to myelin formation (Mirsky and Jessen, 1990). Myelin-forming Schwann cells express myelin-specific molecules including Po, MBP and MAG. In contrast, non-myelin-forming Schwann cells do not express these proteins but exhibit other markers such as GFAP, NGF-R and N-CAM. Some markers will be displayed by both populations, for example, GC, O_4, S100, vimentin and laminin.

1.4.3.1 Schwann cell markers

To complement morphological identification of cell types, biochemical cell-specific markers are now used widely for *in vitro* studies on development and function. Several markers exist which are Schwann cell-specific and some will differentiate between different Schwann cell types. Figure 1.7 shows antigen expression on Schwann cells.

Temporal expression of Schwann cell markers

Precursor Schwann cells express vimentin, laminin, Ran-2, L1, N-CAM and NGF-R. Jessen *et al.* (1989) described the precursor Schwann cell as being NGF-R positive and S100 negative. This precursor is present in rat sciatic nerve at E15. S100 which appears at E16, distinguishes early Schwann cells from precursors and is non-reversible. S100 is expressed by all Schwann cells *in vivo* after E18, and *in vitro*. It is therefore a useful tool to monitor Schwann cell development. Almost immediately after S100, Schwann cells express the surface lipid O_4 (E16)(Mirsky *et al.,* 1990) and this expression is axonally controlled (Jessen *et al.,* 1989). Embryonic Schwann cells also express many proteins which in the mature Schwann cell occur only with the non-myelin-forming phenotype, for example, N-CAM, L1 and NGF-R.

At E18-19 the first GC-positive cells appear

Antigens expressed by myelin-forming Schwann cells;

CNP	MAG
Po	PLP
MBP	0_{11}

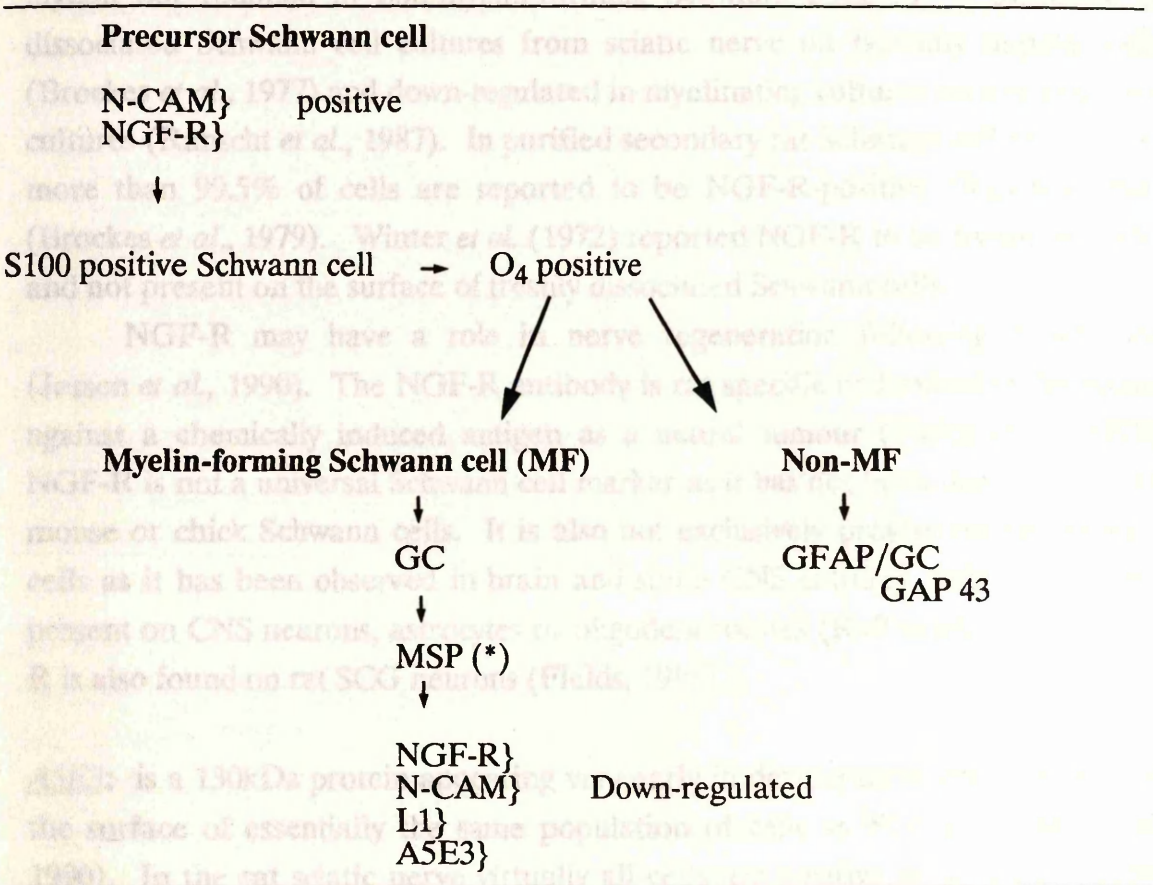
Antigens expressed by non-myelin-forming Schwann cells;

GFAP	L1
NGF-R (Ran-1/217C)	Ran-2
N-CAM	A5E3

Antigens present on both phenotypes;

S100	Vimentin
Laminin	0_4
GC (R-mAb/H8)	

Figure 1.7 Antigen Expression on Schwann Cells



* (MSP = Myelin-specific Proteins)

Figure 1.8 Antigenic Development of Schwann Cells

and these follow the myelin pathway and progress to express Po at around P1. Figure 1.8 outlines the summary of molecule development.

The myelin-forming Schwann cell develops after the non-myelin-forming Schwann cell (Jessen *et al.*, 1989).

NGF-R: is also recognised as 217C or Ran-1 (Ferrari *et al.*, 1991). It is expressed by the majority of cells in rat embryonic sciatic nerve at E15 and by almost all cells by E18 (Jessen *et al.*, 1990). NGF-R is suppressed on Schwann cells which form myelin but retained in non-myelin-forming Schwann cells. It is expressed in dissociated Schwann cell cultures from sciatic nerve on typically bi-polar cells (Brockes *et al.*, 1977) and down-regulated in myelinating cultures such as DRG co-cultures (Ranscht *et al.*, 1987). In purified secondary rat Schwann cell populations more than 99.5% of cells are reported to be NGF-R-positive (Ran-1-positive) (Brockes *et al.*, 1979). Winter *et al.* (1972) reported NGF-R to be trypsin sensitive and not present on the surface of freshly dissociated Schwann cells.

NGF-R may have a role in nerve regeneration following denervation (Jessen *et al.*, 1990). The NGF-R antibody is rat specific and raised in the mouse against a chemically induced antigen as a neural tumour (Fields *et al.*, 1978). NGF-R is not a universal Schwann cell marker as it has not been demonstrated on mouse or chick Schwann cells. It is also not exclusively present on rat Schwann cells as it has been observed in brain and some CNS cultures, although it is not present on CNS neurons, astrocytes or oligodendrocytes (Raff *et al.*, 1979). NGF-R is also found on rat SCG neurons (Fields, 1985).

A5E3: is a 130kDa protein appearing very early in development and is present on the surface of essentially the same population of cells as NGF-R (Jessen *et al.*, 1990). In the rat sciatic nerve virtually all cells are positive at birth but myelin-forming Schwann cells rapidly lose expression.

GFAP (Glial fibrillary acidic protein): is a polypeptide of molecular weight 49kDa (Jessen and Mirsky, 1984), appearing at E18. In Schwann cells, it is located in the perinuclear area of the cytoplasm and extends to the cell processes (Neuberger *et al.*, 1989). It is restricted mainly to non-myelin-forming Schwann cells but is rapidly re-expressed on myelin-forming cells following loss of axonal contact. Unlike most other Schwann cell-associated molecules whose expression is axonally controlled, GFAP is intrinsically programmed (Jessen *et al.*, 1989). GFAP is present in virtually all Schwann cells of the CST, where 99% of all axons are unmyelinated ie. surrounded by non-myelin-forming Schwann cells. GFAP may be

observed in some satellite cells of DRG, but normally this is restricted to cervical or lumbar levels (these ganglia contain the largest neurons) (Jessen and Mirsky, 1984).

Neurofilament protein NF-M: Neurofilament proteins are not restricted to neurons, as was previously thought. Myelin-forming Schwann cells express a cytoskeletal protein of molecular weight 145 which closely resembles the intermediate filament NF-M (Kelly *et al.*, 1992). This protein is expressed prior to Schwann cells acquiring surface galactocerebroside. NF-M co-localises with vimentin, indicating that these proteins may coassemble into intermediate filaments. NF-M may be induced in Schwann cells by elevating intracellular cAMP levels.

GC (Galactocerebroside): A number of specific lipids are associated with the peripheral myelin sheath, the most abundant (about 20%) being the glycosphingolipid galactocerebroside (GC). GC is expressed on the surface of both myelin- and non-myelin-forming Schwann cells in culture (Jessen *et al.*, 1985) and may be used to identify cells in culture in both the CNS and PNS. Expression is regulated by axons in both types of Schwann cell. (Jessen *et al.*, 1985; 1987b).

Freshly isolated Schwann cells in culture display GC only during the first twenty hours *in vitro* (Mirsky *et al.*, 1980), after which it progressively decreases over the following three to four days. Cultured oligodendrocytes also express GC, but unlike Schwann cells, will continue to express the antigen indefinitely. Re-expression in Schwann cell cultures can be induced by cAMP derivatives (Sobue and Pleasure, 1984).

GC appears before the onset of myelination, after the expression of the O_4 sulphatide (Mirsky *et al.*, 1990). Developmentally, *in situ*, GC occurs firstly in the sciatic nerve, appearing at embryonic day eighteen (E18), prior to a 1:1 relationship of Schwann cell:axon. In the CST, where 99% of axons are unmyelinated, GC is first detected at E19, and by P10, over 95% of Schwann cells in this nerve are GC-positive (Po-negative).

By P5, over 60% of Schwann cells in rat sciatic nerve are both GC and Po-positive, although Po-negative Schwann cells are also GC-negative (Jessen *et al.*, 1985). By P35, around 95% of all Schwann cells in the sciatic nerve express GC (both Po-positive and Po-negative cells), showing that Po-negative Schwann cells in the sciatic nerve also acquire GC although at a later developmental stage than Schwann cells from the CST. Appearance of GC in both these nerves seems to coincide with formation of a stable post-mitotic relationship between Schwann cells and axons (Jessen *et al.*, 1985).

Functionally, GC is thought to play a role in the interaction between opposing Schwann cell membranes at the mesaxon or between the mesaxonal-periaxonal junction (Ranscht *et al.*, 1987b). A role in membrane interactions between axons and Schwann cells in mature nerves is also hypothesized. During myelination, reorganisation of the glial membrane occurs, and with respect to GC, the ratio of this glycolipid to cholesterol increases as myelin is compacted.

Antibodies to GC may induce demyelination or inhibit myelinogenesis (Brostoff, 1984).

The monclonal antibody used in this thesis to identify GC was H8 otherwise known as R-mAb, which recognises GC, sulphatide and seminolipid (Bansal *et al.*, 1989).

O antigens

O_4 antibody recognises a glycolipid, probably sulfatide, which is present on both myelin-forming and non-myelin-forming Schwann cells and appears shortly after S100 at E16 and immediately before GC (Mirsky *et al.*, 1990). The O_4 antibody also recognises seminolipid and to some extent, cholesterol (Bansal *et al.*, 1989). Expression is axon-dependent, up-regulated during Schwann cell development and down-regulated on withdrawal of axon contact either by cellular dissociation or nerve transection (Jessen *et al.*, 1987; Eccleston *et al.*, 1987). Intracellular cAMP elevation triggers expression of O_4 and GC (Mirsky *et al.*, 1990; Sobue *et al.*, 1986), and can be induced in both dividing and non-dividing cells (Morgan *et al.*, 1991).

Other "O" antigens: O_7 antibody recognises galactocerebroside. O_7, O_8 and O_9 are all expressed by Po positive myelin-forming Schwann cells from P1 through to P60 (Eccleston *et al.*, 1987). O_{11} is observed on some but not all Po positive cells and is not expressed by non-myelin-forming Schwann cells.

In the CST, O7 binds to the surface of 12% of Schwann cells at P1 and increases to around 95% by P20. O_8 antigen in CST appears after O_7 but also peaked at P20 with around 80% of cells showing positivity. O_9 antigen on CST Schwann cells increases more slowly than O_7 and O_8 and only ever involves up to 40% of the non-myelin forming Schwann cells.

O antigen expression *in vitro* decreases with time and after around 7 days cannot be detected (Eccleston *et al.* 1987).

CELL ADHESION MOLECULES: N-CAM AND L1

N-CAM is an integral membrane glycoprotein which serves as a ligand in cell-cell adhesion (Santoni *et al.*, 1988). The molecule consists of a single polypeptide chain divided into three domains, an amino-terminal region which forms a binding site, a central carbohydrate region, and a carboxy-terminal region associated with the cell membrane (Rutishauser, 1984). Three protein isoforms have been identified which have identical N-terminal regions and differ primarily in the length of their C-terminal membrane-associated and cytoplasmic domains (Rutishauser and Goridis, 1986). N-CAM is expressed on neurons, PC12 cells and glia (Keilhauer *et al.*, 1985), and may be is localised on small, non-myelinated fasciculating axons and axons ensheathed by non-myelinating Schwann cells. Schwann cells in the early stages of myelination, that is, up to the point at which the glial process has turned approximately one and a half loops around the axon, express N-CAM, and the molecule may be subsequently observed in the periaxonal areas and may be detected in compacted myelin, but not at nodes of Ranvier (Martini and Schachner, 1986). During development in the rat sciatic nerve N-CAM is present on all neonatal Schwann cells and is down-regulated on myelin-forming Schwann cells as myelination proceeds. This suppression is axon-dependent and is rapidly reversible after axotomy (Jessen *et al.*, 1987a).

L1: L1 is a 200kDa glycoprotein expressed by Schwann cells prior to the onset of myelination. Its expression is down-regulated on myelin-forming Schwann cells. L1 is an adhesion molecule and member of the immunoglobulin "supergene" family which plays a major role in the Schwann cell-dependent neurite outgrowth of DRG neurons (Seilheimer *et al.*, 1989a), neuronal migration, adhesion, fasciculation (Miura *et al.*, 1991), and the onset and process of myelination (Seilheimer *et al.*, 1989b; Wood *et al.*, 1990). Rat cDNA has been cloned, which compared with mouse L1, has a highly conserved structure, especially in the cytoplasmic domain (Miura *et al.*, 1991).

S100: The S100 protein is a water-soluble, highly acidic protein with a molecular weight of around 20kDa, composed of two sub-units, alpha and beta (Mahadik *et al.*, 1979; Isobe and Okuyama, 1981). S100 was originally thought to be specific to the nervous system (Moore, 1965; Moore *et al.*, 1968), although it has now been found elsewhere. In the nervous system, however, it is present both in the CNS and the PNS, and it is generally agreed that most of the S100 is synthesised by astrocytes, Schwann cells and oligodendrocytes. It may therefore be used in the PNS as a specific marker for Schwann cells. S100 is known to be a calcium-binding

protein and therefore is important to the ionic balance of the cell. It has been suggested that S100 stimulates phosphorylation of some proteins by kinases and inhibits phosphorylation of other proteins, and inhibits microtubule assembly (Moore, 1988).

Ran-2: Ran-2 is a glial surface antigen with a molecular weight of 140kDa present on non-myelin-forming Schwann cells (Mirsky and Jessen, 1984). Ran-2 may be related to N-CAM and L1 as the 14kDa band is similar to one of the multiple bands on both (Mirsky and Jessen, 1984).

Laminin: Laminin is an extracellular matrix glycoprotein expressed by Schwann cells prior to the development of a morphologically-recognisable basal lamina (Cornbrooks *et al.*, 1983; McGarvey *et al.*, 1984). Immunohistochemical staining of cultured (human) Schwann cells showed a distribution of laminin as a punctate pattern on both the somal area and the Schwann cell processes (Scarpini *et al.*, 1986). Expression of laminin is not down-regulated in culture (Scarpini *et al.*, 1986) and is not axon dependent (Cornbrooks *et al.*, 1983) although basal lamina formation is dependent on axonal influence. Fibroblasts do not express laminin. Laminin has been shown to promote peripheral neurite outgrowth *in vitro* (Baron van-Evercooren *et al.*, 1982; Manthorpe *et al.*, 1983). Schiff and Rosenbluth (1986) localised laminin (within the basal lamina) to the lamina lucida and the lamina densa. It is thought that laminin *in vivo* anchors the basal lamina to the Schwann cell and this theory is upheld by the fact that the lamina lucida, immediately adjacent to the plasma membrane, stains heavily with antibodies to laminin.

CNP: 2', 3'-Cyclic Nucleotide 3'-Phosphodiesterase (CNP) is the first myelin-associated polypeptide to be expressed in the CNS. CNP is a dimer of 94-98kDa and may be involved in a metabolic capacity in the Schwann cell as it hydrolyses 2',3'-cyclic nucleotides resulting in 2'-phosphates (Mezei, 1987; Yoshino *et al.*, 1985). It is not observed in compacted myelin but can be immunocytochemically visualised in oligodendrocytes before myelin is evident, and CNP mRNA is present prenatally (Braun *et al.*, 1990). CNP is concentrated in the cytoplasm of non-compacted CNS ensheathment of axons, and in paranodal loops suggesting CNP is not structurally significant in the myelin sheath.

In the PNS, CNP expression does not alter during development (Stahl *et al.*, 1990) and expression does not parallel the myelination process, as it does in the CNS. CNP mRNA is found at maximal quantities in P1 sciatic nerves and then

decreases gradually. CNP is not incorporated into PNS myelin (Puckett *et al.*, 1987).

Po, MBP, and MAG and PLP have been discussed in section 1.3.5.

1.4.3.2. Non-Schwann cell markers.

Thy-1

Since the morphology of Schwann cells can occasionally be confused with that of contaminatory fibroblasts it is useful to have a fibroblast-specific marker such as Thy-1. The Thy-1 antigen was first defined by reciprocal immunisation with mouse thymocytes (Reif and Allen, 1964). The AKR antigen, now renamed Thy-1.1 is a glycoprotein purified from rat brain (Barclay *et al.*, 1975). Thy-1 is used to identify fibroblasts and to distinguish them from Schwann cells; however some neurons have been observed as carrying the Thy-1 antigen. Neonatal DRG neurons express Thy-1 from the first day in culture (Fields *et al.*, 1978). In cultures from neonatal sciatic nerve Thy-1 in conjunction with NGF-R can be used to unequivocally identify the two cell types.

1.4.4 Proliferation of Schwann cells

Proliferation In Vivo;

Schwann cells *in vivo* undergo proliferation during two stages of functional expression: 1) in development during population of peripheral nerves with Schwann cells prior to myelination and 2) following damage to peripheral nerve, in the process of Wallerian degeneration or demyelination (Ratner *et al.*, 1987).

Proliferation during development:

Proliferation of Schwann cells is one of the first events to occur after contact with a growing axon (Aguayo *et al.*, 1976(a); Aguayo *et al.*, 1976(b)), and Weinberg and Spencer, 1976). Axons express a signal, probably axolemmal, which causes an increase in proliferation of Schwann cells *in vivo* (Aguayo, 1976; Terry *et al.*, 1974) and *in vitro* (Wood and Bunge, 1975). In developing rat sciatic nerve the increase in Schwann cell numbers is greatest between E16 and P1 and is due to proliferation within the nerve rather than from further migration from the neural crest (Peters, 1976). More than 70% of rat sciatic nerve Schwann cells have stopped dividing at the time of birth (Asbury, 1967), and in normal adults the proportion of dividing Schwann cells is less than 1% (Friede and Johnstone, 1967).

Wallerian degeneration, demyelination and regeneration:

Interruption of axonal transport *in vivo* either by mechanical trauma (crush injury or axotomy) or by exposure to neurotoxic substances results in axonal degeneration. Wallerian degeneration is characterised by a gradual dissolution of the distal segments of nerve and the compact myelin sheath around the affected axons breaks down (Weinberg and Spencer, 1978). The Schwann cells associated with degenerating axons assume a distinct pattern of behaviour; they ingest myelin debris, acting as facultative phagocytes (Perry and Brown, 1992) and a marked proliferation of Schwann cells occurs in the distal nerve, characteristic of Wallerian degeneration (Romine *et al.*, 1986; Abrahams *et al.*, 1980). Mitosis of Schwann cells during Wallerian degeneration commences at between 24-72 hours following nerve trauma (Crang and Blakemore, 1986; Pellegrino *et al.*, 1986; Abrahams *et al.*, 1980). Autonomic nerves do not myelinate *in vitro* or *in vivo*, and there is very little Schwann cell response in Wallerian degeneration in autonomic nerves (Romine *et al.*, 1986).

Schwann cells are capable of ingesting myelin debris (Weinberg and Spencer, 1978). Phagocytosis by Schwann cells depends on their state of differentiation and proliferative capacity. If cell proliferation is inhibited, for example, by cytosine arabinoside, no phagocytosis is observed (Crang and Blakemore, 1986). Schwann cells phagocytosing myelin may be a stimulus for Schwann cell mitosis (Salzer and Bunge, 1980). Myelin fractions derived from several sources are mitogenic for cultured Schwann cells; however, it seems apparent that phagocytosis and lysosomal processing are required to instigate mitogenesis (Yoshino *et al.*, 1984; Meador-Woodruff *et al.*, 1985).

The Role of the Macrophage:

In peripheral nerve there is a population of resident macrophages estimated to be about 2-4% of the total cell population (Oldfors *et al.*, 1980; Schubert and Friede, 1981). However, myelin associated with nerves undergoing Wallerian degeneration is phagocytosed mainly by invading non-resident myelomonocytic macrophages recruited from the blood (Perry and Brown, 1992). These macrophages play a role in both degeneration and regeneration of peripheral nerves following injury and in initiating the Schwann cell mitoses associated with Wallerian degeneration (Bruck and Friede, 1991; Perry and Brown, 1992; Baichwal *et al.*, 1988; Hann-Bonnekoh *et al.*, 1989; Beuche and Friede, 1984).

Proliferation In Vitro;

Many of the studies into Schwann cell proliferation have been carried out using cell culture techniques. Rat and mouse Schwann cells respond with increased proliferation to contact with neuronal processes (neuritic mitogen) and axolemmal mitogens, as well as to soluble factors present in culture medium. Soluble factors include pituitary extract, FCS, and glial growth factor (GGF). Proliferation in culture may also be induced by specific factors extracted from brain and by membrane-bound factors associated with neurites.

The doubling time for primary cultured Schwann cells in the presence of 10% foetal calf serum is approximately 7-8 days (Raff *et al.,* 1978a). Raff and co-workers postulated that the Schwann cell population divides homogeneously.

Mitogens:

Schwann cell proliferation may be induced *in vitro* by non-specific and specific factors which elevate cyclic AMP (cAMP) levels. Cholera toxin irreversibly ADP-ribosylates the catalytic sub-unit of adenylate cyclase and thereby indirectly raises intracellular cAMP levels (Ratner *et al.,* 1987). At optimal concentrations, cholera toxin reduces the doubling time of Schwann cells to 2 days (Raff *et al.*, 1978a). Schwann cells co-cultured with DRG neurons are only weakly stimulated (Salzer and Bunge, 1980). Elevating cAMP levels using the membrane-permeant derivative, di-butyryl cAMP, also stimulates mitosis, but to a lesser degree than cholera toxin (Raff *et al.*, 1978a). Schwann cell morphology is altered on exposure to factors which raise cAMP levels; they assume a more flattened appearance and lose their characteristic bi-polar shape (DeVries *et al.*, 1982; 1983). The mitogenic effects of cAMP are mediated through the presence of serum or growth factors (Stewart *et al.*, 1991). One effect of cAMP is to increase the density of growth factor receptors in the Schwann cell membrane (Weinmaster and Lemke, 1990). In PNS cultures, agents which elevate intracellular cAMP concentrations cooperate synergistically with Platelet Derived Growth Factor (PDGF) and Fibroblast Growth Factor (FGF). Such agents strongly potentiate Schwann cell expression of the genes encoding the PDGF and FGF receptors (Lemke, 1992).

Pituitary extract in concentrations between 100 to 500*u*g protein/ml., reduced the Schwann cell doubling time to 3 days, but was inhibitory at higher concentrations (Raff *et al.*, 1978). The mitogenic effects of pituitary extract were not accompanied by changes in Schwann cell morphology or intracellular cAMP alterations, unlike those observed with cholera toxin (Raff *et al.*, 1978; Brockes *et al.*, 1979). Treatments with cholera toxin in conjunction with pituitary extract were

considerably more effective than the responses to either treatment alone, suggesting a synergistic effect (Raff *et al.*, 1978b).

FCS in low concentrations (0.1%) has a maintenance effect, while higher concentrations (5 to 15%) have a mitogenic effect. GGF is a protein of molecular weight 30-31kDa, and may be extracted from the brain or pituitary gland. GGF stimulates Schwann cells to divide (Lemke and Brockes, 1984), and is mitogenic not only for Schwann cells, but also for astrocytes and muscle fibroblasts (Brockes *et al.*, 1981).

Axolemmal studies:

Sensory axons are mitogenic for Schwann cells (Wood and Bunge, 1975). Purified Schwann cells are relatively quiescent, however when introduced to purified DRG cultures, 90% of Schwann cells start dividing within 42 hours (Wood and Bunge, 1975). A membrane fraction prepared from similar sensory ganglion neurites is also mitogenic for Schwann cells (Salzer *et al.*, 1980a). Secondary Schwann cell cultures exhibit greater responsiveness to membrane fractions. Salzer and co-workers noted binding of neurite membrane to Schwann cells, while preparations from other sources (3T3 cells) were not mitogenic. The specific stimulation of proliferation by neurite membranes implies localisation of the neurite mitogen to the plasma membrane.

There have been numerous studies into the mitogenic properties of axolemma on Schwann cells. The consensus of opinion is summarised below.

The axolemmal mitogen is probably present on both myelinated and unmyelinated axons and requires close contact with the associated Schwann cell. The signal, which is present on the outer surface of the membrane, is trypsin sensitive and is probably a polypeptide linked to the axolemma via heparin sulphate proteoglycan (Sobue and Pleasure, 1985; Salzer *et al.*, 1980; Salzer *et al.*, 1980a; Salzer *et al.*, 1980b; Salzer and Bunge, 1980; Terry *et al.*, 1974; Wood, 1976; DeVries *et al.*, 1982; DeVries *et al.*, 1983; Meador-Woodruff *et al.*, 1984).

PC12 cells are a source of neurite mitogen for Schwann cells (Ratner *et al.*, 1984), and PC 12 stimulation of Schwann cell proliferation is reduced by 50-60% in the presence of 1mM B-D-xyloside (Ratner *et al.*, 1985).

Termination of Schwann Cell Proliferation

According to Eccleston *et al.* (1989a), the mechanisms underlying cessation of Schwann cell proliferation are poorly understood. One theory is that mitotic inhibitory signals play a part in regulating Schwann cell numbers. *In vitro*,

dissociated sciatic nerve Schwann cell cultures will cease to proliferate when confluence is achieved.

The formation of extracellular matrix, formed by ensheathing Schwann cells, appears to inhibit normal Schwann cell proliferation *in vitro*. In defined medium, where no extracellular matrix forms, continued proliferation occurs (Moya *et al.*, 1980). Basal lamina alone is not inhibitory and *in vivo* Schwann cells proliferate in large amounts of extracellular matrix.

Type I collagen preparations are inhibitory to Schwann cells *in vitro* (Eccleston *et al.*, 1989a), inhibiting DNA synthesis during the early post-natal period. Inhibition is accompanied by a change in morphology, Schwann cells adopting a narrow spindle form.

Aguayo *et al.*(1976c), showed that decreasing the fibre number in neonatal rat cervical sympathetic trunks by killing neurons in the SCG, is followed by a decrease in Schwann cell number, although the ratio of axons to Schwann cells remains normal. This implies that Schwann cell number is regulated by axons.

1.4.5 Migration

Schwann cells migrate from the neural crest into the peripheral nerves. Cells leave the neural crest and migrate along defined pathways to target sites throughout the embryo, where they form a variety of cell types including autonomic and sensory neurons, Schwann cells and melanocytes (Sanes, 1983). Schwann cells begin migrating early and proliferate extensively before differentiating. It is thought that neural crest migration may be regulated by a basal lamina which surrounds the cell-free space through which crest cells move and may define the migratory pathway. Basal lamina also encapsulates the premigratory crest and dissolution of this structure may be the signal involved in triggering migration (Sanes, 1983). In the developing PNS, outgrowth of neurites is closely followed by the appearance of Schwann cells. Actively migrating Schwann cells can move at variable rates, often sporadic, and may remain stationary for long periods. Schwann cells may migrate at rates of up to 114μm per day (Billings-Gagliardi *et al.*, 1974).

Whilst migrating, Schwann cells are ovoid, have not yet developed a basal lamina and have not projected long cytoplasmic processes. On elongation along the axon, the Schwann cell changes shape to become spindle-shaped. Mitotic division occurs as Schwann cells move distally along pioneer sprouts, which provide paths for the axons which grow out later.

1.4.6 Basal lamina

Basal lamina is secreted by Schwann cells subsequent to 1:1 contact with axons (Bunge and Bunge, 1983) and is indicative of Schwann cell differentiation following the stages of migration and proliferation (Billings-Gagliardi *et al.*, 1974; Armati-Gulson, 1980). *In vitro* observations confirm basal lamina is formed only after migration ceases (Armati-Gulson, 1980).

Basal lamina may be clearly observed in electron micrographs of myelinated and unmyelinated fibres and appears as a fuzzy granular deposit covering the surface of the Schwann cell, external to the plasma membrane, and is continuous between adjacent internodes (Raine, 1984). Basal lamina is approximately 80nm. thick, and is composed of type IV collagen, glycosaminoglycans and the glycoproteins laminin and entactin (Schubert, 1978).

Contact between neurons and glia is a requirement for the secretion of basal lamina (Bunge and Bunge, 1983). Schwann cells *in vivo* will produce the basal lamina component laminin, at all stages of development, regardless of any association with axons. Laminin production is necessary but not sufficient on its own for basal lamina formation (Cornbrooks *et al.*, 1983).

	Defined media	Defined media + FCS + ascorbate
Laminin	+ (punctate)	+ + +
HSPG	+ (punctate)	+ + +
Collagen IV	–	+ + +

Table 1.9 Presence of Basal Lamina components

Ascorbic acid and FCS:

DRG co-cultured Schwann cells in defined serum-free medium adhere closely to axons and have short, spindle-shaped morphology. These Schwann cells proliferate due to the mitogenic axonal influence, but do not differentiate, form basal lamina, ensheath or myelinate axons (Moya *et al.*, 1980). Schwann cells appear to be positioned on the outside of axonal fascicles; segregation and engulfment of axons and the classic 1:1 relationship of axon:Schwann cell does not occur. However, on the introduction of serum and ascorbic acid to these cultures, Schwann cells elongate and cease to proliferate. Schwann cell processes extend,

and segregation and ensheathment occurs. Within 4 days, 1:1 relationships form and myelination commences, with the formation of internodes. Schwann cells have now acquired basal lamina. Schwann cells which extend processes into axonal fascicles exhibit patchy or discontinuous deposition of material on their abaxonal surface, which is thickest on myelinating Schwann cells which have achieved 2 or more spiral turns (Wood *et al.*, 1990).

Expression of other Schwann cell markers varies with exposure to serum and ascorbic acid. NGF-R (217C) stains most Schwann cells in defined medium but stains only non-myelin-forming Schwann cells in defined medium plus serum and ascorbate. GC and MAG stain most Schwann cells in defined medium but only show positivity on myelinating Schwann cells in defined serum-rich ascorbate medium. Po protein and MBP are not detected in defined medium but are found in compact myelin in serum and ascorbate-rich media.

The absence of serum and embryo extract may result in the failure of neuronal differentiation and therefore in the inability of the axon to instigate the necessary signal to the Schwann cell (Moya *et al.*, 1980). However, the signal may be delivered correctly, but the Schwann cell may fail to synthesise and/or secrete the components for basal lamina. The fact that cultures maintained in defined medium, once switched to full myelinating medium progress to normal myelination suggests that axonal signals are present and the observation that Schwann cells in defined medium become very granular suggests a build-up of secretory products and therefore a blocking of secretion as opposed to a lack of synthesis (Moya *et al.*, 1980).

It is interesting to note that basal lamina acquisition is a requirement only for Schwann cell myelination and that oligodendrocytes when co-cultured with purified DRG neurons neither produce nor require basal lamina for myelin formation.

CHAPTER 2

MATERIALS AND METHODS

2.1 CELL CULTURE

2.1.1 Dissociated Schwann Cells

The method used to obtain dissociated Schwann cells was modified from that of Brockes *et al.*(1979).

Materials

Dissecting instruments sterilised in 70% alcohol.
Sterile petri dishes.
Sterile bijoux.
Sterile scalpel blades.
1ml syringes.
21g needles.
Sterile centrifuge tubes.
Sterile pipette tips.
Sterile pasteur pipettes.
Tissue culture treated multiwell dishes (or flasks).
Poly-l-lysine coated coverslips or flasks (Appendix 1.5).
Haemocytometer.

Media

1) Standard culture medium:
   DMEM
   FCS 10%
   Glutamine 2mM.
   Penicillin/streptomycin.
2) SATO defined medium : refer to Appendix 1.1.
3) Hank's balanced salt solution (HBSS), without calcium and magnesium, plus antibiotics and glutamine.
4) Liebovitz (L15) medium, plus antibiotics and glutamine.

Enzymes

1) Trypsin 0.25%: 1ml 2.5% trypsin (Flow) diluted in 9mls HBSS without calcium and magnesium.
2) Collagenase 1%: Stock diluted in HBSS, and filter sterilised.
3) SD solution: refer to Appendix 1.2.

Method

Neonatal Sprague-Dawley rat pups between post-natal day 3-5 were killed by cervical dislocation and the pups were sprayed with 70% alcohol prior to dissection. Both sciatic nerves from 10-12 pups were dissected out and collected in a bijoux containing 1ml HBSS. The nerves were transfered to a petri dish and chopped with a scalpel blade. As much chopped nerve as possible was placed into a fresh bijoux and the volume made up to 1ml with HBSS. To this, 1ml of 0.25% trypsin, and 33*u*l of 1% collagenase was added. The nerves were digested at 37^{o}C for 30 minutes, following which as much enzyme as possible was removed and fresh enzymes added as before. The digesting nerves were re-incubated at 37^{o}C

for a further 30 minutes. Enzyme activity was stopped by the addition of 1ml SD solution (Appendix 1.2), and the digested nerves triturated with 1ml syringe and 21g needle 3 times.

The triturated suspension was placed in a sterile centrifuge tube and topped up with L15. 1ml FCS was layered on top of the L15, and the preparation centrifuged at 1200 RPM for 5 minutes. The supernatant was removed and the dissociated cells resuspended in 500*u*l standard or defined medium (Appendix 1.1). The cells were counted using a Neubauer haemocytometer and plated at 20,000 cells per coverslip. The coverslips had previously been washed and sterilised prior to poly-L-lysine treatment which provided a suitable polarised surface enhancing cell adhesion (Appendix 1.5) The cell suspension was allowed to adhere to the culture surface (about 1 hour) at 37^{o}C, 5%CO_2, and fed with the appropriate medium (0.5ml per 13mm coverslip). Cultures required feeding every 3 days.

If purified Schwann cells were required the cultures were treated with cytosine arabinoside (AraC) (Appendix 1.4).

2.1.2 Dorsal root ganglion cultures

Time-mated Sprague Dawley rats were purchased commercially (Interfauna) and embryos used between E15 and E17. One rat was sufficient to generate several cultures. The rat was killed, placed in dorsal recumbency and sprayed with 70% alcohol to sterilise the abdomen. An incision was made and the embryonic sac removed to a 50ml centrifuge tube containing sterile L15 medium. This was transferred to the cell culture laboratory where individual embryos were removed from the sac and placed in fresh L15. The spinal cord was removed from the embryos and placed in sterile L15 with the DRG still intact.

2.1.2.1 *Explant cultures:*

Dorsal root ganglia were removed from E15-E17 rat embryo spinal cord and placed intact on balanced collagen-coated coverslips; 3 ganglia per 13mm coverslip or 6 ganglia per 22mm coverslip. During plating, ganglia were kept moist in a minimal amount of medium and allowed to settle at 37^{o}C. After 45-60 minutes, fresh medium (Sato or myelinating medium plus 200ng/ml 2.5S NGF) was carefully added to each well.

2.1.2.2 *Dissociated cultures:*

Ganglia were removed from E15-E17 rat embryos in the same manner as for explants, on collection being pooled in a small volume (500*u*l) of L15 medium.

Pooled ganglia were transferred to a sterile tissue culture cabinet, and chopped in fresh HBSS (without calcium and magnesium).

Enzymic dissociation:

To ganglia in 500*u*l HBSS the following was added;

33*u*l of stock 1% collagenase (Worthington)

1ml 10% Trypsin (1:250)(ICN Flow).

The method is essentially that of Wood and Williams (1984).

Ganglia and enzymes were incubated at 37°C for 30-40 minutes. The mixture was washed in HBSS (containing 10% foetal calf serum), and centrifuged at 1200 RPM in a Wifug benchtop centrifuge. The cell pellet was resuspended in 1ml HBSS and triturated with a 21g needle (green). The triturated cell suspension was again washed in HBSS, centrifuged and plated onto prepared coverslips in Sato defined medium or myelinating medium plus NGF (200ng/ml) at 20,000 cells per coverslip (coverslips were nitric acid washed, sterilised and collagen coated: Chapter 2.1.5.3).

Media for neuronal cultures:

1) Sato defined media: Apendix 1.1.
2) Myelinating Medium: Appendix 1.3.

2.1.3 Superior cervical ganglion

2.1.3.1 *Explant cultures*

Neonatal Sprague-Dawley rat pups were killed by overdose of halothane anaesthesia. The animals were placed in dorsal recumbency and sprayed with 70% alcohol to sterilise. An incision was made in the throat region and the ganglia were located immediately below the carotid bifurcation and removed to a bijoux containing L15 medium. Sufficient ganglia were pooled, removed to the tissue culture cabinet and each ganglion chopped into four pieces. The chopped explants were placed onto collagen-coated, balanced coverslips in a minimal amount of medium, allowed to settle at 37°C, and fed with medium containing NGF at 200ng/ml.

2.1.3.2 *Dissociated cultures*

SCG were removed in the same manner as for explant cultures and pooled in L15 medium. On completion of dissection the pooled ganglia were removed to the tissue culture hood where they were roughly chopped in HBSS. Trypsin and collagenase were added in the same regime as for dissociated DRG. On completion of enzymic digestion, the ganglia were triturated, washed and plated at

20,000 cells per 13mm coverslip on balanced collagen. Medium was supplemented with NGF at 200ng/ml.

2.1.4 PC12 Cells.

Rat pheochromocytoma cloned cell line was used as a neuronal model (discussed in Chapter 1.2.4). On exposure to NGF these cells differentiate to become very similar to sympathetic neurons. Normal undifferentiated cells are maintained in culture as stock cells (in the absence of NGF) from which differentiated PC12 cells are generated as necessary.

2.1.4.1 *Stock PC12 Cells*

Materials

PC12 cells from frozen stock (approximately 10^6 cells)
Sato medium (refer to Appendix 1.1.)
Hank's Balanced Salt Solution (HBSS)
Vitrogen-coated 75cm^2 tissue culture flasks

Method

PC12 cells were removed from the liquid nitrogen freezer and each ampoule thawed at 37^{o}C. Once thawed, cells were washed twice in HBSS, and resuspended in Sato medium (10mls) and placed in a prepared flask. These cultures were maintained at 37^{o}C in 5% CO_2, and reached confluency after 3-4 days. When approaching confluency the PC12 cells were trypsinised and replated before becoming overgrown and detaching. Using this regime PC12 cells may be maintained for months.

Coated flasks:

Vitrogen is a commercial preparation of collagen. It is stored at 4^{o}C and will gel at 37^{o}C. 140μl cold Vitrogen was added to 1ml cold Sato. This solution was introduced into a 75ml flask and the surface coated evenly. Excess was removed and the flask placed in the 37^{o}C incubator for at least 1 hour before use, allowing the Vitrogen to gel.

Trypsinisation:

PC12 cells adhere very firmly to their substrate and therefore require to be trypsinised prior to passaging. Sato medium was removed and the cell surface washed with HBSS. The HBSS was poured away and 5mls 0.25% trypsin was swirled across the cell monolayer. Excess was removed and the flask placed in the 37^{o}C incubator for several minutes. Cell detachment was carefully monitored. Detached cells were aspirated off in HBSS, washed, centrifuged and replated onto fresh Vitrogen at the required density.

2.1.4.2 *Differentiated PC12 Cells*

Materials

Collagen coated coverslips (method in Chapter 2.1.5)
Sato medium: Appendix 1.1.
Nerve Growth Factor
Stock PC12 cells

Method

PC12 cells were trypsinised and replated onto coverslips at a density of 5-10,000 per coverslip in a volume of 25*u*l. The cells were allowed to settle at 37^{O}C, following which 500*u*l of Sato medium containing 200ng/ml NGF was added to each well. These cells were permitted to differentiate *in vitro* for at least 1 week before conducting experiments. These cultures required feeding 2-3 times a week with Sato plus NGF.

2.1.5 Rat Tail Collagen

Rat tail collagen is a high molecular weight protein extracted from rat tail tendons using dilute acetic acid as described by Bornstein (1958). Type IV collagen is believed to be the primary structural element of the basement membrane (Kleinman *et al.*, 1982). Laminin, which is the other principal component of basement membrane, binds to native but not denatured type IV collagen (Kleinman *et al.*, 1986).

2.1.5.1 *Preparation*

Materials

Six adult rat tails wrapped in sterile gauze and soaked in 70% alcohol for 30 minutes.
1 clamp.
1 pair bone forceps.
Sterile scissors
Sterile petri dish.
Sterile distilled water.
Sterile tweezers.

Method

Collagen fibres were removed from the rat tails by breaking and pulling. The tendons were then cut off and placed in sterile water in the petri dish. At this stage any non-collagenous material was removed. Using tweezers the tendons were teased apart for several minutes until the preparation thickened.

The thickening preparation was transferred into a 100ml bottle containing 0.01% acetic acid (approximately 1 tail to 100mls acetic acid), and allowed to stand at 4^{o}C overnight. If too thick, more acid was added (5-10mls usually sufficient).

The preparation was then centrifuged at 18K at 4^{o}C for 1 hour, the clear supernatant removed and stored at 4^{o}C for up to 12 months. Prior to use, the collagen was dialysed against water and sterility tested using standard microbiological tryptone soya and thioglycollate broths.

2.1.5.2 *Collagen Dialysis*

Materials

500ml bottle sterile de-ionised distilled water (Sigma).
Dialysis tubing: Visking 2-18/32" (Medicell International).
Sterile forceps and scissors.
Sterile petri dish.
Sterile aluminium foil.

Method

Dialysis tubing was boiled in distilled water for 5 minutes, allowed to cool in a sterile hood, and covered with sterile foil. Using sterile instruments, a knot was tied in one end of the tubing and placed into a bottle, keeping hold of the

open end. Using a sterile pasteur pipette the tubing was filled with rat tail collagen to within 5cms. of the top of the bottle, leaving the tubing overlapping the rim of the bottle. The cap was re-placed loosely and covered with sterile aluminium foil. The collagen was dialysed for 36-48 hours at 4°C. The apparatus was removed from the refrigerator and using sterile instruments the tubing was transfered to a large sterile petri dish, still holding open end. The dialysed collagen was carefully poured out into sterile universals and sterility tested.

Dialysed collagen was stored at 4°C for up to 1-2 months.

2.1.5.3 *Coating coverslips*

Coverslips were immersed in concentrated nitric acid for 24 hours, rinsed in running de-ionised water for 30 minutes, soaked overnight in distilled water and sterilised prior to collagen coating.

Method

1) Fluorescent lights were turned off.

2) Mix 1ml riboflavin (flavin mononucleotide - 0.1%, Sigma F8383) with 7mls dialysed collagen. Store at 4°C, wrapped in foil.

3) Using sterile forceps transfer one acid-washed coverslip to each well of a multiwell tissue culture treated dish.

4) Using a 1ml syringe, place a small drop of collagen\riboflavin mix in the centre of each coverslip.

5) Using the syringe plunger spread the collagen evenly across the coverslip.

6) Turn the lights on again, allowing the collagen to gel by ultraviolet light.

7) Once gelled, add sufficient balancing solution to immerse the collagen completely.

2.1.5.4 *Balancing Collagen*

To give a good three-dimensional substrate for cells and processes to adhere to, the collagen coated coverslips should be "balanced" for at least 3 days before use and immediately after collagen has gelled.

Balancing solution

L15 (Liebovitz medium)
Foetal calf serum - 1%
2mM glutamine
Pen/strep.

Method

0.5mls balancing solution was added to each well containing collagen-coated 13mm coverslips. Balancing coverslips were stored in the tissue culture hood for 3 days before plating cells.

2.1.6 Pituitary Extract

Bovine pituitaries were collected as aseptically as possible and homogenised in 2 volumes of 0.15M ammonium sulphate. The pH of the homogenate was adjusted to 4.5 with 10N HCl, and the mix allowed to stand for 2 hours at 4°C. The homogenate was centrifuged at 13,000g for 30 minutes at 4°C, and the supernatant dialysed against 2 changes of sterile distilled water at 4°C. The dialysed extract was centrifuged again for 30 minutes at 13,000g at 4°C, and the supernatant lyophilised. The product was rehydrated in sterile distilled water to 3mg/ml (dry weight of protein), tested for sterility, stored at -20°C, and used at 100μg/ml in cell culture medium.

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2.2 HISTOLOGICAL TECHNIQUES

2.2.1 Light microscopy

Light microscopy was used on a daily basis to examine living cultures. This was carried out with a Wilovert inverted microscope with phase contrast facilities and a variety of objectives, including a dark-ground 2.5X, and 10X and 20X objectives. The eyepieces were 12.5X. A Zeiss Ikon camera was mounted on the microscope enabling photography *in vitro*.

2.2.2 Silver Stain

The method used was essentially that of Karnovsky and Roots cholinesterase method and the modified Tsuji, and Tobin-Gros (1980) silver stain. Materials and solutions may be found in Appendix 2.1.

Cultures were washed in Hanks Balanced Salt Solution (HBSS), and fixed in 4% paraformaldehyde (PF) in 0.1M Phosphate buffer (pH 7.3) (Appendix 2.1), for 1 hour at $4^{o}C$. Following fixation the cultures were washed in 0.1M Phosphate buffer three times. At this stage the cultures may be stored for up to 7 days at $4^{o}C$ in PBS. To commence the silver staining the cultures were incubated in cholinesterase substrate (Appendix 2.1) for 30 mins. at room temperature. They were washed three times in distilled H_2O. To intensify and maintain cholinesterase staining, the cultures were incubated in 0.25% potassium ferricyanide for 10 minutes at room temperature, followed by thorough yet gentle washes in distilled H_2O. Cultures were then incubated in 1% Triton X in 0.1M sodium nitrate for 90 minutes at room temperature, and washed in distilled H_2O. 0.1M silver nitrate was applied to the cultures for 3 hours at $37^{o}C$. The silver nitrate was thoroughly rinsed off with distilled H_2O. Reducer (Quinol solution) was applied for about 7 mins, and rinsed off, before incubating in 5% Hypo for 15-30 mins. The cultures were rinsed in distilled H_2O and counter-stained with 0.2% methylene blue for 90 seconds prior to a final rinse, dehydration, clearing and mounting.

2.2.3 Electron Microscopy

Living cultures were washed in L15 or HBSS and fixed in 2.5% glutaraldehyde in isotonic cacodylate buffer (pH7.3)(Appendix 2.3). Explant cultures were washed in buffer for 30 minutes and post-fixed with 1% osmium tetroxide in isotonic cacodylate buffer for 2 hours. The fixed cultures were washed again for 15 minutes and by this time the explants had usually detached fron the coverslip, if not, they were peeled off using a razor blade. The explants were then dehydrated through alcohols and treated with propylene oxide for 30

minutes, prior to overnight treatment in an araldite resin:propylene mixture (Appendix 2.4). The following day the explants were changed to 3:1 araldite resin:propylene oxide and again left overnight. The propylene oxide was allowed to evaporate and the explants embedded in fresh resin mix which was polymerised at 60^{o}C overnight. Once embedded in Araldite, the cultures were cut on a Reichert-Jung Ultracut E ultramicrotome. Thick sections (1μm) were cut with glass knives and stained with a dye containing 1% methylene blue, 1% azur II in 1% borax. Thin sections (70-80nm) were cut on the ultramicrotome using a diamond knife. These were mounted on 200 mesh copper grids, and stained with uranyl acetate and lead citrate (Appendix 2.5). to enhance contrast. Sections were examined on an AEI 6B electron microscope. Photomicrographs of appropriate areas were taken using Ilford EM grade film. This film was developed using PQ Universal developer at a 1 in 10 dilution for 5 minutes at a temperature of 20^{o}C. Following a rinse in a 1% acetic acid "stop-bath", negatives were subsequently fixed with a 1 in 10 solution of Hypam fixer for at least 5 minutes, followed by thorough washing in running water.

2.3 IMMUNOCYTOCHEMISTRY

Immunocytochemistry may be defined as the identification of a tissue or cellular constituent *in situ* by means of a highly specific antibody-antigen reaction tagged with a visible label.

The techniques involved are invaluable for identifying cytoplasmic and surface markers thus allowing evaluation of cell types in mixed cultures, and also for providing information on the levels of differentiation of neural cells. Surface antigens may also be targeted to provide a specific method to select and kill individual cell populations.

2.3.1 INDIRECT IMMUNOFLUORESCENCE TECHNIQUES

2.3.1.1 Surface Antigens

The method for immunostaining all the surface antigens used in these studies is essentially the same. Primary antibodies are applied to washed living cells for short periods before incubating the cultures in FITC or TRITC conjugated secondary antibodies. As a final step, the cultures are fixed and mounted.

Method:

Coverslips were washed in L15 plus 1% foetal calf serum (FCS), and blocked for 2 minutes with neat FCS. FCS was removed and the cultures incubated in the appropriate primary antibody diluted in L15.

Antibody incubation was 30 minutes at room temperature unless otherwise stated.

Primary antibody was removed and the cultures washed thoroughly in L15. The appropriate secondary antibody diluted in L15 was applied, and incubated for 30 minutes at room temperature. Following a further wash in L15, the cultures were fixed in 95:5% absolute alcohol:glacial acetic acid at -20^{o}C for 20 minutes. The cultures were thoroughly washed in L15 until no evidence of acid remained, given a final wash in H_2O, and the coverslips mounted in 90% glycerol in PBS with 0.1% paraphenylenediamine to reduce fading.

Table 2.1 shows dilutions of primary and secondary antibodies, sources and target antigens.

2.3.1.2 Intracellular Antigens

These antigens are generally cytoplasmic and therefore the plasma membrane has to be disrupted to allow passage of the antibodies to reach their target.

Method:

The cultures were washed in HBSS, and fixed in 4% paraformaldehyde in phosphate buffered saline (Appendix 2.1) at 4^{o}C., for 30 minutes. The cells were permeabilised with 95:5% ethanol:glacial acetic acid for 10 minutes at -10^{o}C, and washed in HBSS until all the acid had visibly been removed. The cultures were blocked with FCS for 30 minutes. Serum was absorbed off, prior to application of the primary antibody diluted in L15 plus 1% FCS. The cultures were incubated overnight at 4^{o}C in a moisture chamber.

Following the overnight incubation, the cultures were allowed to return to room temperature and washed well with HBSS.
The secondary antibody diluted in L15 plus 1% FCS, was applied for 30 minutes at room temperature, and the cultures washed well in L15, followed by three changes of distilled water.

The culture coverslips were mounted in 90% glycerol in PBS with 0.1% paraphenylenediamine to reduce fading.

2.3 1.3 Double Staining

Double staining procedures were carried out in the same manner as normal. Depending on the species of secondary antibodies and the source of primaries, antibodies were incubated together or in sequence.

Primary antibody	Dilution	Target	Source	Secondary antibody
Ran-1 (217C)	1:100	Schwann	P.Kennedy	RAM
R-mAb (H8)	1:5	Schwann	I. Sommer	Mo IgG_3
Po	1:250	Schwann	D. Kirkham	GAR
MBP	1:500	Schwann	J-M. Matthieu	GAR
NF200 (RT97)	1:7500	Axons	Wood & Anderton	GAR
S100	1:900	Schwann	DAKO	GAR
Thy-1	1:100	Fibroblasts	P. Kennedy	GAR
N-CAM	1:100	Schwann	Sigma	GAM IgG_1
NGF-R	1:100	Schwann	Boehringer	GAM IgG_1
GFAP	1:750	Schwann	DAKO	GAR
O_4	1:50	Schwann	I. Sommer	GAM IgM
Laminin	1:1000	Schwann	Sigma	GAR

Notes:

O_4 antibody recognises sulfatide, seminolipid and cholesterol (Bansal *et al.*, 1989).

R-mAb recognises galactocerebroside, monogalactosyl diglyceride, sulfatide, seminolipid and psychosine (Bansal *et al.*, 1989).

GFAP antibody also recognises NF-M.

Po antibody was tested for specificity by immunoprecipitation.

Antibodies were titrated prior to use in order to determine suitable concentrations.

Table 2.1 Table of antibodies for immunofluorescence

2.3.2 PEROXIDASE ANTI-PEROXIDASE TECHNIQUE.

The Peroxidase Anti-Peroxidase (PAP) technique involves the utilisation of three basic reagents: a primary antibody, a secondary antibody and the PAP complex. The PAP complex is comprised of the enzyme peroxidase and an antibody directed against peroxidase. The primary antibody is specific for the antigen. The secondary antibody (termed the "link" antibody) is capable of binding to both the primary antibody and the PAP complex, both of which are produced in the same species of animal. The "link" antibody is added in excess so that only one of its Fab sites (Fragment antigen binding site; see Fig. 2.2) will bind to the primary antibody, leaving the other Fab site free to bind to the antibody in the PAP complex. Peroxidase enzyme is visualised via a substrate-chromagen reaction. Hydrogen peroxide (H_2O_2) plus peroxidase enzyme forms a complex whereby H_2O_2 is decomposed to H_2O and O^{++}, if in the presence of the electron donor 3'-diaminobenzidine tetrahydrochloride (DAB). When observing a stained preparation, deposits of the coloured chromagen indicate the presence of the antigen and represent specific positive staining. Interpretation is a comparison of the specific and non-specific (or background) staining pattern, and comparison of staining intensity of the sample with that of the control. Specific staining may be cytoplasmic, nuclear or surface depending on the antigen.

Advantages of the PAP technique over other immunocytochemical staining procedures include its sensitivity (due to absence of conjugated antibodies). The PAP technique is reported to be 100 to 1000 times more sensitive than indirect methods using either fluorescein or peroxidase-conjugated antisera (Sternberger *et al.*, 1970). Peroxidase is small and therefore does not prevent the binding of antibodies to adjacent sites. Peroxidase is inexpensive, very stable and easily obtainable in highly purified form and there is a wide availability of chromagens which interact with peroxidase. Endogenous peroxidase activity is not a problem as only small amounts are normally present in tissue.

Full details of materials and the complete method may be found in Appendix 2.7.

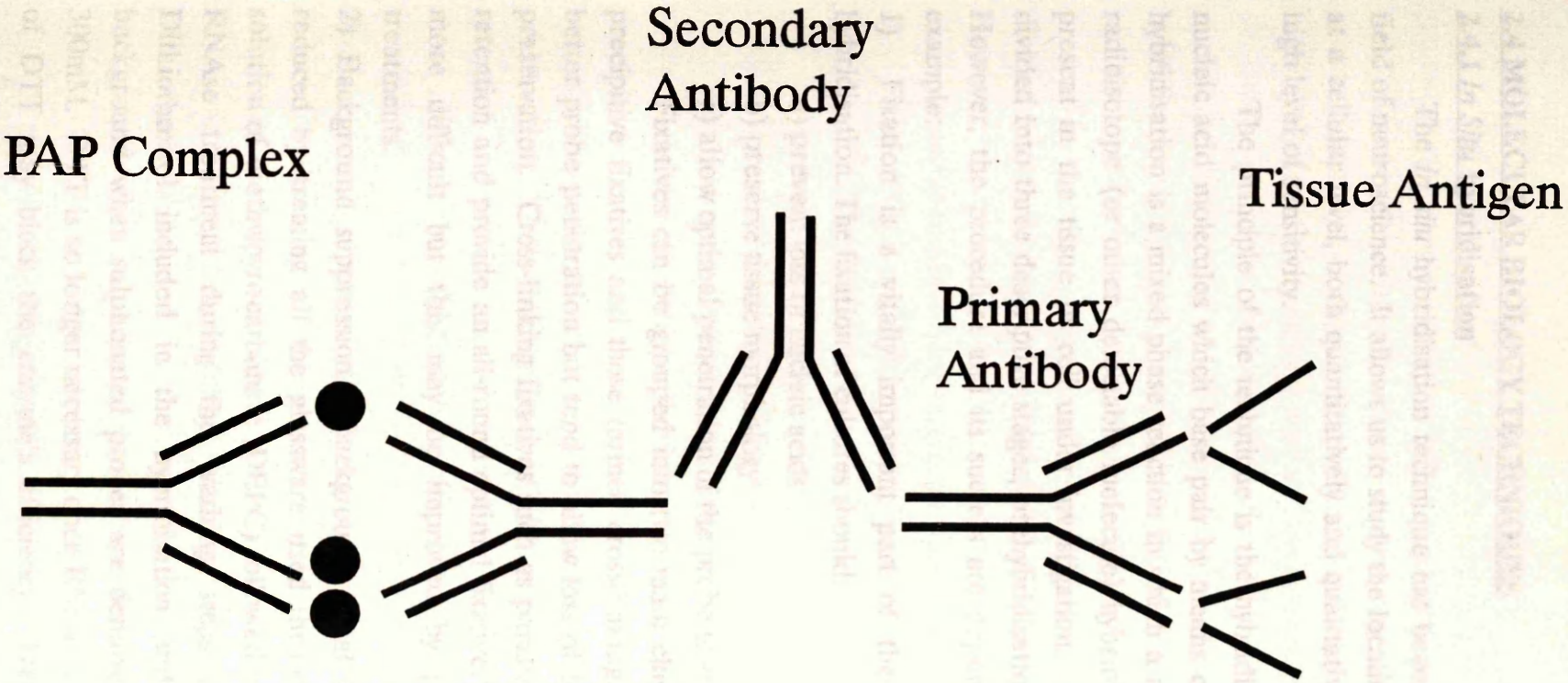


Figure 2.2 Diagrammatic representation of the PAP method

2.4 MOLECULAR BIOLOGY TECHNIQUES

2.4.1 *In Situ* hybridisation

The *in situ* hybridisation technique has become an invaluable tool in the field of neuroscience. It allows us to study the localisation of specific nucleic acids at a cellular level, both quantitatively and qualitatively. Its main advantage is a high level of sensitivity.

The principle of the technique is the hybridisation of two single stranded nucleic acid molecules which base pair by means of hydrogen bonding. *In situ* hybridisation is a mixed phase reaction in which a nucleic acid is labelled with a radioisotope (or other detectable molecule) hybridising to a target nucleic acid present in the tissue or cell under investigation. The technique itself may be divided into three descriptive stages; prehybridisation, hybridisation and washing. However, the procedure and its success are dependant on several factors. For example:

1) Fixation is a vitally important part of the preparatory steps prior to hybridisation. The fixation of cultures should:

a) prevent loss of nucleic acids

b) preserve tissue morphology

c) allow optimal penetration of the probe to its target.

Fixatives can be grouped into two main classes; those which are termed precipitive fixatives and those termed cross-linking. Precipitive fixatives provide better probe penetration but tend to allow loss of RNA and poor morphological preservation. Cross-linking fixatives such as paraformaldehyde give better RNA retention and provide an all-round optimal fixative. Penetration of probe may be more difficult but this may be improved by post-fixative permeabilisation treatments.

2) Background suppression. Background signal and degradation of RNA is reduced by treating all the glassware used throughout the technique with a solution of diethylpyrocarbonate (DEPC) followed by sterilisation (Appendix 3.3). RNAse treatment during the washing steps reduces background signal. Dithiothreitol included in the hybridisation and washing solutions reduces background when sulphonated probes are deployed, up to a concentration of 300mM. DTT is no longer necessary once RNase treatment occurs, as high levels of DTT may block the enzyme's efficiency. Dextran sulphate enhances the hybridisation signal by increasing the hybridisation rate and by serving as a non-specific competitor for the binding of RNA probes to the tissue, thereby increasing the amount of probe capable of hybridising to the tissue RNA. Salmon sperm DNA acts as a non-specific background blocker. Acetylation using acetic

anhydride reduces background signal by blocking basic groups. Lawrence and Singer (1985) found that acetylation of tissues greatly reduced background with probes of more than 1500 nucleotides, but was less effective for smaller probes..

Denhardt's solution contains several ingredients including ficoll, bovine serum albumin and polyvinyl pyrrolidone, which help to decrease non-specific binding to proteins, polysaccharides and nucleic acids.

3) Probe selection and labelling: see section 2.4.3.

Method

1) Prehybridisation

The coverslips supporting the fixed cultures were mounted, surface-up onto DEPC-treated, RNAse-free slides, using Glassbond^{TM}(Loctite). Once firmly attached the slides were placed in each of the following for 5 minutes: absolute alcohol, methylated spirit, saline and phosphate buffered saline.

The slides were transferred into fresh cold 4% paraformaldehyde (Appendix 2.1) for 20 minutes.

Meanwhile, the proteinase K mix was prepared by diluting 10*u*l of 10mg/ml stock in 5mls proteinase K buffer (Appendix 3.4).

Slides were treated with two changes of PBS, each for 5 minutes, excess PBS shaken off and slides placed on filter paper. The proteinase K mix was applied for 7.5 minutes, and the slides placed back into the rack and washed in PBS for 5 minutes. Slides were re-fixed in 4% paraformaldehyde for 5 minutes, during which the triethanolamine mix (Appendix 3.6) was prepared. The mix was placed in a staining dish, a stirrer added, and the slides immersed in the dish. 625*u*l acetic anhydride was added and this was stirred for 10 minutes. The slides were treated with fresh PBS for 5 minutes, followed by saline for a further 5 minutes.

The slides were dehydrated in methylated spirit for 5 minutes followed by two changes of absolute alcohol, each for 5 minutes. Slides were air dried for 1 hour, and the 50^{o}C and 80^{o}C waterbaths switched on in readiness for the hybridisation stage.

2) Hybridisation;

Humidifying solution was prepared in a Nunc vial. This solution is used to moisten tissue taped to the inside of the hybridsation slide box:

50% formamide, 25% SSC (20X), and 25% water.

Hybridisation mix:

stock hybridisation mix:	198.2*u*l.*
DTT:	1.8*u*l.
probe:	20*u*l.

This volume will cover forty 13mm. coverslips.

* Stock hybridisation mix is the same as for Northern blots (Appendix 4.9).

Method

1) Heat hybridisation mix, probe and DTT at 80^{o}C for 2 minutes.
2) Place on ice.
3) Place slides on filter paper.
4) Add 5*u*l hybridisation mix to each coverslip.
5) Cover each with a siliconised coverslip.
6) Place slides in hybridisation box with moist tissue.
7) Seal box with tape.
8) Place box inside double bags and seal.
9) Incubate sealed box at 50^{o}C overnight.

3) Washing

Waterbaths were set to 50^{o}C, 65^{o}C, and 37^{o}C, about one hour before use.

Wash solutions 1, 2, and 3 were decanted into DEPC treated glassware and heated to the correct temperature:

Wash 1: 50^{o}C.

Wash 2: 65^{o}C.

Wash 3: 37^{o}C.

Wash solutions: Appendix 3.1.

Method

The slides were removed from the sealed box which was incubated at 50^{o}C overnight, and placed in wash 1 for 20 minutes to remove the siliconised coverslips.

This was followed by a 20 minute wash in solution 2, and three ten minute washes in wash 3.

Following the above wash steps the slides were treated with 20*u*g/ml RNAse for 30 minutes at 37^{o}C.

The slides were then placed into fresh wash 3 for 10 minutes, followed by another 20 minute wash in wash 2, immersion in wash 4 for 15 minutes at room temperature, followed by wash 5 for 15 minutes.

The slides were dehydrated through dehydration solutions 1 to 4 (Appendix 3.2), followed by 2 changes of absolute alcohol. Slides were air-dried.

Autoradiography/Signal detection

After air drying for 1 hour slides were dry enough to be inserted into an X-ray cassette with X-ray film and subjected to an overnight exposure at room temperature. This allowed evaluation of the success and intensity of the radiolabelling. The film, once processed was used to guage the length of exposure time for autoradiography.

Dipping slides: slides were dipped in a solution of Ilford K5 emulsion mixed 1:1 with distilled water containing 1% glycerol at 42^{o}C. Following dipping in the emulsion mix, the slides were air-dried for 6 hours then stored in light-tight slide boxes containing silica gel, in the dark at 4^{o}C. Exposure time was 4 to 7 days depending on the intensity of signal observed on the radiographic film. The slides were allowed to re-equilibrate to room temperature and developed using Kodak D19 solution for 4 minutes. Development was arrested using 1% glycerol/1% acetic acid for 1 minute. The slides were then fixed in 30% sodium thiosulphate for 2.5 minutes. Following several rinses in water, the slides were air-dried and post-stained with haematoxylin.

2.4.2 Isolation and Preparation of Po cDNA

A cDNA (pSN63c) encoding Po was kindly donated to the group by Dr. Greg Lemke. This was sub-cloned by Dr. Lynn Mitchell (University of Glasgow; Applied Neurobiology Group) into the EcoR1 multiple cloning site of the pGEM4 plasmid resulting in a 4.7kb recombinant containing a 1.85kb insert known as Po-1. This plasmid was digested with excess EcoR1 enzyme at 37°C for 2 hours (Appendix 4.1). Digestion was monitored by electrophoresis on an analytical 1% agarose (BRL) gel (Appendix 4.2), alongside DNA markers. Digested DNA was electrophoresed on a 1% low-melting-point gel (BRL) (Appendix 4.3). Following electrophoresis at 70 volts for approximately 2-3 hours, the Po band was cut out and distributed between several sterile 1.5ml eppendorfs. To these were added 400*u*l TE buffer (Appendix 4.4) and the eppendorfs incubated at 65°C for 15 minutes to melt the agarose. This preparation was phenol extracted twice, followed by chloroform. A final wash with butan-2-ol selectively extracted any remaining water, and the preparation was precipitated in 1/10th volume 7.5M ammonium acetate and 2 volumes ethanol at -20°C. After microfuging at 4°C for 20 minutes the DNA pellet was washed in 75% ethanol, briefly dried under vaccuum and resuspended in TE buffer.

Po linearisation digests from pGEM Po-1 DNA.

To generate a 1550bp antisense fragment, Po-1 DNA was digested with Cla-1 according to manufacturers instructions. A 1024bp sense fragment was generated with Xba-1 enzyme.

The reaction mixture was incubated at 37°C for 1-2 hours. The DNA was phenol-chloroform extracted and precipitated overnight at -20°C. The linearised DNA was ethanol (70%) washed, briefly vaccuum dried and resuspended in TE buffer to a concentration of 1*u*g/*u*l. Stocks of linearised sense and antisense Po DNA were stored at -20°C.

2.4.3 Riboprobe production

Riboprobes were used for *in situ* hybridisation (Cox *et al.*, 1984). Riboprobes are single-stranded RNA molecules produced from a cloned cDNA that has been introduced into a plasmid transcription system. The advantage of these probes over cDNA probes is, because they are single-stranded, re-annealing does not occur, therefore a greater percentage of probe is available for hybridisation. Riboprobes have a high specific activity and form stable hybrids. Post-hybridisation RNAse treatment is required to reduce background as riboprobes are "stickier" than DNA probes and therefore there tends to be a

higher degree of non-specific binding to tissue. Riboprobes were labelled with ^{35}S which tends to attain higher specific activities and greater signals than, for example, ^{3}H-labelled probes, and permits cellular localisation. Riboprobes were synthesised and labelled in reactions involving the use of specifically designed transcription vectors that contained prokaryotic RNA polymerase promoters.

The rat Po cDNA clone was inserted into a pGEM-42 vector and selected by ampicillin and streptomycin. This cDNA was linearised with Cla-1 for the antisense fragment (1500bp) and Xba-1 for sense (1024bp). To produce labelled riboprobes the SP6/T7 transcription kit from Boehringer Mannheim was used. SP6 polymerase transcribes a strand of the insert in one direction while T7 transcribes in the opposite direction, copying the complementary strand of the insert. SP6 RNA polymerase enzyme will recognise its promoter and transcribe in the 5' to the 3' direction until it reaches the site of linearisation. The antisense strand is complementary to cellular mRNA, and is an effective hybridisation probe (DeLeon *et al.*, 1983). The sense strand is similar in length, specific activity, and base composition and provides a suitable negative control. The length of the transcripts was subsequently reduced by alkali hydrolysis treatment.

Method: Briefly, linearised Po cDNA was incubated at $37^{O}C$ in the presence of buffer, nucleotides [UTP, GTP, ATP], isotope (^{35}S CTP), RNAsin and polymerase (T7 for antisense; SP6 for sense) (Appendix 4.5). Following this incubation the template DNA was removed with DNase I. Following a phenol-chloroform extraction the labelled RNA was precipitated with ammonium acetate and ethanol into a pellet and resuspended in 100*u*l water. A 5*u*l sample was added to 5mls Ecoscint in a scintillation vial and activity measured on a scintillation counter. The percentage incorporation was calculated and the RNA re-precipitated and resuspended in 10mM DTT such that the probe concentration was 1ng/*u*l/kb, which in this case was 1.5kb so the probe was resuspended at 1.5ng/*u*l.

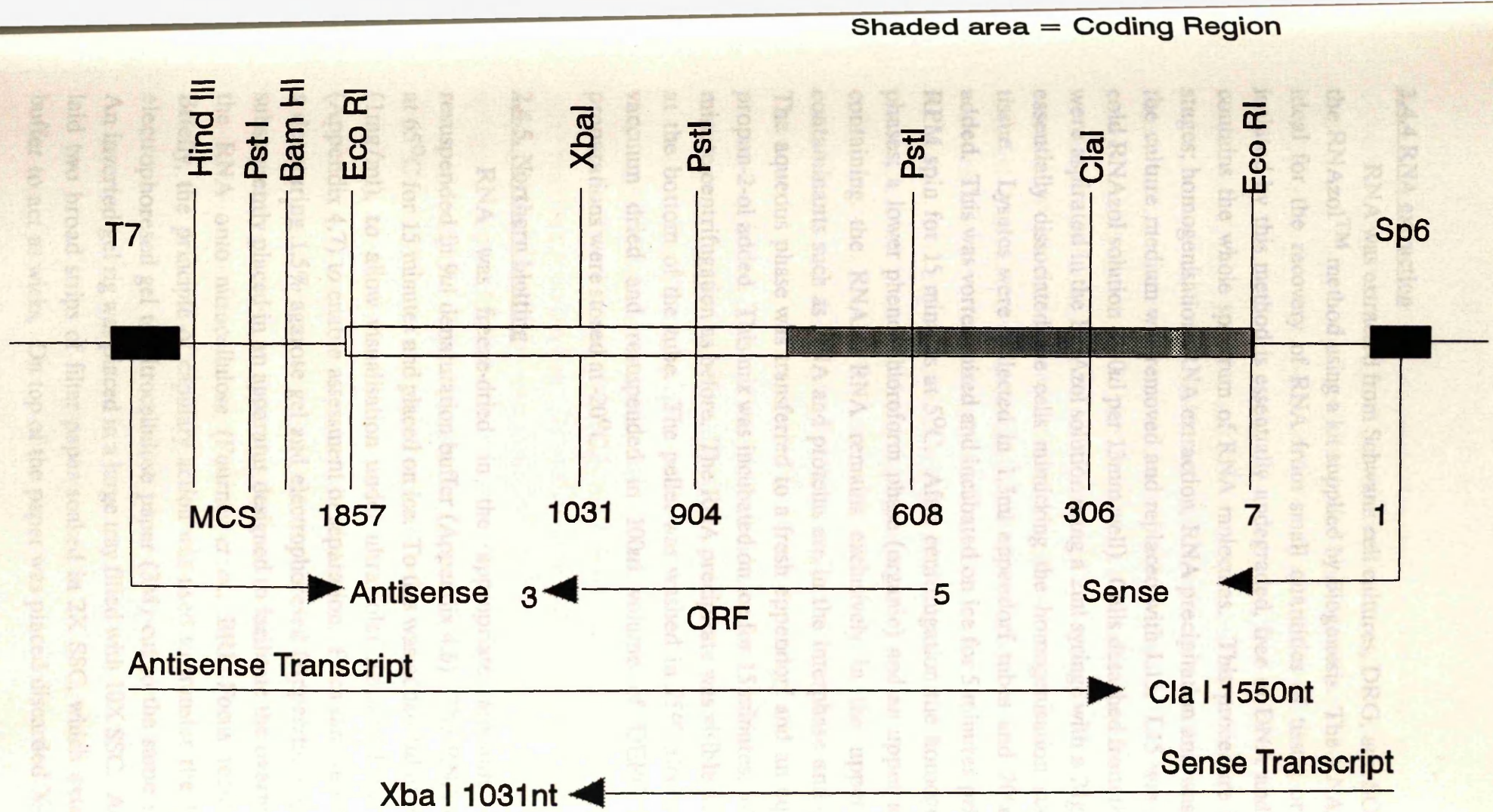


Figure 2.3 Po-1 Transcription Vector

2.4.4 RNA extraction

RNA was extracted from Schwann cell cultures, DRG, and SCG cultures by the RNAzol™ method using a kit supplied by Biogenesis. The RNAzol method is ideal for the recovery of RNA from small quantities of tissue or cells. RNA isolated by this method is essentially undegraded, free of DNA and proteins and contains the whole spectrum of RNA molecules. This procedure involved four stages; homogenisation, RNA extraction, RNA precipitation and washing. Briefly, the culture medium was removed and replaced with L15. L15 was replaced with cold RNAzol solution (250*u*l per 13mm well). Cells detached from the surface and were aspirated in the RNAzol solution using a 2ml syringe with a 21g needle which essentially dissociated the cells mimicking the homogenisation stage for whole tissue. Lysates were collected in 1.5ml eppendorf tubes and 200*u*l chloroform added. This was vortex-mixed and incubated on ice for 5 minutes prior to a 12,000 RPM spin for 15 minutes at 5°C. After centrifugation the homogenate forms 2 phases; a lower phenol-chloroform phase (organic) and an upper aqueous phase containing the RNA. RNA remains exclusively in the upper phase while contaminants such as DNA and proteins are in the interphase and organic phase. The aqueous phase was transferred to a fresh eppendorf and an equal volume of propan-2-ol added. This mix was incubated on ice for 15 minutes, followed by a 15 minute centrifugation as before. The RNA precipitate was visible as a white pellet at the bottom of the tube. The pellet was washed in 75% alcohol, very briefly vaccuum dried and resuspended in 100*u*l volume of DEPC H_2O. RNA preparations were stored at -20°C.

2.4.5. Northern blotting

RNA was freeze-dried in the appropriate amounts (5-10*u*g) and resuspended in 9*u*l denaturation buffer (Appendix 4.6). The RNA was denatured at 65°C for 15 minutes and placed on ice. To this was added 1*u*l ethidium bromide (1mg/ml), to allow visualisation under ultraviolet light, and 1*u*l northern dye (Appendix 4.7) to enable assessment of separation. Each sample was loaded onto a denaturing 1.5% agarose gel and electrophoresed (Appendix 4.6). The gel was subsequently placed in an apparatus designed to facilitate the overnight transfer of the RNA onto nitrocellulose (Fourney *et al.*, BRL Focus technical bulletin). Briefly, the principle of capillary action was used to transfer the RNA from the electrophoresed gel to nitrocellulose paper (3M) cut to the same size as the gel. An inverted gel rig was placed in a large tray filled with 10X SSC. Across this were laid two broad strips of filter paper soaked in 2X SSC, which extended into the buffer to act as wicks. On top of the paper was placed discarded X-ray film which

essentially covered the area of paper on the gel rig leaving a space in the centre on which the electrophoresed gel was placed. A piece of nitrocellulose, cut to the same size as the gel, and previously soaked in 2X SSC and 20X SSC was placed directly on the gel. Four pieces of dry filter paper cut to the same size were placed on top of the nitrocellulose and a bundle of paper towels on top of this. An even weight was placed on the top and the whole apparatus covered with Saran wrap to prevent evaporation. The RNA was allowed to transfer overnight, the apparatus dismantled and the nitrocellulose checked under ultraviolet light for transfer. The gel was also checked with a monitor to assess absence of signal after transfer. The nitrocellulose was allowed to air dry, placed between 2 sheets of filter paper and baked at 80^{O}C for 2 hours to irreversibly bind the RNA to the nitrocellulose. The nitrocellulose was then probed using cDNA ^{32}P probes.

Prehybridisation: The nitrocellulose filter was dampened with distilled water to discard the filter paper, placed in a bag which was then sealed on three sides. To this was added 10-20 mls prewarmed prehybridisation buffer (Appendix 4.8) at 42^{O}C. Air bubbles were teased out and the bag sealed on the fourth side. This was placed in a sandwich box filled with water in a 42^{O}C shaking water bath for 4 hours.

Hybridisation: A cDNA probe (2.4.6) was boiled for 10 minutes to denature and added to 10mls hybridisation buffer (Appendix 4.9). The prehybridisation buffer was discarded and replaced with the hybridisation buffer plus the denatured probe. The bag was re-sealed and the filter hybridised overnight at 42^{O}C in a shaking waterbath. The following morning, the hybridisation mix was discarded and the filter removed from the bag. Hybridisation was checked with a radiation monitor. The nitrocellulose filter was subjected to a regime of washes of increasing stringency (Appendix 4.11).

Autoradiography: The filter was air dried, placed in a plastic bag in an autoradiographic cassette with intensifying screens, and X-ray films inserted on either side of the filter. The cassette was placed in a black bag in a -70^{O}C freezer for 2-5 days. The films were processed in a DuPont Cronex CX-130 processor.

Stripping filters: If required, the probed nitrocellulose filters were stripped and re-probed with different cDNA probes. Filters were stripped by 2 thirty minute incubations with strip buffer (Appendix 4.12) in a 55^{O}C shaking waterbath. Elution of radiolabelled probe was monitored.

2.4.6 cDNA probe production

^{32}P-labelled cDNA probes were used to probe northern blots. The probes were prepared using the random primed DNA labelling kit from Boehringer

Mannheim. The method is modified from Feinberg and Vogelstein (1983; 1984) and is based on the hybridisation of a mix of all possible hexanucleotides to the DNA being labelled. The complementary strand is synthesised from the 3' termini of the random hexanucleotide primer using Klenow enzyme. ^{32}P is incorporated into the newly synthesised cDNA strand. Briefly, 1*u*g Po DNA in 10*u*l water was denatured by boiling for 10 minutes. This was placed on ice and nucleotides, isotope, buffer and Klenow enzyme added (Appendix 4.14). The mix was incubated at 37^{O}C for 30 minutes. 1*u*l dye (Appendix 4.15) was added to the probe mix and this was passed through an agarose bead column which had been eluted with 0.1X SSC. The radiolabelled incorporated probe was eluted in the blue fraction and was measured on a scintillation counter to assess recovery and incorporation. 1-2 x 10^{7}cpm was added to 10mls hybridisation buffer for routine probing of northern blots.

2.5 PROTEIN ANALYSIS

2.5.1 Total protein assay

Albumen was removed from the media samples by capryllic acid treatment. Briefly, the supernatants were diluted 1:30 with 3M acetate buffer and mixed on a roller for a few minutes. To this was added a 1:20 volume of capryllic acid. The mix was stirred for 30 minutes at room temperature and centrifuged for 15 minutes. Supernatants were retained and added to equal volumes of ammonium sulphate. This was mixed for 15 minutes, followed by further centrifugation. The supernatant was collected.

The protein assay was performed using Pierce Chemicals BCA protein assay according to the manufacturers instructions. In brief, 2mls solution A (4% copper sulphate solution) was mixed in a test-tube with 40*u*l solution B (base reagent: sodium carbonate, sodium bicarbonate, BCA detection reagent and sodium tartrate in 0.2N NaOH). 100*u*l sample was added to each test-tube. The basis of the reaction combines the well-known biuret method with a highly sensitive detection method employing bicinchoninic acid (BCA). Briefly, protein reacts with Cu^{2+} in an alkaline medium, yielding Cu^{+}. A purple reaction product is formed by the interaction of two molecules of BCA with one cuprous ion (Cu^{+}). The mix was incubated at 37^{o}C for 30 minutes, diluted 1:10 with water, and optical densities were read on a Pye-Unicam spectrophotometer at 562nm. Protein concentrations were assessed using a standard curve with absorbance at 562nm vs. protein concentration of standards.

2.5.2 SDS-PAGE

Gel apparatus was assembled and the resolving gel prepared. Briefly, 11.95mls water was added to 12.5mls acrylamide (30%) in a beaker on the magnetic stirrer. 3.75mls resolving buffer was added (3M TRIS-HCl) and 0.3mls sodium dodecyl sulphate (SDS). 1.5mls of a 10% ammonium persulphate solution was added. Temed (Tetramethylethylenediamine) was added immediately prior to pouring the gel. The resolving gel was poured and allowed to polymerise for 1 hour. During this time the stacking gel was prepared by mixing 2.5mls acrylamide, 11.3mls water, 5mls TRIS-HCl buffer (0.5M), 0.2mls SDS, 1ml ammonium persulphate and 20*u*l Temed. The resolving gel was washed prior to the stacking gel being poured on top. The reservoir buffer consisted of 0.025M TRIS and 0.192M glycine. The samples were loaded in a TRIS-HCl buffer containing DTT (dithiothreitol), glycerol, SDS and phenol blue. 24*u*g of each sample was added to 15*u*l loading buffer, mixed and boiled for 3 minutes prior to loading. Standards were also prepared and boiled. The samples and standards were loaded onto the

gel and electrophoresed at 10^{o}C, at 500 Volts for 2 hours. The electrophoresed gel was stained with 0.2% Coomassie blue, 30% absolute alcohol and 10% acetic acid on a rotary shaker for 30 minutes, followed by de-staining in a solution of ethanol, acetic acid and water to the ratio of 3:1:7.

CHAPTER 3

A STUDY OF SCHWANN CELLS CULTURED IN THE ABSENCE OF NEURONS

INTRODUCTION

Several previous studies have indicated that expression of many genes in Schwann cells is influenced by contact with, or absence of, axons (see sections 1.3 and 1.4 of Literature review). In this study, the expression of myelin-specific molecules was examined using freshly isolated Schwann cells from neonatal rat sciatic nerve; that is, Schwann cells which are removed from neuronal influence. Morphological studies were conducted to assess optimal culture conditions and to determine the influence of substrate and media on Schwann cells. Following the morphological studies, an immunocytochemical profile for cultured Schwann cells was elucidated. Expression of the major myelin protein Po was monitored *in vitro*, and teased sciatic nerve fibre preparations were used to localise Po mRNA in intact fibres. The aims of these experiments were to elucidate changes in expression of myelin genes in Schwann cells deprived of axonal contact. As Po is a suitable indicator of the myelin-forming phenotype it was important to localise and monitor this gene expression.

Schwann cells from unmyelinated nerve (CST) were also studied as a comparison.

MATERIALS AND METHODS

Full details of materials and methods are described in Chapter 2.

Cell culture

Dulbeccco's modified Eagle's medium (DMEM) plus 10% foetal calf serum (FCS) was used for routine culturing of dissociated Schwann cells. Schwann cells were cultured on sterile poly-L-lysine (PLL)-coated glass coverslips.

Dissociated Schwann cell cultures were prepared essentially according to the method of Brockes *et al.*, (1979). Both sciatic nerves were removed from each 4- to 5- day-old neonatal Spraque-Dawley rat pup and pooled in L15 medium. The nerves were chopped, replenished with HBSS and enzymatically digested with trypsin and collagenase, prior to trituration with a 21g needle. The resultant cell suspension was washed and the cells were plated in 50*u*l DMEM plus FCS at 10,000 per 13mm coverslip. Following adhesion the Schwann cells were fed with a further 450*u*l medium. These Schwann cell cultures are referred to as primary cultures.

Fibroblast monolayers were established by dissociating lung tissue isolated from neonatal rats. The enzymic dissociation was performed in a similar manner to that described for sciatic nerve.

Antisera and immunocytochemistry

Anti-Thy-1 was used to identify fibroblasts and used at 1:100. S100 was used to mark Schwann cells: a polyclonal antiserum (Dako) used at 1:900. NGF-R (Boehringer) at 1:100 was also used to identify Schwann cells in culture. Po was identified using a polyclonal antiserum at 1:250 for immunofluorescence or 1:500 for immunoperoxidase. GC was stained with R-mAb tissue culture supernatant (Ranscht *et al.* 1982) at 1:5 diluted in L15. An antiserum to laminin was obtained from Sigma, and routinely used at 1:1000. NGF-R, O_4, laminin and GC staining was performed on living cells, and for the demonstration of intracytoplasmic antigens such as Po and S100, cultures were fixed in 4% paraformaldehyde for 15 minutes at $4^{o}C$ and permeabilised with 95:5% ethanol:acetic acid at $-10^{o}C$ for 10 minutes. FCS was used for blocking prior to application of primary antibodies. Antibodies were diluted in L15, containing 1% FCS. Secondary antibodies were conjugated to either FITC or TRITC. Preparations were mounted in 90% glycerol in PBS containing 0.1% paraphenylenediamine to reduce fading.

Media and substrates

DMEM plus 10% foetal calf serum was used as the standard medium for Schwann cells and fibroblast cultures, with antibiotics and 200mM glutamine included.

Defined medium (SATO) consisted of a 1:1 mixture of Ham's F12 and DMEM supplemented with glucose, insulin, transferrin, progesterone, putrescine, selenite, antibiotics and glutamine (Appendix 1.1). The standard substrate was poly-L-lysine on 13mm coverslips (Appendix 1.5). Collagen was prepared in the manner described in Chapter 2.1.5, applied to 13mm coverslips, gelled with riboflavin and balanced. Laminin was used as a constituent of the medium and as a coverslip substrate.

In situ hybridisation

Cultures were fixed for 15 minutes in 4% paraformaldehyde at $4^{o}C$ and stored in 70% ethanol prior to ISH (Appendix 2.1 (M)).

Northern blotting

RNA was isolated from cultures using the RNAzol kit (Biogenesis Ltd.). Total RNA was size-fractionated on denaturing formaldehyde gels, transferred to nitrocellulose prior to hybridisation with ^{32}P labelled probes (Chapter 2.4.5).

RESULTS

3.1 Morphology of Schwann cells in vitro

3.1.1 Dissociated neonatal Schwann cells/sciatic nerve (rat).

Dissociated sciatic nerve primary cultures consisted mainly of Schwann cells and fibroblasts. Using neonatal rat pups at 4 to 5 days of age, the usual yield of total cells from one pair of sciatic nerves was in the region of 10^5. Fibroblasts adhered more quickly to the culture substrate, however within 1 hour at 37°C the majority of cells had adhered to the PLL-treated coverslips. For the first 12-24 hours the Schwann cells were visibly indistinguishable from the fibroblasts and had a rounded appearance when observed using an inverted microscope. As time progressed, the Schwann cells adopted a typical bi-polar appearance, extending long processes (Fig 3.1) filled with cytoplasm. Occasionally, a tri-polar shape was observed (Fig. 3.3 large arrowhead). Schwann cell nuclei were normally oval and tended to be central (Fig 3.3 small arrows), however some were observed near the Schwann cell membrane (Fig 3.3 asterisk). Fibroblasts assumed a very flattened amorphous morphology with large concentric nuclei (Fig 3.3 small arrowheads). After several days, the fibroblasts formed a monolayer beneath the Schwann cells (Figs. 3.1b and 3.1c) and it was obvious that fibroblasts had a higher mitotic index than Schwann cells under these conditions.

3.1.2 Purification: secondary Schwann cells

On attaining 80% confluence, Schwann cell cultures were trypsinised, washed and re-seeded. At this time, cultures were purified and expanded. A variety of methods were employed to diminish fibroblast numbers. The antimitotic drug cytosine arabinoside (AraC) decreased the numbers of fibroblasts quite effectively, if only temporarily, at a concentration of 10^{-5}M (Fig 3.2). Once a high level of Schwann cell purity was obtained the cultures were treated with pituitary extract (Chapter 2.1.6) to expand the Schwann cell numbers. Other methods used to purify Schwann cells were "panning", a technique taking advantage of the fact that fibroblasts adhere more quickly to culture surfaces, and complement kills, whereby Thy-1 antibody was utilised to select for fibroblasts.

3.2 Immunocytochemical profile

The immunocytochemical profile is displayed in Table 3.1. Primary Schwann cells displayed the S100 antigen at all times and this antibody could be utilised to distinguish between fibroblasts and Schwann cells (Fig 3.3). Fibroblasts exhibited

Thy-1 antigen (Fig 3.4) whereas Schwann cells did not. Freshly dissociated Schwann cells also displayed bright surface fluorescence with antibodies to GC and O_4 (Fig 3.5a and c). However, expression diminished to undetectable levels within a 3-4 days *in vitro*. Laminin on the surface of Schwann cells was visualised as a fine punctate fluorescence, rather than a solid structure typical of the basal lamina (the enzymic dissociation destroys the basal lamina, which will not re-form under these culture conditions). NGF-R was identified on cultured Schwann cells as a speckled punctate signal (Fig. 3.5b). Po protein was also detected by immunofluorescence (Fig 3.6) and immunoperoxidase techniques in freshly dissociated Schwann cells, but disappeared within the first 2-4 days. When routinely immunostaining with Po antisera, it was observed that an occasional cell, presumably a macrophage, had phagocytosed some Po-positive material which was most probably myelin debris (Fig 3.7).

	Day 1 *in vitro*	**Day 7** *in vitro*
S100	+	+
GC	+	-
O_4	+	-
Laminin	+	+
NGF-R	+\-	+
Po	+	-

Table 3.1 Immunocytochemical profile of cultured Schwann cells

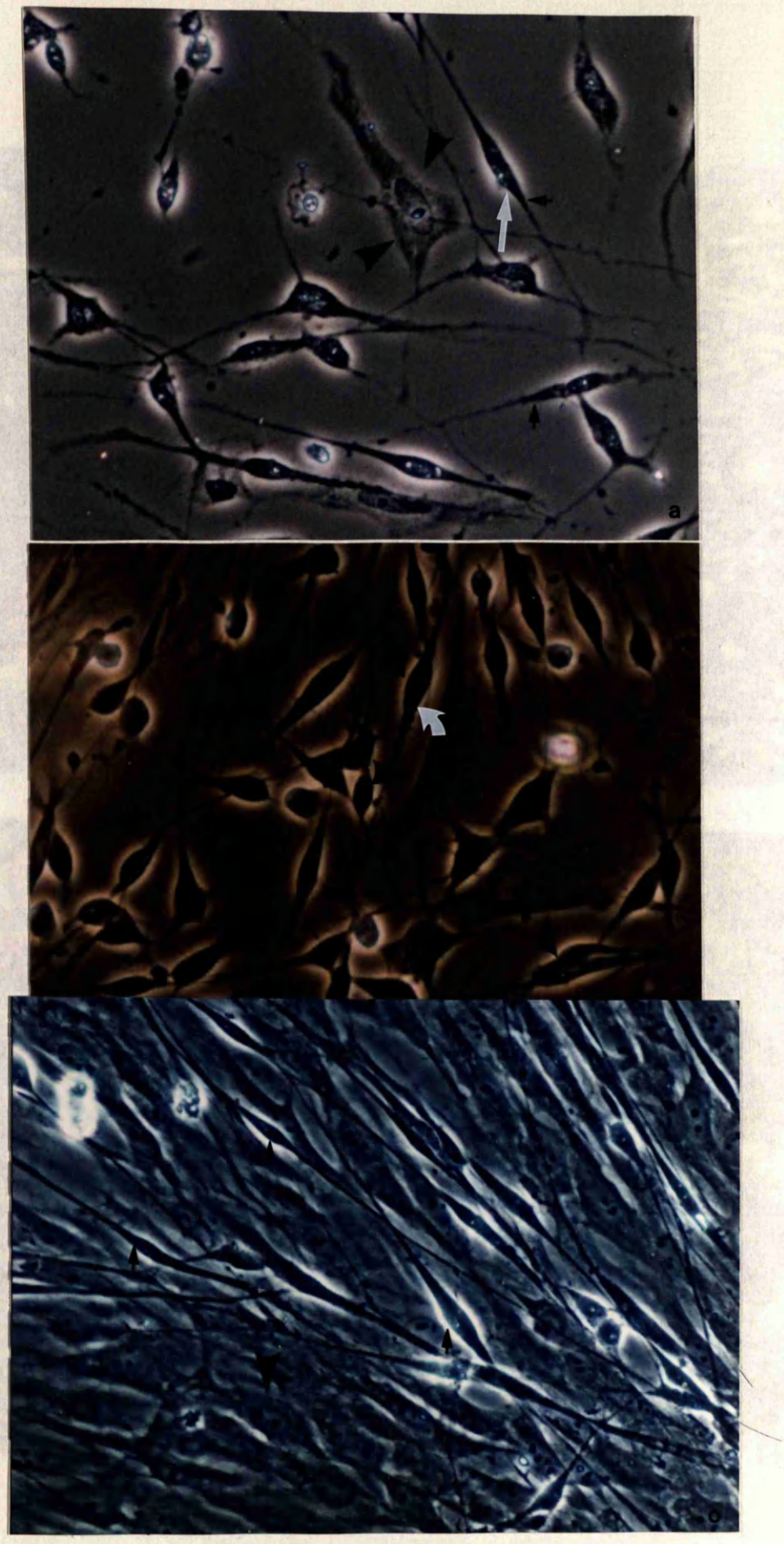


Figure 3.1

Dissociated Schwann cell culture on day 1 (a) showing typical bi-polar appearance of Schwann cells (arrows) and emergence of flattened fibroblastic cells (arrow-heads). By day 3 (b), fibroblasts are proliferating and becoming sheet-like (arrowheads), and Schwann cell processes becoming elongated (curved arrow). By day 7 (c), fibroblasts have formed a confluent monolayer (arrow-head) beneath the Schwann cells (small arrows). x 470.

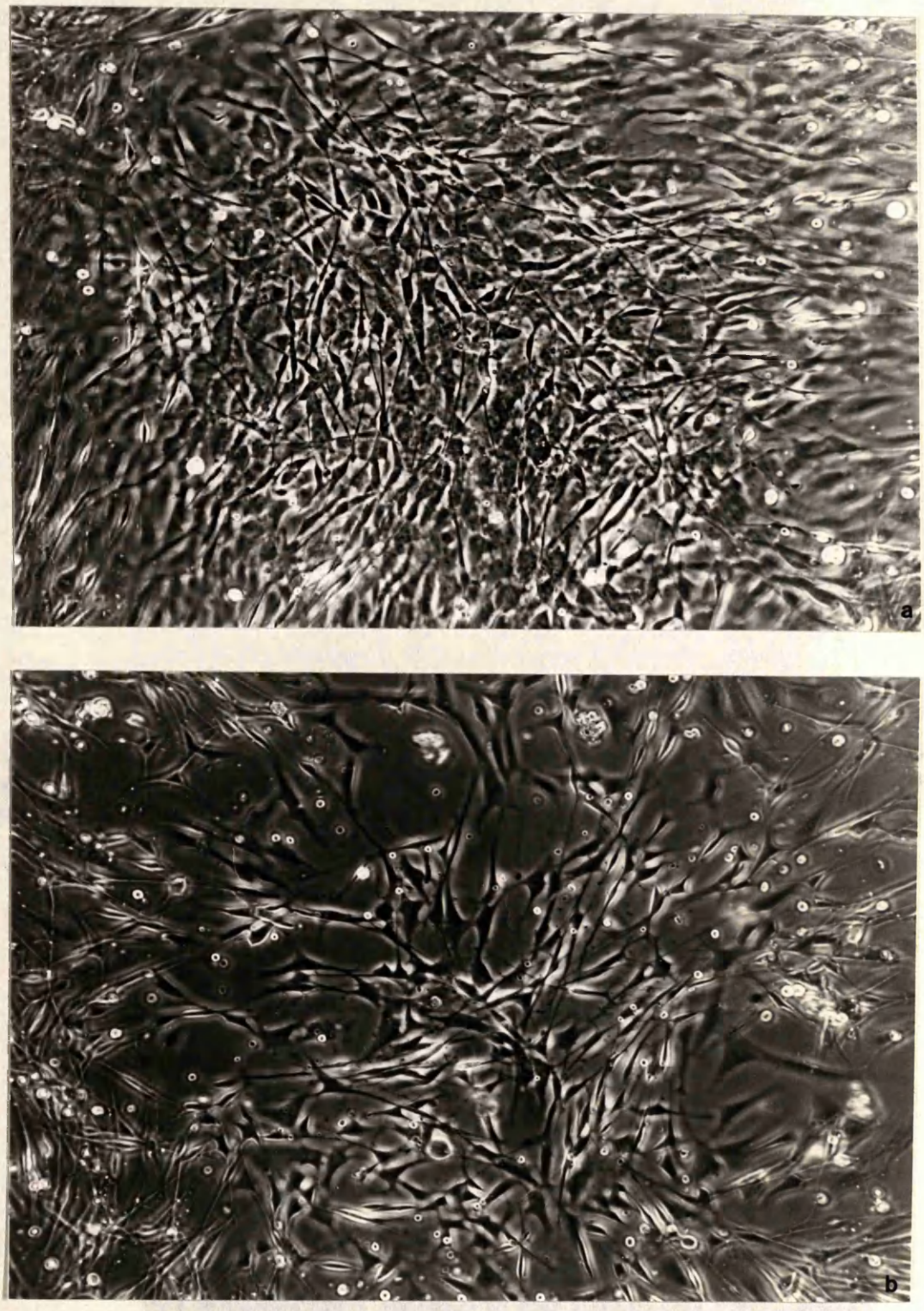


Figure 3.2
Effect of cytosine arabinoside on Schwann cell cultures. (a) normal Schwann cell culture. (b) culture subjected to cytosine arabinoside for 48 hours. Fibroblasts are drastically reduced in number in (b). x 100.

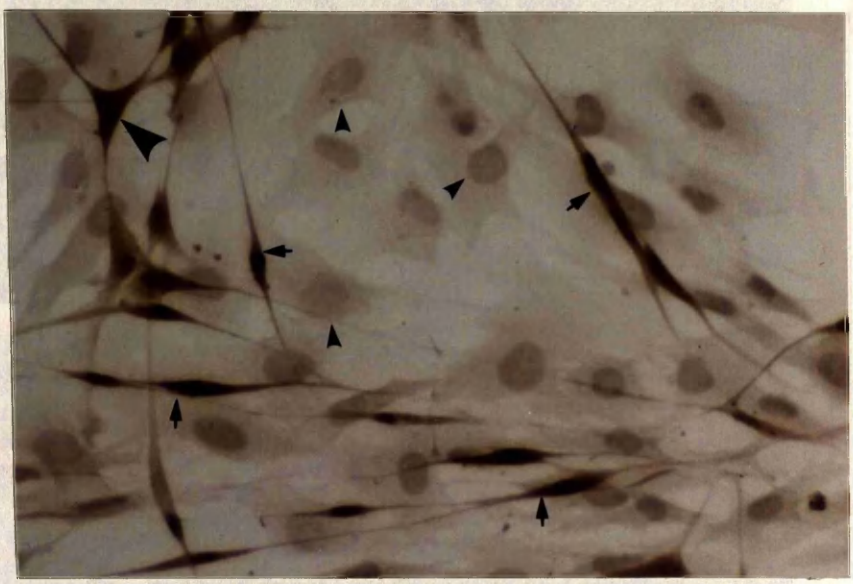


Figure 3.3
Peroxidase anti-peroxidase preparation of day 5 Schwann cell culture immunostained for S100. Large arrowhead indicates tri-polar Schwann cell. Small arrowheads indicate fibroblasts (non-stained). Small arrows indicate normal bi-polar Schwann cells with long fine processes. x 320.

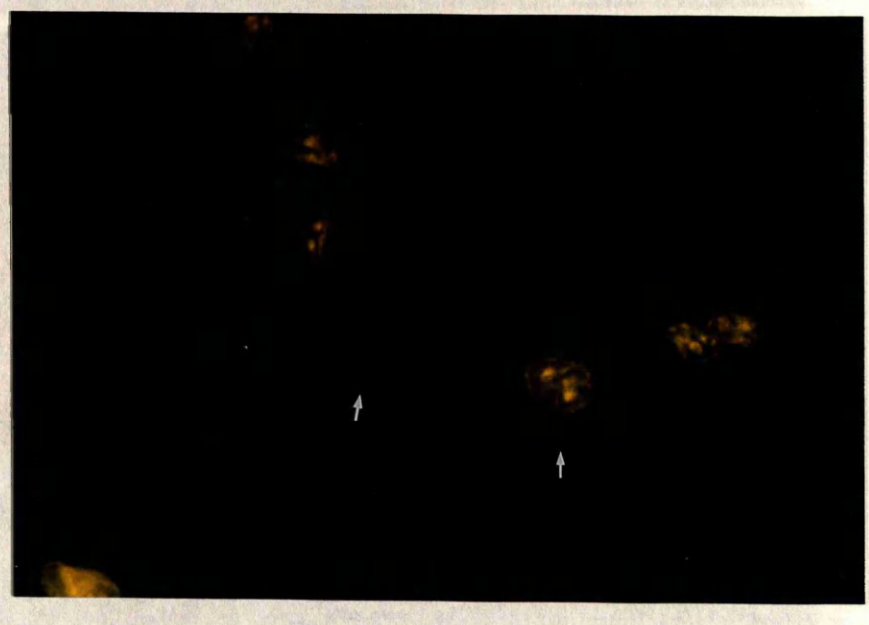


Figure 3.4
Schwann cell culture on day 5 immunofluorescently surface stained with Thy-1 antibody. White arrows indicate fibroblasts. x 320.

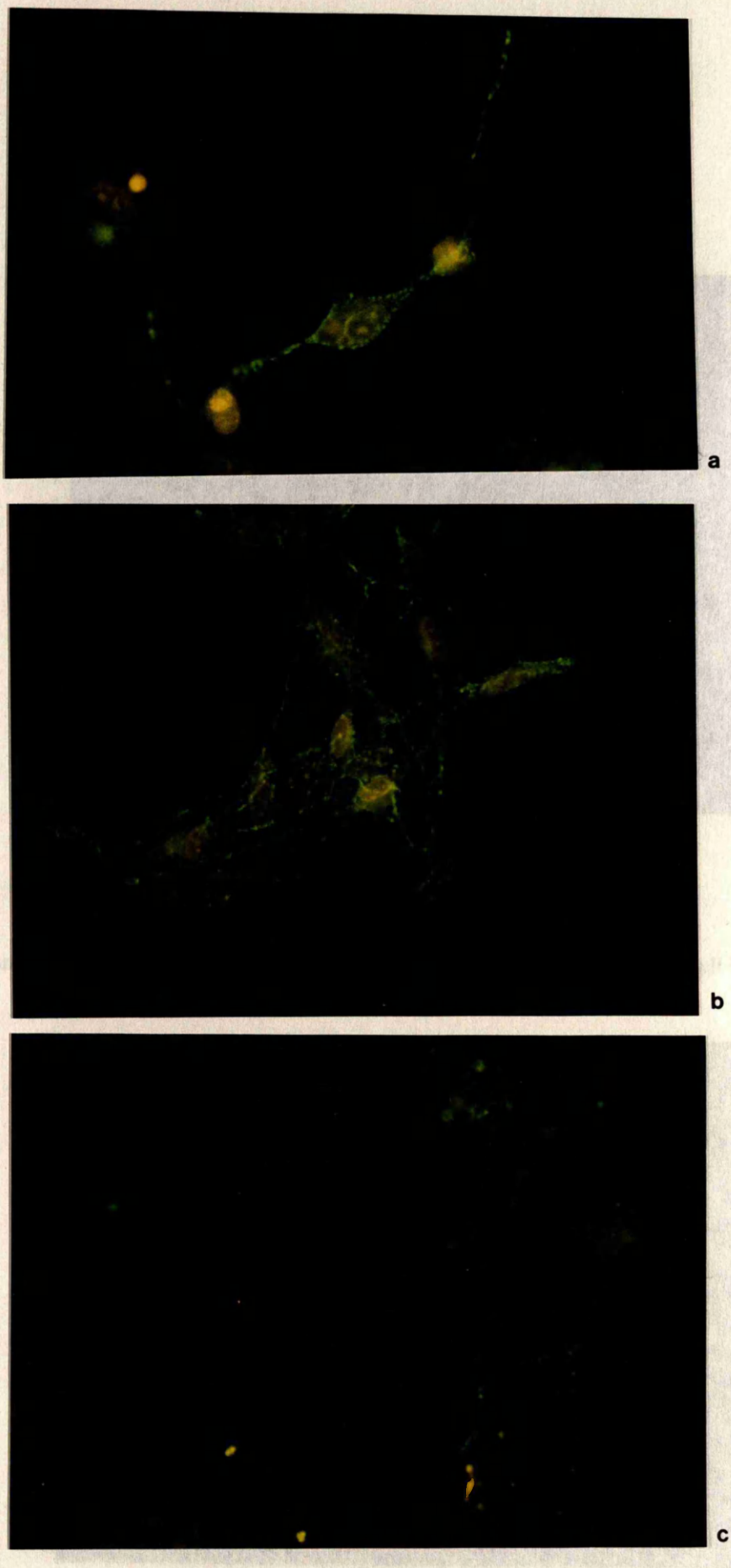


Figure 3.5.
Schwann cell cultures on day 2 *in vitro* immunostained for
GC (a), x 785
NGF-R (b), x 628
and 0_4 (c), x 814.

Figure 3.6.
Schwann cell culture on day 1 immunostained for Po. The Po-positive Schwann cell (arrow) is typically rounded at this early stage of culture. x 785.

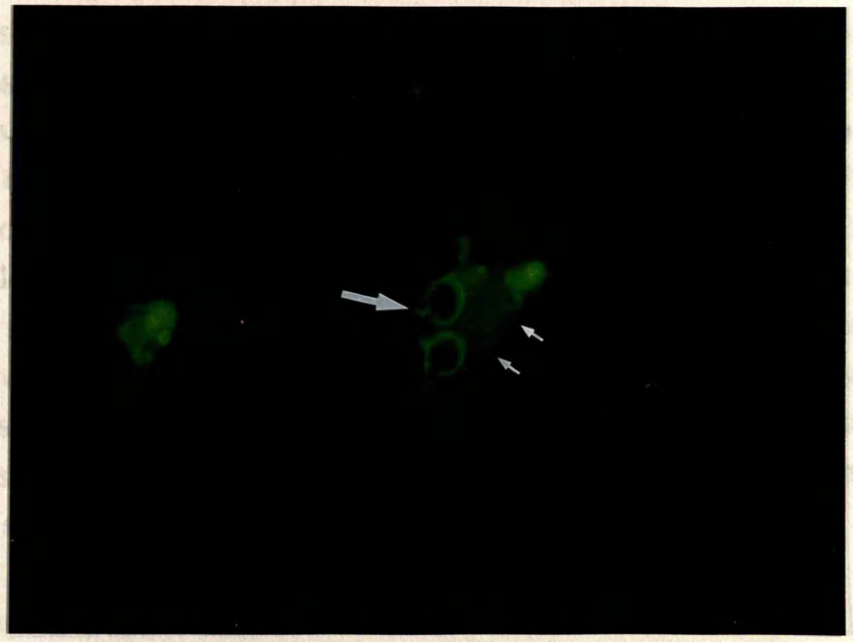


Figure 3.7
Phagocytosed myelin debris fluorescently marked with Po. This cell is most probably a macrophage. x 785.

3.3 Localisation of Po mRNA

3.3.1 Dissociated Schwann cells/sciatic nerve

Freshly dissociated neonatal Schwann cells were cultured for 4 to 5 hours in DMEM plus FCS, fixed and probed for Po mRNA by ISH. Cells were also probed for MAG and MBP mRNAs but the signals for these myelin proteins were extremely weak. Po mRNA was easily detectable and as it is the major myelin protein expressed at the time of formation of the myelin sheath, was chosen to represent the myelin-forming phenotype. Although at this time (4 to 5 hours following plating) the Schwann cells were still rounded in shape, a strong focal signal was observed around the nuclei of 8 to 20% of the cells (Fig. 3.8). Greater than 95% of these cells were identified as Schwann cells when similar cultures were examined for S100 by immunofluorescence.

3.3.2 Dissociated Schwann cells/cervical sympathetic trunk

Schwann cells were dissociated from young adult (25-30 day old) rat CST, cultured for 4 to 5 hours and examined for Po mRNA by ISH. Compared to Schwann cells derived from freshly dissociated sciatic nerve, no significant signal was observed. Schwann cells from these cultures expressed S100 but not GC antigen.

3.3.3 Teased fibre preparations

Nerve fibres from sciatic nerve and CST were loosely teased in PBS onto PLL-coated slides and air dried. These preparations were probed for Po mRNA by *in situ* hybridisation. Po mRNA was concentrated focally in the mid-internodal perinuclear cytoplasm of sciatic nerve teased fibres (Fig 3.9). The signal remains obvious throughout adulthood unlike that of MAG mRNA which is markedly reduced following myelination (Griffiths *et al.*, 1990). In teased unmyelinated fibres from the CST hybridised with Po probe, no focal signal was evident. The signal observed was comparable to that seen in fibres hybridised with sense probe.

The strong signal observed with sciatic nerve teased fibres also showed that signal intensity is related to fibre size. Larger axons exhibit a greater signal for Po than do the smaller axons (Fig 3.10).

3.4 Down-regulation of Po mRNA

Temporal changes in Po mRNA levels were examined in dissociated neonatal Schwann cell cultures. When such cultures were examined for Po mRNA by ISH 4 to 5 hours after plating (day 0), a strong focal signal was observed around the

nuclei of 8 to 20% of cells (Fig. 3.11). After 1 day *in vitro* the signal intensity of the grains had decreased (Fig. 3.12) and by day 5 the decrease was very marked (Fig. 3.13). By day 7, only a basal level of signal was observed. These results were confirmed by northern blotting of total RNA extracted from sciatic nerves of 5 day-old rat pups (equivalent to day 0 *in vitro*) and from Schwann cells after 10 days in culture in either standard DMEM or SATO. Contaminating cells (fibroblasts) of these cultures were also probed for Po. Specific hybridisation was observed only in sciatic nerve and absent in day 10 Schwann cell cultures (Fig. 3.14). Fibroblasts were also negative.

3.5 Effect of media on dissociated Schwann cell cultures

3.5.1 Morphology/Immunology

Dissociated Schwann cell cultures were also set up and maintained in a chemically defined medium (SATO). It was observed from these cultures that fibroblasts were inhibited and their mitotic rate decreased. Schwann cells adopted a more spindly appearance (Fig. 3.15), but still displayed similar immunocytochemical characteristics to those observed in standard DMEM medium. Due to its effect of inhibiting fibroblasts this defined medium was utilised in further experiments (Chapter 7).

3.5.2 Effect of laminin on Schwann cell expression

Schwann cells *in vivo* in intact nerve are associated with basal lamina, which is a sign of differentiation in Schwann cells. Dissociated Schwann cells from sciatic nerve possess no basal lamina as it is enzymically removed during preparation. Extrinsic laminin was added at a concentration of 10μg/ml to defined culture medium to determine if this influenced expression of the Po gene. Addition of laminin to the culture medium did not prevent down-regulation of Po mRNA. However, when compared with standard medium containing 10% FCS, with and without laminin, after 1 day in culture, the number of expressing cells observed by ISH was greatest in serum-containing medium with laminin and lowest in defined medium. By day 7, the mRNA levels per cell were approximately similar.

3.6 Effect of substrate on dissociated Schwann cell cultures

For most experiments Schwann cells were cultured on a substrate of PLL. Schwann cell morphology and antigens were compared using PLL, dried collagen, riboflavin-gelled collagen, and a commercial basal lamina preparation, Matrigel. Dissociated Schwann cells derived from sciatic nerve tended to attach poorly to

gelled collagen, although attachment was better on ammoniated dried collagen. Schwann cells cultured on collagen had a slightly more spindly appearance, with longer, finer processes than on PLL. Schwann cells adhered well to Matrigel. The immunocytochemical profile remained similar regardless of substrate, so most experiments were carried out using PLL as it gave the best overall results for viability and ease of use.

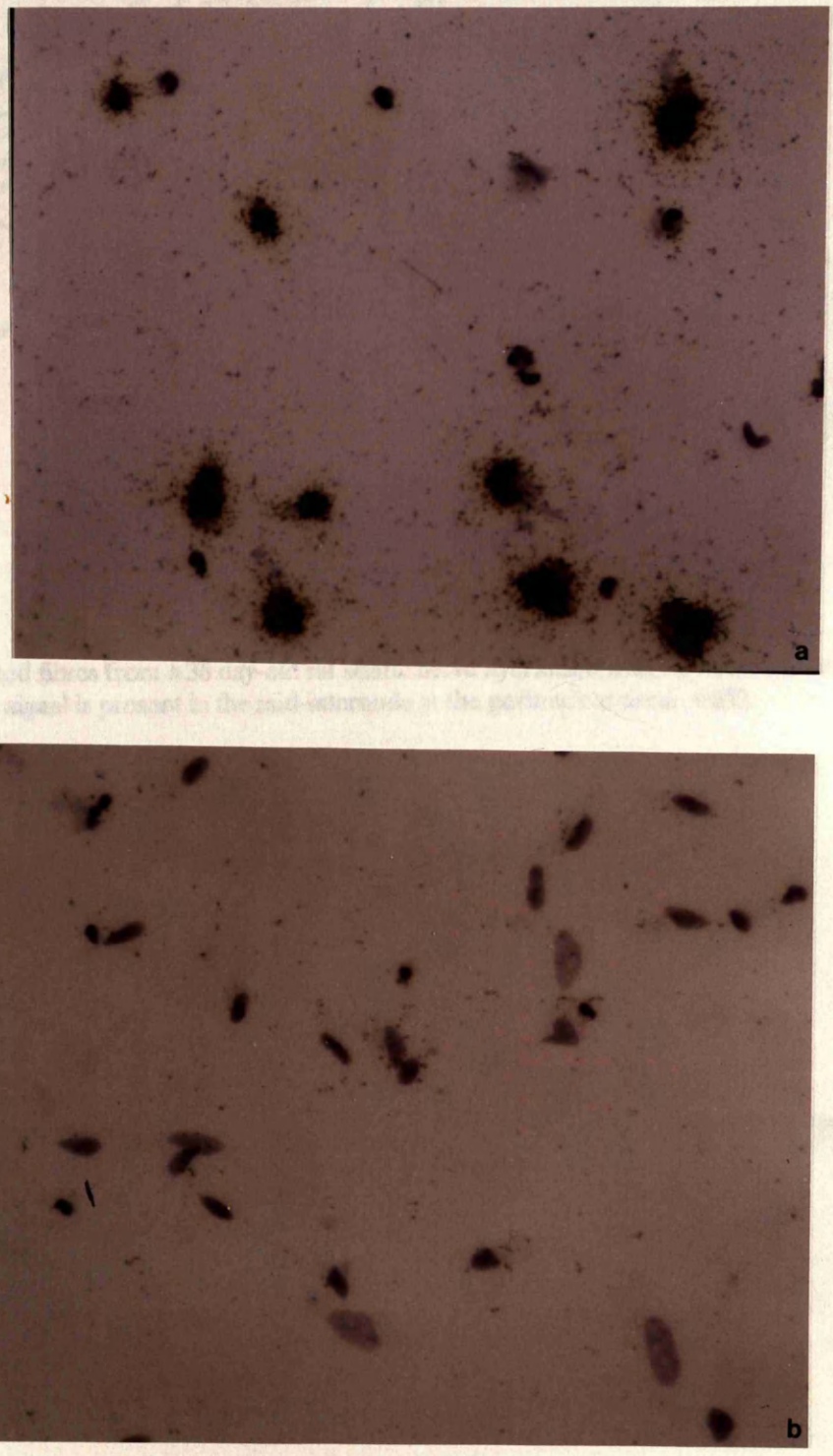


Figure 3.8

Day 0 Schwann cell cultures from sciatic nerve hybridised with Po antisense probe (a), and a sense control (b). Counterstained with haemalum. A strong signal is observed for Po mRNA in (a). x 320.

Figure 3.9
Teased myelinated fibres from a 36 day-old rat sciatic nerve hybridised with Po antisense probe. The signal is present in the mid-internode at the perinuclear area. x 350.

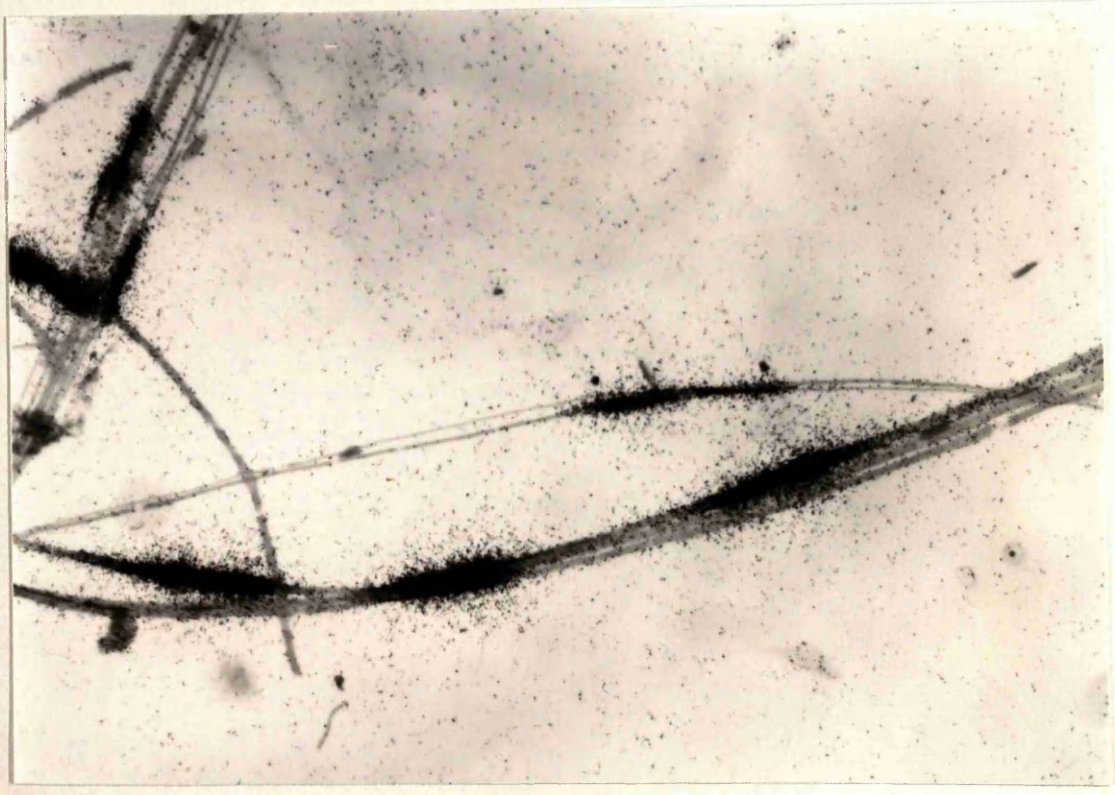


Figure 3.10
Teased myelinated fibres from 36 day old rat sciatic nerve show Po mRNA expression is related to fibre size. x 450.

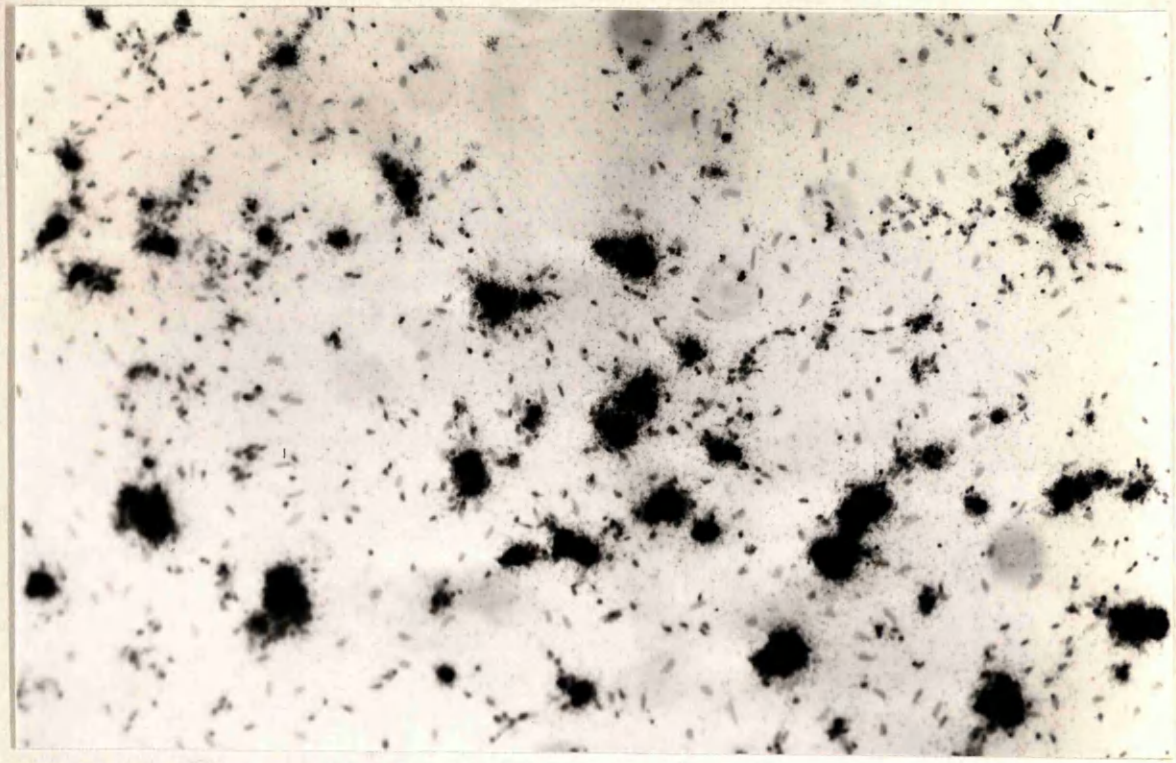


Figure 3.11
Dissociated neonatal Schwann cells from rat sciatic nerve hybridised with Po probe at 5 hours post-plating. A strong signal is observed around Schwann cells. x 320.

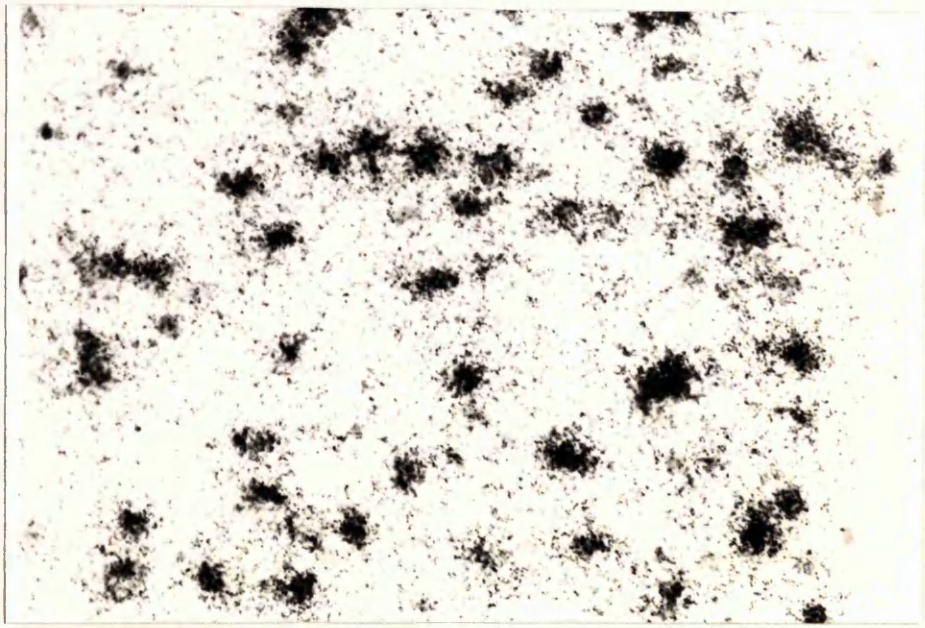


Figure 3.12
Dissociated neonatal Schwann cells from rat sciatic nerve hybridised with Po probe at 24 hours post-plating. Signal has decreased noticeably, but the message is still obvious. x 200.

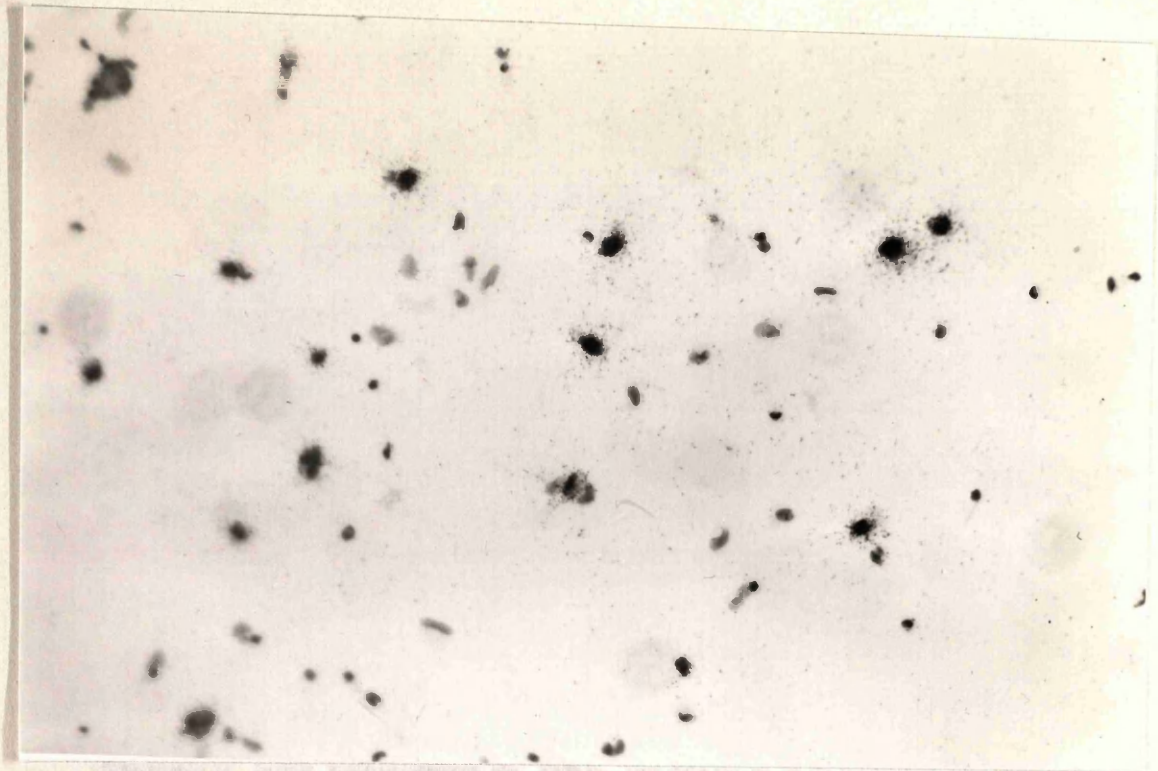


Figure 3.13
Dissociated neonatal Schwann cells from rat sciatic nerve hybridised with Po probe at 5 days *in vitro*. Signal is now drastically reduced. x 320.

Figure 3.14

Northern blots showing RNA extracted from young adult rat sciatic nerve (lane 1), RNA extracted from P5 rat sciatic nerve (the age used to establish cultures) (lane 2), and RNA from Schwann cell cultures at day 10 *in vitro* (lane 3). All hybridised with Po probe. These results confirm the down-regulation of Po in dissociated sciatic nerve cultures.

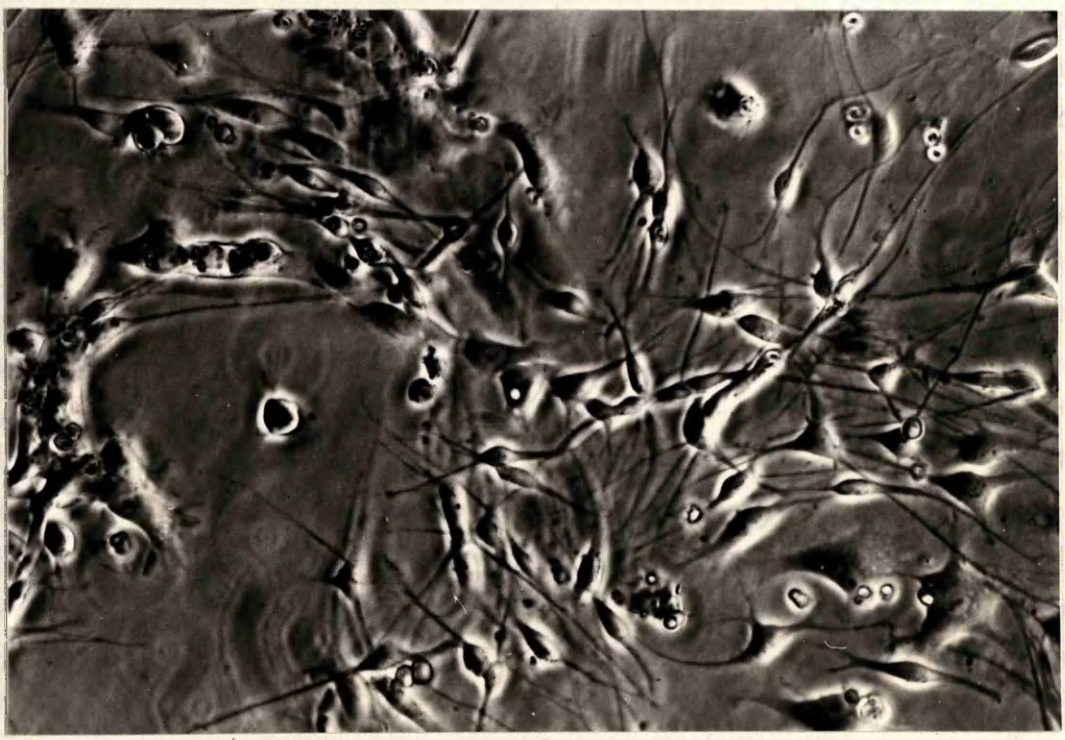


Figure 3.15

Morphology of dissociated neonatal Schwann cells cultured in DMEM (a), and defined Sato medium (b). Processes tend to be spindlier in defined medium cultures and fibroblasts are not as prevalent. x 200.

DISCUSSION

Neonatal rat sciatic nerves are in the early stages of myelination and so mechanical dissociation following enzymic digestion is not hampered by vast quantities of compacted myelin. Older animals produced very poor yields of Schwann cells and enzymatic dissociation resulted in unhealthy cultures with extensive myelin debris.

Typical cultured Schwann cells display several morphological characteristics including a small spindle-shaped soma with an oval nucleus, a bi-polar configuration with tapering processes extending 50-100*u*m in opposing directions, and have a tendency to associate with each other even in the absence of neurons (Varon and Manthorpe, 1982; Brockes *et al.*, 1979; Raff *et al.*, 1978; Mirsky *et al.*, 1980). All these features were noted during the course of my experiments with dissociated sciatic nerve Schwann cells, however exceptions were observed, with the most notable being the occasional tri-polar Schwann cell.

Examination of dissociated neonatal Schwann cell cultures showed at least two morphologically distinct cell types. Schwann cells were generally identifiable 2-3 days following plating, by their bi-polar morphology. Adjacent Schwann cells were occasionally observed to form networks or longitudinal associations with each other. The perinuclear Schwann cell region was estimated to be generally within 5-10*u*m. Fibroblasts tended to assume a quite different morphology, forming a monolayer beneath the Schwann cells if permitted. These were larger flattened cells, often greater than 20*u*m in diameter. The fibroblast nucleus was larger than the Schwann cell nucleus and sometimes contained more than one nucleolus. Fibroblasts, if uninhibited by antimitotic drugs, tended to form a confluent monolayer beneath the Schwann cells, and were noted to proliferate to a greater extent than Schwann cells. Brockes and colleagues (1979), showed Schwann cells divided slowly in standard serum-containing culture medium while fibroblasts divided rapidly. Brockes *et al.,* (1977), showed using tritiated thymidine that in 2-3 day cultures, 80-90% of the Thy-1-positive cells labelled with isotope whilst only 15-30% of Ran-1 positive (NGF-R) cells labelled. Therefore, either all the Schwann cells were dividing very slowly, or possibly a sub-population of Schwann cells was dividing more quickly. These studies were conducted in the presence of 10% foetal calf serum, which itself is mitogenic, particularly to fibroblasts. Raff and colleagues demonstrated the doubling time for cultured Schwann cells is 7-8 days in standard medium containing 10% FCS. Antimitotic drugs such as cytosine arabinoside are known to diminish fibroblast populations in sciatic nerve cultures and have been used to purify Schwann cell cultures to greater than 99%. It was my impression that the percentage of Schwann cell purity obtained by this method was

substantially less than this figure, and also that Schwann cell proliferation was affected by the drug, although to a far lesser degree than fibroblasts. Indeed, Schwann cells cultured in 10% foetal calf serum and subjected to cytosine arabinoside treatment displayed a mitotic index of 6% (Eccleston *et al.,* 1987), approximately half that observed by Raff. The effectiveness of this drug is thought to be influenced by the initial plating density (Brockes *et al.,* 1979) and it was my impression that the age of the animals dissected was a critical factor in the percentages of cell types present in cultures.

Thy-1 complement kills were attempted, with varying degrees of success to inhibit fibroblasts. On some occasions, most of the Schwann cell population was also depleted, presumably due to some toxic contaminant in the complement. Thy-1 may be trypsin sensitive which may affect its ability to "knock-out" fibroblasts in dissociated cell suspensions.

Following experiments in which fibroblast numbers were substantially controlled by antimitotic drugs, the introduction of pituitary extract into the culture medium did seem to increase Schwann cell numbers, however it also stimulated remaining fibroblasts to divide rapidly, thus resulting in fibroblast overgrowth once again.

I found the most effective and simple method of controlling fibroblasts, and at the same time encouraging healthy survival of Schwann cells was the use of a defined medium (Sato N_2). Schwann cell morphology was more spindly than in DMEM, with less cytoplasm apparent in the soma, although the immunocytochemical profile appeared similar regardless of medium.

Unequivocal identification of cell types within dissociated neonatal sciatic nerve cultures was facilitated by the use of specific immunocytochemical markers. Table 3.1 shows the results of this study. Several markers (such as GC) used to identify Schwann cells are dependent on axonal contact for maintenance. Removing sciatic nerve and dissociating the cells effectively removes axonal contact. Following axotomy *in vivo*, Schwann cells down-regulate myelin genes to resemble non-myelin-forming Schwann cells. S100 antibody will identify all Schwann cells regardless of source and status, including Schwann cells from the CST. In sciatic nerve cultures S100 is continually expressed throughout the duration of culture. Freshly dissociated Schwann cells display GC, and O_4 as surface markers. Both disappear after several days *in vitro*, demonstrating dependence on axonal contact for expression. CST Schwann cells from young rats are negative for GC. The major myelin protein Po, is also expressed in some freshly dissociated sciatic nerve Schwann cells, as are MBP and MAG. All three genes are down-regulated in dissociated neonatal sciatic nerve cultures. Some

Schwann cell specific markers are up-regulated, for example, NGF-R, N-CAM and GFAP. All the above antigens were expressed solely by Schwann cells in these cultures, while Thy-1 was localised in fibroblasts.

Dissociated Schwann cells from actively myelinating sciatic nerve expressed Po mRNA in high abundance; however, due to the loss of axonal contact down-regulation occurred within 2 days. The addition of laminin to the medium to simulate some of the conditions necessary for myelination, that is, to compensate for the loss of basal lamina, resulted in an initially higher level of signal but did not prevent down-regulation. Rutkowski and colleagues (1990) reported that cultured secondary Schwann cells expressed basal levels of Po mRNA, and Po protein throughout the culture period. This is in contradiction to my findings. However, Rutkowski's secondary Schwann cells had been expanded with cholera toxin treatment which elevates intracellular cyclic AMP levels. As cyclic AMP induces myelin protein genes, this pretreatment may have influenced gene expression. I found that RNA of other myelin proteins such as MBP and MAG showed only weak hybridisation with their relevant riboprobes. The MAG gene is expressed early in myelination and then downregulated. MAG protein is associated with axon/Schwann cell interaction and uncompacted myelin.

Po mRNA was localised in the mid-internodal perinuclear cytoplasm of Schwann cells in teased sciatic nerve fibres. The signal remained fairly intense in preparations from various ages of animals throughout adulthood (Griffiths *et al.*, 1989). In cultured Schwann cells the signal was observed as intense foci of grains around the Schwann cell nuclei; precise cellular localisation under these conditions was difficult as the cultures were immature and the Schwann cells had not yet flattened. By the time they had assumed their flatter polar arrangement the signal had already decreased significantly. Expression of myelin protein genes in Schwann cells is therefore regulated by some form of axonal influence, thought to depend on contact (Mirsky *et al.*, 1980; LeBlanc *et al.*, 1987; Trapp *et al.*, 1988; Mitchell *et al.*, 1990).

Neither dissociated Schwann cells nor teased preparations from the cervical sympathetic trunk hybridised with Po probe. Indeed, no hybridisation was expected as this nerve remains more than 99% unmyelinated. Dissociated Schwann cells from the CST of young rats did not express GC. This molecule is axonally regulated and downregulated in sciatic nerve Schwann cells with time in culture (Mirsky *et al.*, 1980; Morrison *et al.*, 1991). Northern blots confirmed *in situ* results of Po down-regulation and also showed that fibroblasts did not hybridise with Po probe.

These results showed clear evidence that expression of many myelin proteins, especially Po, is regulated by axonal influences. The weight of evidence from this and other studies seem to indicate that axonal contact is necessary, however there may be some form of diffusable signal albeit only over short distances. This will be investigated in subsequent chapters.

CHAPTER 4

ESTABLISHMENT OF NEURON-SCHWANN CELL CO-CULTURES CAPABLE OF MYELINATION

INTRODUCTION

Prior to studying Schwann cells cultured in the presence of neurons, it was necessary to establish optimal culture conditions in which myelination could be achieved *in vitro*. Myelination requires a precise set of conditions and many external factors are critical for myelination to proceed. Firstly, various substrates were assessed, including PLL and several types of collagen. Media supplements including serum and nerve growth factor levels were altered to optimise viability. The age of animals used was a critical factor; embryonic and neonatal cultures were compared. Dissociated cultures were compared with explanted neuronal cultures. Other factors studied were incubation temperature, and CO_2 levels. Optimal conditions were assessed by neuronal morphology, silver staining to assesss axonal networks and myelination was monitored immunocytochemically and by electron microscopy.

MATERIALS AND METHODS

Cell culture

Dorsal root ganglia were dissected from embryonic and neonatal Sprague-Dawley rats (Chapter 2.1.2). Explanted DRG cultures were established in a similar manner to Bunge and colleagues (1967, 1989). Briefly, the spinal cords were removed from the embryos together with ganglia, which were separated and placed onto substrate-coated coverslips (13mm or 22mm) in tissue culture wells. Neonatal DRG's were dissected from the spinal column, chopped and placed on substrate-coated coverslips as explants. Both types of DRG were also dissociated by a modified method of Wood and Williams (1984). The ganglia were pooled, enzymatically digested with trypsin and collagenase, triturated and plated at 20,000 cells per coverslip on substrate-coated coverslips. (See Chapter 2.1.2.1 and 2.1.2.2 for full methods).

Substrates

PLL was used to coat coverslips (Appendix 1.5). Various types of collagen were prepared, including commercial solutions and home-made rat-tail collagen (Chapter 2.1.5) which was either ammoniated and air-dried or riboflavin-gelled and balanced (Chapter 2.1.5.3 and 2.1.5.4).

Media

Myelinating medium was based on modified Eagle's medium (MEME), supplemented with 2mM Glutamine and Penicillin\Streptomycin. FCS was added at various concentrations and with various combinations of donor horse serum, chick embryo extract and human placental serum. Medium was supplemented with 2.5S or 7S NGF in varying concentrations.

Antisera and immunocytochemistry

Axons were stained with RT97 (Wood and Anderton, 1981) ascitic fluid (at 1:7500), which recognises a phosphorylated epitope on the 200kDa neurofilament protein. Po was identified using a polyclonal antiserum at 1:250. Cultures were fixed for 15 minutes with cold 4% paraformaldehyde, and permeabilised with ice-cold 95:5% ethanol:acetic acid at -15^{o}C for 10 minutes. FCS was used neat for blocking prior to application of primary antibodies. Antibodies were diluted in L15 medium containing 1% FCS. Secondary antibodies were conjugated to either FITC or TRITC. Single and double labelling was conducted. Stained cultures were mounted in 90% glycerol in PBS containing 0.1% paraphenylenediamine to

reduce fading. Po was also immunostained using the peroxidase anti-peroxidase technique (Chapter 2.3.2).

Silver staining

Cultures were silver-stained to demonstrate axons according to the method of Tsuji and Tobin-Gros (1980), full description in Chapter 2.2.2.

Electron microscopy

Cultures were washed in HBSS and fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer at 4^{o}C. Processing and embedding was performed as described in Chapter 2.2.3.

RESULTS

4.1.1 Substrates

Several preparations were tested using E17 dorsal root ganglion explants. Observations were made on initial explant attachment, explant flattening, neuronal morphology and maintenance of the cultures through to myelination.

PLL was applied to nitric acid-washed coverslips and air-dried. Commercially obtained collagen (Sigma) was applied to coverslips and as it was fairly fluid was also air-dried under sterile conditions. PLL and collagen were used in conjunction. Home-made collagen was prepared as described in Chapter 2.1.5.1 and dialysed as described in Chapter 2.1.5.2. This preparation was applied to coverslips in minimal amounts and as it was fairly viscous in texture was spread evenly across the culture surface. This was used either immediately following ammoniation and washing, or riboflavin gelled and balanced for 3-4 days as described in Chapter 2.1.5.4. On occasion, laminin was added to the collagen prior to application.

Air-dried commercial collagen did not provide sufficient matrix for neurons to adhere to. Fibroblasts and Schwann cells survived fairly well on this substrate, but neurons tended to be washed off during feeding or prior to immunofluorescence or silver staining. Remaining neurons tended to be granular with accentric nuclei. Cultures did not proceed to myelination. PLL resulted in similar cultures and did not provide suitable adherent properties for neurons. PLL plus collagen gave no improvement. A commercial basal lamina preparation Matrigel was discarded as it was extremely granular which interfered with observations of the cultures. Matrigel also tended to wash off very easily thereby causing loss of cultures.

Home-made ammoniated collagen was deemed unsuitable. Rigourous washing with HBSS to eradicate the ammonia from the gel resulted in damaged collagen and risk of contamination, and in the event of inadequate washing, the high pH was toxic to all cell types. Home-made riboflavin-gelled collagen gave the best results particularly if balanced for 3-4 days prior to use (Chapter 2.1.5.4). Balancing the collagen gave improved explant adherence and resulted in a three-dimensional structure providing better axonal attachment. Explants tended to flatten-out to reveal neurons which were morphologically healthy. Axonal networks thrived in the three-dimensional surface forming an intense web. PLL treatment of the coverslips prior to collagen coating and balancing resulted in marginally better attachment of explants, however the collagen layer tended to detach along with the cultures after around 7 days *in vitro*. There were several problems maintaining the collagen until myelination occurred. Application and

spreading of the collagen was a critical factor as any damage to the three-dimensional structure resulted in holes appearing in the collagen after culturing had commenced.

Ganglia which adhered quickly tended to remain attached. Re-attachment of detached explants was unsuccessful. Adherence was improved if balanced coverslips were allowed to dry slightly, but not dry out, immediately prior to plating ganglia. This improved the stickiness of the collagen.

Substrate	Attachment	Flattening	Axonal network	Morphology
PLL	+	+	+	+
Sigma collagen	+	+	+	+
Dialysed collagen	+	+	+	+
PLL+collagen	+	+	+	+
Ammoniated coll	++	++	++	+
Riboflavin coll	+++	+++	+++	+++
Matrigel	+	+	+	+
Laminin+Rb coll	++	++	++	++

Table 4.1 Influence of substrate on DRG cultures

4.1.2 Media

The medium used was based on MEME, supplemented with FCS, donor horse serum and chick embryo extract in various combinations shown in Table 4.2. The results as assessed by general morphology and eventual myelination showed that optimal conditions were achieved with 10% FCS, 10% horse serum and 5% chick embryo extract. Significantly more myelinated internodes were observed when 5% of the horse serum was substituted with 5% human placental serum. It was frequently observed with these cultures that in the higher concentrations of serum there tended to be much more fibroblast activity and subsequent loss of monolayers due to over-confluency. In higher serum concentrations there also appeared to be some cell death and neurons often became granular and generally unhealthy.

Penicillin/streptomycin was the preferred antibiotic, Gentamycin seemed to be slightly inhibitory to myelination as less myelinated internodes were observed when Gentamycin was incorporated into the medium.

Antimitotic drugs were avoided following observations that cytosine arabinoside was toxic to neurons, and cultures treated with fluorodeoxyuridine plus uridine were less viable than if drugs were omitted from the medium.

It was observed that feeding twice a week or three times a week seemed to make little difference to morphology and viability, however in actively myelinating cultures the pH of the medium tended to become acidic very quickly and hence was replaced more quickly, that is, every alternate day. In some experiments all the medium was removed and replaced with fresh medium, and in others half the spent medium was removed and replaced with fresh. The optimal regime appeared to be to remove 2/3 of the spent medium and replace with fresh.

4.1.3 NGF

Two types of Nerve Growth Factor were assessed; 2.5S and 7S. These were used at a range of concentrations (0, 50ng/ml, 100ng/ml, 200ng/ml and 400ng/ml) and either continually or initially only. The cultures were assessed for morphologically healthy neurons, good outgrowth and neurite network. Neurites and axons were immunostained with anti-NGF antibody and the cultures were also silver-stained to assess axonal networks. Schwann cells were identified with S100 immunofluorescence.

By 2 days *in vitro* all the cultures appeared fairly similar. NGF concentrations or type did not show any variation in adherence of explants or outgrowth. Continuous NGF resulted in healthier cultures generally. With regards to the 7S NGF the cultures looked very similar regardless of concentration, however when these cultures were immunostained for NF200, it did appear that the staining was more intense in the 200 and 400 concentrations than in the 50 and 100ng/ml. It also became apparent with time that more Schwann cells were present in media containing higher concentrations of NGF. Neuronal morphology seemed best with 2.5S NGF and in particular at 200ng/ml. At 50 and 100ng/ml the neurons were granular and possessed accentric nuclei. 400ng/ml gave no better results than 200ng/ml.

4.1.4 Embryonic DRG versus neonate DRG

To assess optimal age of embryos for DRG cultures destined to myelinate, I set up explant cultures from embryonic dorsal root ganglia aged E15, E16 and E17. I also assessed neonatal DRG's and investigated if the location of DRG, that is, lumbar, sacral or cervical made any significant difference. Assessments were made on neuronal morphology and outgrowth.

The age of animals affected the size and type of neurons in the cultures. At E15 there tended to be more neurons with larger cell bodies, which are purported to be more involved in myelination. In younger embryos there also tended to be slightly less fibroblast activity. For future experiments I decided to use E16 embryos as these produced viable, healthy cultures and were relatively easy to set up.

There appeared to be no significant differences between cultures derived from embryonic lumbar, sacral or cervical ganglia, therefore subsequent cultures were established from mixed ganglia.

4.1.5 Explants versus dissociated cultures

E16 DRG explants were compared with dissociated ganglia from the same source. Explant cultures tended to have better neuronal viability than dissociated cultures. Indeed, the survival rate of early cultures was far higher in explants. Dissociated cultures tended to require dense initial seeding, and required more ganglia due to loss of neurons during washing, plating and feeding. There was very little loss of material in the explants and with experience a high rate of initial attachment was achieved. Explant cultures under optimal conditions flattened down to reveal groups of neurons which were easily observable. Dissociated neurons tended to aggregate to from small groups and these survived fairly well. Isolated neurons in dissociated cultures tended to die or be morphologically poor. Explants survived well with only initial NGF supplementation during the first few days following plating. Subsequent to this they could survive with no extra NGF, however it was my impression that cultures with a constant supply of exogenous NGF achieved slightly better morphology and higher levels of myelination. Dissociated neuronal cultures required constant supplementation with NGF to maintain reasonable morphology.

4.1.6 External factors: Temperature and CO_2

Four temperatures were examined to ascertain the effects of temperature on myelination *in vitro*. At 37^{o}C it was observed that the collagen tended to begin disintegrating after around a week in culture. As this was thought to be due to enzymic action in the cultures, the temperature of the incubator was reduced to 36^{o}C. Collagen tended to last longer at this temperature with no adverse effects on neuronal morphology or myelination. Cultures incubated at 35^{o}C or 36.5^{o}C showed no improvements over those at 36^{o}C.

Cultures were incubated at four levels of CO_2. Optimal results were observed at 6% CO_2, however neurons also survived at 4% CO_2 and 5% CO_2. At

7% CO_2 cultures became quite acidic and morphology suffered with cells becoming granular and an increase in cell death.

DISCUSSION

Although of necessity some damage is incurred to the tissues during excision and dissection, the degree of damage is less for explants than for dissociated cultures. Explants do not suffer the rigours of enzymatic digestion followed by dissociation by trituration. A further advantage of the explant over dissociated cells is that the original cytoarchitecture and organotypic relationship of cells is maintained as closely as possible resembling the associations *in vivo*. However, the size of the explants must be small enough to facilitate adequate gaseous exchange and permit nutrition via the medium. Centrally located cells within the explants frequently become less effectively nourished and may become necrotic. This was observed in my explant cultures to a certain degree, however this feature is more pronounced in SCG explant cultures than DRG cultures as will be shown in Chapter 6. With dissociated cultures the arrangement of component cells and their viability is altered, there are also usually significant losses of cells due to the many steps involved. Enzymatic dissociation alters the antigenic features of cell types; for example, NGF-R is trypsin sensitive and this antigen cannot be detected in freshly dissociated Schwann cells. This may lead to confusion over cell types and identification techniques.

Visualisation of cell types may initially be easier in dissociated cultures, however my observations that explanted DRG cultures flatten over time and migration of cells from the explant enabled the formation of a fairly consistent monolayer by 2 to 3 weeks in culture. Neurons tended not to migrate to the same extent as non-neuronal cells, however they seem to be pulled by their association with Schwann cells so that the net effect of migrating cells is to flatten and stretch the explant across the substrate matrix. Non-neuronal cells proliferate in dissociated cultures and in the outgrowth of explants. There is a lag phase consisting of attachment and outward migration, an exponential growth phase and a stationary phase which is reached at confluency due to density or contact inhibition.

Mixed neural cultures not only undergo an increase in the number of non-neuronal cells but also a shift in the relative proportions of cell types present. Growth is promoted by serum, CO_2 and growth factors in the medium and growth rates of individual cell types vary. An important factor to consider when establishing dissociated cell cultures is the initial plating density as this affects the viability of individual cell types.

The microenvironment of cells in culture is controlled by medium, substratum and gaseous exchanges. Serum and embryo extract contain many

undefined constituents, some of which enhance cell growth and differentiation, however some may be inhibitory or even toxic. Constituents include variable amounts of hormones, growth factors, transport proteins, substratum-modifying proteins, vitamins, trace elements and unknown elements. Thyroid hormone, growth hormones, steroid hormones and insulin may all be present in any batch of commercial serum and are known to have significant effects on the development of the nervous system (Bottenstein, 1985).

Neuronal cultures respond and adhere to substrata which mimic the extracellular matrix *in vivo*. Explanted cultures have the advantage of conserving some of their natural extracellular matrix in culture. Enzymatically dissociated cultures lose the matrix and may require a more complex substratum to maintain viability. It was my impression that dissociated cells degraded the collagen layer more quickly than explants. This may be due to residual amounts of active trypsin or collagenase in plated cells or may reflect the requirement for a more substantial matrix.

Ascorbate is required for optimal collagen production, but has no effect on laminin synthesis (Bottenstein, 1985). Ascorbic acid acts as a co-factor for prolyl hydroxylase which forms, from collagen-bound proline, the hydroxyproline residues essential for the formation and stabilisation of the collagen triple helix. Ascorbic acid has been used to induce extracellular matrix production by cultured Schwann cells leading to myelination in the absence of serum (Carey and Todd, 1987, Eldridge *et al.*, 1989).

Laminin enhances the growth of processes of cultured human embryonic sensory neurons *in vitro*. Antibodies directed against laminin suppress all neurite growth (Baron Van Evercooren *et al.*, 1982).

Nerve growth factor exerts two main effects; (a) a neurotrophic action promoting neuronal survival and maintaining the state of differentiation in mature animals (Bunge and Bunge, 1975) and (b) neuronotropic effect in guiding axons (Campenot, 1977; Gundersen and Barrett, 1980). The neurotrophic effects of nerve growth factor are restricted to cells derived from the neural crest including sensory and sympathetic neurons and especially during development *in vivo*. The relatively high levels of NGF in sympathetic and sensory ganglia result from NGF accumulation by retrograde axonal transport rather than by local synthesis (Heumann *et al.*, 1984; Korsching and Thoenen, 1985). NGF is synthesised by Schwann cells which ensheath fibres and also by the target cells of sensory and sympathetic neurons (Bandtlow *et al.*, 1987).

Although sensory cultures are reported to survive without exogenous NGF following the embryonic stage, my results showed definite benefits of continuous

application. Optimal neuronal morphology was achieved with 200ng/ml 2.5S NGF, so this was adopted for all further experiments. The intense staining of neurofilaments with the higher concentrations of 7S NGF may indicate that this type of NGF in higher concentrations promotes neurofilament production resulting in thicker axons rather than causing more prolific neurite outgrowth. Neurofilament proteins have been shown to increase in response to NGF (Richter-Landsberg *et al.*, 1985).

Dickson *et al.* (1986) studied the effects of NGF on expression of neurofilament genes in rat PC12 cells. Addition of NGF produced increases in mRNA corresponding to NF68. Expression was dependent on NGF. This shows reversible activation of neuron-specific genes occurs during NGF-induced differentiation.

CHAPTER 5

A STUDY OF SCHWANN CELLS CO-CULTURED WITH SENSORY NEURONS IN MYELINATING MEDIUM

INTRODUCTION

This study examines the expression of myelin-associated molecules in Schwann cells cultured with DRG neurons under myelin-forming conditions established in Chapter 4. Special reference is made to the major myelin protein Po and its expression before and during myelination. Po is an integral membrane glycoprotein accounting for around 50% of the total myelin protein (Lees and Brostoff, 1984) and is believed to be important in the periodicity of the myelin lamellae (Lemke and Axel, 1985). As I have already shown (Chapter 3), the presence or absence of competent axons and/or neuronal networks significantly influences the expression of the Po gene in Schwann cells. Schwann cells derived from myelinating nerve rapidly down-regulate Po expression when cultured without neurons (Mirsky *et al.*, 1980; Lemke and Chao, 1988; Morrison *et al.*, 1991). The spatial relationship between neurons, axons and myelinating Schwann cells is a complex process and takes place as an orderly sequence of events. The appearance of certain myelin-specific molecules is indicative of early myelination events. Very little is known about the axonal signal(s) which initiate up-regulation of myelin genes.

MATERIALS AND METHODS

Media and culture substrate

Normal myelinating medium consisted of modified Eagle's medium (MEME) supplemented with 10% FCS, 10% donor horse serum, 5% chick embryo extract, 2mM glutamine, glucose, antibiotics and 200ng/ml NGF (2.5S) (Appendix 1.3). Neurons were cultured on 13mm or 22mm glass coverslips coated with rat tail collagen which was mixed with riboflavin to promote gelling. Once gelled, the coverslips were balanced for 3 to 4 days in a medium consisting of L15, FCS, glutamine and antibiotics (Chapter 2.1.5 3 and 2.1.5.4).

DRG cultures

Explant cultures were established in a similar manner to Bunge and colleagues (1967, 1988) with some modifications developed in Chapter 4. Dorsal root ganglia were removed from 16-day embryos (E16) and plated (3 ganglia per 13mm coverslip or 6 ganglia per 22mm coverslip) onto riboflavin-gelled collagen-coated coverslips in a small amount of medium to encourage adherence. After an hour, the culture well was filled to the appropriate level with complete medium. Cultures were maintained in myelinating medium and fed 2-3 times per week by removing 80% of spent medium and replenishing with the same amount of fresh medium.

Antisera and immunocytochemistry

Axons were stained with ascitic fluid RT97 at 1:7500. RT97 recognises a phosphorylated epitope on the 200kDa neurofilament protein. S100 was used to identify Schwann cells in co-cultures at 1:900, and NGF-R also identified Schwann cells at 1:100. Po protein was identified using a polyclonal antiserum developed within our group, which was used at 1:250 for immunofluorescence or 1:500 for immunoperoxidase. GC was stained with tissue culture supernatant R-mAb (H8) (Ranscht *et al.*, 1982) at 1:5, and the monoclonal O_4 (Sommer and Schachner, 1981) which detects sulphatide on Schwann cells (Mirsky *et al.*, 1990) was used at 1:50. A commercial antiserum to laminin (Sigma) was used at 1:1000.

NGF-R, GC, O_4 and laminin staining were performed on washed, living cultures which were post-fixed with ice-cold ethanol. For the demonstration of intracellular antigens, including intracytoplasmic laminin, cultures were fixed with 4% paraformaldehyde and permeabilised with ice-cold 95:5% ethanol:acetic acid prior to application of neat FCS to block non-specific reaction, and incubation overnight at $4^{o}C$ with the primary antisera.

Secondary antisera were conjugated to FITC or TRITC for either single or double labelling of antigens. Preparations were mounted in 90% glycerol in PBS containing 0.1% paraphenylenediamine to reduce fading.

Po was also immunostained using the peroxidase anti-peroxidase technique (Chapter 2.3.2).

Silver staining

Explant cultures were silver-stained to demonstrate axon networks using the method of Tsuji and Tobin-Gros (1980). (Full method in Chapter 2.2.2.)

In situ hybridisation

Cultures were fixed for up to an hour in 4% paraformaldehyde at 4^{O}C and stored in 70% ethanol prior to *in situ* hybridisation (Chapter 2.4.1). ^{35}S-labelled antisense and sense probes were prepared as described in Chapter 2.4.3.

Northern blotting

RNA was extracted from the cultures using the RNAzol kit (Biogenesis Ltd.). 10*u*g samples of total RNA were size-fractionated on formaldehyde denaturing gels and transferred to nitrocellulose prior to hybridisation (Chapter 2.4.5) with ^{32}P-labelled probes (Chapter 2.4.6).

Electron microscopy

Cultures were washed in HBSS and fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer at 4^{O}C for 1 hour. The explants were processed intact on their coverslips according to the method in Chapter 2.2.3 to the dehydration stage with 70% ethanol. At this point the explants were embedded in an agar sandwich and the specimen detached from the coverslip. The area containing the dehydrated explant was excised from the gel and processed through the remainder of the schedule to embedding in Araldite$^{(TM)}$.

RESULTS

5.1 Morphology

Explants from E16 rats were placed intact onto three-dimensional gelled collagen. Within a few hours the explant was observed to have produced processes and adhered to the surface (Fig. 5.1). By 24 hours individual cells were observed to be migrating out from the body of the explant (Fig. 5.2a and b). As the processes extended radially the central clump of cell bodies tended to flatten. In these conditions, in the absence of antimitotic drugs, fibroblasts proliferated rapidly and by 5 days *in vitro* the neurite-Schwann cell population was surrounded by fibroblasts and fibroblast processes (Fig. 5.3a). The fibroblasts tended to form a monolayer beneath the Schwann cell-neuron population. Fibroblasts extended further radially than Schwann cells and eventually covered the entire collagen coated surface, forming connections between separate explants. Neurons were observed to migrate from the centre of the explant after 4-5 days (Fig. 5.3b). By 2-3 weeks in culture axons, neurites and Schwann cell processes had formed a complex network across the collagen (Fig. 5.4). Individual neurons became visible with identifiable nuclei and nucleoli (Fig 5.5). Neurons tended to aggregate in groups and assumed a fairly granular appearance. By the third week in culture, randomly myelinated internodes were observed under phase contrast microscopy.

5.2 Immunocytochemical profile

Virtually as soon as processes and cells started to emanate from the explant body immunocytochemical identification was possible. Established cultures stained by silver techniques or immunostained for neurofilaments demonstrated profuse axonal meshworks (Figs. 5.6 and 5.7). Schwann cells were positively identified by S100 (Fig. 5.8). Figure 5.9 identified Schwann cells expressing GC. The randomly myelinated internodes observed in mature cultures were confirmed by staining with Po antiserum. Double labelling with RT97 to identify neurofilament and Po to identify myelin showed which areas of an individual axon were myelinated (Fig 5.10). NGF-R was down-regulated in Schwann cells which were in association with axons or neurites. O_4 was present on the same population of Schwann cells as GC.

5.3 Po mRNA expression

The temporal expression of Po mRNA was studied once it was apparent that under these culture conditions myelination *in vitro* was possible. By 24 hours in culture, a few weakly positive Schwann cells were present in the outgrowth around the explant bodies when examined by *in situ* hybridisation with Po antisense probe (Fig. 5.11). Explants probed with the sense riboprobe were negative. By 4 to 5

days an obvious signal was present around the centrally-located neuronal cell bodies which were essentially negative (Fig. 5.12). As the neurite-Schwann cell outgrowth increased with time in culture, the intensity of signal and area occupied by Po mRNA-expressing cells markedly increased (Figs. 5.13 and 5.14). At the periphery of the cultures where the fibroblast outgrowth extended further than the Schwann cell-neurite outgrowth silver grains became very sparse indicating no significant signal associated with fibroblasts.

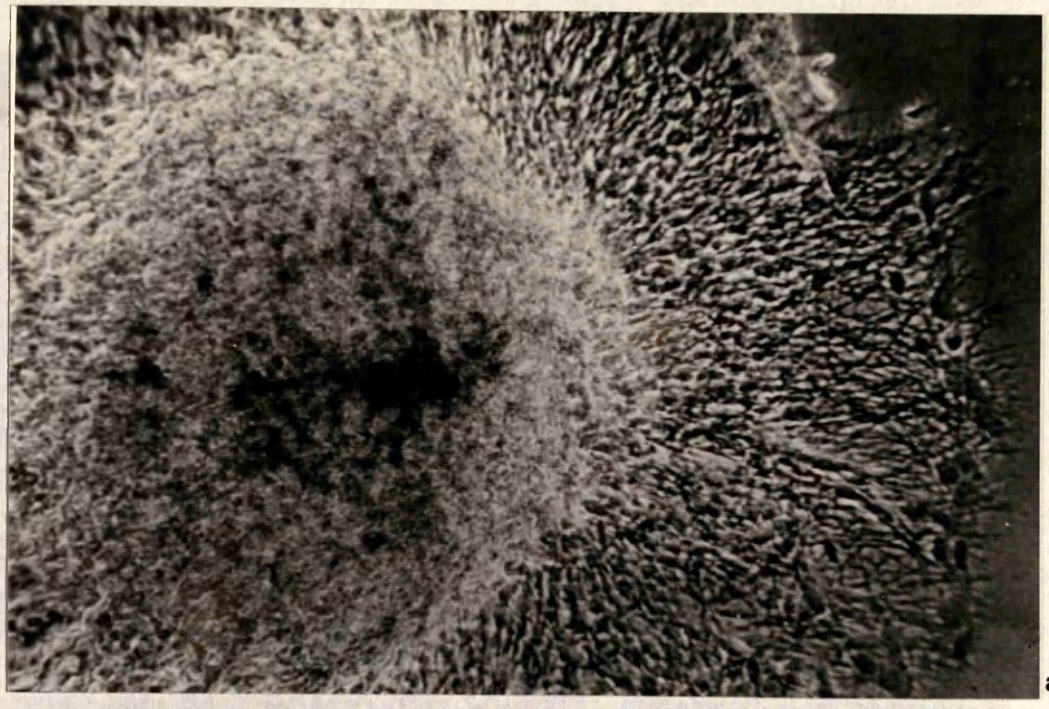
The area of Schwann cell outgrowth tended to be covered in silver grains and because of the high density of Schwann cells it was not possible to identify individual Schwann cells, as in freshly dissociated sciatic nerve cultures. Over the majority of Schwann cells the silver grains were scattered in a diffuse manner unlike the intense focal signal associated with Schwann cells freshly isolated from myelinating nerve. Some clustering of very intense signal was observed in the areas of the culture nearest the neuronal cell bodies.

Expression of Po mRNA under these conditions was confirmed by northern blotting. Total RNA was isolated from 7- and 21- day-old cultures, size-fractionated on formaldehyde denaturing gels and transferred to nitrocellulose. The nitrocellulose filters were hybridised with Po which showed a specific signal at 1.9-2.0kb. This signal increased in intensity by 57% between the two time points. RNA extracted from E16 ganglia (the age used to establish the cultures) revealed no specific Po message. It was observed during these experiments that a less intense band at 4kb was also present (Fig. 5.15).

5.4 Electron microscopy

Electron microscopy studies confirmed the presence of compact myelin in mature cultures (Fig 5.16a and b). Various degrees of association between Schwann cells and axons were observed. Bundles of axons were enveloped by a Schwann cell process(es); in other cases a 1:1 relationship was present, sometimes with complete ensheathment and in some instances compacted lamellae had formed. Basal lamina was a prominent feature with many Schwann cells displaying a continuous or discontinuous layer. Microtubules and neurofilaments were observed in smaller and larger axons. Fibroblasts formed a monolayer beneath the Schwann cells and neurons, and the collagen substrate was visible beneath the fibroblasts.

Figure 5.1
Dorsal root ganglia at less than 12 hours *in vitro*. Peripheral outgrowth is emerging from the body of the explants. Dark-ground photography. x 25.



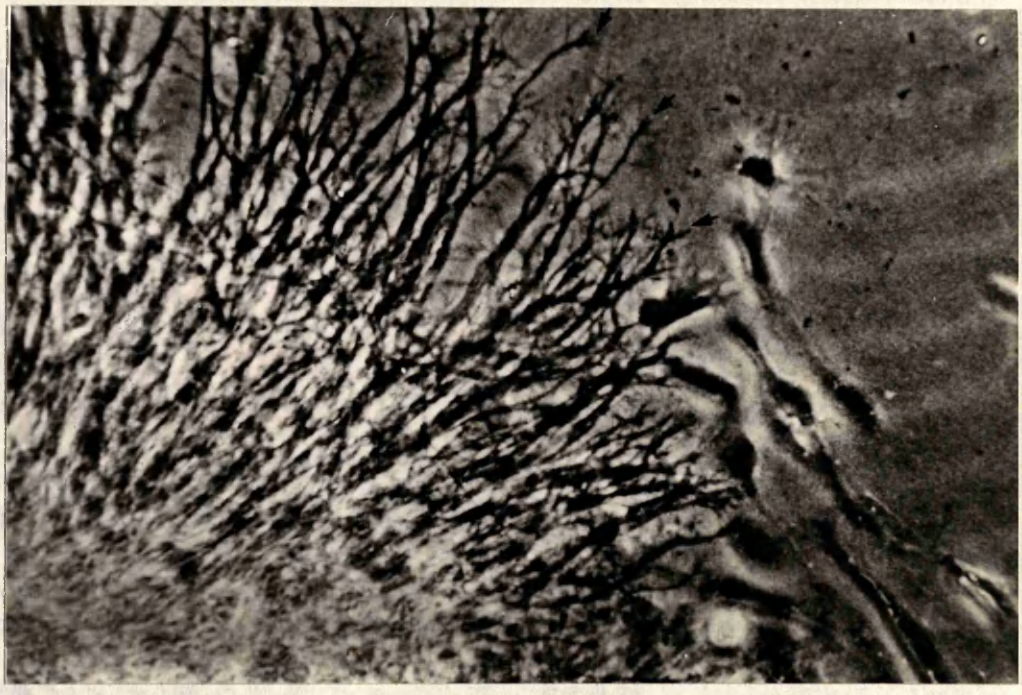


Figure 5.2

Dorsal root ganglion explants at 24 hours *in vitro*. (a) Schwann cell, fibroblast and neurite outgrowth is emerging rapidly. Explants are now firmly attached to the substrate, x 100. (b) Neurite growth cones emerging (arrows), x 200.

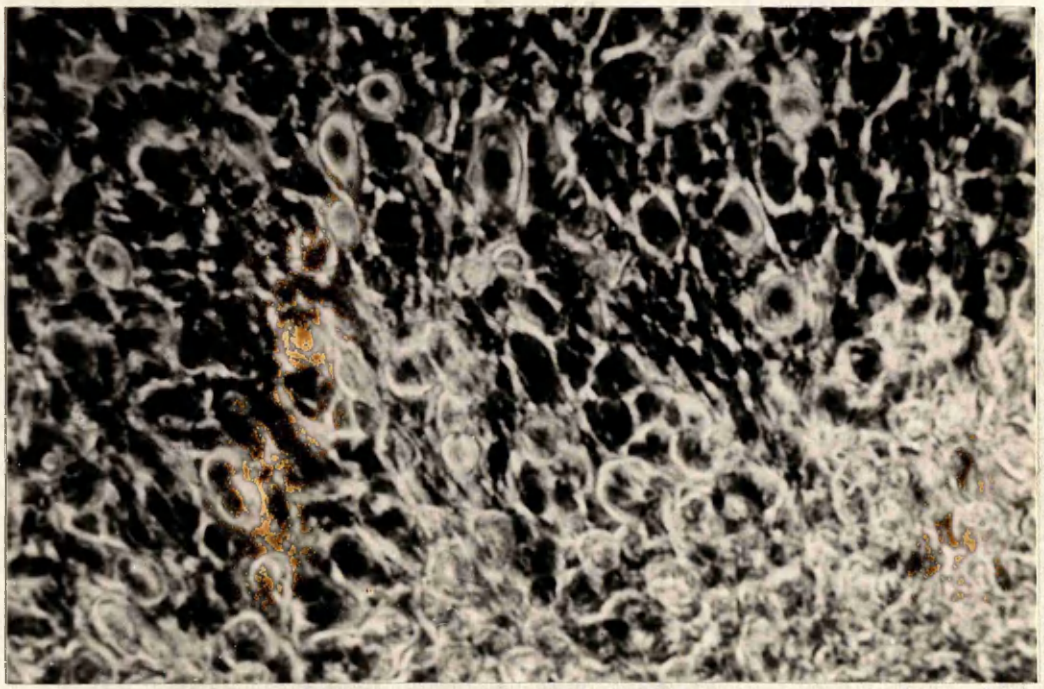


Figure 5.3
A day 5 DRG culture showing (a) established outgrowth of cells and processes towards periphery of explant, x 100, and (b) flattening of central part of explant with neurons becoming distinguishable (white arrow), x 200.

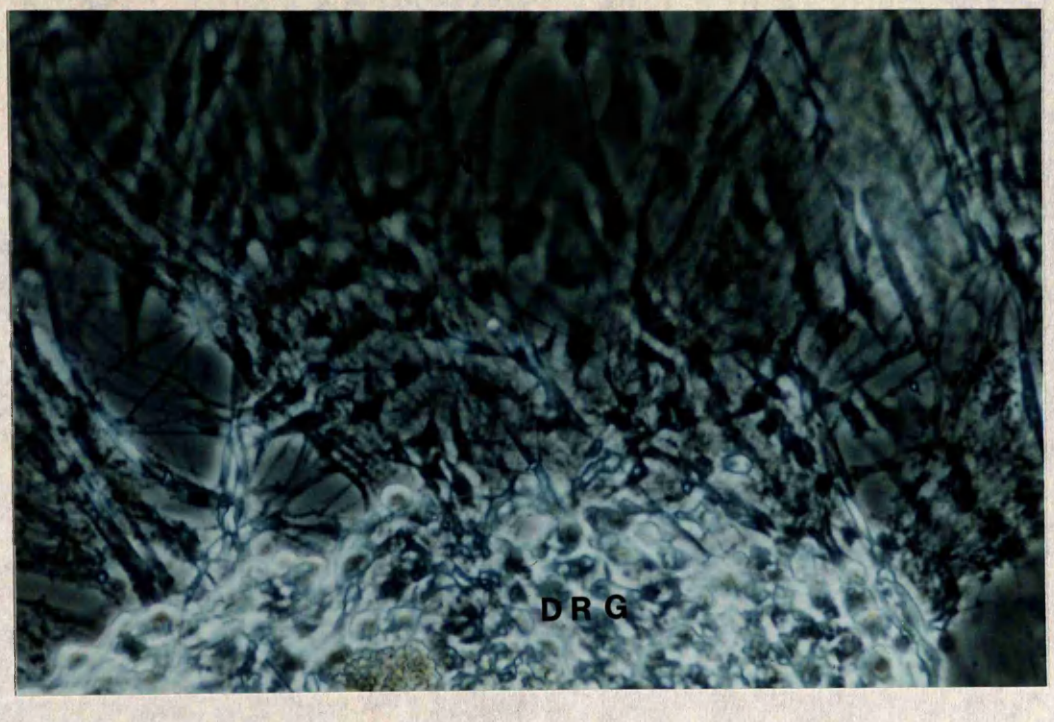


Figure 5.4
A 14 day DRG culture showing flattening explant (DRG), with confluent outgrowth and profuse neurite network (N). x 250.

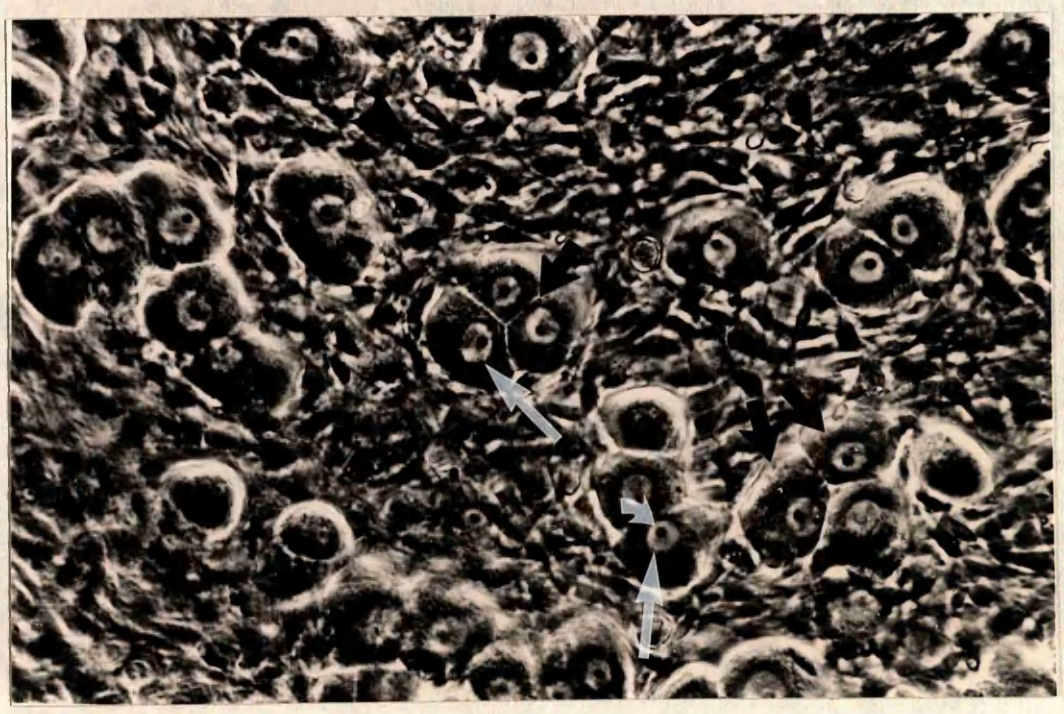
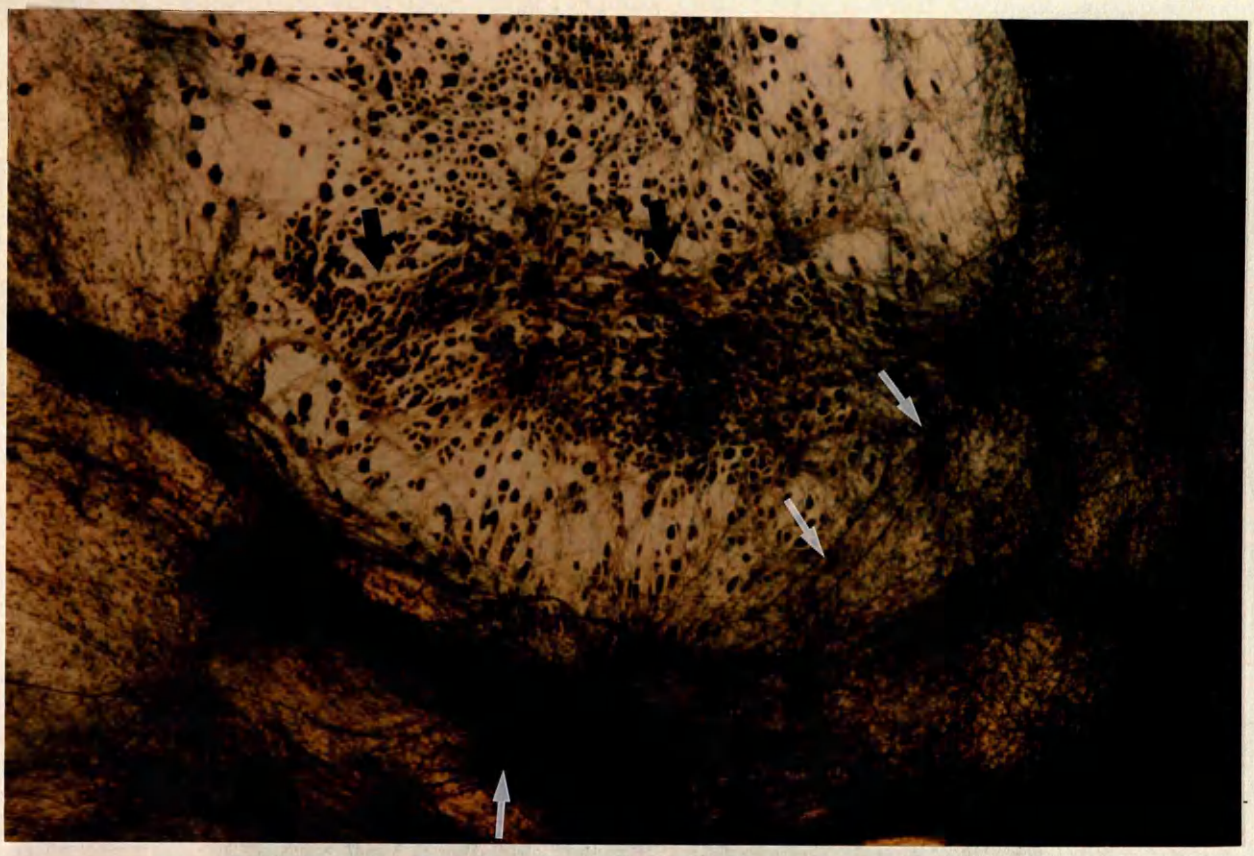


Figure 5.5
A 21 day DRG culture showing individual neurons tending to aggregate (arrows). Note the nuclei (white arrows) and nucleoli (curved arrows). A confluent bed of Schwann cells and fibroblasts encompass the neurons (arrowheads). x 200.



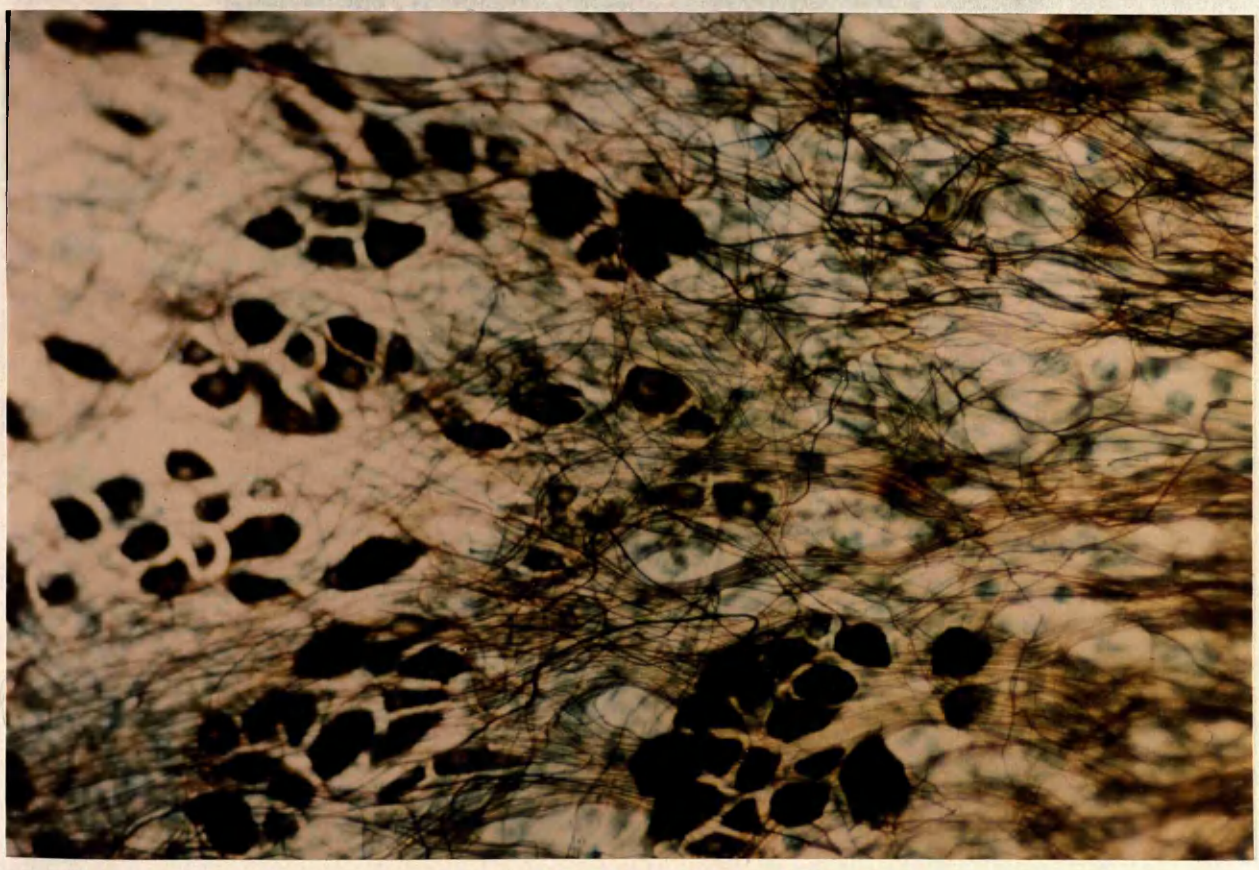


Figure 5.6

DRG culture at 31 days *in vitro* silver stained to demonstrate axonal networks. (a) shows a concentration of neurons in the centre of the frame (arrows), with profuse non-neuronal cell growth and axonal networks to the periphery (white arrows), x 235. (b) shows enlargement of detail to demonstrate individual neuronal morphology and underlayer of Schwann cells and fibroblasts counterstained with methylene blue, x 470.

Figure 5.7
DRG culture at 31 days *in vitro* immunostained with RT97 to demonstrate the 200kDa neurofilament within axonal networks. Rhodamine optics. x 785.

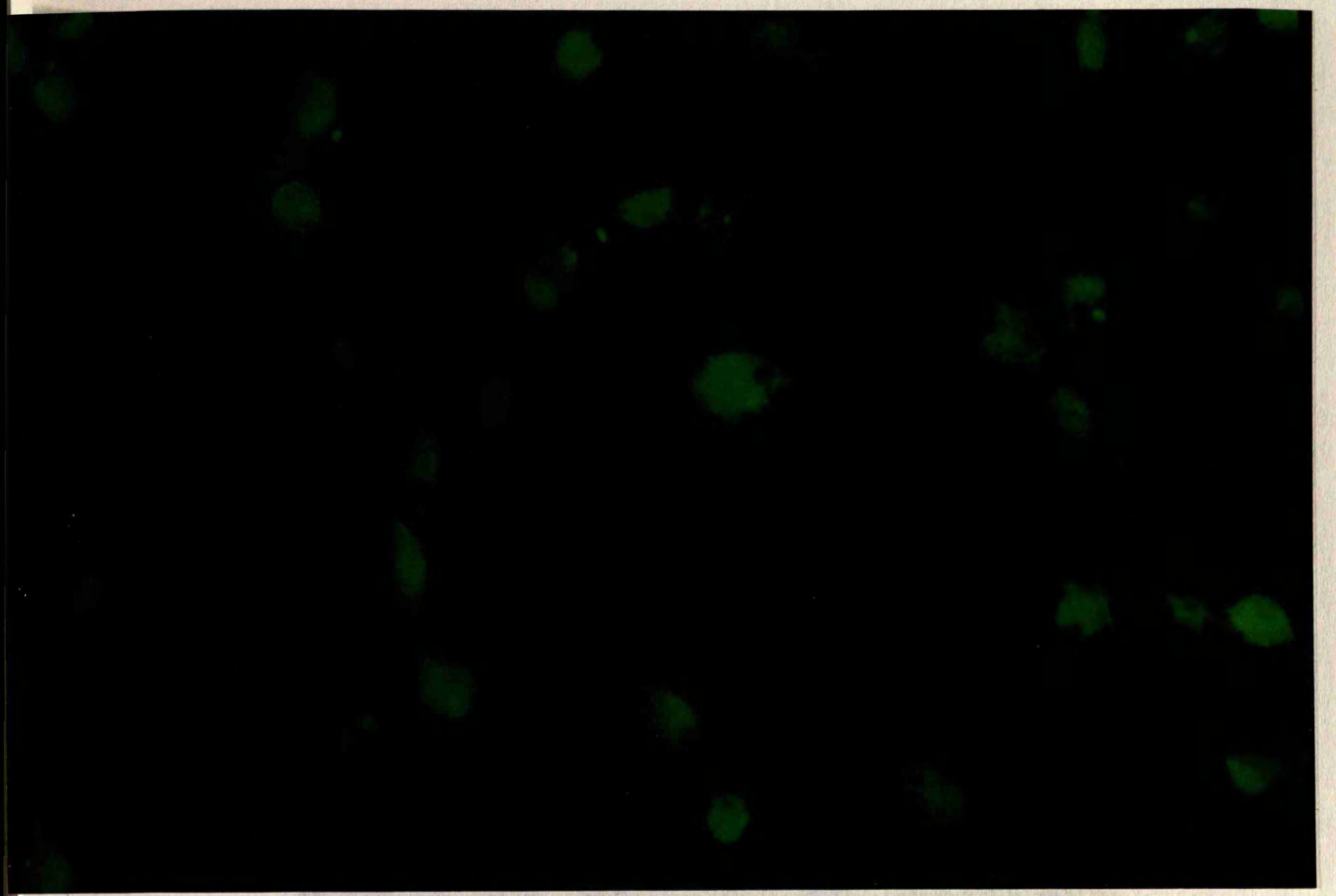


Figure 5.8
DRG culture at 10 days *in vitro* immunostained for S100 to identify Schwann cells. x 625.

Figure 5.9
DRG culture at 10 days *in vitro* immunostained for galactocerebroside with R-mAb to demonstrate maintenance of expression under myelinating conditions. x 625.

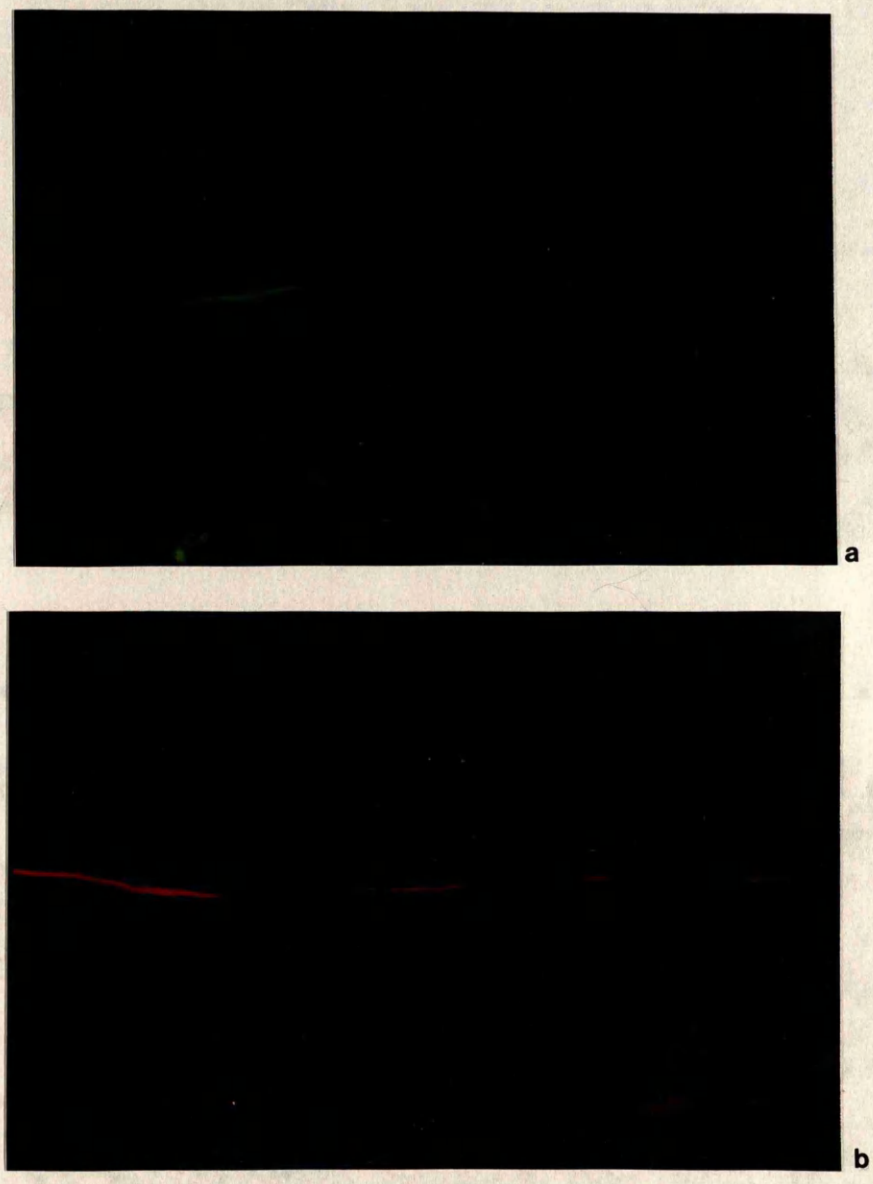


Figure 5.10

Day 31 DRG culture double-labelled with Po and NF200 antisera to demonstrate areas of myelin along one axon. (a) shows myelinated area labelled with Po conjugated to FITC. (b) shows corresponding areas of same axon labelled with NF200 antiserum under rhodamine optics. Unmyelinated areas are more heavily stained than myelinated zones; this may reflect a reduced penetration of antibody into the myelinated axon . x 355.

Figure 5.11
Autoradiogram of a day 1 DRG culture hybridised with Po, showing very little signal. x 200.

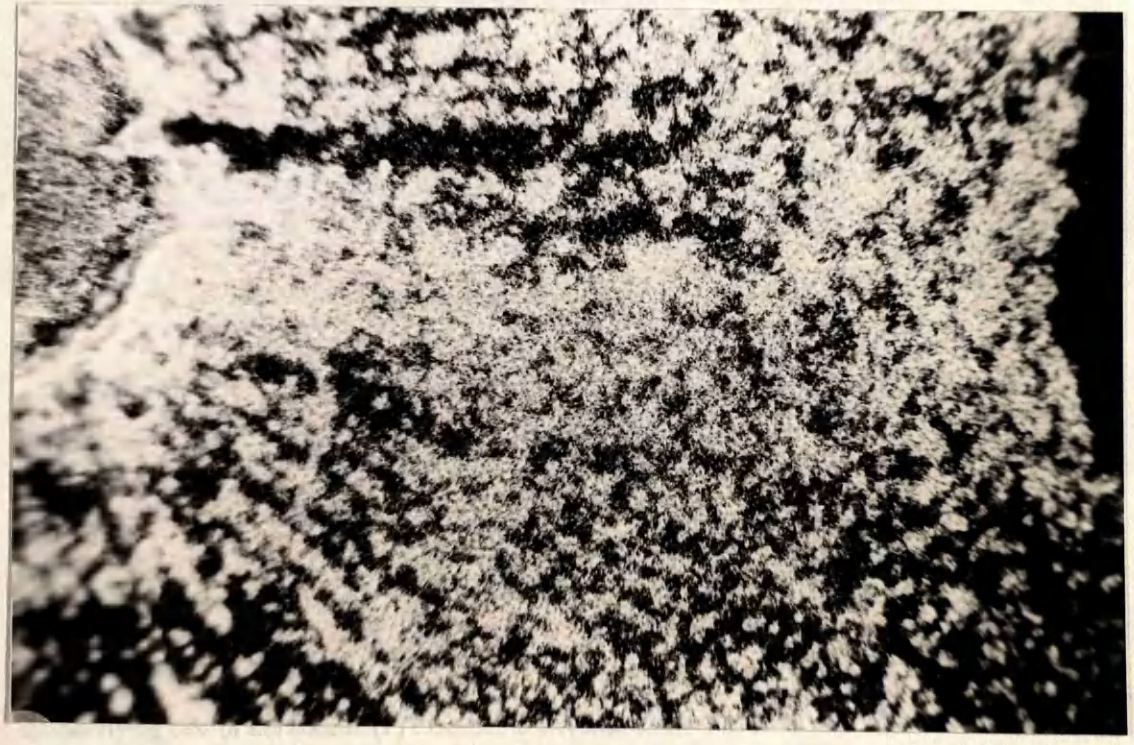


Figure 5.12
A day 5 DRG culture hybridised with Po by ISH, showing increased expression of signal. x 200.

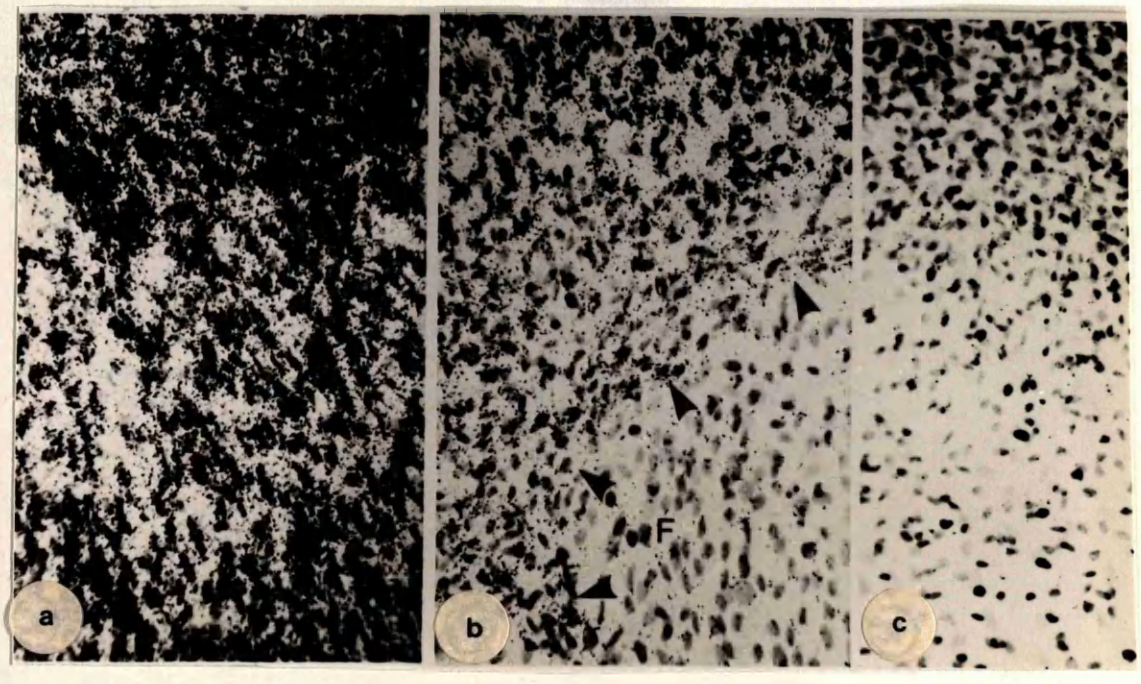


Figure 5.13

A mature DRG explant culture in the third week of culture hybridised with Po by ISH, showing intense signal (a). (a) is from an area near the neuronal cell bodies showing strong signal particularly in the upper portion where the grains show a more focal, clumped appearance. (b) is from the periphery of the same culture at the edge of the major Schwann cell outgrowth (arrowheads). An underlying bed of fibroblasts (F) extends further outwards. Signal is confined to the Schwann cell area and is less intense than the signal observed nearer the neuronal cell bodies. (c) represents the sense control. Exposure time 4 days. x 160. Counterstained with haemalum.

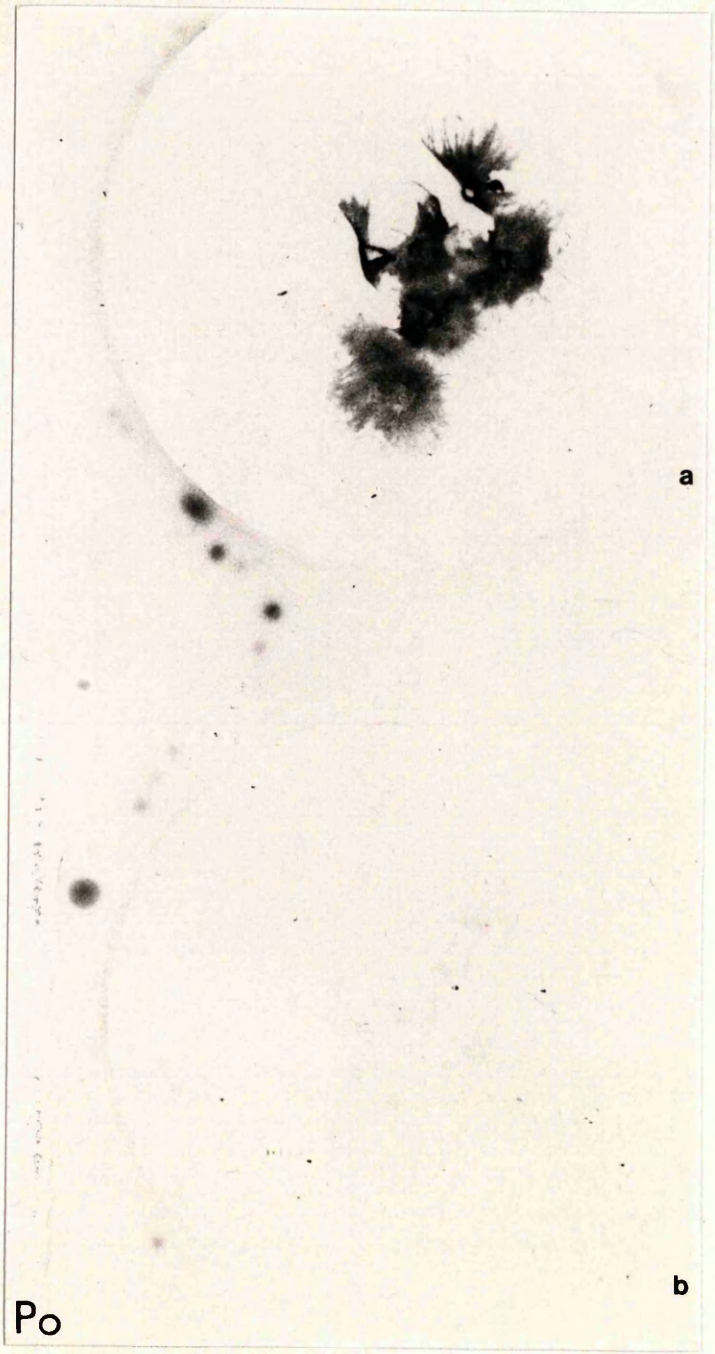


Fig. 5.14
In situ hybridisation of day 4 DRG explants. (a) shows 5 explants hybridised with Po antisense probe. (b) shows similar coverslip treated with Po sense probe. x 8.

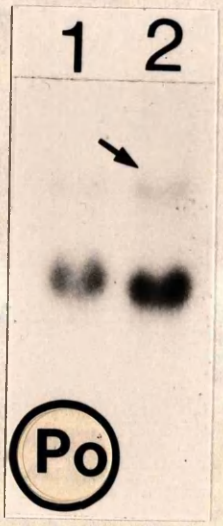


Figure 5.15

RNA extracted from DRG cultures in myelinating medium. Northern blot probed for Po showing increased message between days 7 (lane 1), and 21 (lane 2) *in vitro*. Po mRNA has increased by 57% between the two time points. Note the presence of the 4kb band at 21 days.

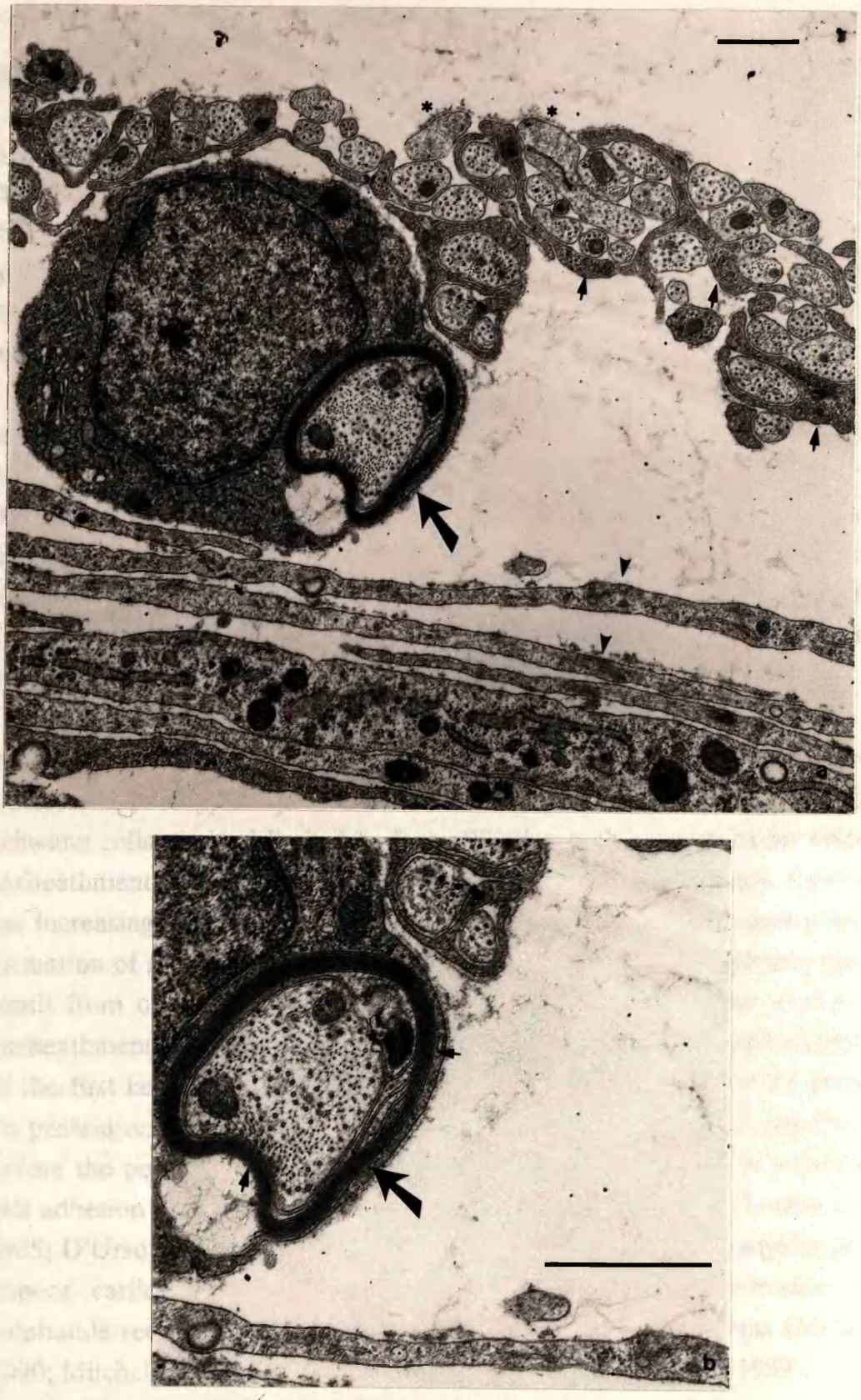


Figure 5.16

Electron micrograph of a 31 day myelinated DRG culture (a), showing compact myelin (large arrow), fibroblasts (arrowheads), basal lamina (asterisks) and Schwann cell processes (small arrows). Scale bar = 1μm. Enlargement of lamellae (b), scale bar = 1μm.

DISCUSSION

The phenotypic expression of Schwann cells cultured in myelinating medium in the presence of neurons is entirely different from that observed in Chapter 3 where Schwann cells were cultured in the absence of neurons. The use of antimitotic drugs was avoided in these experiments so that there could be no question of interference with early expression of myelin-related genes. There is also the role (for example, secretion of growth factors) of fibroblasts in the myelination process to be considered.

E16 DRG's contain no significant levels of Po mRNA, as confirmed by the northern blots of pooled E16 DRG's. Baron *et al.* (1989), established that E18 was the earliest point at which Po mRNA could be detected in the rat sciatic nerve. DRG's cultured in full myelinating medium incorporating foetal calf serum and chick embryo extract displayed increasing expression of Po mRNA from 4 to 5 days onward. Compact myelin was not observed, however, until the 14th day *in vitro*. The increase in expression of Po mRNA in these cultures would appear to be attributable solely to the presence of neurons and axons.

Basal lamina is vital for PNS myelination. The appearance of basal lamina may be in response to the neuronal influence (Bunge *et al.*, 1982b). From the electron microscopy study it was observed that by 7-10 days *in vitro* the majority of Schwann cells attained limited surface affiliation with axons with no evidence of ensheathment. This association between Schwann cells and axons together with the increasing expression of Po mRNA, both occurring several days prior to the formation of myelin lamellae, suggest that induction of myelin protein genes may result from only very minimal Schwann cell-axon contact rather than extensive ensheathment. Po protein was not detected in these experiments until formation of the first lamellae, however Martini *et al.* (1988) demonstrated the presence of Po protein on Schwann cell membranes prior to the formation of myelin, but not before the point of the 1:1 Schwann cell:axon relationship. Po is attributed with cell adhesion properties, possibly justifying its early expression (Lemke and Axel, 1985; D'Urso *et al.*, 1990; Filbin *et al.*, 1990). MAG is the only myelin protein to appear earlier than Po, however the glycolipid galactocerebroside and the sulphatide recognised by O_4 also indicate early myelination events (Mirsky *et al.*, 1990; Mitchell *et al.*, 1990; Gupta *et al.*, 1990; Owens and Bunge, 1989).

Schwann cells cultured in the absence of neurons fail to express MAG, however Schwann cells in neuron co-cultures produce MAG after 7 days *in vitro*. MAG expression occurs prior to the onset of myelination and its production is in response to neuronal influence. Prior to the formation of compact myelin, MAG may be observed along the entire length of the axon, however in mature PNS

myelin sheaths MAG is only to be found in the paranodes and the Schmidt-Lanterman incisures. In electron microscopy studies, MAG has been localised in Schwann cell processes which have surrounded the axon by 1.5 turns (Martini and Schachner, 1986).

MAG has been shown to be involved in the process by which the Schwann cell engulfs an axon destined to be myelinated and also seems to establish the extent of the future internode (Owens and Bunge, 1989). The periaxonal location of MAG implicates this molecule in the maintenance of the volume of periaxonal Schwann cell cytoplasmic collar inside compacted myelin lamellae (Trapp and Quarles, 1982; Trapp *et. al.*, 1984). MAG may also maintain uncompacted membranes by homophilic interaction and must be removed from the membrane for compaction mediated by Po to occur (Trapp, 1988).

In vitro studies have also accounted for the hypothesis of MAG having a recognition or binding function with the axonal surface (Arquint *et al.*, 1987; Salzer *et al.*, 1987). The receptor for MAG resides on the surface of large diameter axons. This receptor may be an immunoglobulin-like molecule or an integrin-like molecule constituting the axonal ligand for MAG (Owens and Bunge, 1990).

MAG is expressed prior to basal lamina formation but its function is enhanced once basal lamina is deposited by the Schwann cell. This requires differentiation of Schwann cells involving polarisation (Bunge *et al.*, 1986). Polarisation allows a critical number of MAG molecules to interact with receptors on the surface of the axon. Enveloping basal lamina is deposited and abaxonal and adaxonal surfaces are restricted to contacting components of extracellular matrix and axon.

GC is also an early marker of myelination, but it is interesting to note that exclusion of GC does not affect MAG expression, and MAG will mediate the initiation of axon engulfment although the process of myelination will not proceed further without GC (Owens and Bunge, 1990). Cultures of dorsal root ganglion neurons and Schwann cells may be depleted of GC using anti-GC antibody (Ranscht *et al.*, 1987). These cultures will assemble basal lamina and initiate the 1:1 stage but will proceed no further even in myelin promoting medium. Anti-GC does not interfere with formation of the mesaxon but does prevent its elongation (Ranscht *et al.*, 1987). The accumulation of subsequent myelin proteins which accompany the compaction of myelin is prevented, however the ensheathment of smaller axons and the deposition of extracellular matrix is unaffected. The segregation of larger axons is also unaffected. In the presence of this antibody the process of mesaxon formation is initiated, but extensive mesaxon elongation and compaction does not occur. There is evidence that Schwann cell nuclear

movements in the early stages of myelin deposition indicate an active progression of the inner lip of the Schwann cell spiral over the axonal surface (Bunge *et al.*, 1986b). The mechanism of mesaxonal elongation appears to require that the inner lip slips by the next Schwann cell surface layer. Removal of GC from this interface may prevent the necessary membrane slippage and "freeze" the process of spiral growth. The mechanism by which GC is removed from the Schwann cell surface seems to be by internalisation of the GC/anti-GC antigen-antibody complex, preventing interaction of adjacent cell surfaces during elongation of Schwann cell membranes. These GC-negative Schwann cells express MAG but not Po (Owens and Bunge, 1990). At the 1:1 step, the inner axon-related cytoplasmic process (which has MAG located on it) had passed under the outer mesaxon but had not completed a full turn around the axon. The MAG presence in this single cytoplasmic process apposed to axons in GC-positive Schwann cells implicates MAG further in the initial envelopment of the axon. Upon removal of the antibody the Schwann cell proceeds to form myelin (Ranscht *et al.*, 1987). GC depletion may prevent advancement of the inner axon-related cytoplasmic process due to the alteration of physical properties in the Schwann cell membrane, that is, removal of lipids. Similar suppression experiments with Po have ruled out the Po molecule as having an early role in myelination.

In further cell culture studies on the role of MAG in myelination, Owens *et al.* (1990) introduced MAG cDNA by way of a recombinant retrovirus into primary cultured Schwann cells. These cells constitutively expressed MAG on their surface without the normal requirement for axonal contact to induce expression. In co-cultures, a higher than normal level of MAG was observed. However, unlike normal Schwann cells, transfected Schwann cells associated with non-myelinated axons or axons undergoing Wallerian degeneration also express high levels of MAG. These results suggest a post-transcriptional mechanism modulating MAG expression during myelination.

By immunostaining cultures with antibody specific to L-MAG, Owens showed L-MAG was transiently expressed at the earliest stages of myelination in normal cultures. In short-term co-cultures with sensory neurons, infected Schwann cells expressing only L-MAG (not S-MAG) segregated and ensheathed large axons after four days *in vitro*, providing exogenous basal lamina was added. Morphologically-normal myelin was subsequently formed.

Experiments performed with Schwann cells infected with antisense Po RNA indicated that restricting the level of Po expression inhibits spiralling of the Schwann cell membrane and subsequent compaction (Owens and Boyd, 1991). These cultures did not express Po or MBP, but did express GC, MAG and

deposited a basal lamina. Inhibition of MAG expression with a MAG antisense retrovirus prevents Schwann cells from segregating large axons and engulfing them (Owens and Bunge, 1991). The use of Po and MAG antisense viruses further strengthens the evidence that these two molecules regulate myelination at different stages. Po appears to be necessary prior to compaction for the progression of Schwann cell membrane after the initial envelopment of the axon, mediated by MAG.

In conclusion, a simplified version of myelination events would involve firstly, neurons and Schwann cells in close proximity. The initial envelopment of the axon requires MAG, and further progression of the spiral of Schwann cell membrane requires Po. Schwann cells depleted of GC express MAG, and elicit basal lamina but do not progress to Po expression or myelin compaction. Po and MAG are transiently co-expressed during the spiralling process, however compaction of the myelin membrane requires increased levels of Po, insertion of Po into the Schwann cell membrane, and the exclusion of MAG such that in mature myelin segments MAG may only be found in the paranodes and Schmidt-Lanterman incisures. Studies limiting the level of Po expression allow persistence of MAG and therefore a subsequent lack of compaction (Owens and Boyd, 1991). As with Po, MBP is also found within the myelin membrane. While axonal interaction may up-regulate expression of MAG by Schwann cells, it transpires that the molecule cannot function as an axon segregator nor promote the initial investment of axons until basal lamina is elicited (Owens and Bunge, 1989). The deposition of basal lamina is vital to Schwann cell differentiation and myelination will not proceed in its absence. Induction is dependent on neuronal interaction (Bunge *et al.*, 1982b).

CHAPTER 6

SCHWANN CELLS CO-CULTURED WITH SYMPATHETIC NEURONS

INTRODUCTION

Results from Chapter 5 showed that under certain conditions it was possible to achieve myelinated DRG cultures. Myelin-specific molecules were studied in these cultures and associations between Schwann cells and axons monitored by electron microscopy. In this chapter, a similar study was conducted studying events in cultures obtained from the superior cervical ganglia and stellate ganglia of 4- to 5-day-old postnatal rats.

SCG cultures are reported not to myelinate (Estridge and Bunge, 1978; Johnson *et al.*, 1980), although, *in vivo*, the SCG supports a proportion of myelinated postganglionic fibres (Heath, 1982: Kidd and Heath, 1988). Bearing this in mind, I wished to elucidate the immunocytochemical profile of these cultures and establish if myelin protein mRNA could be detected in the absence of myelin proteins.

The SCG plays an important role in the development of the cervical sympathetic trunk (CST). The CST is a unifascicular autonomic nerve composed mainly of preganglionic unmyelinated nerve fibres which ascend and synapse in the SCG.

Optimal conditions for SCG cultures were assessed in the same manner as for DRG cultures. Morphology was studied in explant cultures and also in dissociated cultures. An immunocytochemical profile was established and the presence of Po mRNA examined using *in situ* hybridisation and northern blotting techniques.

MATERIALS AND METHODS

Cell culture

SCG were located immediately below the carotid bifurcation, removed and pooled in L15. SCG from 8-10 rats (16-20 ganglia) were generally sufficient. The capsules were stripped and the explants chopped into 4 pieces. At this stage explants were either placed directly onto moist riboflavin-gelled, balanced collagen-coated coverslips, or enzymatically dissociated in the presence of trypsin and collagenase (Chapter 2.1.3.2).

Stellate ganglia were removed from 4- to 5-day-old rat pups and prepared in the same manner.

Media

Myelinating medium was used to establish explant cultures (Appendix 1.3). 2.5S NGF was incorporated at 200ng/ml.

Antisera and immunocytochemistry

Axons were stained with RT97 ascitic fluid at 1:7500. S100 was used to identify Schwann cells at 1:900. NGF-R and GC immunostaining were examined, as were O_4 and laminin. Po polyclonal antibody was used for immunofluorescence and the PAP technique (Chapter 2.3.2).

NGF-R, GC, O_4 and laminin immunostaining were all performed on living cultures; primary antibodies were applied for 30-60 minutes, and post-fixed with ice-cold methanol. For the demonstration of intracytoplasmic antigens such as Po, cultures were fixed in 4% paraformaldehyde and permeabilised with ice-cold 95:5% ethanol:acetic acid prior to application of a FCS block followed by overnight incubation with the primary antibody. Preparations were mounted in 0.1% paraphenylenediamine in 90% glycerol/10% PBS to reduce fading.

Electron microscopy

Cultures were washed in HBSS and fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer. They were processed and embedded in Araldite^{TM} as described in Chapter 2.2.3.

Silver staining

Cultures were silver stained in the same manner as for DRG cultures, described in Chapter 2.2.2. The method is modified from that of Tsuji and Tobin-Gros (1980).

In situ hybridisation

Cultures were washed in HBSS and fixed for 1 hour at 4^{O}C in 4% paraformaldehyde in PBS. Coverslips were stored in 70% ethanol prior to mounting face-up on RNAse free slides using Glassbond^{TM}. The coverslips were probed with ^{35}S-labelled sense and antisense riboprobes for Po.

Northern blotting

Total RNA was isolated from the culture using the RNAzol kit from Biogenesis. The preparation was size-fractionated on formaldehyde denaturing gels and transferred to nitrocellulose prior to hybrisdisation with ^{32}P-labelled cDNA probes for Po.

RESULTS

6.1 Morphology of SCG explant cultures

The optimal age of neonatal animals which provided good cultures was assessed as being post-natal days 2 to 4. SCG explant cultures consist of sympathetic neurons, Schwann cells and fibroblasts. There tended to be a higher level of cell death in these cultures compared to DRG explant cultures. Neonatal SCG explants being larger than embryonic DRG explants required to be chopped into at least four pieces. If the explants were too large neuronal cell death occurred in the centre of the explant, appearing as dark patches (Fig. 6.1). Peripheral outgrowth from the SCG explants was not as abundant as in DRG explants (Fig. 6.2a), although many features were similar, for example, the emergence of growth cones (Fig. 6.2b). SCG explants failed to flatten out in the same manner as DRG cultures, and this resulted in cable networks of axons emanating radially, from the explants which at times did not connect with either the monolayer or the collagen substrate (Fig. 6.3a). A feature of SCG cultures was the prevalence of fat cells (Fig. 6.3b), presumably due to the intricate nature of the dissection with cross-contamination occurring. By 4 days *in vitro* the SCG explants were surrounded by polymorphic fibroblasts and axonal processes (Fig. 6.1). By 1 to 2 weeks in culture, bundles of axons or neurites had almalgamated to form thick fascicles which terminated within the collagen matrix but were at times aerial due to the lack of explant flattening (Fig. 6.3). When compared to DRG explant cultures there appeared to be fewer Schwann cells present. The addition of NGF was required throughout the culture period, and FCS was required at least initially to improve explant adherence. 10% foetal calf serum produced healthier cultures than 20% serum. It was also observed that commercial preparations of chick embryo extract were as good as, if not better, than home-made preparations. On attaining confluence, the cultures tended to lift away from the substrate; therefore decreasing the number of fibroblasts and encouraging longer survival of the cultures. However, AraC when added to young cultures adversely affected adherence and flattening of the explants. Benefits were greater if antimitotics were applied after around 8 days *in vitro*.

SCG cultures failed to form myelin in the presence of myelinating medium over an experimental period of 4 weeks.

6.2 Morphology of SCG dissociated cultures

SCG were dissociated in the same manner as DRG cultures. This resulted in some loss of neurons, with remaining neurons aggregating in clumps. Neurons within

these clumps remained morphologically healthy while single neurons tended to become very granular and eventually died. Schwann cells were present in these cultures, although to a lesser extent than in dissociated DRG cultures. Fibroblasts proliferated extensively if not deterred by the use of antimitotic drugs.

6.3 Morphology of stellate ganglia cultures

Stellate ganglia were chopped and placed as explants onto riboflavin-gelled collagen. These explants adhered well, flattened down and produced plentiful neurites extending well beyond the non-neuronal outgrowth. There appeared to be less fibroblast activity than in DRG explant cultures.

Dissociated stellate ganglia cultures tended to generate more fibroblasts than explants. Neurons aggregated in small clumps; however there appeared to be considerable neuronal cell loss and death in dissociated cultures.

6.4 Immunocytochemical profile of SCG cultures

Staining of axons with RT97 antibody was less intense than observed in DRG cultures. Silver staining was also less intense in SCG cultures. Schwann cells were positively identified using S100 antibody, and this staining confirmed previous impressions that Schwann cells were less prevalent in SCG cultures than in DRG cultures. GC immunoreactivity was observed in SCG cultures derived from P4 rat pups. Po protein was not detected either by immunofluorescence or immunoperoxidase techniques. NGF-R was present on Schwann cells. Fibroblasts were identified using anti-Thy-1 antiserum.

6.5 Po mRNA expression

SCG explant cultures grown in myelinating medium were probed for Po mRNA by *in situ* hybridisation techniques. After 1 day *in vitro*, only a very occasional Schwann cell in some explants hybridised with Po antisense probe. This was confirmed using dissociated cultures. In time-course experiments, it was observed that by 7 days in culture Po mRNA was present in the Schwann cells immediately surrounding the central explant neuronal bodies (Fig. 6.4). The appearance and distribution of grains was similar to that observed in DRG cultures although the intensity was significantly lower.

Explanted and dissociated stellate ganglia cultures were probed for Po mRNA. Both cultures displayed a positive signal, however there was high background in the explant cultures. Dissociated cultures showed some positive Schwann cells, but the majority of cells were negative.

Northern blotting of SCG and stellate cultures confirmed the Po message expression and also displayed the lower intensity of signal compared to DRG cultures. Using Quantimet analysis the signal present in SCG samples was only 57% of that observed for DRG cultures of similar duration in myelinating medium. The 4kb band observed in previous blots tended to be more intense in samples derived from SCG (Fig. 6.5).

RNA isolated from SCG of 4 day-old rat pups produced a strong signal when hybridised for Po mRNA on Northern blots.

6.6 Electron microscopy

Electron micrographs of SCG explant cultures showed that the central part of the culture consisted of neuronal cell bodies, and axonal and neuritic processes (Fig. 6.6). Immediately distal, fibroblasts and Schwann cells were observed and on the periphery of the explants there tended to be fewer axon terminals and more fibroblasts. Neurons had large nucleoli and many Nissl granules. Axolemmal surfaces demonstrated no signs of the basal lamina formation indicative of early myelination events.

Figure 6.1

Superior cervical ganglion explant culture at 4 days *in vitro*. The explant shows good outgrowth of Schwann cells, fibroblasts and neurites (arrows). The centre of the explant body exhibits dark necrotic patches indicating neuronal cell death (white arrows). x 100.

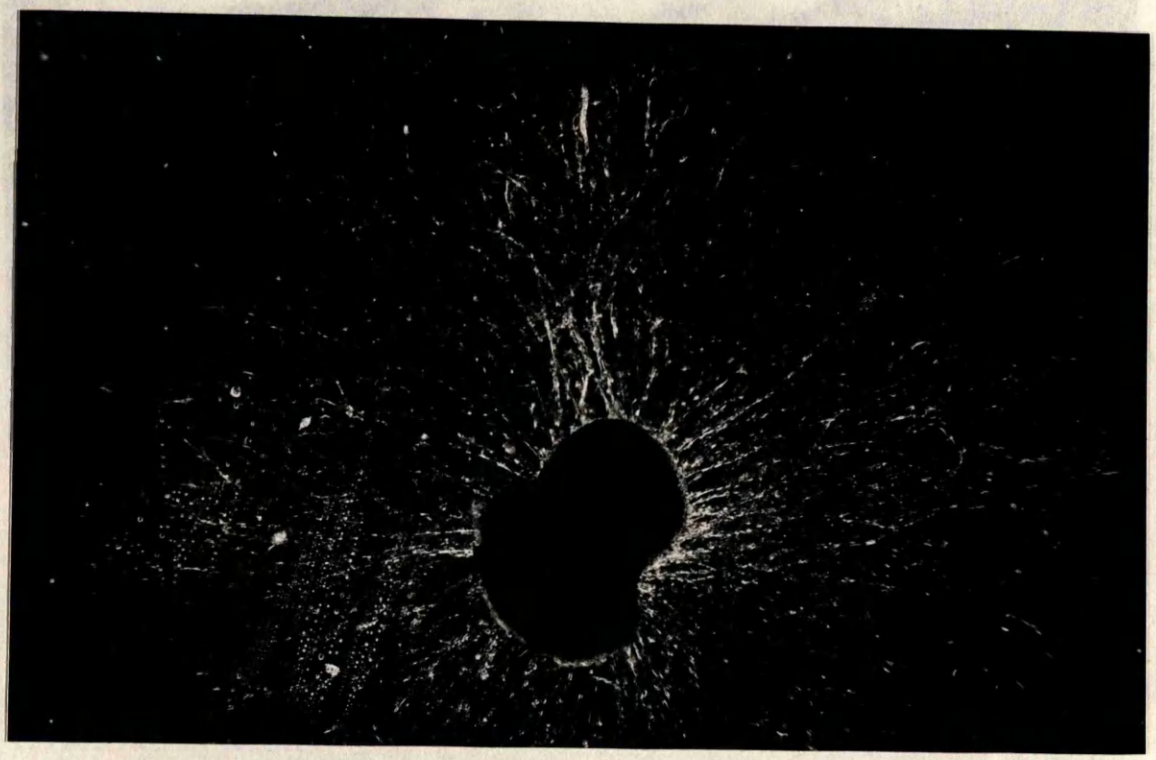


Figure 6.2

A typical SCG explant (a) at 3 days *in vitro* demonstrating less intense outgrowth than a typical DRG explant cultured for the same period of time. Dark-ground photography, x 30. (b) SCG growth cones on periphery of a 5 day explant (arrows). x 218.

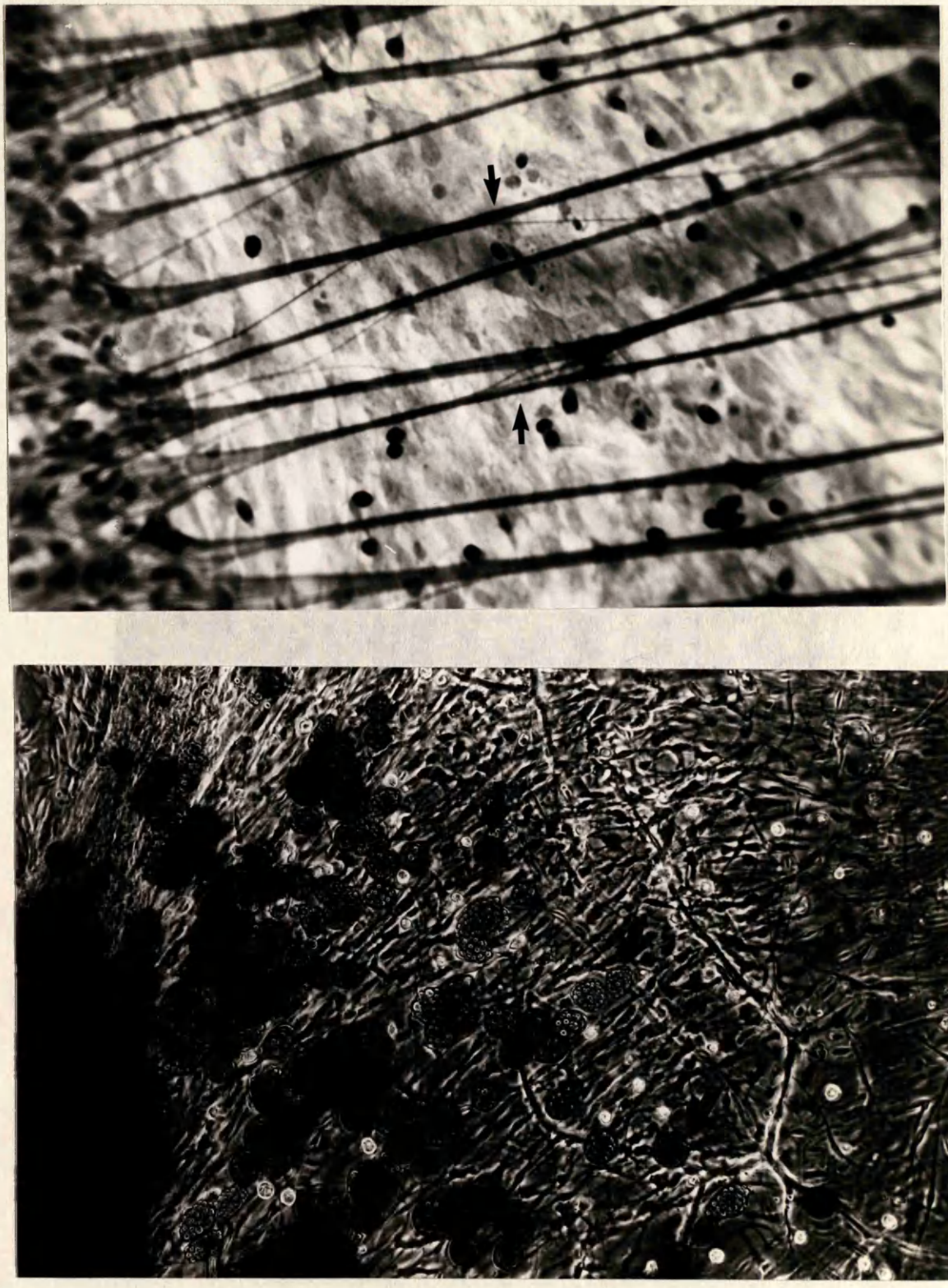


Figure 6.3

An SCG explant at 14 days *in vitro* demonstrating unflattened morphology. Note the cables of neurites extending from the centre of the explant which are not in contact with the collagen matrix for part of their course (arrows). (b) One feature noted to be prevalent in SCG cultures was the presence of fat cells (arrows). x 218.

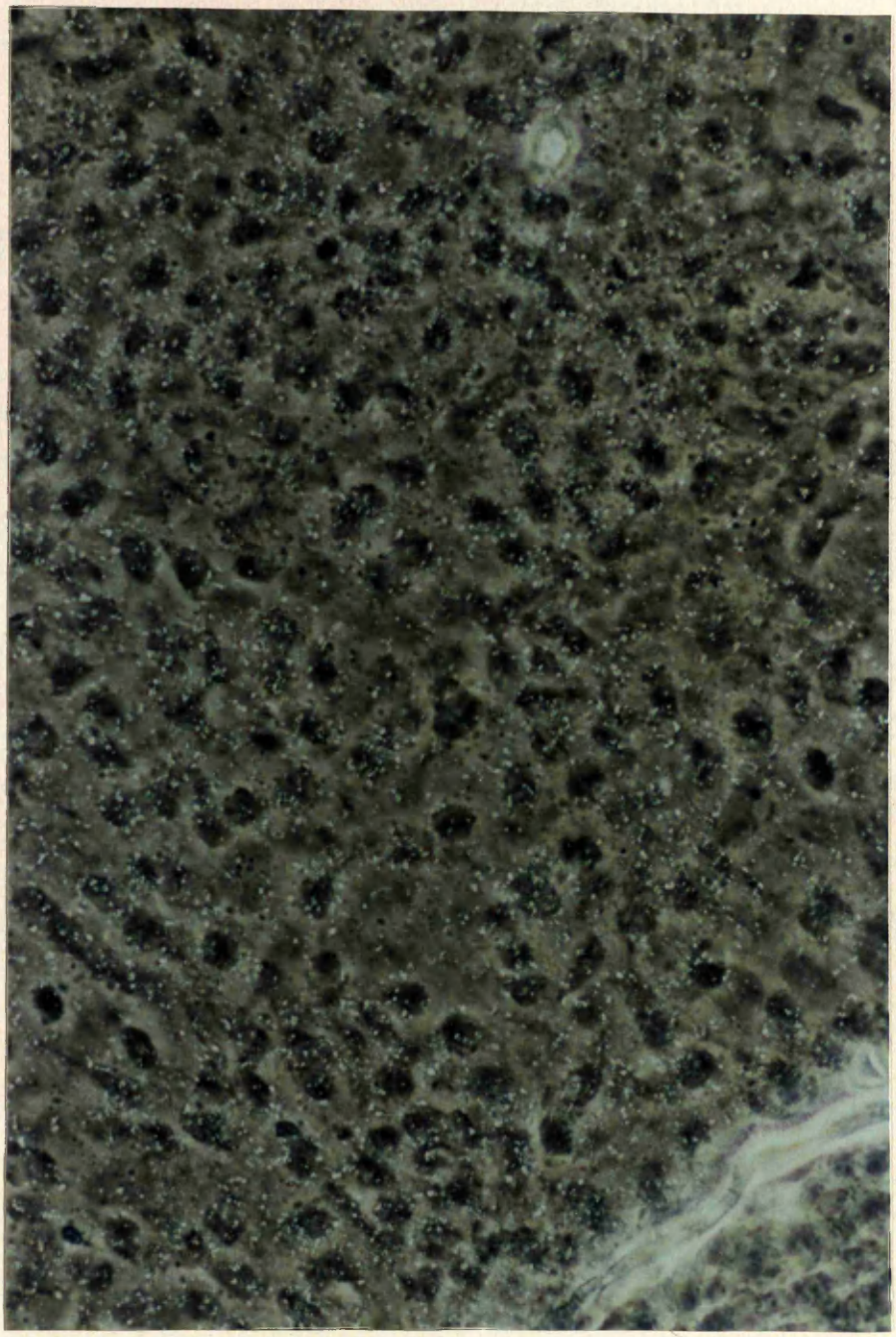


Figure 6.4
SCG explant culture at 7 days *in vitro* probed for Po mRNA by *in situ* hybridisation. Phase optics. The silver grains appear as white dots. x 320.

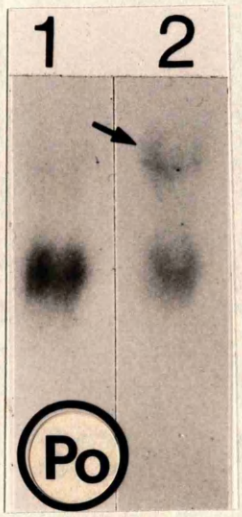


Figure 6.5

Northern blots of DRG (lane 1), and SCG (lane 2) explants cultured for 14 days in myelinating medium, hybridised with Po probe. The signal is less intense in the SCG, however note the more prominent 4kb band in the SCG lane (arrow).

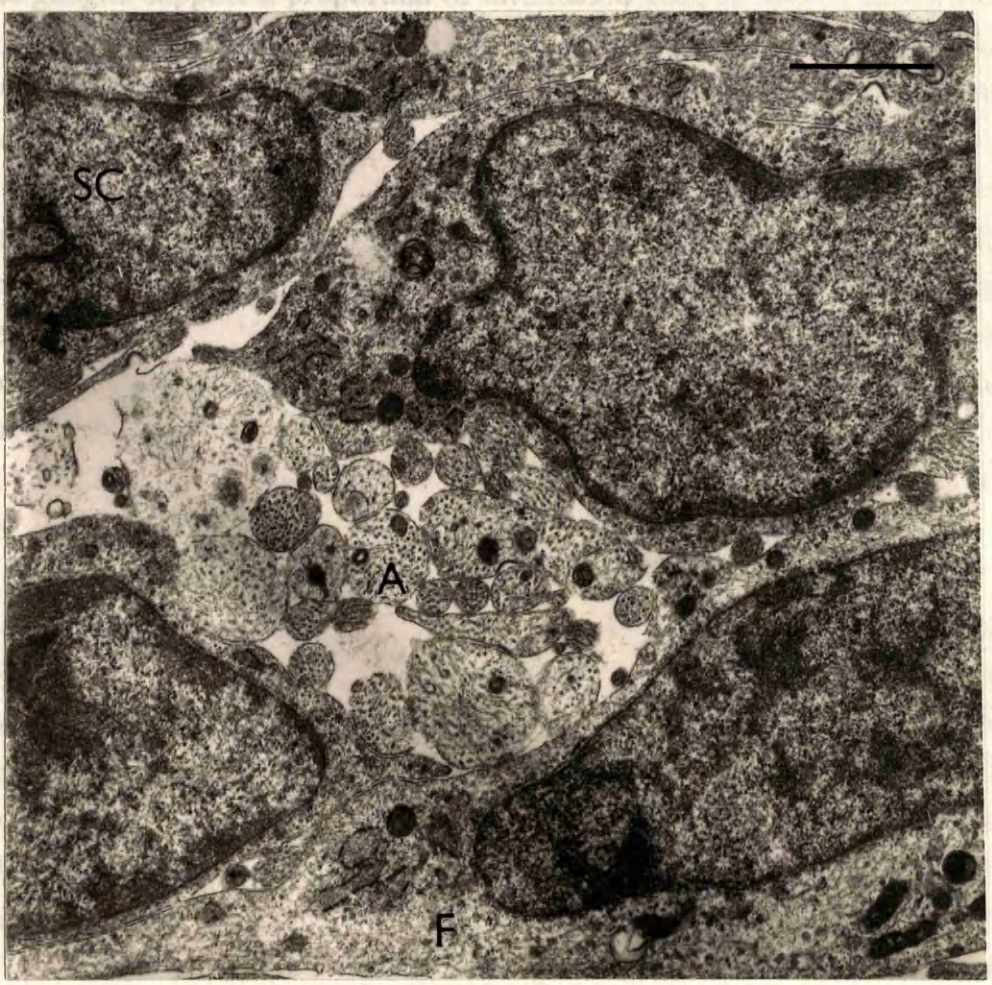


Figure 6.6
Electron micrograph of cultured SCG explant. Schwann cells, fibroblasts and axons are observed, but basal lamina is absent. Scale bar = 1μm.

DISCUSSION

Cultures derived from SCG are reported not to myelinate (Estridge and Bunge, 1978; Johnson *et al.,* 1980). Myelinated fibres in the CST are insignificant, amounting to less than 0.5% (Jessen *et al.,* 1985). The major myelin protein Po was not detected in either SCG or stellate ganglia cultures. However, Po mRNA was detected in these cultures both by *in situ* hybridisation and northern blotting techniques, although in far lower abundance than observed in DRG cultures in Chapter 5. This expression may be explained by the fact that *in vivo* the SCG and stellate ganglia support a proportion of myelinated post-ganglionic fibres (Heath, 1982; Kidd and Heath, 1988). The myelinated fibre population of the SCG is highly unusual in that many axons have a double myelin sheath (Kidd and Heath, 1988).

It has been reported that culturing dissociated neonatal SCG in the presence of human placental serum and chick embryo extract resulted in increased choline acetyltransferase activity (Johnson *et al.,* 1981). Adrenergic neurons derived from the SCG of young rats develop several cholinergic properties of neurons when cultured under appropriate conditions. This tends to suggest that myelinating medium does induce some differentiation in these cultures, but this effect may be confined only to neurons. There is no evidence to suggest that Schwann cells differentiate to myelin-forming cells in these cultures.

Non-myelin-forming Schwann cells of the peripheral nervous system have been reported to express the growth-associated-protein GAP 43 *in vitro* (Curtis *et al.,* 1992). However, no GAP 43 immunoreactivity has been found in sympathetic neuron/Schwann cell co-cultures (Meiri *et al.,* 1988). Mature myelin-forming Schwann cells do not express GAP 43, but if Schwann cells are removed from axonal contact this molecule is up-regulated. However, in culture, GAP 43 is not rapidly up-regulated in Schwann cells which have been involved in myelin formation *in vivo*. GAP 43 has been observed at low levels in some Schwann cells in DRG cultures (Woolf *et al.,* 1990), although it is not clear whether this is taken up from neurons/axons or if it is being synthesised by the Schwann cells. Curtis and colleagues demonstrated that Schwann cells can synthesise GAP 43 in the absence of axons both *in vivo* and *in vitro*. They also deduced that during development GAP 43 is confined to non-myelin-forming Schwann cells. Unfortunately, I did not have antibody or probe available for GAP 43 to examine this aspect in my cultures.

The presence of the higher molecular weight 4kb band of Po mRNA in addition to the main 1.9-2.0kb observed in northern blots may be significant as this higher band is consistently more intense in samples derived from SCG cultures

than DRG cultures. An identical band was observed in chickens by Barbu (1990), particularly in early embryogenesis when it appeared as the major band. It is possible that its presence in my cultures represents an early-expression species of Po mRNA whereas in myelinating and adult Schwann cells the 1.9-2.0kb size predominates.

The results from this chapter indicate that Schwann cell expression of the major myelin protein is regulated at the level of post-transcription. Why these Po mRNA-expressing Schwann cells do not elicit Po protein in the presence of myelinating medium is open to speculation. Perhaps these Schwann cells are intrinsically programmed to cease myelin differentiation on the production of mRNA, or perhaps the problem lies within the axon. Are the necessary signals between axon and Schwann cell being withheld for some reason? The presence of the 4kb band and its involvement in early embryongenesis may suggest that SCG Schwann cells are themselves fairly immature or primitive, however the fact that no GAP-43 immunoreactivity was observed in sympathetic cultures infers that the Schwann cells in these cultures are mature, since mature myelin-forming Schwann cells do not express GAP-43.

I have not, however answered the question of why these cultures do not form myelin when it is apparent that myelin gene expression of Po occurs. SCG cultures were grown on collagen in myelinating medium and theoretically all the requirements for myelination were present. Po mRNA was expressed, however basal lamina was lacking. It is interesting to note that Schwann cells from these cultures have the potential to form myelin since when transplanted into spinal ganglia cultures, they proceeded to form myelin (Roufa *et al.*, 1985). Roufa concluded that these Schwann cells when in the presence of SCG neurons only, are less "driven" than when exposed to sensory neurons.

CHAPTER 7

PART 1

SCHWANN CELL\DRG CO-CULTURES IN DEFINED MEDIUM

INTRODUCTION

Results from Chapter 5 showed that under certain conditions it was possible to achieve myelinated DRG cultures. Myelin-specific molecules were studied in these cultures and associations between Schwann cells and axons monitored by electron microscopy. In this chapter, a similar study was conducted studying events in a defined medium. It has been reported previously that Schwann cells in DRG cultures proliferate but fail to differentiate in defined medium (Moya *et al.*, 1980).

Defined medium differs from myelinating medium in that it contains no serum or chick embryo extract. Both FCS and CEE contain many defined and undefined proteins, hormones and growth factors, which vary between batches. Chemically defined medium contains specific hormones and vitamins etc, in specified amounts, allowing continuity between batches.

From the results in this chapter it appears that axonal contact alone is insufficient to induce full Schwann cell differentiation progressing to myelination.

The aims of this study were to examine Schwann cell gene expression in defined medium co-cultures and compare the results with the expression observed in myelin-forming cultures, with a view to identifying the mechanisms involved in myelin formation *in vitro*.

MATERIALS AND METHODS

Media

DRG cultures were maintained in SATO N_2 defined medium described in Appendix 1.1. This is a chemically-defined medium based on equal parts DMEM and Ham's F12 media, supplemented with ascorbic acid, growth factors, hormones, glucose and antibiotics. Cultures were also prepared with full myelinating medium as a control comparison (Appendix 1.3). All media were supplemented with 200ng/ml 2.5S NGF.

Substrates

Cultures were maintained on 13mm or 22mm glass nitric acid-washed coverslips coated with riboflavin-gelled collagen which had been balanced for 3-4 days prior to plating (Chapter 2.1.5.3/2.1.5.4).

Cultures

DRG explant cultures were established in the same manner as in Chapter 5. DRG's were removed from E16 Sprague-Dawley embryonic rats, cleaned of excess tissue and placed directly onto collagen-coated coverslips. The explants were bathed in a small amount of medium containing FCS and NGF at 37°C/5% CO_2. Once attachment was achieved the cultures were fed with either defined medium or myelinating medium. Cultures were maintained for several weeks and fed with fresh medium 2-3 times per week by removing 80% spent medium and replacing with fresh.

Antisera and immunocytochemistry

Axons were stained with ascitic fluid RT97 at 1:7500. S100 was used to identify Schwann cells in co-cultures at 1:900, and NGF-R also identified Schwann cells at 1:100. Po protein was stained using a polyclonal antiserum developed within our group which was used at 1:250 for immunofluorescence or 1:500 for immunoperoxidase. GC was stained with tissue culture supernatant R-mAb (Ranscht *et al.*, 1982) at 1:5, and the monoclonal O_4 (Sommer and Schachner, 1981) which detects sulphatide on Schwann cells (Mirsky *et al.*, 1990) was used at 1:50. A commercial antiserum to laminin (Sigma) was used at 1:1000.

NGF-R, GC, O_4 and laminin staining were performed on washed living cultures and post-fixed. For the demonstration of intracellular antigens, including intracytoplasmic laminin, cultures were fixed with 4% paraformaldehyde and

permeabilised with ice-cold 95:5% ethanol:acetic acid prior to application of a foetal calf serum block and incubation overnight with the primary antisera.

Secondary antisera were conjugated to FITC or TRITC for either single or double labelling of antigens. Preparations were mounted in 90% glycerol in PBS containing 0.1% paraphenylenediamine to reduce fading.

Po was also immunostained using the peroxidase anti-peroxidase technique (Chapter 2.3.2).

Silver staining

Explant cultures were silver stained to demonstrate axon networks using the method of Tsuji and Tobin-Gros (1980). (Chapter 2.2.2.)

In situ hybridisation

Cultures were fixed for up to an hour in 4% paraformaldehyde at $4^{o}C$ and stored in 70% ethanol prior to *in situ* hybridisation (Chapter 2.4.1). ^{35}S-labelled antisense and sense probes were prepared as described in Chapter 2.4.3.

Northern blotting

RNA was extracted from the cultures using the RNAzol kit (Biogenesis Ltd.). 10*u*g samples of total RNA were size-fractionated on formaldehyde denaturing gels and transferred to nitrocellulose prior to hybridisation (Chapter 2.4.5) with ^{32}P-labelled probes (Chapter 2.4.6).

Electron microscopy

Cultures were washed in HBSS and fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer at $4^{o}C$ for 1 hour. The explants were processed intact on their coverslips according to the method in Chapter 2.2.3 to the dehydration stage with 70% ethanol. At this point the explants were embedded in an agar sandwich and the specimen detached from the coverslip. The area containing the dehydrated explant was excised from the gel and processed through the remainder of the schedule to embedding in Araldite$^{(TM)}$.

RESULTS

7.1 Morphology of DRG explants maintained in defined medium

Explants from E16 rats were placed intact onto three-dimensional riboflavin-gelled balanced collagen. Within an hour the explants had adhered to the substrate and within the following few hours non-neuronal cells and neurites were observed migrating radially out from the body of the explant. At this stage the cultures in defined medium were indistinguishable from those initiated in myelinating medium. By 24 hours *in vitro*, explants maintained in defined medium could be identified from those in normal myelinating medium. In defined medium there was notably less fibroblast activity, although Schwann cells and neurites emerged from the explants (Fig. 7.1). As time progressed a fine intricate meshwork of neurites became established and Schwann cells were abundant and morphologically healthy (Fig. 7.2). Throughout the culture period fibroblast proliferation appeared to be inhibited by the defined medium, and fibroblasts were present as individual cells rather than confluent sheets of cells. By 2-3 weeks some Schwann cells were observed to be in association with neurites to a limited extent.

Dissociated DRG cultures were maintained in defined medium. These cultures also demonstrated fewer fibroblasts, intricate networks of axons, substantial numbers of Schwann cells and individual healthy neurons with accentric nuclei (Fig. 7.3).

7.2 Immunocytochemical profile

Once DRG cultures were established in defined medium, Schwann cells were identified with S100 antibody. Double staining with S100 and RT97 demonstrated the intricate pattern of Schwann cells and neurites (Fig. 7.4). Fibroblasts were identified using Thy-1 antiserum. Fourteen-day-old cultures were immunostained for GC and the sulphatide detected by the monoclonal antibody O_4. GC was expressed on many cells, as was O_4 (Fig. 7.5). 14-day cultures were also immunostained for surface and cytoplasmic laminin. A very fine or punctate surface deposit of laminin (Fig. 7.6) and a more intense cytoplasmic accumulation were observed. NGF-R and N-CAM surface staining was present on all or the vast majority of Schwann cells. Po and MBP proteins were at no time detected in defined media cultures either by immunofluorescence or immunoperoxidase.

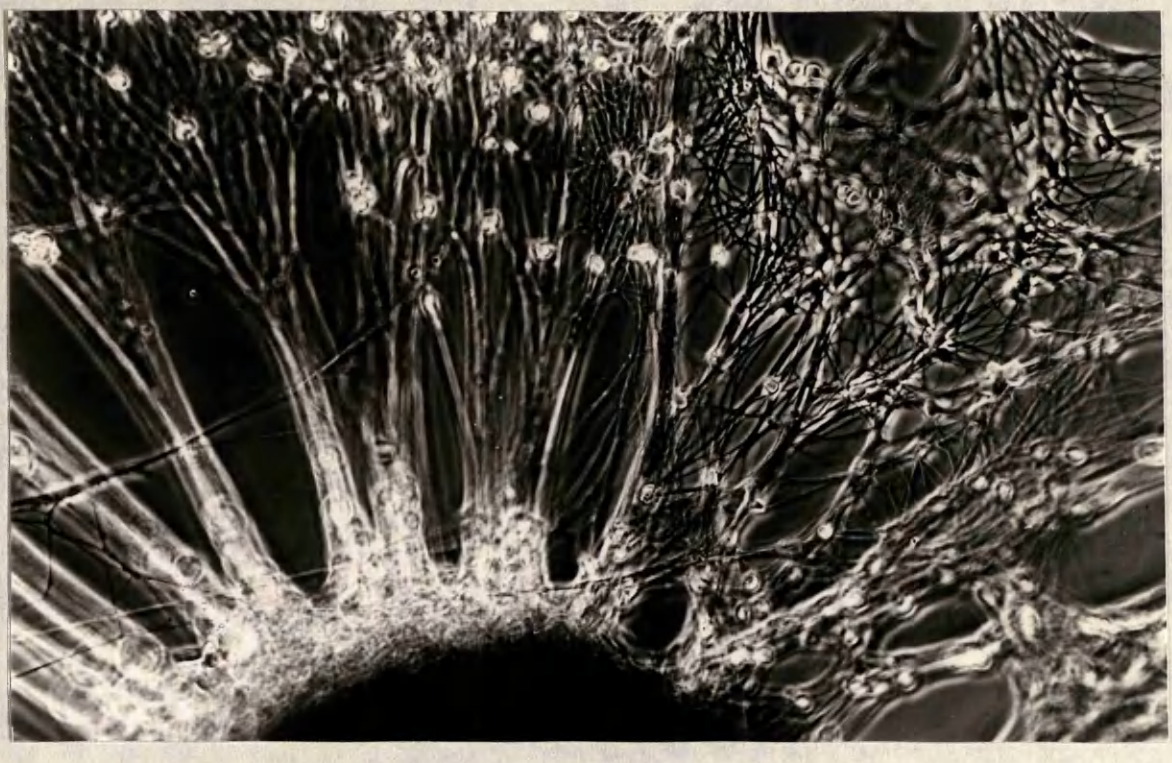
7.3 Po mRNA expression

DRG cultures maintained in defined medium were processed for *in situ* hybridisation at various time points and probed with ^{35}S-radiolabelled Po

antisense and sense probes. Po mRNA was expressed by most Schwann cells with similar intensity to that observed in DRG cultures maintained in myelinating medium prior to the formation of myelin lamellae (Fig 7.7). Northern blotting confirmed these results. Figure 7.8 shows a comparison of 21-day cultures in defined and myelinating medium probed with Po cDNA probes labelled with ^{32}P.

7.4 Electron microscopy

DRG cultures maintained in defined medium at various time points up to 4 weeks revealed no myelinated fibres when examined by electron microscopy. Schwann cells exhibited degrees of association with axons varying from focal contact to complete envelopment of an axon and the formation of primitive mesaxons (Fig. 7.9). Basal lamina was absent from virtually all Schwann cells, but on a very occasional cell, a small, almost punctate area of fuzzy material was present on the external plasmalemma. This could represent an abortive attempt to form basal lamina.



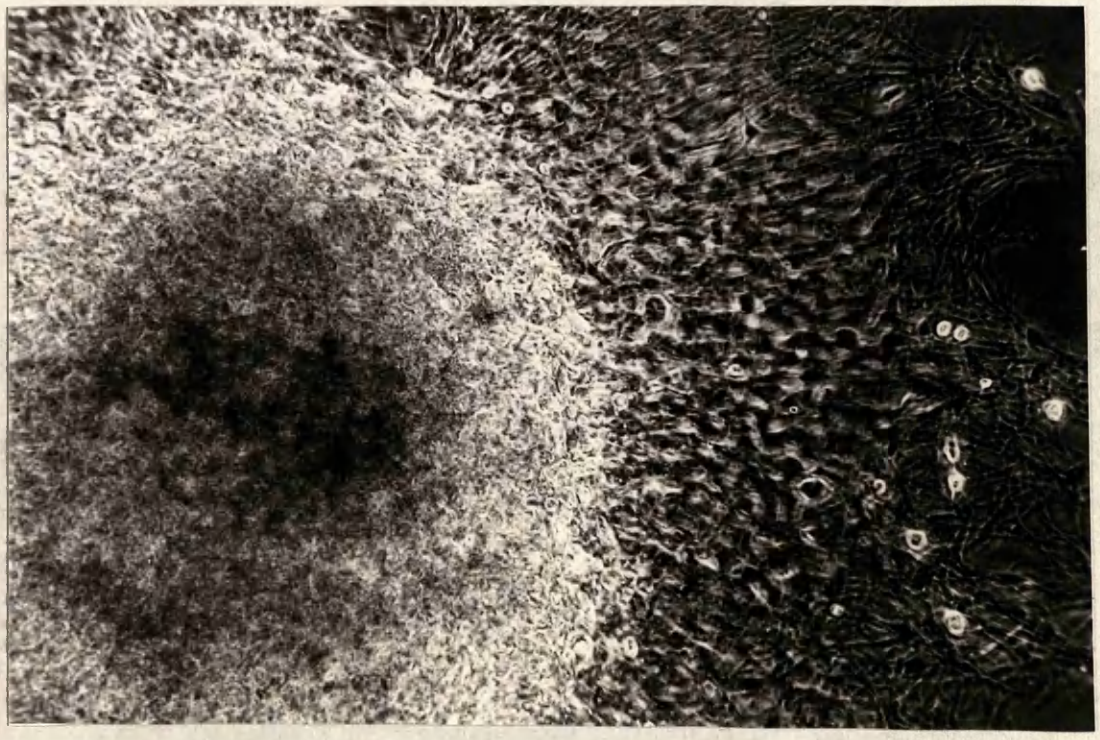


Figure 7.1
DRG explants at 48 hours *in vitro* cultured in (a) defined medium, (b) myelinating medium. Note the lower levels of fibroblasts in (a). x 100.

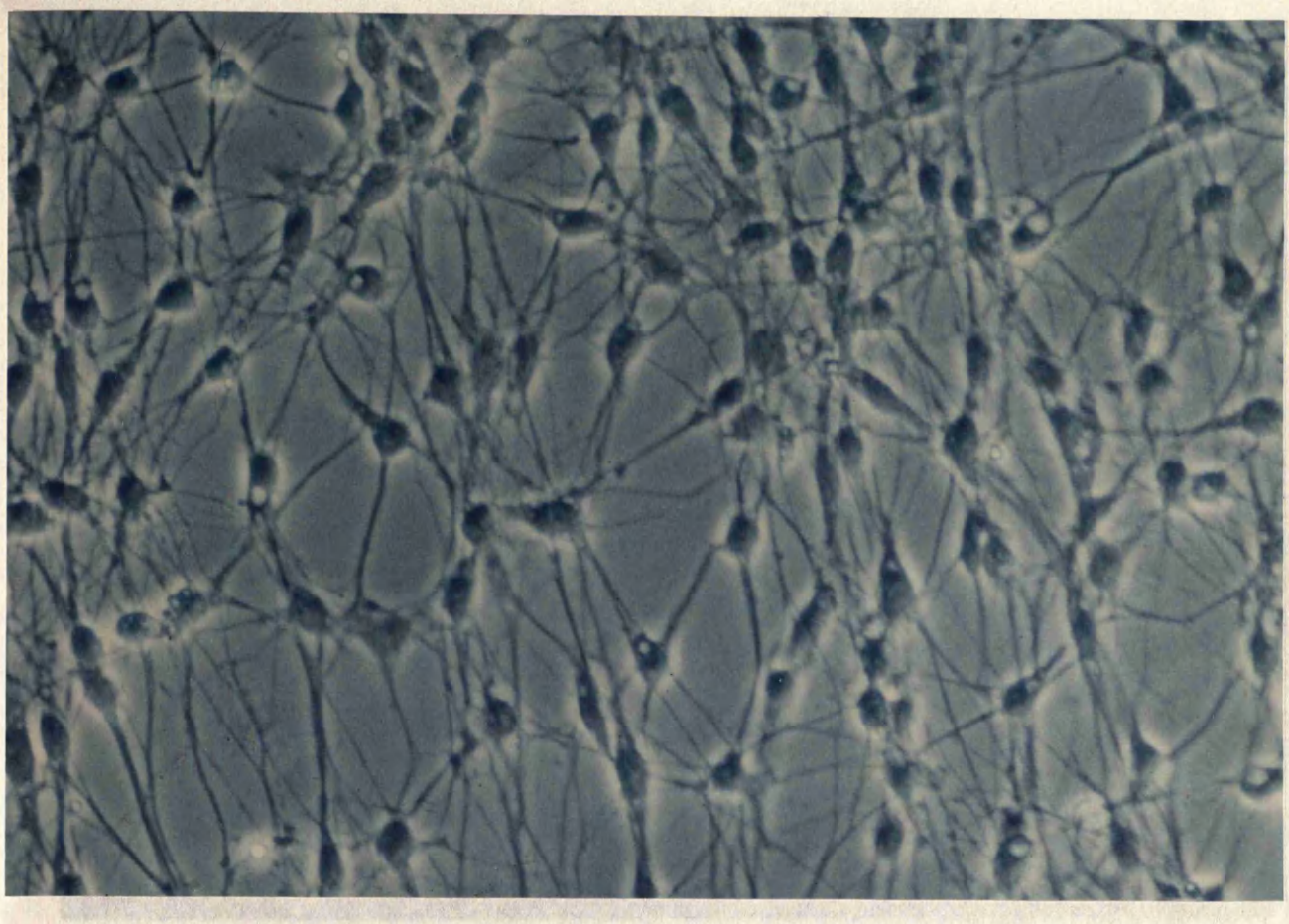


Figure 7.2
DRG explant cultured for 10 days in defined medium. Compare to outgrowth of similar culture in myelinating medium (Figs. 5.3(a) and 5.4). x 500.

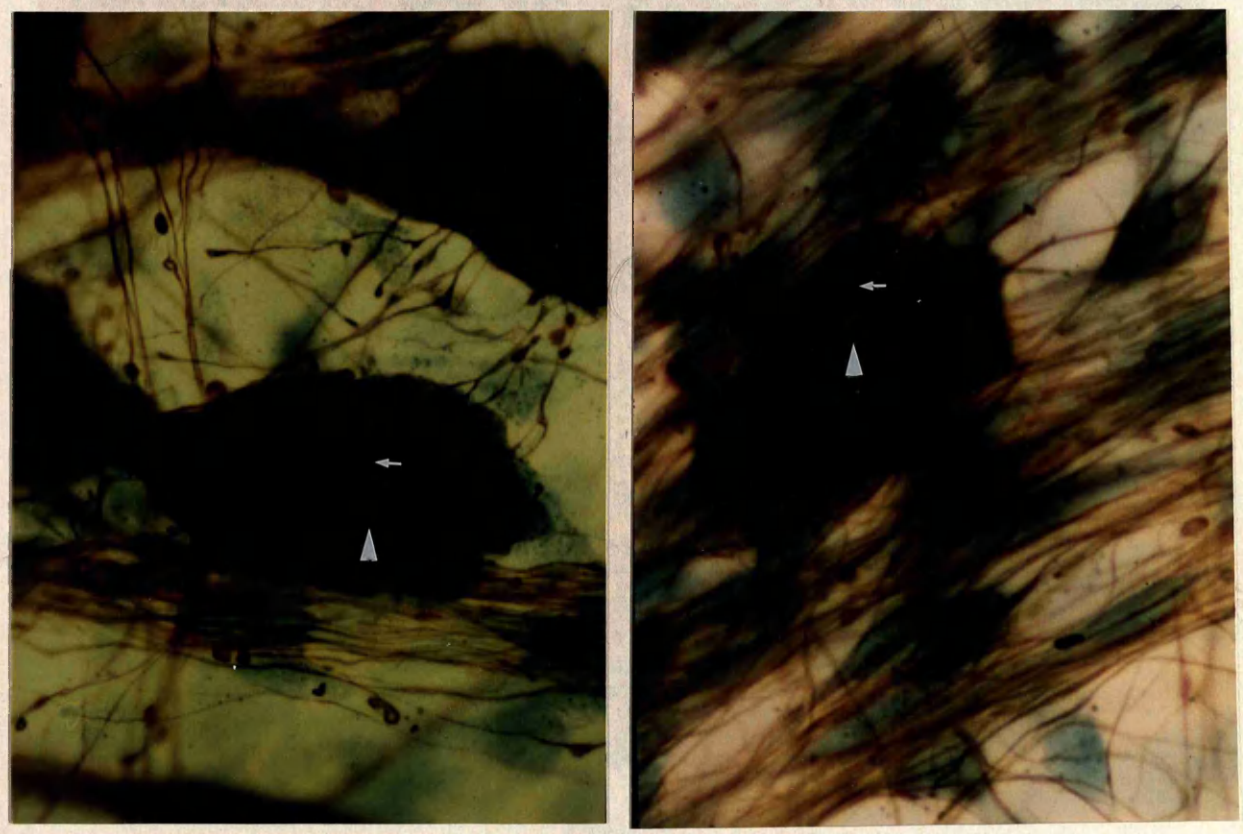


Figure 7.3
Silver stained dissociated DRG cultures at 14 days *in vitro* in defined medium, showing neuronal morphology and position of nuclei (arrowheads) and nucleoli (arrows). x 785.

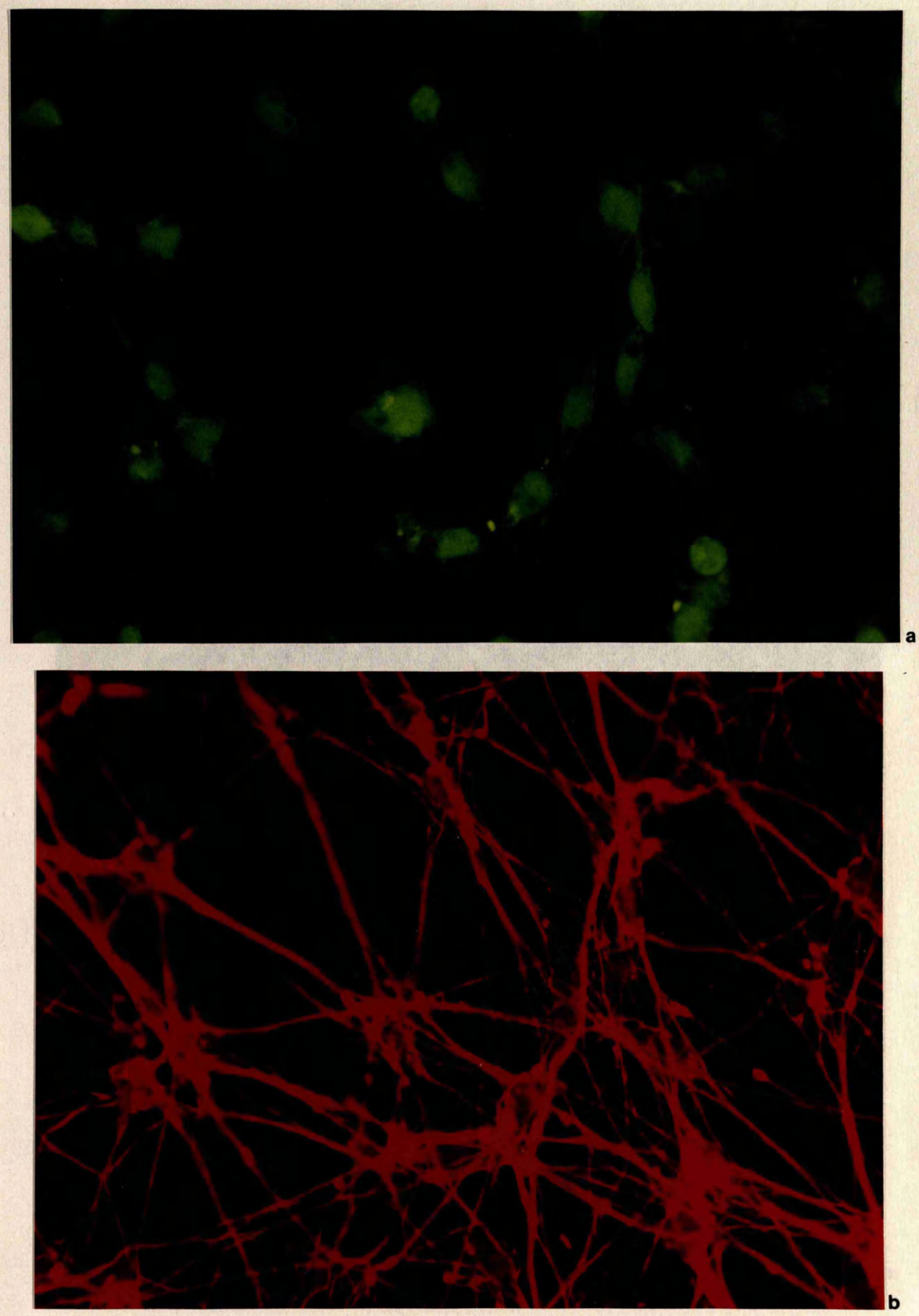
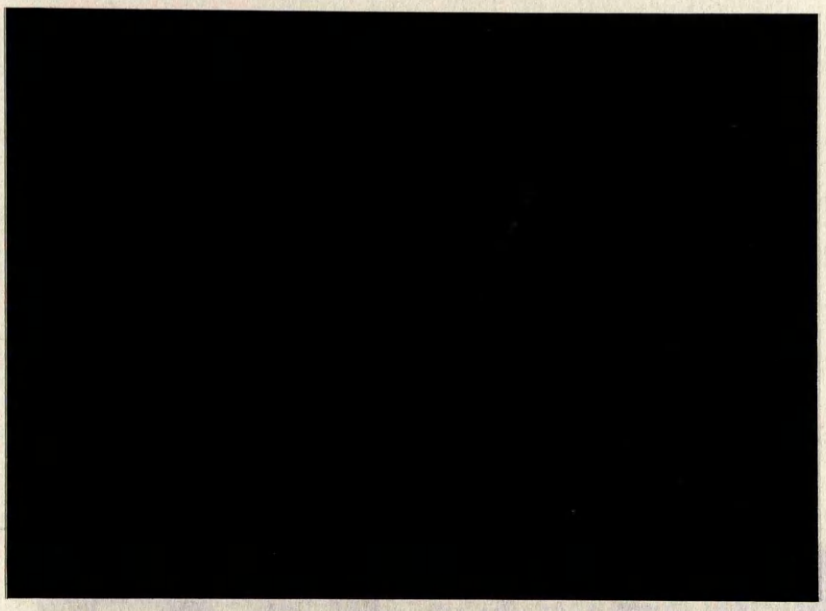


Figure 7.4
Double labelling of defined medium DRG explant cultures, (a) S100, (b) RT97. Note the intricate network of neurites throughout the culture. x 712.



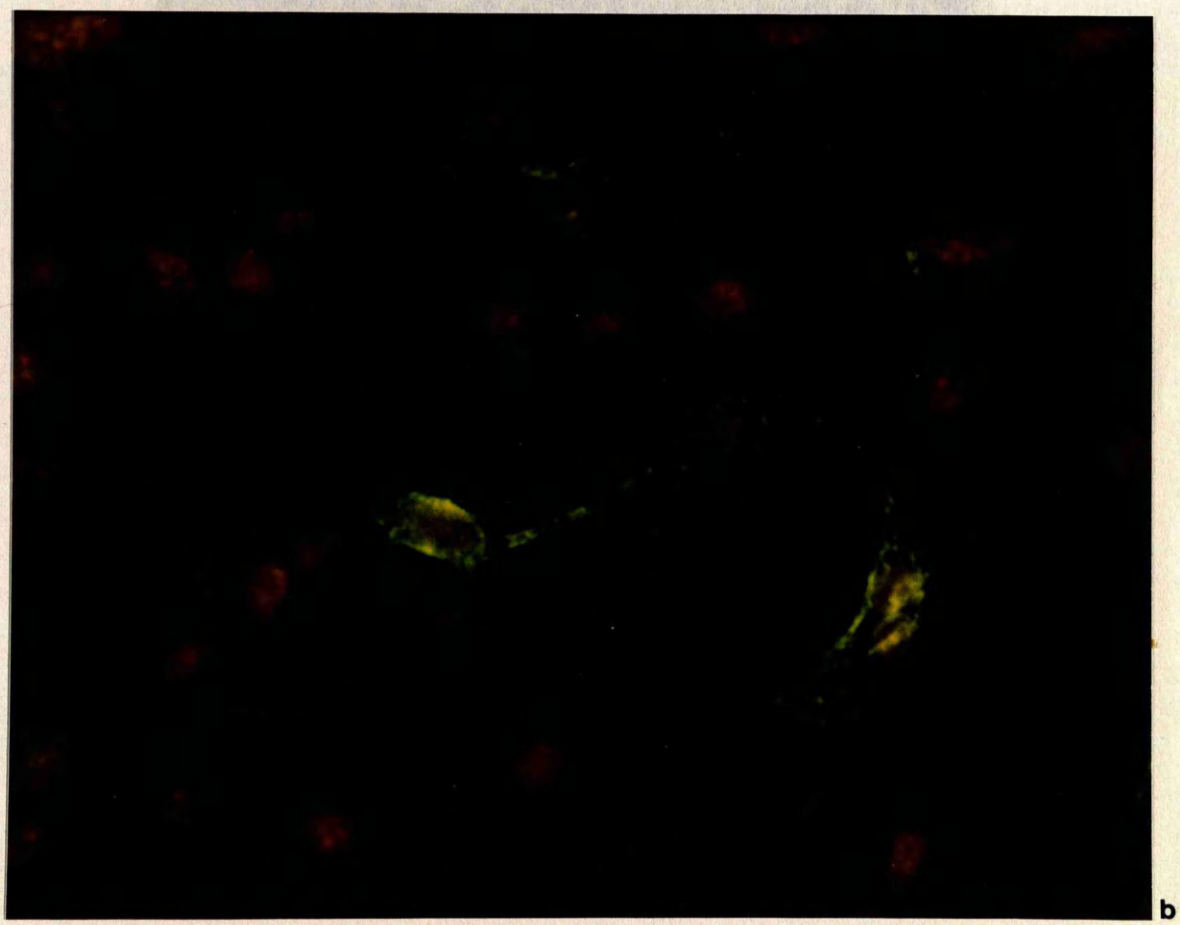


Figure 7.5
DRG explants cultured for 14 days in defined medium immunostained with O_4 (a) x 800, and for GC (b) x 1285.

Figure 7.6
14 day DRG culture in defined medium immunostained for surface laminin. x 800.

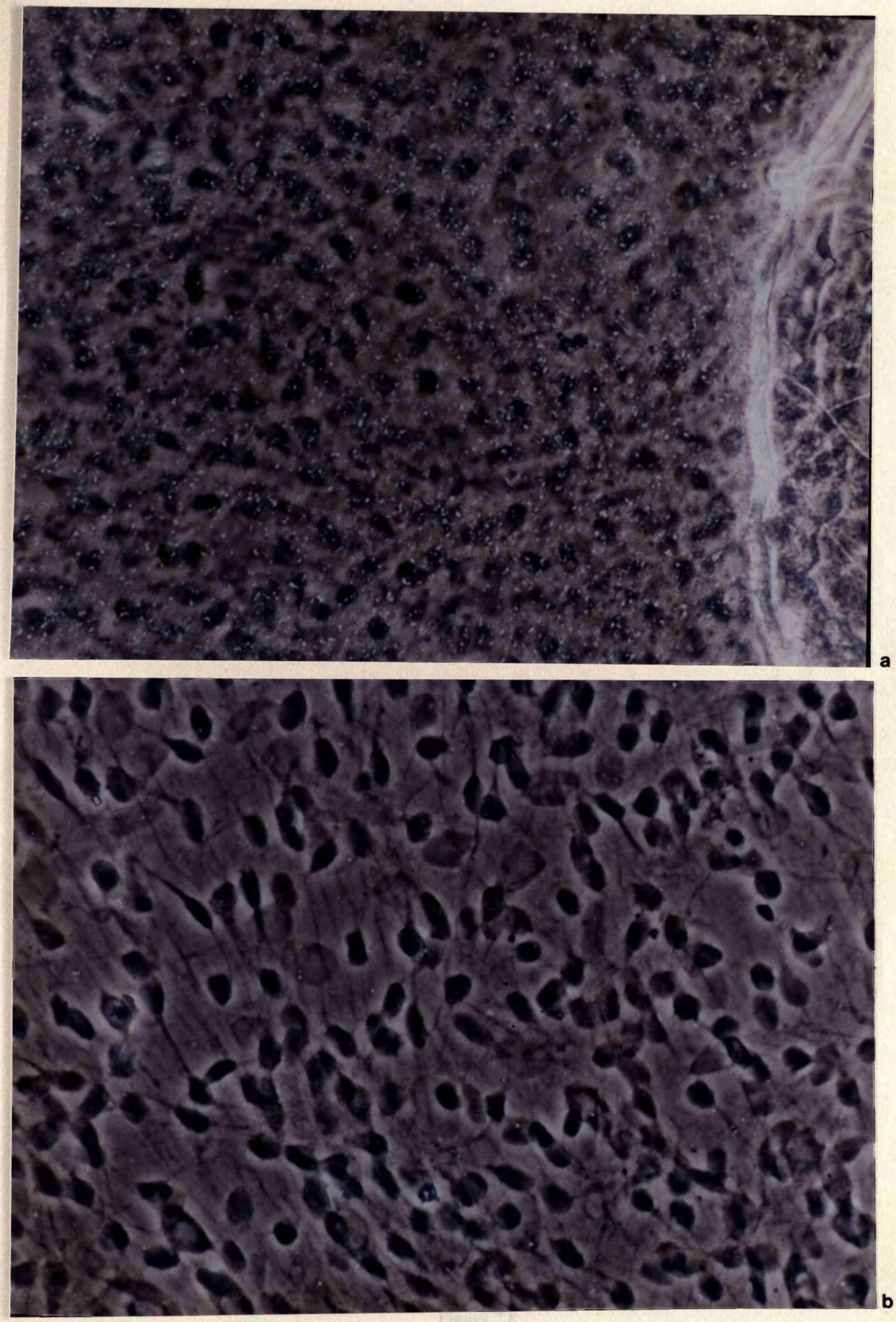


Figure 7.7

In situ hybridisation of 14 day cultures maintained in defined medium. Probed for Po mRNA. (a) antisense, (b) sense control. Phase optics. Silver grains appear as white dots. x 500.

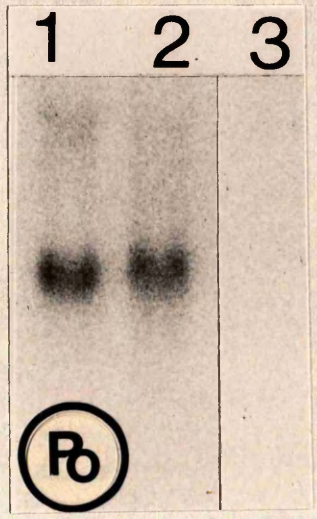


Figure 7.8
Northern blots of 21 day DRG cultures maintained in myelinating medium (lane 1), and serum-free defined medium (lane 2). Probed for Po mRNA. Similar levels of expression are observed between the two cultures. Lane 3 represents Schwann cells cultured for several days in the absence of neurons.

Figure 7.9

Electron micrograph of 21 day DRG culture in defined medium. Note the Schwann cell nucleus (SN), axons (A), and Schwann cell processes (SP). Some axons are associated with and occasionally surrounded by Schwann cell processes which lack basal lamina (arrow). Scale bar = 0.5μm.

CHAPTER 7
PART II
SUPPLEMENTATION WITH EXOGENOUS LAMININ OR MATRIGEL

INTRODUCTION

In the absence of basal lamina deposition resulting from culture in defined medium, I attempted to induce myelination by providing exogenous supplies of the basal lamina component laminin, or by exposure to a commercial basement membrane preparation, Matrigel.

MATERIALS AND METHODS

E16 DRG explant cultures were established in the same manner as for Chapter 7, Part I. Cultures were maintained for 14 days in defined medium and subsequently supplemented with either laminin at 10 or 20*u*g/ml or Matrigel$^{(R)}$.

DRG cultures were also established on collagen substrates which were either mixed with Matrigel prior to gelling or coated with laminin prior to collagen coating.

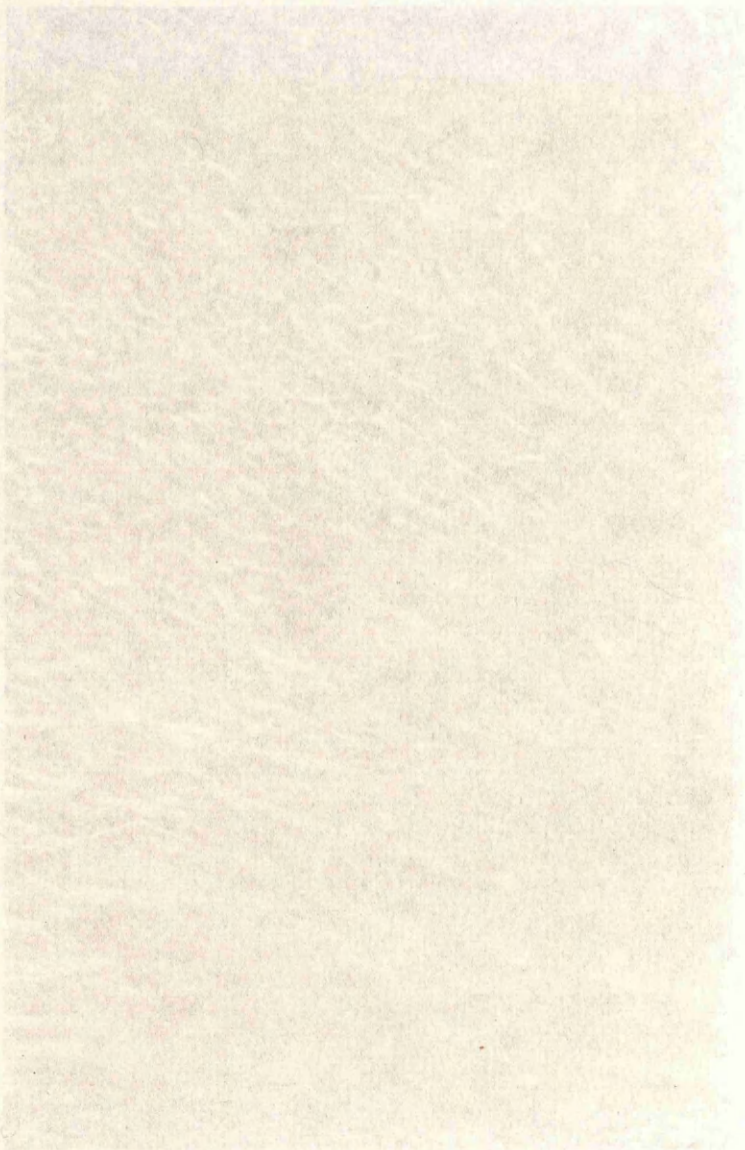
Immunocytochemistry, electron microscopy, *in situ* hybridisation and northern blotting were performed as described in Chapter 7, Part I.

RESULTS

Addition of laminin to the culture medium appeared to enhance the alignment and elongation of Schwann cells along axons (Fig. 7.10). Po mRNA expression in terms of intensity and location was comparable to that observed in defined medium alone, and in some cultures basal lamina was observed by electron microscopy. An occasional Schwann cell expressed Po protein by immunofluorescence and limited degree of myelination was noted; however to a significantly lesser degree than previously observed in myelinating medium. No evidence of myelination was observed in cultures where 10*u*g/ml laminin was mixed with the collagen substrate prior to riboflavin gelling. Po mRNA signal was comparable in intensity and distribution to that observed with defined medium alone.

Experiments with Matrigel were unsuccessful in eliciting myelin. When added to the collagen substrate, the substrate became so granular that it became difficult to visualise cells on the surface. The collagen also tended to detach from the surface causing loss of explants. Matrigel added to the defined medium produced no beneficial changes to the cultures. Basal lamina deposition could not be clearly observed, and immunocytochemistry and Po *in situ* hybridisations

became difficult to assess due to high background levels. In none of the circumstances applied in these experiments did cultures progress to myelination under the influence of Matrigel.



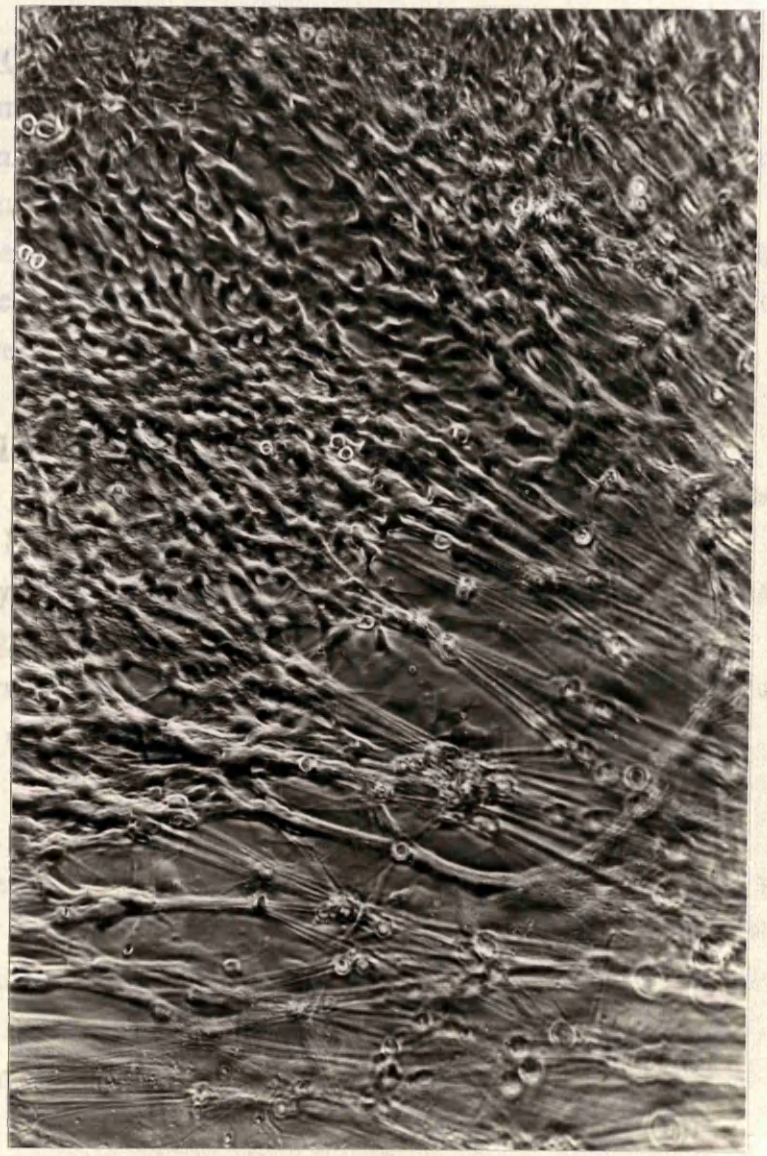


Figure 7.10
DRG defined medium cultured with exogenous laminin. Schwann cells elongate and show alignment along axons. x 100.

CHAPTER 7
PART III
SCHWANN CELL/DRG CO-CULTURES MAINTAINED IN DEFINED MEDIUM AND SWITCHED TO MYELINATING MEDIUM

INTRODUCTION

From the first part of these experiments it became apparent that myelination would not proceed in the absence of foetal calf serum and chick embryo extract components even although neurons were present. To elucidate if such cultures were still capable of forming myelin I exposed cultures maintained in defined medium for 21 days to full myelinating medium previously known to support myelination.

MATERIALS AND METHODS

E16 DRG explants were established in an identical manner to those in Chapter 7, Part I. After monitoring these cultures for 21 days and establishing the lack of any evidence of the formation of myelin the cultures were re-fed with myelinating medium described in Appendix 1.3.

Immunocytochemistry, electron microscopy and *in situ* hybridisation techniques were performed exactly as for Part I.

RESULTS

After one week's exposure to myelinating medium the first myelinated internode could be observed by light microscopy and was confirmed by immunostaining with Po antiserum (Fig. 7.11) and by electron microscopy (Figs. 7.12 and 7.13). It was observed that this method induced synchronous initiation of myelination *in vitro*. Over the following week the number of myelinated fibres increased and cultures resembled normal myelin-forming DRG cultures continually maintained in myelinating medium.

Figure 7.11
Po immunostaining of DRG explant culture 10 days after switching from defined medium to myelinating medium. A section of myelinated axon is clearly visible (white arrows). x 650.

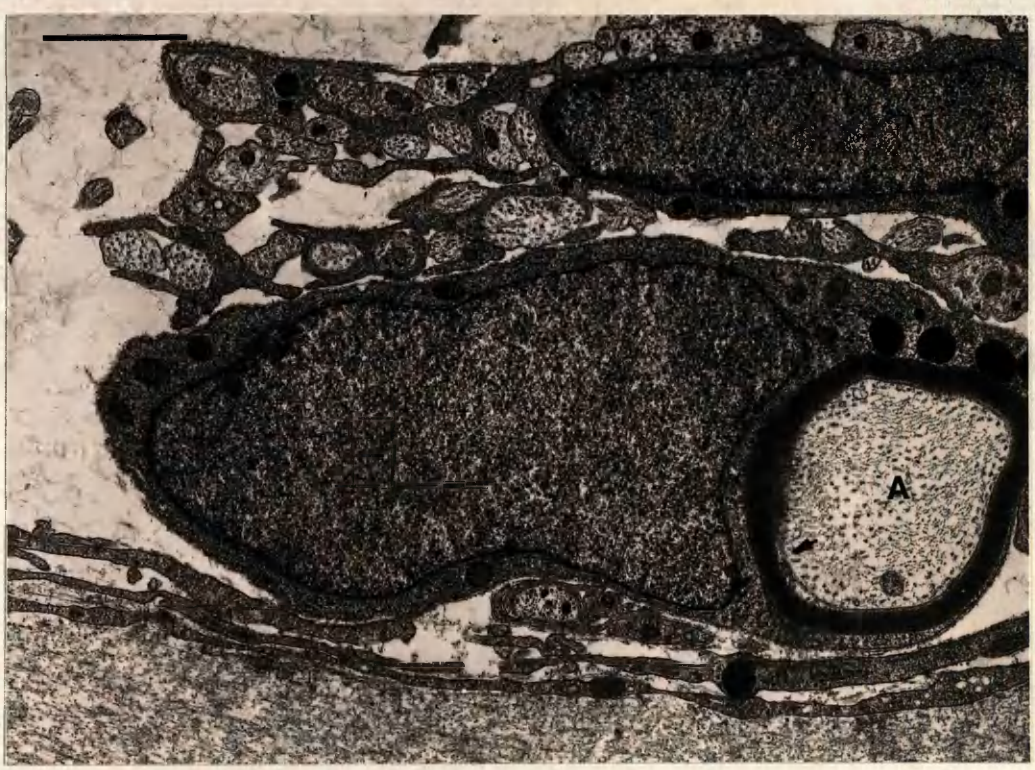


Figure 7.12
Electron micrograph of DRG explant culture 14 days after switching from defined medium to myelinating medium, showing an axon clearly encompassed by compact myelin (arrow). Figure 7.13 shows more detail of the lamellae. Scale bar = 1μm

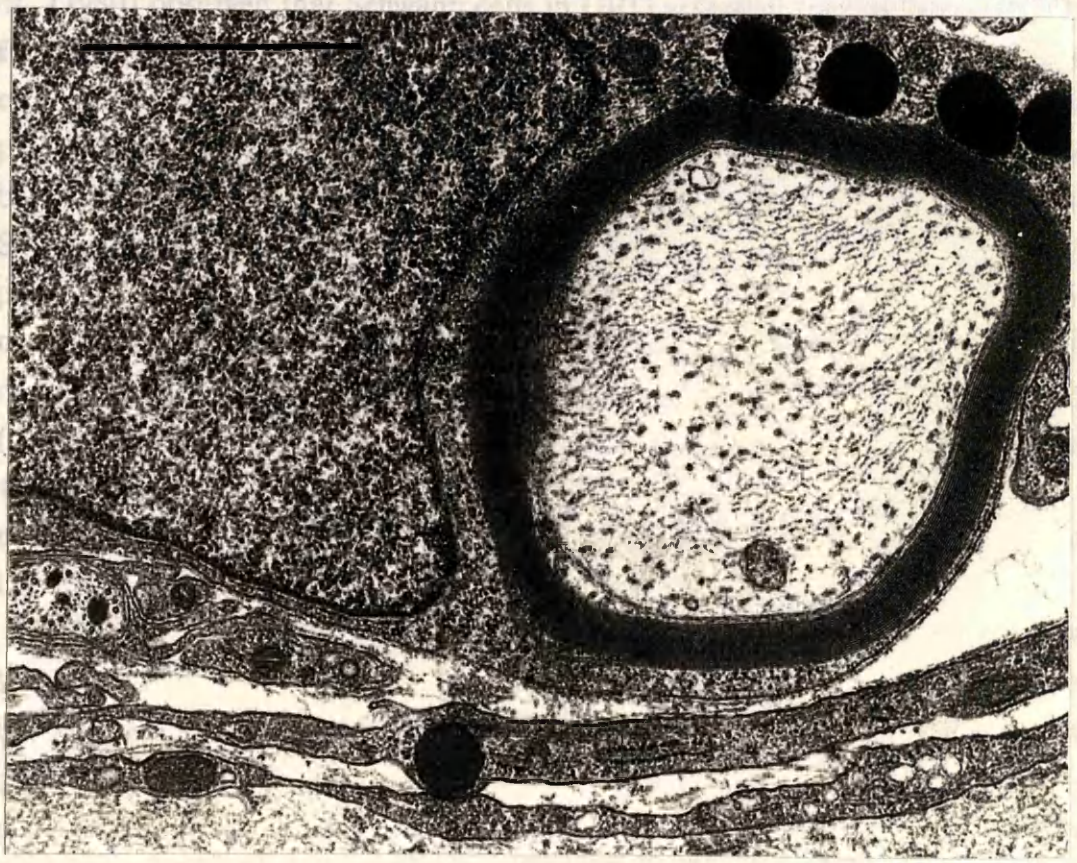


Figure 7.13
Enlargement of electron micrograph from Figure 7.12 showing compact lamellae and morphologically normal myelin. Scale bar = 1μm

DISCUSSION OF CHAPTER 7. PARTS I. II AND III.

Myelin-forming Schwann cells from neonatal sciatic nerve in isolation from neurons down-regulate expression of myelin-specific molecules such as the myelin proteins, GC and O_4 (Mirsky *et al.*, 1980; 1990; Eccleston *et al.*, 1982). From the results in this chapter and previous studies by Bunge and Eldridge, it is apparent that axons are not the only prerequisite for myelin formation. DRG co-cultures maintained in a chemically-defined medium do not form either a basal lamina or myelin. However, such cultures retain the ability to form myelin if conditions are altered favourably. GC and O_4 are expressed by Schwann cells in DRG defined medium cultures therefore Schwann cells must be at a higher level of differentiation than quiescent Schwann cells derived from sciatic nerve. Moya *et al.,* (1980) observed that Schwann cells in DRG cultures maintained in SATO N_2 for several weeks became granular in appearance due to the presence of distended cisterns of granular endoplasmic reticulum suggesting that Schwann cells in the presence of DRG neurons in defined medium are synthesising metabolic products but are for some reason failing to secrete these products. My findings that Schwann cells in such cultures express significant levels of Po mRNA but not protein support the hypothesis of full differentiation being withheld in the absence of serum factors. The presence of laminin on Schwann cell surfaces observed in my defined medium experiments correlates with the results of Cornbrooks *et al.* (1983), and further strengthens the hypothesis that Schwann cells co-cultured with sensory neurons in defined medium synthesise basal lamina components but are unable to secrete the products or organise them into the characteristic basal lamina structure.

Contact between Schwann cells and axons is required for the deposition of basal lamina prior to myelin formation. The absence of serum factors in defined medium may result in a failure of neuronal differentiation which hinders the ability of the axon to initiate the appropriate signal to the Schwann cell to cease proliferation, elongate along the axon and produce basal lamina leading to myelin formation. An alternative theory may be that the axonal signal(s) is delivered correctly but not processed by the Schwann cell, resulting in failure of synthesis and/or secretion of basal lamina components. The fact that exposure of these cultures to myelinating medium results in the formation of myelin lamellae so rapidly suggests that the problem lies at the secretory stage rather than the earlier synthetic stage. Bunge *et al.* (1982), demonstrated, using radiolabelling and SDS-PAGE techniques, that Schwann cells in defined medium and not related to axons released approximately four times less polypeptides than Schwann cells in co-cultures in myelinating medium. Within 2 days of switching cultures from defined

medium to myelinating medium, a four-fold increase in labelled polypeptides being released was noted, resembling the normal pattern of polypeptides produced in myelinating cultures. Carey and Bunge (1981) also noted that in N_2 medium the accumulation of Schwann cell-derived proteins, including those destined for incorporation into the extracellular matrix, is drastically reduced.

These results demonstrate a major difference between Schwann cells of the peripheral nervous system and the myelin-forming cells of the central nervous system, oligodendrocytes. It is known that oligodendrocytes will form myelin in cultures maintained in defined medium (Kleinschmidt and Bunge, 1980; Bunge *et al.*, 1982) and basal lamina deposition is not a prerequisite for CNS myelination. Peripheral myelination would therefore seem to require a greater degree of interaction between axons and myelin-forming cells than is necessary for central myelination. These findings also suggest that defined medium is a sufficient source of precursors for the biosynthesis of myelin lipids, glycolipids and certain glycoproteins, some of which are common to both CNS and PNS myelin.

The axon surface provides a mitogenic signal to the Schwann cell (Wood and Bunge, 1975; Salzer and Bunge, 1980; Salzer *et al.*, 1980a and b), as do the serum components present in myelinating medium. A combination of both axonal and medium factors is required for differentiation leading to myelination, possibly with some synergistic interaction occurring.

SCG neurons cultured in defined medium do not acquire cholinergic properties observed in cultures supplemented with serum and chick embryo extract (Bunge *et al.*, 1982). This would suggest that defined medium affects neuronal differentiation, and some factor(s) in serum or embryo extract exerts a differential effect on both neurons and Schwann cells.

Induction of extracellular matrix production, that is, basal lamina deposition, in Schwann cell co-cultures in defined medium has been achieved by supplementing the defined N_2 medium with ascorbic acid (Carey and Todd, 1987; Eldridge *et al.*, 1987) and fetuin. Ascorbic acid is required for the production of type IV collagen by Schwann cells. Fetuin glycoprotein is the major protein in foetal calf serum. Supplementation of cultures exposed to ascorbic acid with fetuin significantly enhanced myelin formation. Ascorbic acid alone induced some myelin formation, however fetuin in isolation did not. A degree of synergy must operate resulting in optimal rates of myelin formation. Eldridge *et al.* (1987) deduced that ascorbic acid could substitute for chick embryo extract in the presence of foetal calf serum resulting in optimal myelin formation, and attributed the efficacy of embryo extract to ascorbic acid being the active component. This was subsequently confirmed by treating chick embryo extract with ascorbic acid

oxidase which destroys ascorbic acid. Myelin formation was significantly reduced if treated embryo extract was used in the culture medium. It is interesting to note that Eldridge and co-workers could not promote the formation of myelin using ascorbic acid and fetuin and deduced that the activity of serum must be attributable to some other macromolecular serum component. Again, some degree of synergistic action was observed between serum and ascorbic acid, and basal lamina production has been reported to be more uniform in the presence of serum. This may be due to some serum factor(s) buffering the ascorbic acid from oxidation, thereby maintaining concentrations of ascorbic acid in the medium for longer periods of time. Ascorbic acid exogenously supplied to cultures requires to be replenished at 24 hour intervals as it degrades very rapidly *in vitro*.

Myelin formation was observed in co-cultures of neurons and Schwann cells in defined medium exposed to exogenous supplies of a basement membrane preparation, but only by Schwann cells contacting the matrix (Carey *et al.*, 1986). Eldridge *et al.* (1989), confirmed these results using exogenous basal lamina gels and further demonstrated that individual components of basal lamina elicited no induction of myelination, except in the case of laminin. In my experiments I found that addition of laminin as a medium constituent produced minimal amounts of myelin in some of the cultures, however when incorporated into the collagen substrate laminin failed to elicit basal lamina deposition. Eldridge and co-workers used laminin at 50μg/ml which is a higher concentration than I used.

From the results in this chapter and from Chapters 3 and 5 it would seem that the expression of Po above basal levels is likely to be due to the neuronal influence. In defined medium, axonal signals can induce Po mRNA expression to a certain degree. Full up-regulation however, is usually associated with the rapid membrane expansion accompanying myelination. Is this due to further axonal signalling or due to Schwann cell events? The results suggest that Po expression can be regulated at several stages of synthesis. Owens and Bunge (1989) showed axonal interaction up-regulated the cell surface expression of MAG and GC but not Po. MAG, in fact, appears to be over-expressed in co-cultures lacking basal lamina, possibly due to the ease with which Schwann cells can contact the larger axons capable of inducing the expression of myelin-specific components without the constraints of basal lamina. Following basal lamina deposition Schwann cells are committed to interact with either larger or smaller axons, the latter unable to upregulate either MAG or GC.

Differentiation of the Schwann cell involves polarisation (Bunge *et al.*, 1986). This is achieved by the deposition of the enveloping basal lamina such that

abaxonal and adaxonal surfaces of Schwann cells are restricted to contacting components of the extracellular matrix and axon, respectively. While axonal interaction can up-regulate MAG, it may be that this molecule cannot function in its segregating role and initiation of myelin wrapping until Schwann cell basal lamina is formed. Polarisation allows a critical number of MAG molecules to interact with receptors on the surface of an axon destined to be myelinated. MAG may mediate the process by which the Schwann cell engulfs the axon and establishes the extent of the future internode. This critical role for MAG early in myelination does not exclude it from having further functions later in the process.

Trapp (1988) suggested that MAG must be removed from the Schwann cell membrane before compaction mediated by Po may occur. In defined medium no Po protein could be detected suggesting that the myelination process has been suspended at the stage immediately prior to membrane expansion, Schwann cell polarisation and basal lamina deposition.

CHAPTER 8

AXONAL SIGNALS: CONTACT OR DIFFUSIBLE FACTORS?

INTRODUCTION

The relationship between Schwann cells and axons destined to be myelinated is highly complex and involves a series of interactions some of which have been defined in detail, for example the role of certain cell adhesion molecules and some growth factors. The signal(s) initiating these relationships and the subsequent formation of myelin have not yet been clearly elucidated, however the weight of evidence suggests that such signals operate via contact between the Schwann cell and the axon destined to be myelinated. However, from my electron microscopy studies in previous chapters, it transpires that only a very minimal level of contact is required for expression of myelin proteins. Up-regulation of the Po gene appears to require only a limited amount of Schwann cell-axon contact and occurs prior to ensheathment. I therefore embarked on several experiments to establish if myelination events could be switched on by diffusible factors. The experiments entailed various methods of culturing Schwann cells and neurons in the same medium but not in direct contact. A second approach exposed Schwann cells from different sources to DRG-conditioned myelinating medium.

MATERIALS AND METHODS

Cell culture

E16 DRG explant cultures were established on riboflavin-gelled collagen coated coverslips and maintained in myelinating medium incorporating chick embryo extract and foetal calf serum described in Appendix 1.3.

Dissociated neonatal rat sciatic nerve Schwann cells were plated onto PLL-coated coverslips or Poly-L-lysine subsequently coated with collagen and riboflavin gelled. Schwann cell cultures were initiated in standard DMEM medium containing 10% foetal calf serum.

Neonatal SCG explants were established on collagen-coated coverslips and maintained in myelinating medium.

Antisera and immunocytochemistry

Axons were stained with RT97 ascitic fluid (Wood and Anderton, 1981) at 1:7500 which recognises a phosphorylated epitope on the 200kDa neurofilament protein. Schwann cells were identified with S100, a polyclonal commercial antiserum used at 1:900. NGF-R was also used to identify Schwann cells. Po protein expression was examined using a polyclonal antiserum at 1:250. Galactocerebroside was detected with monoclonal cell culture supernatant at 1:5 (Ranscht *et al.*, 1982).

NGR-R and galactocerebroside were detected on living cells and cultures were post-fixed. Intracytoplasmic markers were applied following fixation of cultures in 4% paraformaldehyde and permeabilisation in 95:5% ethanol:acetic acid. Foetal calf serum was used for blocking prior to application of primary antibodies. Secondary antisera were conjugated to either FITC or TRITC for single or double staining of antigens. Stained preparations were mounted in 90% glycerol in PBS containing 0.1% paraphenylenediamine to reduce fading.

In situ hybridisation

Cultures were fixed in 4% paraformaldehyde and stored in 70% ethanol prior to ISH with ^{35}S-labelled antisense and sense probes for Po. Full method in Chapter 2.4.1.

RESULTS

8.1 Diffusion studies

8.1.1 DRG and Schwann cells

Several techniques were established in an attempt to culture neonatal Schwann cells derived from rat sciatic nerve in the same medium as DRG explant cultures. The first method entailed dissociated Schwann cells cultured on PLL-coated 13mm coverslips for 5 days (therefore down-regulated for GC and Po expression) and established 1-2 week DRG explants cultured on collagen-coated 13mm coverslips being placed alongside each other in the same well of a 6-well tissue culture plate. The well was replenished with 1.5ml myelinating medium plus 200ng/ml NGF. The cultures were re-fed every 2 days by removing 1ml spent medium and replacing with fresh. After 3, 5 and 7 and 14 days each pair of coverslips were fixed in 4% paraformaldehyde and processed for *in situ* hybridisation with Po probe. At each time point a pair of coverslips were also immunostained for GC and Po protein expression. The coverslips with the DRG explants showed similar intensity and distribution of signal when hybridised to Po probe as was observed in Chapter 7, Part I, and Chapter 5. These cultures also exhibited GC although no Po protein was detected. The corresponding Schwann cell coverslip which had been incubated in the same medium showed no signal for Po by *in situ* hybridisation, and no evidence of GC nor Po expression. Schwann cells were identified in these cultures using S100 antibody.

8.1.2 DRG and freshly dissociated Schwann cells

The previous experiment was repeated using established DRG cultures on collagen as before, but this time freshly dissociated Schwann cells were placed in the well alongside the DRG coverslip. This was to ascertain if co-incubation could prevent the initial down-regulation expected with dissociated neonatal sciatic nerve Schwann cells. Co-incubation did not prevent down-regulation of Po mRNA in the Schwann cell coverslip, as assessed by *in situ* hybridisation, nor was down-regulation of GC prevented. The results for the DRG coverslips were as before.

8.1.3 DRG and Schwann cells

Another approach was attempted in an effort to induce expression of myelin-specific molecules by diffusion from DRG cultures. DRG explants were established on collagen coated 22m coverslips and maintained for 10-14 days in myelinating medium. Three sterilised glass beads were placed on the edge of each DRG coverslip and held in place by sterile vaccuum grease. A freshly plated

22mm PLL-coated coverslip of dissociated Schwann cells was inverted and placed on top of the glass beads. The level of medium was topped up until both coverslips were immersed in myelinating medium. The medium was replaced every 2-3 days. After 7 days the structure was dismantled and each pair of coverslips processed for *in situ* hybridisation and immunofluorescence. The results obtained were as before, DRG cultures expressed Schwann cell Po mRNA and surface GC, while the Schwann cell cultures could not be induced to express either.

8.2 Conditioned medium studies

8.2.1 Schwann cells exposed to DRG-conditioned medium

Schwann cells were established from dissociated neonatal rat sciatic nerve on PLL-treated 13mm coverslips and maintained in myelinating medium. At 5 days *in vitro*, having established down-regulation of Po mRNA by ISH and GC, the cultures were re-fed with pooled, freshly thawed DRG-conditioned medium collected from cultures which had previously formed myelin. After 5-7 days of incubation with conditioned medium the Schwann cells were fixed and processed for *in situ* hybridisation with Po probe or surface immunostained for GC. No up-regulation of myelin expression was observed in any of the cultures. The experiment was repeated on a smaller scale using freshly collected conditioned medium from current myelinating DRG cultures, however the results were the same as before.

In an attempt to prevent down-regulation of the myelin-forming phenotype, Schwann cells were dissociated from neonatal rat sciatic nerve and immediately bathed in fresh DRG-conditioned medium. They were maintained in DRG-conditioned medium for up to 2 weeks, however on testing at several time points the normal rate of down-regulation was observed.

8.2.2. Protein analysis of conditioned medium

To determine if significant amounts of protein are taken up by cultures from the medium, protein estimations (Chapter 2.5) were performed on myelinating medium, balancing solution and samples of DRG-conditioned media taken at 4 and 9 days *in vitro*. Balancing solution, myelinating medium and day 4 conditioned medium showed similar levels of total protein (around 2.4mgs/ml) while day 9 conditioned medium showed substantially less (1.6mgs/ml). Following these results SDS-PAGE analysis was performed on the samples (Chapter 2.5.2) and the results are shown in Figure 8.1. As expected, balancing solution exhibits less of the lower molecular weight bands, while both aliquots of conditioned media look very similar to fresh myelinating medium.

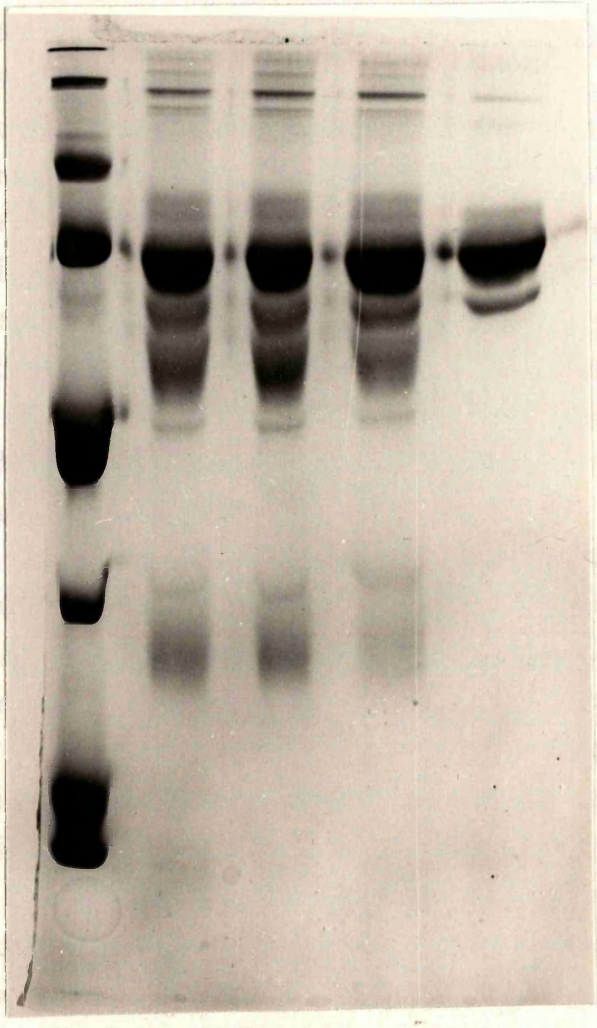


Figure 8.1
SDS-PAGE results. Lane 1 shows protein standards. Lane 2 shows DRG-conditioned medium from 9-day cultures. Lane 3 represents DRG-conditioned medium from day 4 cultures. Lane 4 is fresh myelinating medium and lane 5 is balancing solution.

DISCUSSION

In this chapter I attempted to induce the myelin-forming phenotype in cultured Schwann cells without direct axonal contact. It has been thought for some time that myelination requires direct axon/Schwann cell contact, however the possibility of diffusible factors being involved has never been completely ruled out. In Chapter 4 I showed by electron microscopy, immunocytochemistry and *in situ* hybridisation techniques that only a very minimal contact between Schwann cells and sensory axons is required to induce up-regulation of the myelin-forming phenotype (Morrison *et al.*, 1991).

In DRG cultures which have produced myelin or have the potential to myelinate, only those Schwann cells in direct contact with axons display the GC antigen (Mirsky *et al.*, 1980; Jessen *et al.*, 1987b). As this is an early marker of Schwann cell differentiation and initiation of myelination this is possibly the strongest evidence to suggest that direct axonal contact is vital for Schwann cell myelination. Under none of the conditions investigated in this series of experiments did I detect any evidence of up-regulation of myelin markers such as GC and Po. No Po mRNA could be detected suggesting absence of the myelin-forming phenotype in these cultures.

Expression of sulphatide is up-regulated by axon-Schwann cell signals (Mirsky *et al.*, 1990), therefore in retrospect, it might have been interesting to look for O_4 in these cultures. O_4 is detectable on the Schwann cell surface 2 days prior to GC. Available evidence indicates that Schwann cells bind O_4 at around the time when basal lamina is deposited. O_4 is a sulphatide and as such binds selectively to laminin (a component of basal lamina) (Cornbrooks *et al.*, 1983; Bunge *et al.*, 1986; Eldridge *et al.*, 1989; Mirsky *et al.*, 1990). The appearance of sulphatide in the Schwann cell membrane could indicate imminent basal lamina formation. Cyclic AMP may be a second messenger in the axonal induction of myelination. Elevation of cAMP induces Schwann cells to express surface GC and O_4 (Mirsky *et al.*, 1990). Cyclic AMP analogues also increase the synthesis of basal lamina components in Schwann cells *in vitro*.

It may also have been interesting to study possible changes in mitotic indices in these cultures; an indication of differentiation if the percentage of cells in a post-mitotic state increases.

I conclude that although the concept of diffusible factors cannot be ruled out completely, the conditions and results of these experiments confirm previous impressions that contact is required for up-regulation of myelin specific molecules.

CHAPTER 9

A STUDY OF THE EFFECT OF PC12 CELLS ON SCHWANN CELLS

INTRODUCTION

PC12 cells are derived from a rat pheochromocytoma clone; a neural crest-derived adrenergic tumour cell line (see Chapter 1.2.4). Exposure of cultured PC12 cells to NGF induces differentiation to cells resembling sympathetic neurons (Greene and Tischler, 1976), exhibiting many of the properties of mature terminally differentiated sympathetic neurons (Guroff, 1985). The cells cease division and adopt many characteristics of neurons including the projection of neurite-like processes which reach 500-1000*u*m in length (Guroff, 1985), and the increased production of transmitter-synthesising enzymes (Greene and Shooter, 1980; Thoenen and Barde, 1980). These effects are entirely reversible upon the withdrawal of NGF (Dickson *et al.*, 1986).

In Chapter 6 it was shown that sympathetic neurons derived from various sources induced Schwann cells to produce Po mRNA, although no Po protein or evidence of myelination was detected. The aims of this chapter were to show if differentiated PC12 cells induce the same response as sympathetic neurons in Schwann cells derived from sciatic nerve.

MATERIALS AND METHODS

Full details of materials and methods are described in Chapter 2.

Cell culture

Stock PC12 cells were maintained in Sato defined medium in Vitrogen-coated flasks. PC12 cells adhered firmly to the culture surface and required to be trypsinised prior to passaging. PC12 cells were maintained for many months in this manner. Prior to experimental use PC12 cells were trypsinised and replated onto collagen-coated or PLL-coated coverslips and induced to differentiate with the introduction of NGF (200mg/ml) into the medium. These cells were maintained in NGF-containing medium and permitted to differentiate for at least 1 week before conducting experiments with Schwann cells.

Schwann cells were dissociated from neonatal rat sciatic nerve in the same manner as described in Chapter 3. They were maintained in either Sato or DMEM media.

Antisera and immunocytochemistry

Schwann cells were identified with S100 antibody. The myelin-forming Schwann cell phenotype was examined with antibodies to GC and Po. Double-staining was performed using RT97 to identify neurofilament expression by PC12 cells. N-CAM surface antigen was detected using anti-N-CAM at 1:100. All procedures were as described in Chapter 2.

In situ hybridisation

Cultures were fixed with cold 4% paraformaldehyde and subjected to *in situ* hybridisation techniques (Chapter 2.5.1). ^{35}S-labelled antisense and sense probes for Po were prepared as described in Chapter 2.4.3.

RESULTS

9.1 Morphology of PC12 cells

PC12 cell cultures were established from frozen stocks kindly donated by Dr. Julian Dow, Department of Cell Biology, The University of Glasgow. The cells were quickly thawed and washed in HBSS to remove all traces of DMSO. The cells were seeded onto Vitrogen-coated 25cm^2 tissue culture flasks. They adhered quickly to the culture surface and began dividing efficiently within 24-48 hours. PC12 cells appeared healthy with a rounded morphology and good viability (Fig. 9.1). After several days and regular feeding with Sato defined medium a near-confluent monolayer of PC12 cells was formed. Contaminating cells were non-existent, as is to be expected in a cloned cell line. Once the cultures reached 80% confluency, they were trypsinised off the flasks and re-seeded onto collagen-coated 13mm coverslips. Once attached to the substrate they were fed with Sato defined medium plus 200ng/ml NGF. PC12 cells immediately responded to the NGF by ceasing to divide and projecting neurite-like processes within 12 hours. After several days in the presence of NGF an established network of processes was observed (Fig. 9.2).

9.2 Immunocytochemical profile

Stock PC12 cells not subjected to NGF treatment showed no significant immunostaining with RT97 (Fig. 9.3), however, NGF-differentiated PC12 cells demonstrating profuse neurite-like processes showed positive staining with RT97 (Fig.9.4). Neither non-differentiated nor differentiated pure PC12 cell cultures demonstrated any positivity for Schwann cell antigens such as S100, GC or Po. Differentiated PC12 cells exhibited N-CAM expression by immunocytochemistry (Fig. 9.5).

9.3 Addition of Schwann cells to differentiated PC12 cells

PC12 cells were established as before and maintained in NGF-containing medium for 1 week. Schwann cells were dissociated from neonatal rat sciatic nerve and seeded onto the PC12 cultures on day 8. The cultures were allowed to settle and maintained in Sato plus NGF for several days. The PC12 cells maintained their neuronal networks (Fig. 9.6) and the Schwann cells extended their processes and resembled normal bi-polar cultured Schwann cells. Schwann cells tended to align and elongate under these conditions (Fig. 9.7a and b). Fibroblast monlayers formed after addition of Schwann cells to PC12 cultures (Fig. 9.7a). Immunostaining of these cultures with S100 antibody positively identified Schwann cells and RT97 distinguished Schwann cell processes from PC12 neurites (Fig. 9.8).

Po protein was not detected after 2-3 days *in vitro*. GC was also down-regulated. NGF-R and N-CAM were observed in Schwann cell/PC12 co-cultures (Fig. 9.9a and b, and Fig. 9.10a and b).

In situ hybridisation of these cultures with antisense probe showed some evidence of Po mRNA as compared to hybridisation with sense probe. No further evidence of the myelin-forming phenotype was detected, either by phase microscopy, electron microscopy or immunocytochemistry. Schwann cell processes did not ensheath or myelinate PC12 neurites in these experiments. The experiment was repeated using full myelinating medium, however, the results were not appreciably different.

Dissociated Schwann cells were also added to non-differentiated PC12 cells as a control. The Schwann cell down-regulated for myelin-forming phenotype, and the PC12 cells appeared to have no effects on the Schwann cells.

9.4 Addition of PC12 cells to established Schwann cell cultures.

Dissociated Schwann cells were established from neonatal rat sciatic nerve as before and seeded onto PLL-coated or collagen-coated 13mm coverslips. These cultures were permitted to down-regulate for the myelin-forming phenotype over a period of 1 week, at which time NGF-differentiated PC12 cells were trypsinised and re-plated onto the Schwann cell cultures. Unfortunately, the Schwann cells survived better on PLL than collagen while the PC12 cells seemed to attach better to the collagen substrate. Examination of these cultures using immunocytochemical techniques revealed normal bi-polar Schwann cells with S100, and after several days RT97 demonstrated neurite-like processes on the PC12 cells. Neither GC nor Po antigens were detected. *In situ* hybridisation using Po antisense probes revealed no significant message in these cultures.

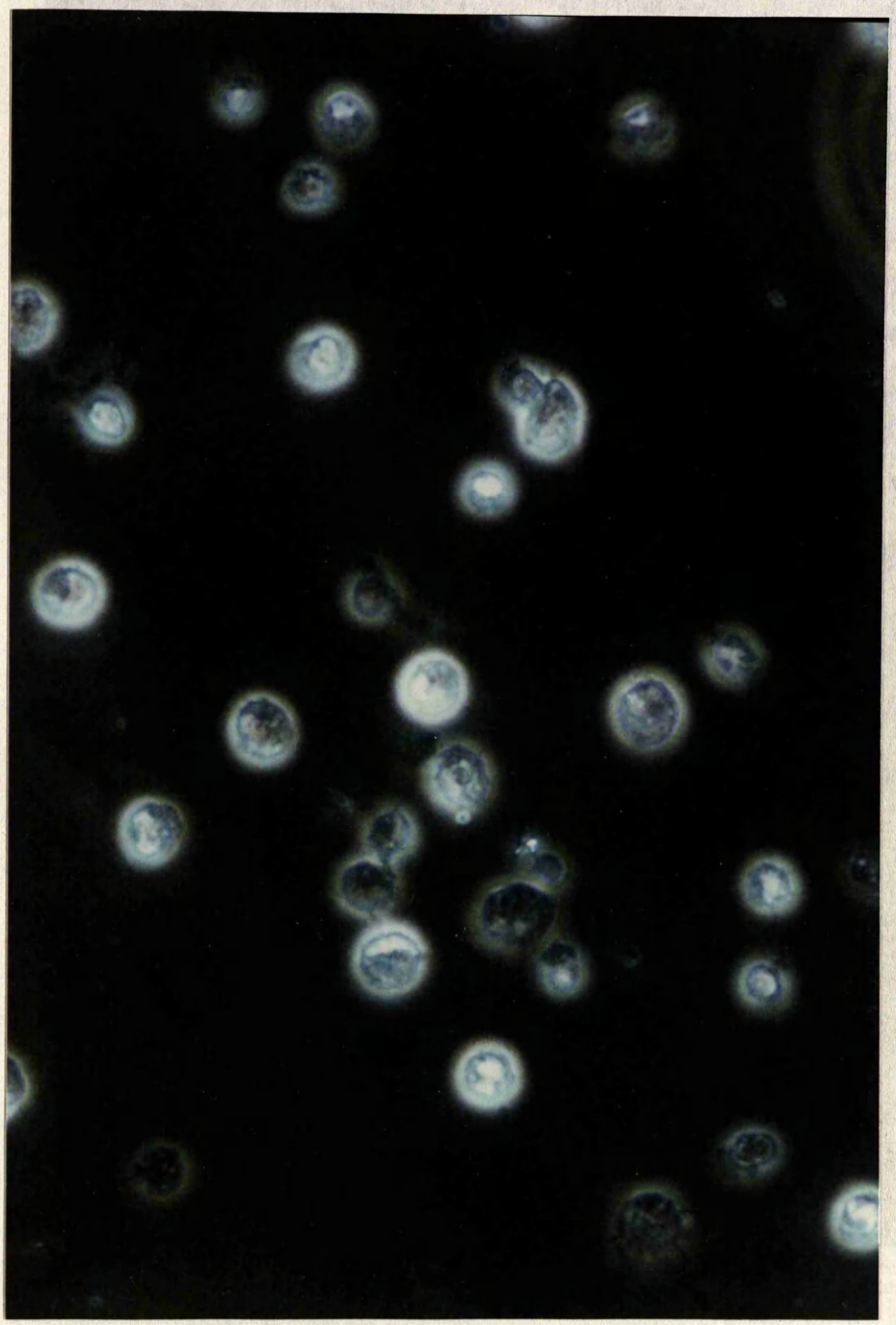


Figure 9.1
PC12 cells at 2 days *in vitro* cultured on collagen in defined medium without NGF. x 750.

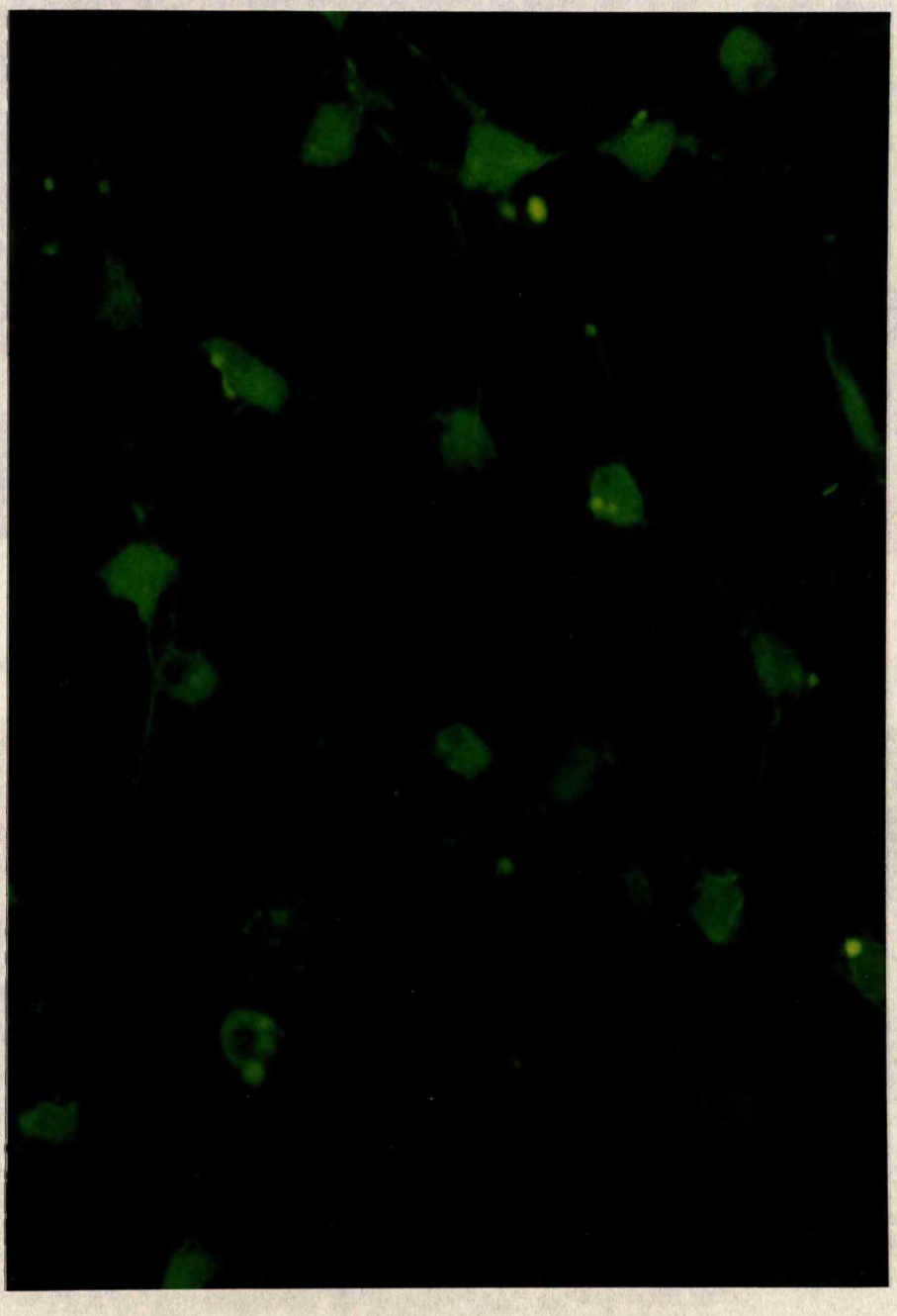


Figure 9.2
PC12 cells cultured for 7 days in the presence of NGF immunostained for RT97 (FITC) to demonstrate neuronal processes. x 750.

Figure 9.3
Undifferentiated PC12 cells immunostained for RT97 after 7 days *in vitro*. Rhodamine optics. x950.

Figure 9.4
NGF-differentiated PC12 cells immunostained for RT97. Rhodamine optics. x 950.

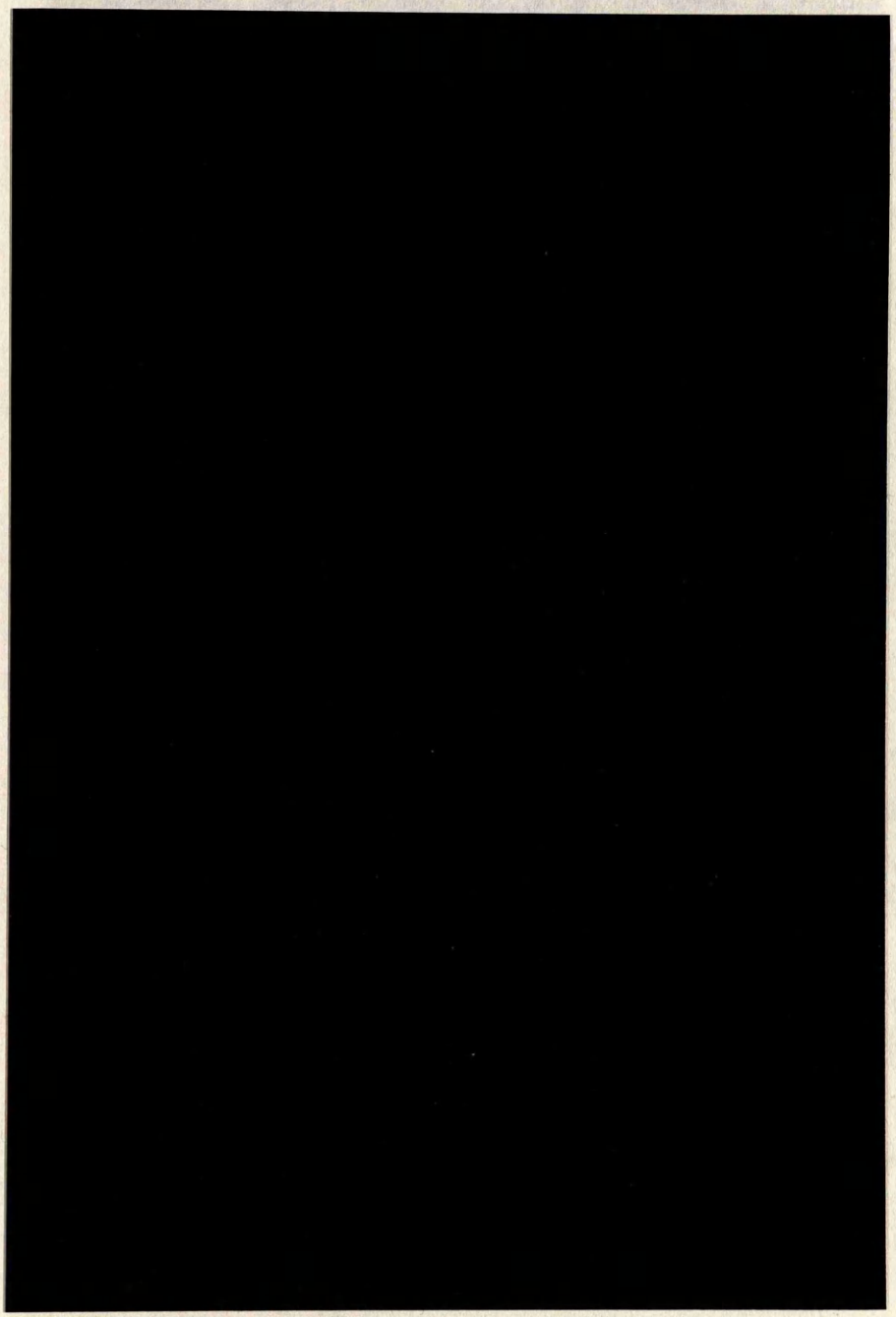


Figure 9.5
NGF-differentiated PC12 cells immunostained for N-CAM (FITC). x 850.

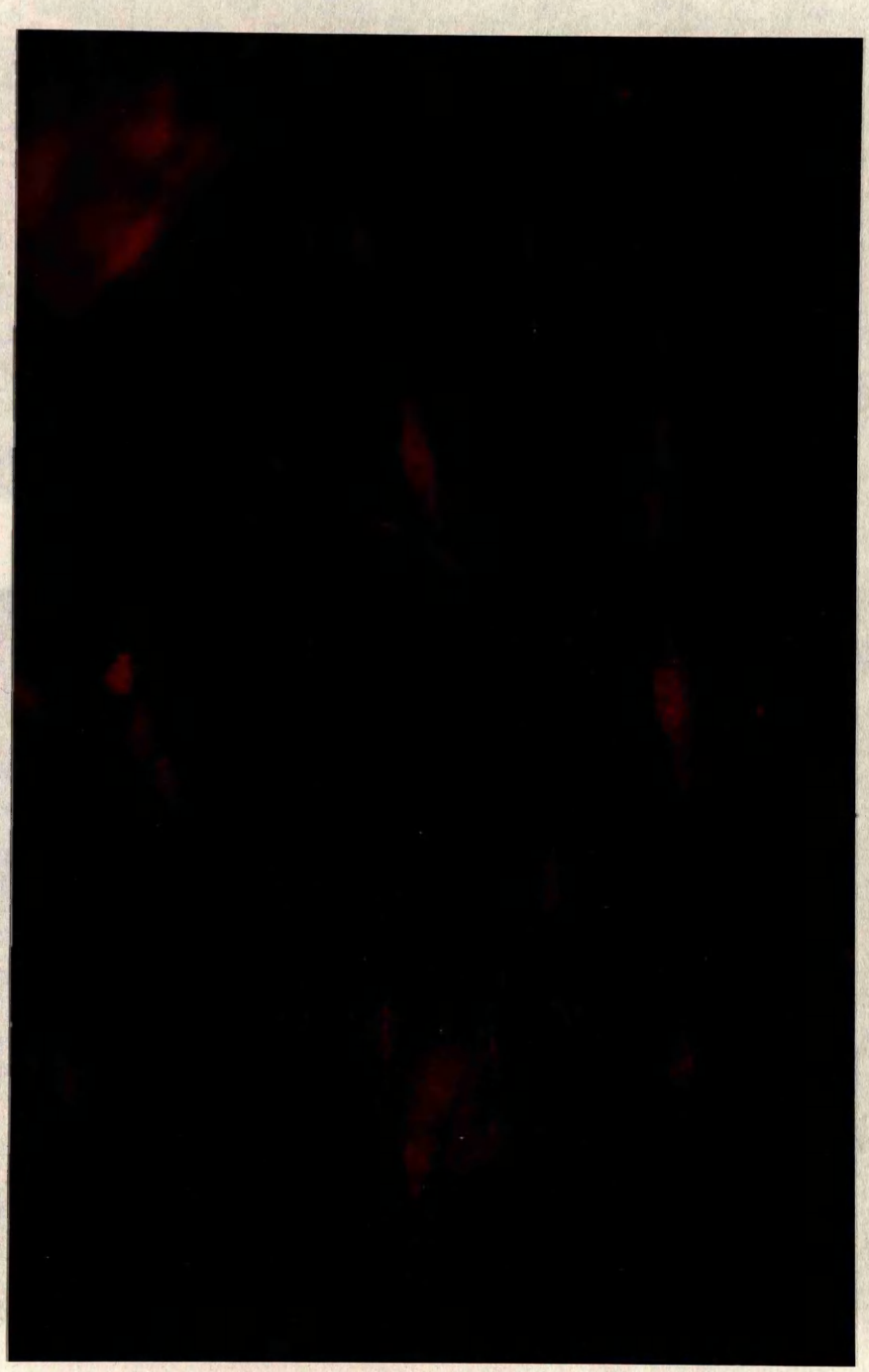
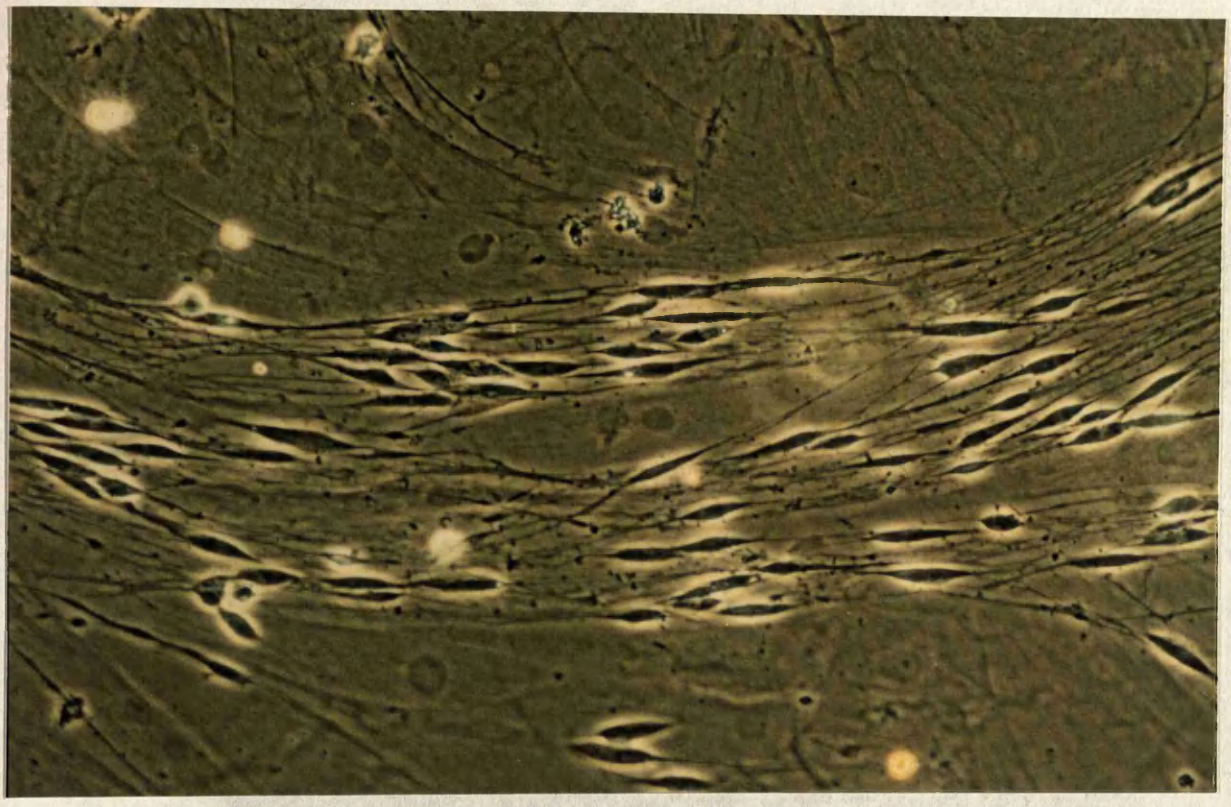


Figure 9.6
PC12/Schwann cell co-culture immunostained with RT97 to show maintenance of neuronal processes following addition of Schwann cells. PC12 cells were maintained in NGF-containing medium for 7 days prior to addition of freshly dissociated neonatal Schwann cells. Rhodamine optics. x 800.



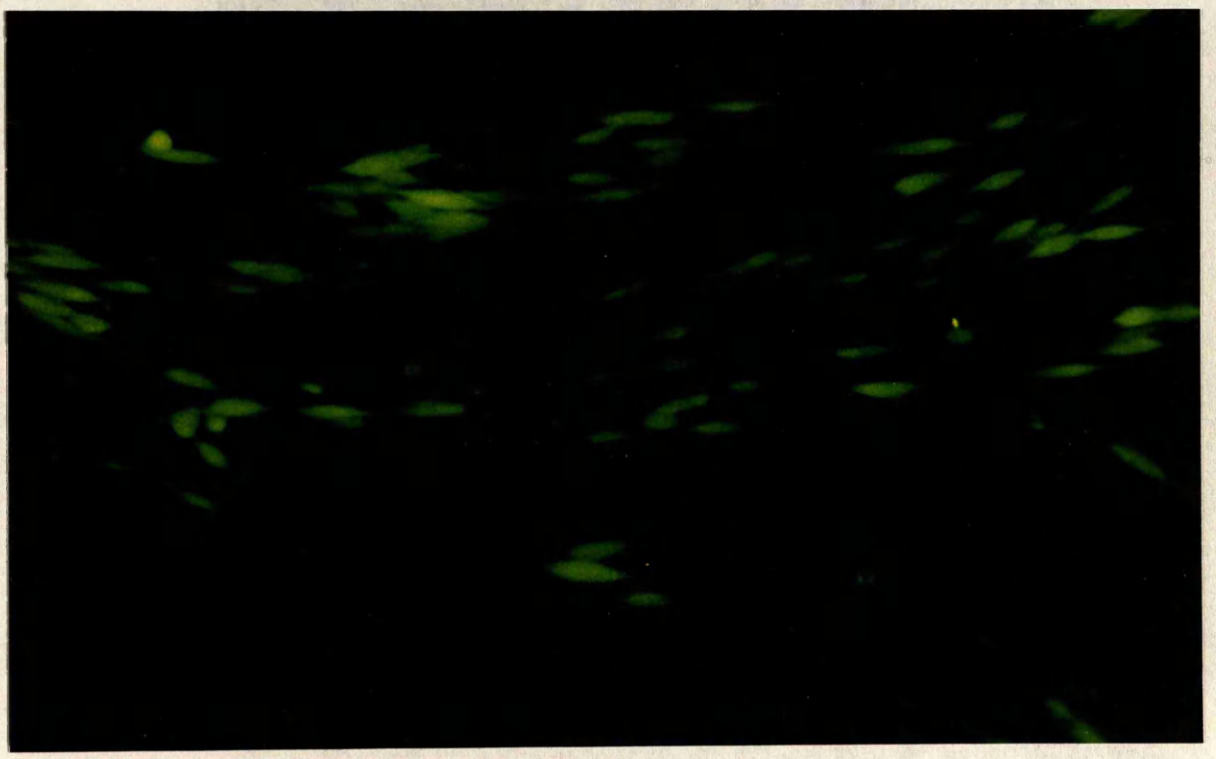


Figure 9.7
Schwann cells added to NGF-differentiated PC12 cells demonstrating alignment and elongation of Schwann cells. (a) phase microscopy. (b) S100 immunostaining (FITC). x 650.

Figure 9.8
Schwann cell/PC12 co-culture double immunostained with S100 (FITC) and with RT97 (rhodamine optics). x 750.

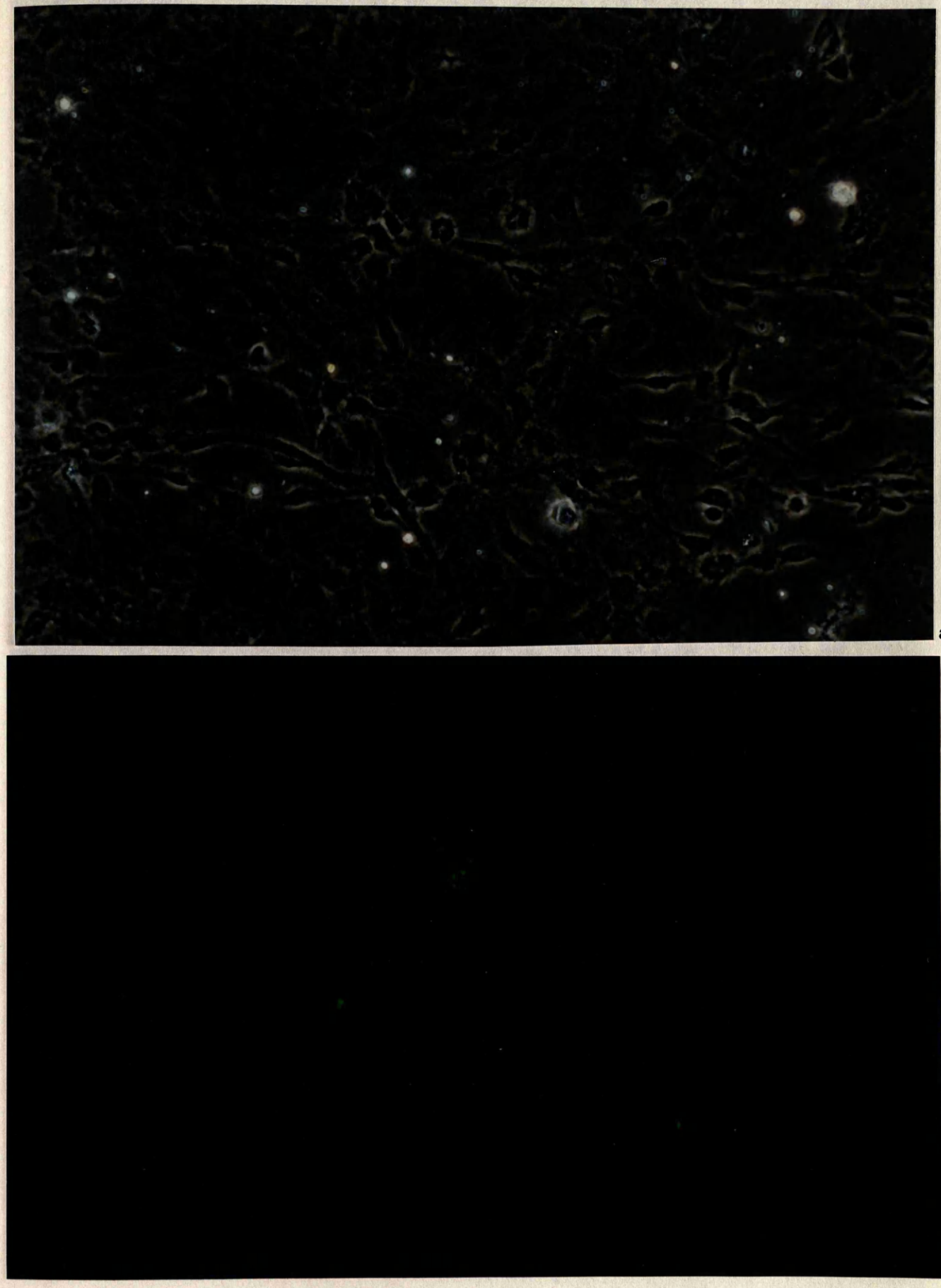
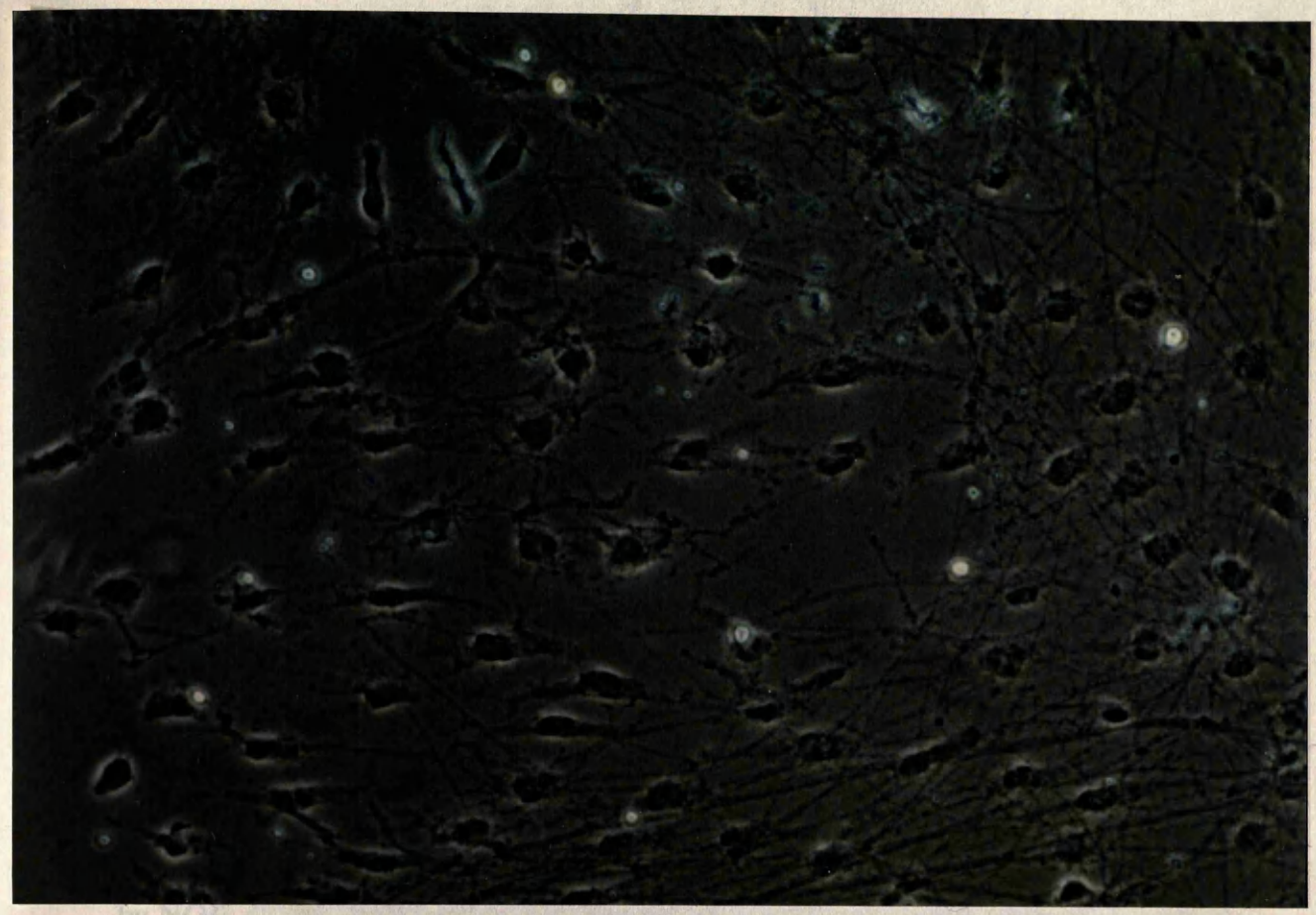


Figure 9.9
Schwann cells and differentiated PC12 cells maintained for 7 days *in vitro* immunostained for NGF-R. (a) phase microscopy. (b) NGF-R immunofluorescence. x 650.



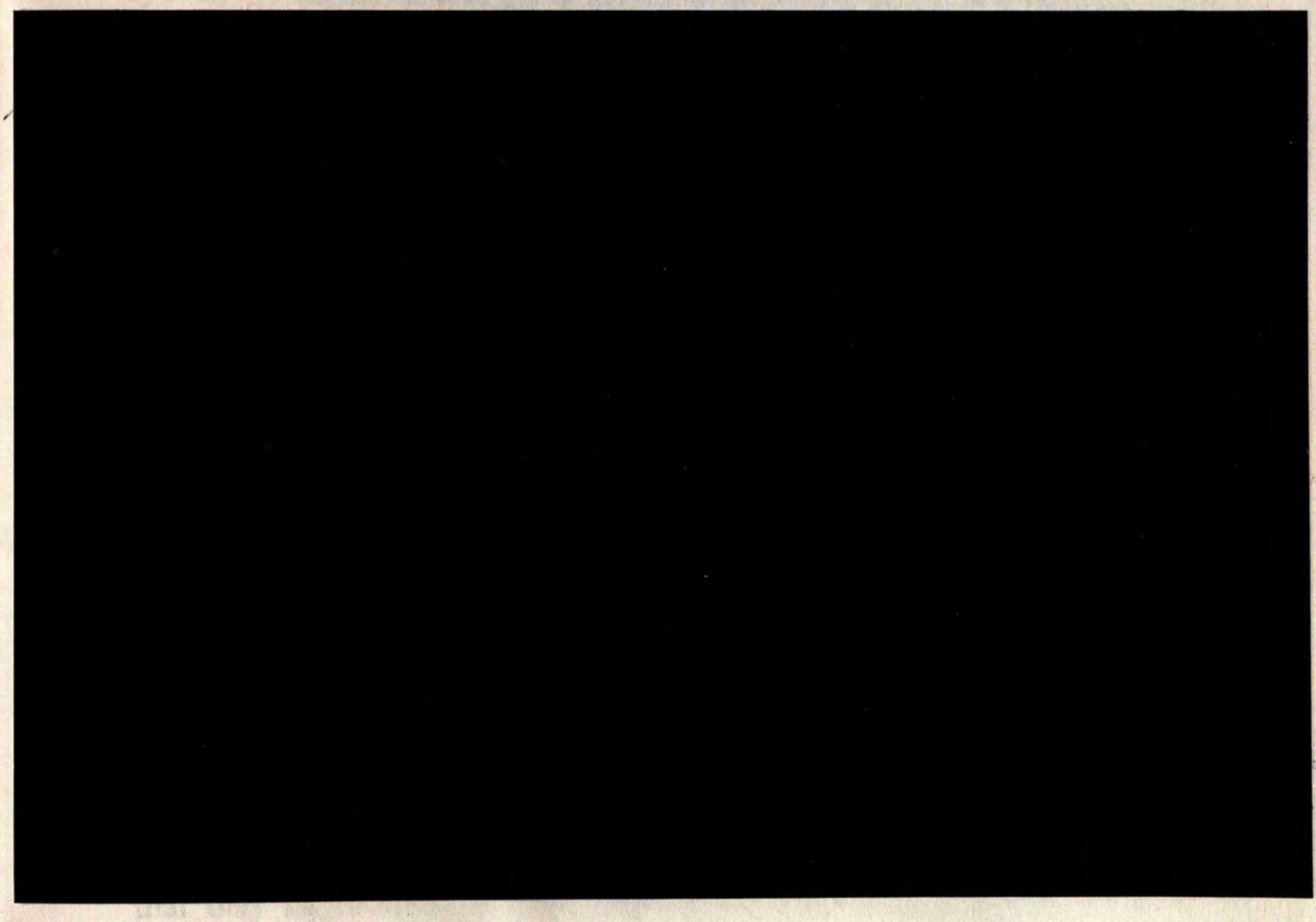


Figure 9.10
Schwann cells and differentiated PC12 cells maintained for 5 days *in vitro* immunostained for N-CAM. (a) phase microscopy. (b) N-CAM immunofluorescence. x 650.

DISCUSSION

There is evidence to show that NGF may influence the expression of proteins at transcriptional, post-transcriptional and post-translational levels in PC12 cells (Dickson *et al.*, 1986). One of the effects of NGF noted during my experiments with PC12 cells was the presence of the 200kDa neurofilament protein as identified by RT97 antibody. Neurofilament proteins are expressed by cells committed to become neurons; it was thought that mammalian neurofilament proteins were found only in neurons (Lee and Page, 1984), however some have recently been identified in myelin-forming Schwann cells (Kelly *et al.*, 1992). RT97 recognises a phosphorylated epitope on the 200kDa neurofilament protein. This protein, along with other neurofilament proteins was shown to increase in response to NGF-induced differentiation in PC12 cells (Lee and Page, 1984). NGF has been shown previously to elicit changes in phosphorylation events within these differentiating cells (Halegona and Patrick, 1980). Dickson *et al.* (1986) observed increased rates of neurofilament gene transcription (NF68) in PC12 cells induced by NGF. Thy-1 and N-CAM levels also increase in PC12 cells in response to NGF (Dickson *et al.*, 1986; Prentice *et al.*, 1987). I found N-CAM to be expressed on NGF-differentiated PC12 cells. Prentice *et al.*, (1987) found that only a 140kDa N-CAM species was expressed by naive PC12 cells while NGF-differentiated PC12 cells displayed the 140kDa species and a 180kDa N-CAM. N-CAM 180 has been suggested to be involved in neurite extension (Pollerberg *et al.*, 1985). Prentice and colleagues concluded that NGF activates a neuron-specific splicing mechanism and that N-CAM expression is controlled by NGF.

No evidence of myelin was observed in any cultures containing Schwann cells under the influence of PC12 cells. There are also, as far as I am aware, no reports of any other workers achieving Schwann cell myelination of PC12 neurites. There have been reports of Schwann cells myelinating non-axonal structures (Ernyei and Young, 1966) although Field *et al.*, (1968) dispute this. This non-myelination may be due to the relatively small diameter of the neurites in PC12 cultures. There has been a correlation between myelination and axon diameter, inferring that only axons greater than a certain diameter (1*u*m) are destined to be segregated by Schwann cells and myelinated (Ribchester, 1986). Myelin sheath thickness and the number of myelin lamellae is determined by the calibre of the axon (Friede, 1972). It appears to be peculiar to the peripheral nervous system that only larger axons become myelinated, although the critical diameter for myelination in the peripheral nervous system is around 1 micron, in the central nervous system it is 0.3*u*m or less (Matthews, 1968).

Ratner *et al.*, (1984) demonstrated NGF-differentiated PC12 cells to be a source of neurite-derived surface mitogen, stimulating Schwann cell division. Proliferation of Schwann cells occurred where PC12 cells were in contact with Schwann cells, and PC12 membrane preparations were shown to be mitogenic. Intact PC12 cells were less potent mitogens than DRG neurites, however both mitogens were assumed to be surface components with protein constituent(s) since both were heat and trypsin sensitive. Proliferation of Schwann cells is essential in the early stages of myelination, however, for ensheathment and myelination to progress the Schwann cells must cease dividing, differentiate and elicit a basal lamina. Differentiation, basal lamina formation and ensheathment was not observed by Ratner and colleagues. My findings concur with these results.

PC12-induced Schwann cell proliferation is independent of the presence of serum. Both my work and Ratner's findings using Sato chemically defined medium showed similar levels of proliferation when compared to serum-containing medium. I have previously shown that serum components are necessary to achieve myelination *in vitro*, which leads me to conclude that although signals from the neuron or PC12 cell are vital to trigger myelination, the requirement for external factors is criucial for myelination to progress. If it could be assumed that PC12 neurite diameter was not a problem, it seems entirely feasible that the correct conditions may be attained in which Schwann cell myelination of PC12 cell neurites could be achieved. However, this brings us back to the comparison between NGF-differentiated PC12 cell and sympathetic neurons. There appears to be some factor lacking or an inhibition of signal common to both, resulting in the inability in translation of myelin proteins. From my studies, it would appear that the major myelin protein Po is transcribed in these cultures (see Chapter 6) as seen by the presence of Po mRNA by *in situ* hybridisation techniques, but is not translated into protein. Therefore, if PC12 cells were capable of inducing differentiation of Schwann cells one would expect to observe a noticeable signal in hybridisation experiments with Po antisense probe.

CHAPTER 10

GENERAL DISCUSSION

GENERAL DISCUSSION

This study has examined peripheral myelination *in vitro*, with specific regard to the behaviour of Schwann cells under myelin-forming and non-myelin-forming conditions, and the expression by those Schwann cells of myelin-specific molecules. Myelination in the peripheral nervous system is a highly complex process and is an absolute requirement for the efficient functioning of the vertebrate nervous system. During the differentiation of myelin-forming Schwann cells, expression of myelin-specific genes is induced. Some of these genes encode proteins which have roles in the induction of myelination, the initial formation of the myelin membrane, and in its wrapping and compaction around the axon, and finally the maintenance of the compact myelin formed. Po is the major structural protein in peripheral myelin, and due to its abundance and relative ease of detection was chosen to represent the myelin-forming phenotype in Schwann cells. Po appears to function as a homophilic adhesion molecule promoting the formation of the intraperiod line of myelin (Filbin *et al.*, 1990) which is important for the periodicity of the lamellae (Lemke and Axel, 1985). These factors may explain the appearance of Po early in the myelination process.

Several previous studies have indicated that the expression of many genes in Schwann cells is influenced by the presence or absence of axons. Myelin-specific protein genes are rapidly and markedly down-regulated following nerve transection *in vivo* and removal from axons in dissociated cell culture (Mirsky *et al.*, 1980; LeBlanc *et al.*, 1987; Trapp *et al.*, 1988; Lemke and Chao, 1988; Mitchell *et al.*, 1990). Demonstrating this down-regulation in Chapter 3, I showed that Schwann cells from actively myelinating neonatal sciatic nerve when cultured for up to 7 days in the absence of axonal influences, down-regulate their Po mRNA signal as expected, such that by 7 days *in vitro*, the signal compared to that *in vivo* was negligible. These results seem to indicate that axons are necessary for maintenance of myelin protein gene expression. However, other studies have shown that Po mRNA and protein continue to be detected in the absence of axons, albeit at basal levels (Poduslo *et al.*, 1985c; Rutkowski *et al.*, 1990). Poduslo and colleagues demonstrated that adult cat sciatic nerve Schwann cells are capable of continued synthesis of Po *in vivo* in permanently transected nerves, suggesting that axonal association is not an absolute requirement for specifying myelin protein synthesis. Rutkowski and colleagues reported that cultured secondary Schwann cells continued to express basal levels of Po mRNA and protein, however in these cultures cholera toxin had been used to expand cell numbers, and this may have influenced gene expression. On examining Schwann cells from several sources, I

found that Schwann cells of unmyelinated nerves such as the cervical sympathetic trunk did not appear to express significant levels of Po mRNA at any time. With regard to the findings of Poduslo *et al.* (1985) that axonal influences may not be an absolute requirement for myelin gene expression, experiments were performed to attempt compensation for axonal loss by supplementing sciatic nerve Schwann cell cultures with laminin, which has been reported to be an active component of basal lamina (Eldridge *et al.*, 1989). Neither laminin added to the culture medium nor laminin used as a substrate prevented the down-regulation of Po mRNA in dissociated sciatic nerve cultures, however there appeared to be a greater number of Schwann cells expressing Po mRNA over the first 2 days *in vitro*, and there also appeared to be a more intense signal associated with expressing cells. The fact that extraneous laminin had little effect is not altogether surprising, since Schwann cells themselves synthesise laminin, and express the antigen in culture from around 24 hours *in vitro* onwards (Cornbrooks *et al.*, 1983; McGarvey *et al.*, 1984). Laminin is sensitive to trypsinisation and is lost during dissociation, therefore its presence following plating demonstrates continued synthesis in the absence of axons.

On examination of Schwann cells in association with neurons cultured in a full myelinating medium (Chapter 5), I observed increasing expression of Po mRNA from around 4 days *in vitro* onwards, although initial DRG cultures established from E16 embryos showed only a very occasional cell expressing Po mRNA. Northern blot analysis of E16 DRG's indicated that Po mRNA was essentially absent. This concurs with Baron *et al.* (1989) who demonstrated E18 to be the earliest time in the rat at which Po mRNA could be detected. The timing of appearance of Po mRNA in my cultures correlates with the expression pattern *in vivo*. Up-regulation of the Po gene (and other myelin-specific molecules such as GC) occurs early in myelination since the electron microscopic study on DRG cultures revealed that by 7-10 days *in vitro*, only a limited surface affiliation of Schwann cells with axons was present, with no evidence of ensheathment. Indeed, the first compact myelin was not observed *in vitro* until day 14. These studies would indicate that induction of myelin genes may result from only very minimal Schwann cell-axon interaction.

DRG cultures maintained in a chemically defined medium do not form either a basal lamina or myelin (Bunge *et al.*, 1982; Morrison *et al.*, 1991). However, Schwann cell proliferation and neurite outgrowth readily occur, and some degree of axon ensheathment was observed in my cultures. It therefore appears that axonal influences are not the sole prerequisite for myelination. If culture conditions are altered favourably these cultures proceed to form myelin,

thereby retaining their myelin-forming capacity. If DRG cultures in defined medium are supplemented with ascorbic acid and serum (Eldridge *et al.*, 1987), or exogenous basement membrane or laminin alone (Carey *et al.*, 1986; Eldridge *et al.*, 1989), Schwann cells acquire a basal lamina and myelination proceeds in the normal fashion. In my experiments I observed the retention of capacity of these cultures to form myelin when I transferred them from defined medium into full myelinating medium. This results in some degree of synchrony of myelination *in vitro*. *In situ* hybridisation studies of DRG cultures in defined medium showed that Po mRNA is readily expressed, although no protein was detected either by immunofluorescence or peroxidase techniques. Brunden and Brown (1990) published similar results, however they did detect minimal amounts of Po protein and also reported lysosomal degradation. Other reports of Po mRNA expression in the absence of protein have been described in cultured Schwann cells transformed with SV40 virus (Chen *et al.*, 1987), and in perineurial Schwann cells of the rat trigeminal ganglion *in vivo* (Lamperth *et al.*, 1989).

The expression of Po mRNA in defined medium DRG cultures along with expression of GC, O_4 and laminin, suggest that Schwann cells co-cultured with sensory neurons in defined medium are capable of synthesising basal lamina and myelin components but are unable to secrete the products or organise them into the characteristic structures. Full differentiation is somehow being prevented, although all the basic requirements seem to be present. Either some form of inhibition is occurring or a further signal or signals is lacking from the system.

The results from the defined medium experiments and similar studies (Brunden and Brown, 1990) suggest that Schwann cells can regulate the levels of Po at the translational or posttranslational stage although current evidence suggests that in normal myelination the major control operates at a transcriptional level (LeBlanc *et.al.*, 1987; Trapp *et.al.*, 1988).

Cultures of SCG are not reported to myelinate (Estridge and Bunge, 1978; Johnson *et al.*, 1980), and myelinated fibres in the CST amount to less than 0.5% *in vivo*. Cultures derived from the SCG and stellate ganglia expressed Po mRNA although at lower levels compared to DRG cultures. The presence of the higher molecular weight band of Po in addition to the main 1.9-2.0kb signal in the northern blots was more prominent in SCG preparations than in DRG culture. Barbu (1990) reported a similar pattern, particularly in early embryogenesis where the 4kb band was the major band. It may be that the presence of this band in my cultures represents an early-expression species of Po mRNA, whereas in myelinating and adult Schwann cells the 1.9-2.0kb size predominates. This may suggest that the SCG and stellate ganglia are in a more primitive state than sensory

ganglia, perhaps due to lack of signalling to differentiate. The experiments by Roufa *et al.*, (1985) in which SCG Schwann cells when transplanted into spinal ganglia cultures elicited myelin demonstrate that these Schwann cells retain the capacity to form myelin and that the signal produced by the spinal neurons is not emitted by SCG neurons. Therefore Schwann cells are extremely vulnerable to environmental factors in addition to axonal contact.

The requirement for basal lamina deposition prior to myelination is as necessary as the axonal influence, however there is a possibility that basal lamina formation from synthesised components is initiated by an axonal signal. It is interesting to note in Brunden and Brown's study (1990), Schwann cell/neuron co-cultures deprived of NGF show continued survival of Schwann cells but poor survival of neurons and neurite outgrowth is inhibited. Schwann cells in these cultures do not contain appreciable levels of Po mRNA, and little or no Po glycoprotein can be detected (Brunden *et al.*, 1990). Since NGF does not directly affect the level of Po mRNA in cultures of Schwann cells (Lemke and Chao, 1988), the lack of Po mRNA expression in co-cultures deprived of NGF must be due to the effects of NGF depletion on neurons, and not on Schwann cells. These results indicate that the axonal influence may be the primary factor in signalling myelination. Schwann cell contact with intact healthy axons would appear to be essential for up-regulation of myelin-specific molecules judging by the results in Chapter 8, where under none of the circumstances tried could I induce myelin gene expression through DRG conditioned medium rather than direct axonal contact.

Although axonal signals are obviously a major factor in myelination, their exact nature and mechanisms have not as yet been elucidated. The expression of myelin-specific molecules is a temporal process regulated primarily by axonal contact with the Schwann cells, but is not clear whether a single signal initiates the chain of Schwann cell differentiation or whether a series of signals exists, with each step dependent on the previous one. Thomson *et al.*, (1991) showed evidence that the axonal signal maintaining (and possibly initiating) expression of the Po gene is a relatively stable, probably axolemmal-associated molecule (Griffiths *et al.*, 1991) not dependent on continuous fast anterograde or retrograde axonal transport. Future studies may identify the nature of signalling molecule(s) involved in inducing myelin-specific genes.

In rat dorsal root ganglion neuron cultures and Schwann cells from sciatic nerve, in defined serum-free medium, Schwann cells proliferate but do not

differentiate. Under these conditions with axonal contact, MAG and GC are upregulated but Po protein is not. Placing the cultures into medium which promotes myelination results in an upregulation of Po and myelination proceeds (Owens and Bunge, 1989). MAG has been shown to be involved in the process by which the Schwann cell engulfs an axon destined to be myelinated and also seems to establish the extent of the future internode (Owens and Bunge, 1989). The periaxonal location of MAG implicates this molecule in the maintenance of the volume of periaxonal Schwann cell cytoplasmic collar inside compacted myelin lamellae (Trapp and Quarles, 1982; Trapp *et. al.*, 1984). MAG may also maintain uncompacted membranes by homophilic interaction and must be removed from the membrane for compaction mediated by Po to occur (Trapp, 1988).

In vitro studies have also provided for the hypothesis of MAG having a recognition or binding function with the axonal surface (Arquint *et. al.*, 1987; Salzer *et al.*, 1987). The putative receptor for MAG resides on the surface of large diameter axons. This receptor may be an immunoglobulin-like molecule or an integrin-like molecule constituting the axonal ligand for MAG (Owens and Bunge, 1990).

MAG is expressed prior to basal lamina formation but its function is enhanced once basal lamina is deposited by the Schwann cell. This requires differentiation of Schwann cells involving polarisation (Bunge *et al.*, 1986). Polarisation allows a critical number of MAG molecules to interact with receptors on the surface of the axon. Enveloping basal lamina is deposited and abaxonal and adaxonal surfaces are restricted to contacting components of extracellular matrix and axon.

In electron microscopy studies, MAG has been localised in Schwann cell processes which have surrounded the axon by 1.5 turns (Martini and Schachner, 1986). However, in compacted myelin MAG can be visualised only in Schmidt-Lanterman incisures, and it seems apparent that MAG must be removed from the membrane before compaction occurs (Trapp, 1988).

GC is also an early marker of myelination, but it is interesting to note that exclusion of GC does not affect MAG expression, and MAG will mediate the initiation of axon engulfment although the process of myelination will not proceed further without GC (Owens and Bunge, 1990). Cultures of dorsal root ganglion neurons and Schwann cells may be depleted of GC using anti-GC antibody (Ranscht *et al.*, 1987). These cultures will assemble basal lamina and initiate the 1:1 stage but will proceed no further even in myelin promoting medium. Anti-GC does not interfere with formation of the mesaxon but does prevent its elongation (Ranscht *et al.*, 1987). The mechanism by which GC is removed from the Schwann

cell surface seems to be by internalisation of the GC/anti-GC antigen-antibody complex, preventing interaction of adjacent cell surfaces during elongation of Schwann cell membranes. These GC-negative Schwann cells express MAG but not Po (Owens and Bunge, 1990). At the 1:1 step, the inner axon-related cytoplasmic process (which has MAG located on it) had passed under the outer mesaxon but had not completed a full turn around the axon. The MAG presence in this single cytoplasmic process apposed to axons in GC-positive Schwann cells implicates MAG further in the initial envelopment of the axon. Upon removal of the antibody the Schwann cell proceeds to form myelin (Ranscht *et al.*, 1987). GC depletion may prevent advancement of the inner axon-related cytoplasmic process due to the alteration of physical properties in the Schwann cell membrane, that is, removal of lipids. Similar suppression experiments with Po have ruled out the Po molecule as having an early role in myelination.

In further cell culture studies on the role of MAG in myelination, Owens *et al.* (1990), introduced MAG cDNA by way of a recombinant retrovirus into primary cultured Schwann cells. These cells constitutively expressed MAG on their surface without the normal requirement for axonal contact to induce expression. In co-cultures, a higher than normal level of MAG was observed. However, unlike normal Schwann cells, transfected Schwann cells associated with non-myelinated axons or axons undergoing Wallerian degeneration also express high levels of MAG. These results suggest a posttranscriptional mechanism modulating MAG expression during myelination.

By immunostaining cultures with antibody specific to L-MAG, Owens showed L-MAG was transiently expressed at the earliest stages of myelination in normal cultures. In short-term co-cultures with sensory neurons, infected Schwann cells expressing only L-MAG (not S-MAG) segregated and ensheathed large axons after four days *in vitro*, providing exogenous basal lamina was added. Morphologically-normal myelin was subsequently formed.

In conclusion, although many of the mechanisms of myelination have been elucidated, the triggers which initiate the process remain somewhat of an enigma. A competent axon delivers a signal to the Schwann cell which induces the Schwann cell to align itself along the axon forming a 1:1 relationship, cease proliferation and begin differentiation. The Schwann cell becomes up-regulated for myelin-specific markers, membrane expansion occurs, basal lamina is deposited and myelin formation proceeds in an orderly sequence of events.

The competent axon must be of a certain diameter prior to myelination and it is thought that the putative myelinating signal is associated with the axolemma

(Griffiths *et al.*, 1991). In the CNS, however, axon calibre appears to be less crucial to initiation of myelination.

Basal lamina deposition, although a requirement for PNS myelination, is not necessary for expression of Po mRNA as observed from my results in Chapter 7 (Morrison *et al.*, 1991: Brunden and Brown, 1990). DRG co-cultures expressed similar levels of Po mRNA in defined medium when lacking basal lamina as in similar myelin-forming cultures. Po protein however was not detected in defined medium co-cultures, although Brunden and Brown detected basal amounts. These events suggest that since myelination does not occur in the absence of basal lamina, myelin protein gene expression and myelin sheath synthesis by Schwann cells may be separable events (Brunden and Brown, 1990). It has been postulated that basal lamina plays a role in maturation and polarisation of the Schwann cell providing the mechanically correct structure inducing the progression of myelination (Bunge *et al.*, 1986). Each subsequent step appears to depend on the step immediately preceeding it, a prime example being the role of Po in compaction. This step cannot proceed until MAG has been extruded from the membrane.

Once compact myelin has been formed the integrity of the axon is responsible for maintenance of the myelin sheath. The axon, therefore is of crucial importance before, during and following myelin formation. Environmental factors such as medium and the type of neurons are secondary to this. Finally, it would appear from the literature and this study that all Schwann cells regardless of status and origin retain the capacity to differentiate into myelin-forming cells given the appropriate conditions.

APPENDICES

APPENDICES

1.1 SATO defined medium.

1:1 Ham's F12:DMEM		(Flow)
glucose	4.5g/L.	(BDH)
glutamine	2mM.	(Flow)
penicillin/streptomycin	1:50	(Flow)
bovine insulin	10*u*g/ml.	(Sigma I6634)
human transferrin	100*u*g/ml.	(Sigma T1147)
BSA pathocyte	0.0286%.	(I. Sommer)
progesterone	0.2*u*M.	(Sigma P8783)
putrescine	0.1*u*M.	(Sigma P5780)
thyroxine	0.45*u*M.	(Sigma T0397)
selenite	0.224*u*M.	(Sigma S5261)
tri-iodothyronine	0.5*u*M.	(Sigma T5516)

Fresh ascorbic acid was added such that the final concentration was 50*u*g/ml.

1.2 SD solution

Soy-bean trypsin inhibitor	0.52mg/ml	(Sigma T6522)
DNAse 1 (bovine pancreas)	0.04mg/ml	(Sigma D0876)
bovine serum albumin (fraction V)	3mg/ml	(BDH)

1.3 Myelinating medium

MEME (Eagle's Modification of Essential Medium)		(Flow)
Horse serum (heat inactivated)	10%.	(Sigma)
Foetal calf serum (heat inactivated)	10%.	(Flow)
Chick embryo extract	5%.	(Flow)
Glucose	3.2mg/l.	(BDH)
Glutamine	2 mM	(Flow)
Penicillin/Streptomycin	2mls/100mls media.	(Flow)

(If available add human placental serum).
Ascorbic acid was added such that the final concentration was 50*u*g/ml.

1.4 Anti-mitotic drugs

Cytosine arabinoside (Sigma C1768)

The final concentration was 10^{-5}M. A stock solution of 100X was made up in L15 medium and stored at -20°C. This was diluted 1 in 100 with culture medium prior to use and used in 48 hour pulses, repeated every 48 hours for 3 pulses.

Fluorodeoxyuridine (FdU)

FdU	(Sigma F0503)
Uridine	(Sigma U3003)

Both were used together at a final concentration of 10^{-5}M. A 100X stock solution was made up in L15 medium and stored at -20°C.

1.5 Poly-L-lysine (Sigma P5899)

A stock solution of 100*ug*/ml in sterile distilled water was stored at -20^{o}C. Sterile coverslips were immersed in this for at least 5 minutes, washed in sterile distilled water and allowed to air dry in a sterile cabinet prior to seeding cultures. Flasks were coated by swirling the PLL across the culture surface, removing excess and air drying.

2.1 Silver stain materials:

A) Cholinesterase substrate

0.76g sucrose
1ml d.d. H2O.
6.5mls maleate buffer
0.5mls sodium citrate
1ml potassium ferricyanide
1ml copper sulphate

The above solutions may be mixed and stored overnight at 4^{o}C. Acetylthiocholiniodide (Sigma A5751) at 5mg/10mls was added immediately prior to use.

B) Maleate buffer (0.1M.)
11.6g maleic acid (Sigma M0375) in 400mls in H_2O.
7g sodium hydroxide pellets were used to achieve pH 6.
Final volume was made up to 500mls with distilled H_2O.

C) Sodium citrate (0.1M).
2.94g tri-sodium citrate dihydrate in 100mls distilled H_2O.

D) Potassium ferricyanide (0.005M) (Sigma P8131).
0.165g in 100mls distilled H_2O.

E) Copper sulphate (0.03M) (Sigma C7631).
2.394g in 50mls distilled H_2O.

F) Sodium nitrate (0.1M). (Sigma S5506).
0.85g in 100mls distilled H_2O.

G) Triton X (1%).
Add 1ml Triton X 100 to 100mls. Sodium nitrate (0.1M). Heat gently to mix.

H) Reducer.
Quinol (hydroquinone) (Sigma H9003) 0.028g.
Sodium sulphite (Sigma S0505) 0.032g.
Mix together in 10mls distilled H_2O, and store in the dark.

I) Hypo
5% Sodium thiosulphate in distilled H_2O. (Sigma S7143).

J) Methylene blue.
Make a 2% stock in distilled H_2O. Dilute 1:100 to give 0.02%.

K) Silver nitrate
0.17g in 10mls distilled H_2O. Never handle with metal instruments and store in the dark.

L) Phosphate buffer. (0.2M).
(1) 0.4M NaH_2PO_4 (6.24g/100mls d.d. H_2O).
(2) 0.4M Na_2HPO_4 (5.66g/100mls d.d. H_2O).
Mix 9.5mls (1) with 40.5mls (2) and make up to 100mls. with distilled H_2O.
Adjust to pH 7.4.

M) Paraformaldehyde (4%) in 0.1M Phosphate buffer.

Add 1g paraformaldehyde to 10mls distilled H_2O and 2 drops 1N NaOH. Dissolve by heating to 60°C and stirring. Cool and add 12.5mls 0.2M phosphate buffer and make up to 25mls. with d.d. H_2O. Adjust pH to 7.3. Paraformaldehyde should be made fresh but can be stored at 4°C for 24 hours.

Dehydration Schedule:

1 X 70% alcohol 30-60 seconds.
1 X 90% alcohol 30-60 seconds.
2 X 100% alcohol 60 seconds.

Clearing: Xylene or Citroclear

Mounting: DPX

2.2 TRIS buffer (0.5M), pH 7.6:

TRIS 6.05g.
NaCl 8.5g.
Distilled water 950mls.
HCl (1N) 40mls.

2.3 Isotonic cacodylate buffer, pH7.3

Sodium cacodylate	16.05g.
Sodium chloride	3.8g.
Calcium chloride	0.055g.
Magnesium chloride	0.102g.

Made up to 1 litre with distilled water and pH adjusted to 7.3 with 1N hydrochloric acid.

2.4 Araldite resin mix:

Araldite	30g. (CY212)
DDSA	25.2g. (Dodecenyl succinic anhydride)
DMP 30	1.2mls. (2,4,6-tri(dimethylaminomethyl) phenol)
Dibutyl phthalate	1ml.

2.5 Reynolds lead citrate:

Trisodium citrate	1.33g.
Lead nitrate	1.76g.

Each dissolved in 15mls distilled water and mixed together. Precipitate dissolved with 8mls 1N NaOH. Made up to 50mls with distilled water.

2.6 2.5% Glutaraldehyde

25% glutaraldehyde (Agar Scientific) diluted 1 in 10 with isotonic cacodylate buffer (Appendix 2.3). Cultures were washed in L15 and placed in fixative. Fixed cultures were stored at 4°C in 2.5% glutaraldehyde prior to processing for electron microscopy.

2.7 Peroxidase anti-peroxidase technique

Materials:

TRIS buffer (0.05M), pH 7.6: refer to Appendix 2.2.

Hydrogen peroxide (3%): Hydrogen peroxide 10mls
Distilled water 90mls

Normal goat serum.

DAB: Weigh out 50mg DAB in fume cupboard and add 50mls. distilled water. Shake to mix and store in the dark until ready to use.

Buffer: A: Potassium Dihydrogen Phosphate (0.2M).
1.36g in 50mls distilled water.
B: Disodium Hydrogen Orthophosphate (0.2M).
2.84g in 100mls distilled water.
Pour A into B until pH equals 7.3.

Method:

1) Fix the cultures in 4% paraformaldehyde.
2) Wash the cultures in water for 30 minutes.
3) Block endogenous peroxidase with 3% hydrogen peroxide.
4) Incubate at room temperature for 20 minutes.
5) Wash in water for 20 minutes.
6) Add 100*u*l normal goat serum to 1ml TRIS buffer.
7) Incubate each coverslip with 30*u*l diluted goat serum.
8) Leave at room temperature for 2 hours.
9) Make up primary antisera. Antibodies are diluted in TRIS with 1% normal goat serum.
10) Remove 10% serum block from coverslips and replace with 30*u*l diluted antiserum.
11) Incubate in moisture chamber overnight at 4 C.
12) Allow coverslips to return to room temperature.
13) Wash coverslips with TRIS buffer for 30 minutes.
14) Apply link antiserum diluted in 1% normal goat serum in TRIS.
15) Incubate slides for 1 hour at room temperature in link antibody.
16) Wash off incubated link with 6 changes of TRIS buffer over a 20 minute period.
17) During washes, make up PAP. Dilutions are made in 1% normal goat serum.
18) Incubate slides in PAP for 30 minutes at room temperature.
19) Make up DAB and phosphate buffer as directed in Materials 4) and 5).
20) Wash slides in TRIS buffer - 6 changes over 20 minutes.
21) Place slides in a staining dish containing phosphate buffer for 1 minute. Meanwhile, make DAB up to 100mls with remaining phospate buffer and filter.
22) Transfer slides to DAB/phosphate in a staining dish and incubate for 20 minutes at room temperature.
23) Remove slides and add 330*u*l of 3% hydrogen peroxide to the DAB solution. Replace slides for 3-5 minutes monitoring microscopically for a reaction.
24) When reaction is complete ie. background remains unstained, wash slides well in water, dehydrate, clear and mount. If required, slides may be counterstained prior to dehydrating.

3.1 ISH wash solutions:

1) 62.5mls. SSC (20X) (Appendix 4.16).
1.85mls. water.
2.5mls. DTT (added immediately prior to use).

2) 4mls. SSC (20X).
12mls. water.
20mls. formamide.
Add 4mls. DTT immediately prior to use.

3) 0.5 M NaCl (29.25g.).
10mM Tris pH 7.5 (1.211g.).
5mM EDTA (1.861g.).
Make up to lL. with water.
Adjust to pH 7.5 and filter.

4) 50mls. SSC (20X).
450mls. water.

5) 2.5mls. SSC (20X).
497.5mls. water.

3.2 ISH dehydration solutions:

D1 75mls. ethanol
162.5mls. water
12.5mls. 6M ammonium acetate

D2 150mls. ethanol
87.5mls. water
12.5mls. 6M ammonium acetate

D3: 200mls. ethanol.
37.5mls. water.
12.5mls. 6M ammonium acetate.

D4: 237.5mls. ethanol.
12.5mls. 6M ammonium acetate.

3.3 Preparation of glassware for ISH

To minimise background in the ISH procedure and eliminate RNAse contamination, glassware was subjected to the following regime. All glassware, including slides, was immersed 6% sulphuric acid with 6% potassium dichromate for 24 hours. This was rinsed in running tap water for 4 hours, followed by several rinses in distilled water. The glassware was then soaked in DEPC-water (0.01% DEPC in distilled water) overnight and dried at 60^{o}C. The glassware was wrapped in aluminium foil and dry-sterilised in a 180^{o}C oven. Pasteur pipettes were immersed in DEPC water overnight, rinsed in distilled water and dry-sterilised. Plastics, including eppendorfs and pipette tips were also immersed overnight in DEPC water, rinsed and dried in an 80^{o}C oven. These were subsequently autoclaved. Slide boxes were treated with acid solution as for glassware, rinsed and dried at 60^{o}C. Coverslips were immersed in 1N hydrochloric acid overnight, rinsed thoroughly in distilled water, separated and oven dried at 60^{o}C. They were treated with a siliconising solution (Repelcote) to facilitate removal following

hybridisation and to discourage probe binding. They were briefly rinsed in distilled water and sterilised in a 180°C oven.

3.4 Proteinase K buffer

0.05M Tris-HCl
0.005M EDTA
(pH 7.6)

Proteinase K was added at 0.002%.

3.5 DTT (dithiothreitol) Sigma D0632 F.Wt. 154.2

1M: dissolve 3.09g of DTT in 20mls of 0.01M sodium acetate (pH 5.2). Sterilise by filtration. Dispense into aliquots and store at -20°C.

3.6 Triethanolamine Sigma T1502 F.Wt. 185.7

1M: Dissolve 185.7g in 1 litre of DEPC treated distilled water. Dilute to 0.1M for use in ISH. 400mls was used in prehybridisation treatments with 625*u*l acetic anhydride added at 5 minute intervals. Treatment of slides lasted 10 minutes, with constant stirring, in a fume cupboard.

4.1 Plasmid digestion EcoR1 enzyme at 37°C for 2 hours (BRL).

4.2 1% Agarose gel (BRL 5510UA) (denaturing gel/northern blots)

1g agarose in 74mls SDW microwaved for 4 minutes. Re-adjust volume to 74mls with water and when cooled slightly add 10mls MOPS (10X) and 16mls formaldehyde. Pour gel.

4.3 1% Low melting point gel (BRL 5517UA) (for isolation of DNA)

0.5g agarose melted in Tris acetate buffer.

4.4 Gel Buffers

TE buffer: TRIS 10mM
EDTA 1mM

Made up with distilled water, and pH adjusted to 8.0.

TAE buffer: TRIS acetate 0.04M
EDTA 0.001M

50X stock: 242g TRIS base
57.1mls glacial acetic acid
100mls 0.5M EDTA (pH 8.0).

4.5 Riboprobe production (using T7/SP6 Transcription kit from Boehringer Mannheim.

	Antisense (Cla 1)	Sense (Xba 1)
DNA	3.0*u*l	3.0*u*l
Water	2.3*u*l	5.8*u*l
Buffer (10X)	2.0*u*l	1.5*u*l
Nucleotide mix*	3.0*u*l	1.5*u*l
Isotope (S^{35} dCTP)	7.5*u*l	2.5*u*l
RNAsin	1.0*u*l	0.5*u*l
Polymerase	1.2*u*l(T7)	1.2ul (SP6)

*Nucleotide mix: UTP 2*u*l
GTP 2*u*l
ATP 2*u*l

4.6 Denaturation buffer: A solution containing 1X MOPS, 50% formamide and 2.2M formaldehyde.

Formamide	250*u*l
Water	117*u*l
Formaldehyde	83*u*l
MOPS(10X)	50*u*l

Denaturing gel:

Distilled water	74mls
Agarose (BRL 5510UA)	1g.

Microwave for 4 minutes and add:

MOPS (10X)	10mls
Formaldehyde	16mls.

4.7 Northern gel dye: A solution containing 0.01% bromophenol blue, 0.01% xylene cyanol and 30% sucrose.

bromophenol blue	1*u*l
xylene cyanol	1*u*l
sucrose	3mls

Made up to 10mls with 1X MOPS buffer (Appendix 4.13).

4.8 Prehybridisation buffer (Northern blots). A solution containing 50% formamide, 5X SSC, 5X Denhardt's solution, 50mM phosphate buffer and 0.1% SDS:

Formamide	10mls.
SSC (20X)	6mls. (Appendix 4.16)
Denhardt's solution (100X)	1ml. (Appendix 4.10)
Sodium phosphate buffer *(1M)	1ml.
SDS (10%)	200ul
Distilled water	1.8mls

* Sodium phosphate buffer (1M):

1M $Na_2H_2PO_4$	30.5mls.
1M Na_2HPO_4	19.5mls.

Mix and pH to 7.0.

The above buffer was made up and stored at 4°C in a foil-wrapped bottle. The following were added to decrease non-specific binding:

Salmon sperm DNA	100ug/ml
Poly A	10ug/ml
Poly C	10ug/ml

These components were mixed in a conical flask, boiled for 10 minutes, plunged in ice and added to the prehybridisation buffer.

4.9 Hybridisation buffer (Northern blots). A solution containing 50% formamide, 5X SSC, 1X Denhardt's soultion, 20mM phosphate buffer, 0.1% SDS and 10% dextran suphate.

Formamide	50mls
SSC (20X)	25mls
Denhardt's	1ml
Sodium phosphate buffer (1M)	2mls
SDS (2%)	5mls
Dextran sulphate	10g.

To the above was added the ssDNA/Poly A/Poly C mix described for prehybridisation buffer. The volume was made up to 100mls with distilled water.

4.10 Denhardt's solution (100X)

Ficoll	10g.
Polyvinylpyrrolidone	10g.
Bovine serum albumen	10g.

These components were mixed in 500mls water and stored in frozen aliquots.

4.11 Stringency washes (Northern blots)

3X SSC 0.1%SDS	4 x 5 minute washes at 65°C
0.5X SSC 0.1%SDS	2 x 30 minute washes at 65°C
0.1X SSC 0.1%SDS	1 x 30 minute wash at 65°C

4.12 Strip buffer. A solution containing 70% formamide, 0.1mM EDTA, 10mM TRIS and 0.2% SDS.

Formamide	140mls
EDTA (400mM)	50*u*l
TRIS (0.1M, pH 7.4)	20mls
SDS (10%)	4mls
Distilled water	35mls

4.13 (10X) MOPS buffer (3-[N-Morpholino] propanesulphonic acid)

MOPS	0.2M
sodium acetate	50mM
EDTA	10mM

Made up with distilled water, pH adjusted to 7.0 with acetic acid, autoclaved and stored at $4^{O}C$ in foil-wrapped bottles.

4.14 Po cDNA probe labelling

Po DNA	9*u*l
GTP	1*u*l
ATP	1*u*l
TTP	1*u*l
nucleotide buffer	2*u*l
^{32}P dCTP	5*u*l
Klenow	1*u*l

4.15 DNA-probe-labelling column dye

Dextran blue	0.02%
phenol red	0.02%
ssDNA carrier	100*u*g/ml

Made up in sodium phosphate buffer (Appendix 4.8)

4.16 20X SSC

3M NaCl
0.3M Trisodium citrate

Dissolved in distilled-deionised water, pH adjusted to 7.0.

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